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PROCEEDINGS OF THE 3rd ANNUAL CONFERENCE ON ENVIRONMENTAL TOXICOLOGY

25, 26, AND 27 OCTOBER 1972

DECEMBER 1972

AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO

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13. ABSTRACT

This report is a compilation of the papers presented at the Proceedings of the 3rd Annual Conference on Environmental Toxicology, sponsored by the SysteMed Corporation and held in Fairborn, Ohio on 25, 26, and 27 October 1972. Major technical areas discussed included biologic threshold limits; toxicology of halogenated solvents, aerosol propellants, and fire extinguishants; and pyrolysis and rocket exhaust products.

Key words:

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FOREWORD

The Third Conference on Environmental Toxicology was held in Fairborn, Ohio on 25, 26, and 27 October 1972. Sponsor was SysteMed Corporation under the terms of Contract F33615-70-C-1046 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Arrangements were made by the Toxic Hazards Research Unit of SysteMed Corporation, and the papers presented at this Conference by personnel of SysteMed Corporation represent research conducted under the cited contract. Harold C. Hodge, Ph.D., University of California, San Francisco Medical Center, School of Medicine, Department of Pharmacology, served as Conference Chairman, and Mrs. Lois Doncaster, SysteMed Corporation, served as Conference Coordinator.

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Commander
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Good morning, ladies and gentlemen. It's my distinct pleasure to welcome you to the Third Conference on Environmental Toxicology. General Schafer sends his best wishes and regards for a successful conference. Much as he regrets it, he cannot be here to address you this morning because of a Commander's Conference which preempted his attendance here. Last year he was able to participate in this conference for its entire duration and is truly sorry to miss it at this time. This is actually the eighth in a series of annual toxicology conferences conducted by the Aeromed Labs in-house contractor who operates our Toxic Hazards Research Unit for the Toxic Hazards Division. The first five meetings were called Conference on Atmospheric Contamination in Confined Spaces and although they were heavily oriented towards space cabin toxicology, artificial atmospheres, and altitude physiology, they were still truly addressing environments, however exotic they may be, and the influence of these environments on the outcome of toxic injury. With the deletion of the military man in space mission, and the concomitant reorientation of national priorities, the unique inhalation exposure capabilities of the Toxic Hazards Division have been reoriented to emerging challenges in the area of environmental toxicology. Since we feel that the Toxic Hazards Research Unit represents a national asset, we have originated a much broader program in environmental toxicology through cooperative efforts with the National Institute of Occupational Safety and Health of HEW and one that embraces the entire field of toxic industrial chemicals and their occupational exposure in the everyday working environment. Other joint interest programs with the Department of Transportation, the Federal Aviation Agency and the United States Navy have added to the variety and scope of our research and by getting involved in these programs, we have also fulfilled research needs of a special Air Force interest. In doing these specific efforts, we have gained increased confidence in our facilities, equipment and scientific capabilities. We have recently improved our facilities in electron microscopy including a capability for cell membrane morphology. We plan to institute computer aided image scanning morphometric techniques to reduce the tedious aspects of this quantitative morphological approach. In the area of characterizing the chemical environment, our analytical capability has been expanded by a new DuPont mass spectrometer coupled with a dedicated computer to interpret chemical reaction kinetics and thermal degradation processes. All in all, the past one has been a reasonably good year and I'm confident that it will be reflected in the presentations

here. I cannot think of a better way to disseminate new scientific information than through these conferences. These conferences are a living, face-to-face encounter wherein everybody gets a chance to discuss the meaning of the data, either immediately or at the open forum sessions that are provided. This free exchange of ideas, criticisms and information has always been the strongest justification for these conferences. Finally, I'd like to take this opportunity to introduce to you the Vice Commander of the Aerospace Medical Laboratory recently arrived, Colonel Frederic Doppelt. He's an old friend and associate from the years of the Manned Orbiting Laboratory Program and therein had substantial responsibility for planning the expansion of the facilities here at the Toxic Hazards Division in support of the Manned Orbiting Laboratory Program. Dr. Doppelt was certified by the American Board of Internal Medicine in 1964 and in Aerospace Medicine in 1966. He spent 4 years in the Manned Orbiting Laboratory Program, was Chief of Professional Services at the March Hospital subsequent to that and just returns to us from an advisory role to the Republic of Korea Air Force.

In closing, let me wish you a rewarding exchange and we certainly hope to reconvene again next fall. Thank you.

SESSION I

BIOLOGIC THRESHOLD LIMITS

Chairman

Seymour L. Friess, Ph. D.
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AMRL-TR-72-130

PAPER NO. 1

RATIONALE FOR THE USE OF BIOLOGIC THRESHOLD LIMITS IN THE CONTROL OF WORKER EXPOSURE*

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Industrial hygienists and others concerned with safeguarding the health of workers have long recognized the many shortcomings and real limitations of industrial air standards as a means of controlling worker exposure.

NEED FOR BIOLOGIC THRESHOLD LIMITS (BTL's)

Measurement of air-borne concentrations are not always a reliable index of exposure in at least the following 5 situations:

1. When percutaneous absorption represents a significant or the major contributory route to body burden; (e. g. certain particulate carcinogens such as benzidine and derivatives). That skin absorption plays a large role in worker exposure is seen by the fact that of the more than 550 TLV-listed substances, approximately 125, or 23%, bear the "Skin" notation, indicating some exposure by this route not measured by determining air levels; CS_2 is a notable example.

2. When exposure is intermittent, or characterized by frequent, brief intermittent peaks, and air sampling, unless continuously recorded, does not conform to such intermittency; in these instances, air measurements indicate a falsely low exposure for slow-acting, cumulative substances; for fast-acting substances, fail to detect the critical peaks.

3. In situations of mixed exposures when there is metabolic interaction (potentiation, synergism, antagonism), as in the synergistic action of alcohol on the chlorinated hydrocarbon solvents.

*Also presented at XVII International Congress on Occupational Health, Buenos Aires, Argentina, September 1972.

4. Individual peculiarities of work habits; e.g. the mouth breather vs. the nose breather; carelessness in handling substances.

5. Added exposure from "moonlighting", off-the-job activities, or unsuspected dietary sources, e.g. lead, from illicitly distilled alcohol; carbon monoxide, from exposure to dichloromethane-containing paint strippers.

It is obvious from these examples that there is a need for other methods for monitoring worker exposure in many industrial situations than those provided by measuring air concentrations.

The Biologic Threshold Limit Concept. From the following premises on which the BTL concept rests, it is clear that the monitoring of worker exposure for changes in biologic (body) constituents offers, in most industrial situations, a superior method of controlling worker exposure and assuring a safer work-place than do air standards.

1. The worker is his own individual collector, registrar, and hence, monitor of his particular exposure(s).

2. Biologic measurements reflect

a) The worker's own individual and characteristic work habits.

b) His characteristic metabolism of industrial chemicals, including

c) Hereditary differences

d) His particular eating and drinking habits, and hence, his own individual, characteristic response.

3. Biologic measurements reflect the worker's over-all intake by all routes of entry simultaneously (mouth, skin, eye), and is thus independent of the manner in which exposure occurred.

4. The BLTV is the counterpart of the industrial air standard (TLV) and hence provides a biologic limit to the intake or to the response obtained at the industrial air limit.

5. Most important, different, and unique, BTLs protect the worker with preexposure burden or unrelated job exposure.

Nature of the BTLs. Biologic Threshold Limits represent limiting amounts of substances to which the worker may be exposed without hazard to his health or well-being as determined in his tissues and fluids or in his exhaled breath.

Some Biologic TLs and Their Corresponding TLVs. Table I shows that there are several different types of biologic measurement currently used for Biologic TLs, whether it be inorganic or organic substances that are being measured.

TABLE I

SOME BIOLOGIC TLVs AND THEIR CORRESPONDING TLVs

Substance	Biologic Source	Biologic TLV	TLV
	INORGANIC		
Arsenic	Urine	1 mg/L	0.5 mg/m^3
Arsenic	Hair	400 ppm	0.5 mg/m^3
Fluorides	Urine	5 mg/L	2.5 mg/m^3
Lead	Urine	0.15 mg/L	0.15 mg/m^3
Lead	Urinary dALA	2-4 mg/100 ml	0.15 mg/m^3
Lead	Blood	0.08 mg/100 g	0.15 mg/m^3
Mercury	Urine	0.15 mg/L	0.05 mg/m^3
Mercury	Blood Serum	LDH Isozyme, F5	0.05 mg/m^3
	ORGANIC		
Benzene	Urine, as Phenol	200 mg/L	C 25 ppm
Benzene	Breath	1 ppm (at 1 hr.)	C 25 ppm
DDT	Urine, as DDA	3 ppm	1 mg/m^3
Org. Phosphate Insect.	Blood, as CHE	50% Red'n	0.1 mg/m^3
Tetraethyl Pb	Urine Pb	0.1	0.1 mg/m^3
Toluene	Urine, as Hippuric Ac.	3000 mg/m^3	100 ppm

These measurements fall into two broad categories:

1. Those that measure exposure and indirectly body burden (as arsenic, fluorides, lead, mercury, and benzene), in blood, urine, hair and breath;

2. Those that measure response from exposure (as changes in urinary metabolites as d-amino levulinic acid for effects from lead, or changes in blood enzymes, as inhibition of activity of cholinesterase for organic phosphate insecticides).

It is obvious that BTLs based on measurements of response of the worker to his exposure are superior to those that merely indicate the magnitude of his exposure. Thus measurement of urinary d-amino levulinic acid (dALA) for lead, which measures the worker's bodily reaction to lead exposure is superior as an indicator of how the worker is personally responding to his environment than the level of lead in blood, which in any individual case may vary widely from the norm.

Lead Effect on Porphyrin Synthesis. A good example of the way response to a toxic agent may be determined in an industrial worker, is lead's interference with the synthesis of heme, the red pigment in hemoglobin of the red blood cell. Figure 1 shows one of the ways by which lead interferes with the action of the enzyme that makes the essential pyrrole precursor of heme, d-amino levulinic acid accumulate, spill over into the urine, where it can be measured.

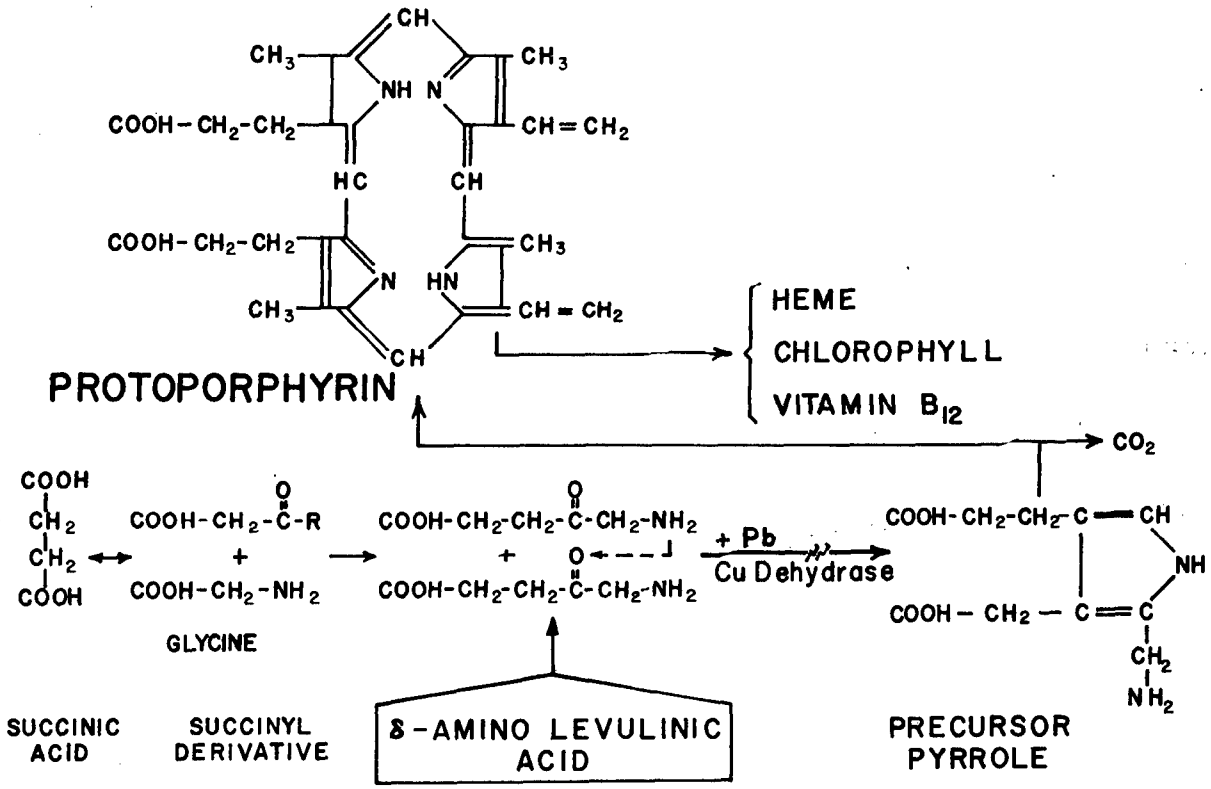

Figure 1. LEAD EFFECT ON PORPHYRIN SYNTHESIS.

Because dALA's increase in the urine is one of the earliest signs of the body's reaction to the toxic effects of lead, the measurement of dALA in the urine of lead workers is especially valuable in detecting overexposure to lead while the changes are still reversible, or conversely, equally valuable in demonstrating lack of effects from lead exposure.

Table II shows the various categories of biologic response to lead with corresponding BTLs for industrial workers for 5 biologic indicators, as recommended in the international conference on inorganic lead (Kehoe, 1971).

TABLE 2

CATEGORIES OF BIOLOGIC RESPONSE FOR LEAD[+]

Test	Normal	Acceptable	Biologic TLV	Excessive	Dangerous
Blood Lead	<40 μg/100 ml	40-80 μg/100 ml	80 μg/100 ml	80-120 μg/100 ml	>120 μg/100 ml
Urinary Lead	<80 μg/L	80-150 μg/L	150 μg/L	150-250 μg/L	>250 μg/L
Urinary Coproporphyrin	<150 μg/L	105-500 μg/L	500 μg/L	500-1500 μg/L	>1500 μg/L
Urinary d-Amino Levulinic Acid	<0.6 mg/100 ml	0.6-2 mg/100 ml	2 mg/100 ml	2-4 mg/100 ml	>4 mg/100 ml
Erythrocyte d-Amino Levulinic Dehydrase	0.92[+]-0.16*	0.42-0.38	0.40-0.2	0.38-0.2	<0.2

* Activity expressed as μmoles porphobilinogen synthesized per ml packed red blood cells per hour incubation.

[+] After Lane et al., Brit. Med. J. 4, 501 (1968).
Conference on Inorg. Pb., A.E.H. 23, 245 (1971).

Changes in Serum [illegible] from Overexposure to Inorganic Mercury. Another good example of the way response [illegible] toxic agent may be detected in a marker is by measuring changes in an enzyme, or fractions [illegible] of isozymes). Figure 2 shows very clearly what large changes occur in the F5 fraction of lactic acid dehydrogenase (LDH) that pours out of the kidney into the serum when there is overexposure above the TLV for inorganic mercury, or when for reasons of hypersusceptibility, a worker hyperreacts from an exposure below the air standard. For monitoring response to organic mercury, serum phosphoglucose isomerase is the indicator of choice (Taylor, et al., 1969).

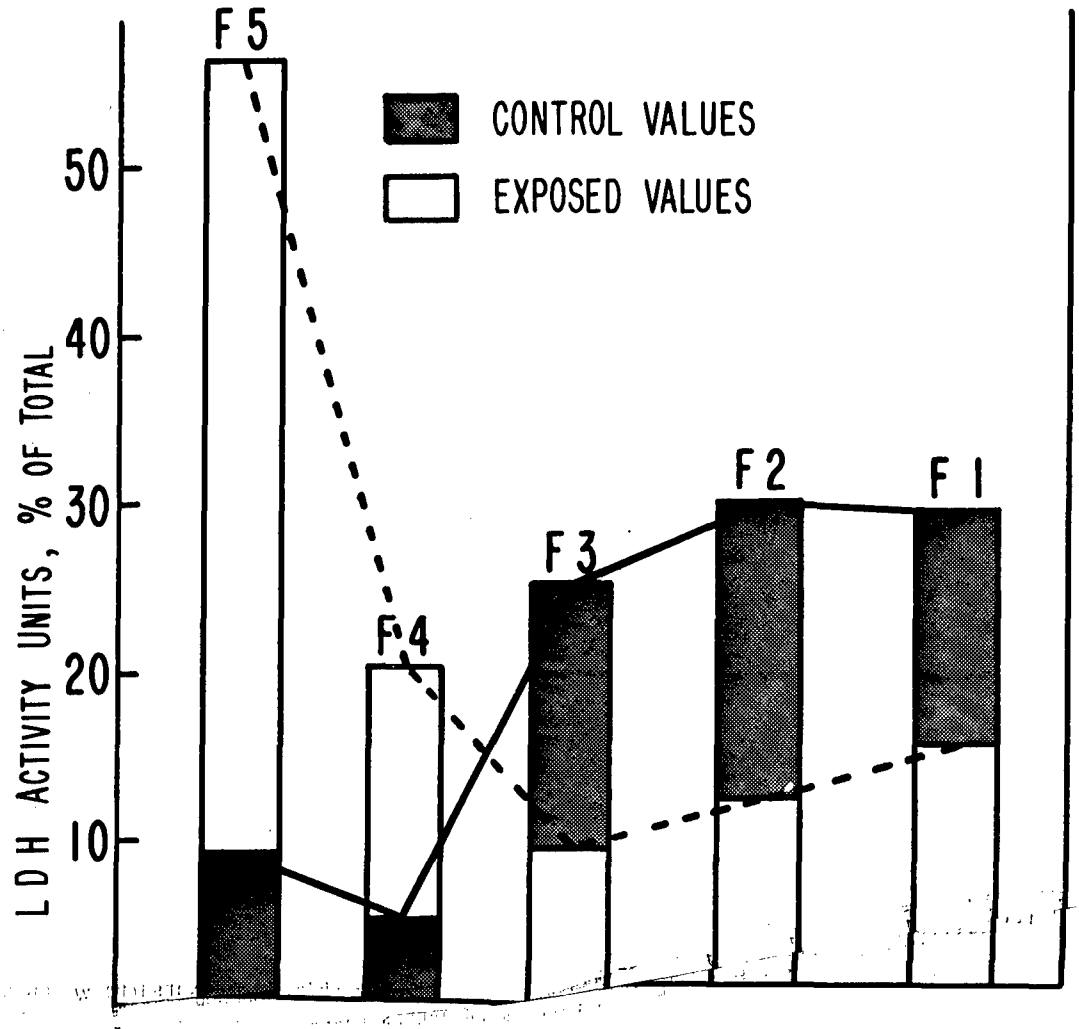

FIG. 2. SERUM (RENAL) LACTIC ACID DEHYDROGENASE ISOZYME PATTERN IN MERCURY MINERS
J. T. MOUNTAIN, DIVISION OF OCCUP. HEALTH, CIN'TI., 1967.

We have presented some BTLs for both inorganic and organic substances, and two examples which illustrate how the measurement of biologic constituents represent a valid and superior indicator of a worker's own individual and characteristic exposure, or in some instances, response to that exposure. The next subject for discussion is the application of the BTLs to the industrial scene and their limitations.

Application and Limitations of BTLs. Unlike air standards, which, theoretically at least, apply to all substances, BTLs apply to a somewhat lesser number of substances. BTLs apply to those substances that have an appreciably finite residence time in the body and which 1) appear per se in body tissues or fluids, (e.g., arsenic in urine, hair); or 2) appear as metabolites, (e.g., phenol from benzene); 3) appear in breath, (e.g., all volatile liquids, vapors, and some gases whose main route of excretion is via the lungs, such as halogenated hydrocarbon solvents; 4) cause alteration of in kind, or amounts, of some accessible body constituent, (e.g., methemoglobin from cyanogenic chemicals, aniline and derivatives, nitrobenzene and derivatives); 5) cause alteration in activity of an enzyme of critical biologic importance, (e.g., increase in the F5 fraction of LDH by inorganic mercury; 6) cause alteration in some readily quantifiable physiologic function, (e.g., changes in F.E.V. (forced expiratory volume) from overexposure to isocyanates); 7) cause changes in behavioral patterns.

Substances Not Lending to Biologic Monitoring. 1) Those substances which are constituents common to the body or which convert to same, e.g., SO_2, chloride phosphate, and which create no alteration in body composition or function; 2) fast-acting substances such as skin irritants, that decompose or combine with skin components, e.g., bis-chloromethyl ether; 3) substances with predominantly sensitizing properties.

Biologic TLs as Action Limits. With the limitations just noted, how are the BTLs applied? BTLs are best applied as a set of two action limits: 1) a warning limit; 2) a limit for medical intervention, to be followed by these actions:

Warning Limit:

1) If a single individual or a few (depending on group size) are found to exceed the warning BTL, the finding shall be verified. If verified, the cause should be investigated by obtaining the history of work habits and outside work exposures, and if no apparent cause is discovered, the finding shall be considered idiosyncratic, and the individual should be removed from the exposure, and if necessary, tests shall be made to determine whether the causes are of genetic origin.

2) If a significant group of exposed workers is found to exceed the warning BTL, corrective action should be taken to reduce the exposure. (The warning BTL should be set so as to include the experimental error incurred by the use of analytic procedures, such procedures to be recommended in the practice). Thus, a 'warning' limit is a signal to be on guard that an undesirable situation may be close at hand.

Limit for Medical Intervention. A BTL to be used as a test of compliance cannot be based solely on a 'warning' limit, where seeming infractions upon further investigation may be due to causes unrelated to job exposure, but must be based on a second BTL, which, if exceeded, is likely to lead to adverse physiologic or biochemical changes in the majority of exposed workers, and is thus a cause for medical intervention.

Cyanogenic Chemical as Examples of Warning and Medical Intervention Limits. Table 3, showing the BTLs for some cyanogenic chemicals, provides a good example of how the two limits are used in practice. The control of worker exposure to the entire group of cyanogenic chemicals, -- aniline and its numerous industrial derivatives, nitrobenzene and its derivatives -- is provided by the application of BTLs rather than by air monitoring (TLVs) by one of the major United States chemical industries. The reason, the major exposure is percutaneous, not respiratory. A relatively simple single biochemical procedure is used to monitor all the cyanogenic chemicals and their mixtures (Koniecki and Linch, 1958). Aromatic amines are diazotized, coupled with Chicago acid (8-amino-1-naphthol-5,7-disulfonic acid) and light transmittance read at wavelength appropriate to the amine being determined. (Nitroaromatics are first reduced to amines before diazotizing, by formamidine sulfinic acid (thiourea dioxide) under alkaline conditions). Differing BTLs are assigned to each aromatic amine and nitro compound and their derivatives singly or as a group, as shown in table 3. An alternative procedure is methemoglobin analysis with values greater than 10% considered warning limit (Wetherhold, et al., 1959).

TABLE III

BIOLOGIC THRESHOLD LIMITS FOR CYANOGENIC CHEMICALS
ROUTINE ANALYSIS FREQUENCY - 60 DAYS

	mg/L Urine		
	Warning Limit	Medical Intervention	Recommended Revised TLV
Aniline	10	20	5 ppm
Chloroanilines	10	20	1 ppm
Chloroaminotoluenes	10	20	5 ppm
Nitrobenzene	25	50	5 ppm
o-Nitrotoluene	25	50	10 ppm
Nitroanilines (Dust)	10	20	6 mg/m^3

Clearly, the medical intervention limit is a cause for extensive, corrective action, both for workers and the environment. The situation (company) may be considered out of compliance until all involved workers return to at or below the 'warning' or lower BTL.

Difficulties in Establishing BTLs. An answer to the question as to why, if biologic monitoring is a superior way of controlling worker exposure, are there so few BTLs; of the now more than 600 (combined USSR and USA) substances with industrial air standards, fewer than a dozen BTLs for inorganic substances and only double this number for organic substances have been proposed for use, and of this number even fewer are routinely used.

The reason so few BTLs have been established are many. Chief among them is the difficulty of establishing norms, and the corresponding BTL, individual differences among workers being the main factor. This, in turn, requires the study of large groups over a relatively long period of time. Further, the development of a biologic method is commonly more difficult than are air-sampling and analysis methods, and, moreover, obtaining a suitable biologic specimen often involves more difficulty than an air sample, and once obtained, may pose problems of preservation because of instability.

Trichloroethylene (TCE) serves to illustrate how individual differences play such a large part in establishing a BTL. Figure 3 shows that the metabolic pathway of TCE in man involves a series of interdependent steps, each of which can be rate-modified in any individual leading to different amounts per unit time of the metabolites being measured in the BTL. The more steps involved in the production of the metabolite indicator, the greater the chance for individual variation. For example, if trichloroacetic acid (TCA) is used as the biologic indicator of exposure to TCE, greater variation may be expected in the results than if trichloroethanol is used. For this reason, and because 1) trichloroethanol is excreted in larger amounts than TCA, 2) excretion of TCA is slow and variable, leading to uncertain determination of peak excretion values, trichloroethanol is the indicator of choice.

Other factors posing difficulties in obtaining reliable biologic data or in interpreting the data to judge compliance, are functional derangements in the organs of metabolism and excretion, either hereditary or acquired, -- in the case of TCE, renal and hepatic dysfunction. Drugs, including alcohol, commonly interfere with normal metabolism and excretion of inhaled industrial substances; disulfuram for example (figure 3) increases pulmonary excretion 3-fold. Accordingly, before compliance can be judged, a history of drug-taking, including alcohol intake, should be obtained prior to the biologic measurement. If positive, the biologic measurement on the worker should be deferred until the drug has been cleared from the body, usually 49 hours after taking.

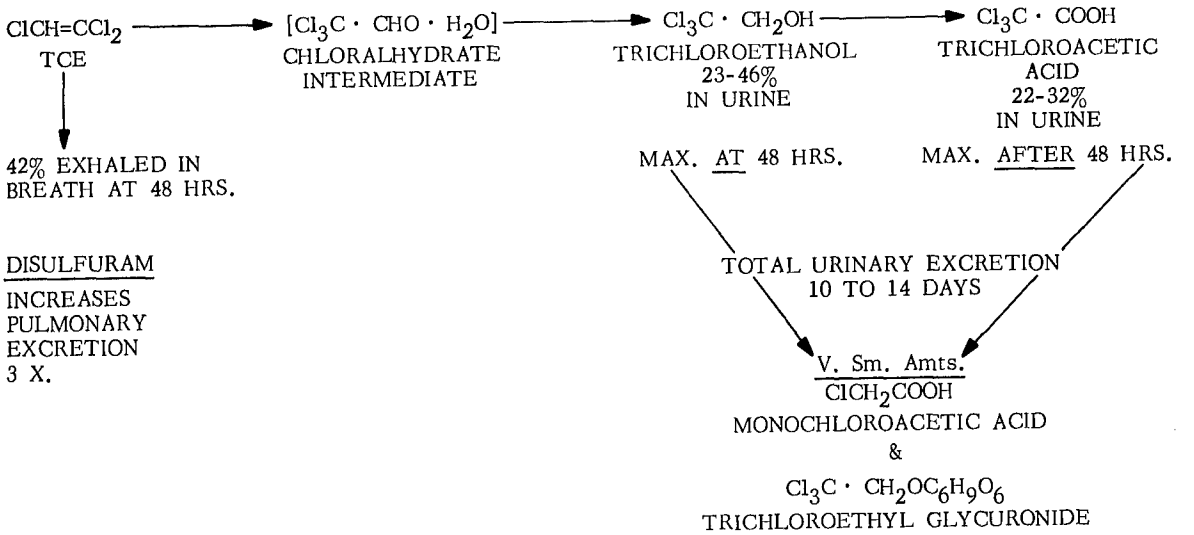

FIGURE 3. - HUMAN METABOLIC PATHWAY OF TRICHLOROETHYLENE (TCE)

SUMMARY & CONCLUSIONS

Because of several shortcomings of air monitoring as a true measure of worker exposure, there is a real need for biologic measurements which supply for most substances a far more exact index of exposure. Measurements of airborne concentrations as indices of exposure are unreliable in the following 5 situations. 1) When percutaneous absorption represents a major contribution to body burden; 2) When exposure is intermittent, and air-sampling method does not register the intermittent peaks; 3) In mixed exposures when there is metabolic interaction; 4) Individual peculiarities of work habits leading to abnormal intake; and 5) Additive, off-the-job exposure.

Biologic monitoring offers a remedy for all 5 deficiencies of air sampling by A) using the worker as his own collector and monitor of his particular exposure; B) reflecting faithfully the worker's personal and work habits; C) measuring the over-all industrial exposure by all routes of entry; and D) registering any pre-exposure burden or off-the-job exposure along with the industrial exposure.

Biologic measurements furnish 2 types of information: 1) an index of exposure; 2) an index of response. Obviously, the latter is superior, as it is capable of detecting the first, early signs of metabolic derangement, while it is still reversible, whereas measurement of exposure, even at the biologic limit, may or may not indicate a hazardous exposure in the individual case.

Biologic measurements may be either biochemical or physiologic. The biochemical measures A) changes in amount of some critical biochemical constituent, as methemoglobin from overexposure to cyanogenic substances; B) changes in activity of a critical enzyme, as lactic acid dehydrogenase patterns from overexposure to inorganic mercury; C) determining in blood, urine, hair, nails, in body tissues and fluids the amount of substance to which the worker was exposed, as the amount of lead in blood; D) determination of the amount of the metabolite(s) of the substance in tissues and fluids, as phenol in the urine from exposure to benzene; E) determination of the amount of substance in the exhaled breath, as content of trichloroethylene in the breath at a known time since exposure. Physiologic measurements assess changes in some physiologic function, as measurement of forced expiratory volume for change in lung function from overexposure to isocyanates.

Despite the superiority of biologic monitoring for controlling worker exposure, difficulties in establishing biologic threshold limits, chiefly because of difficulties in establishing worker norms, has limited the number to a few dozen in practical, routine use.

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DISCUSSION

MR. WANDS (National Academy of Sciences): Herb, I would like to ask one thing about the various biologic values which you showed in your slides. Are these official recommendations at this point or are they merely numbers pulled out of the literature?

DR. STOKINGER (U. S. Public Health Service): These are the best available information that was produced by Dr. Elkins many years ago, and some of them have been improved since. At the present time, NIOSH is developing about 7 of these on an official basis and the first is the one I showed you on lead - the 5 categories of response to lead. These will be included in criteria documents along with the air standards, but they have not been incorporated yet. I don't think the criteria document for lead has been released yet. But that's the way they will come out. They will probably not come out as separate documents. They will be included as alternative ways of monitoring various elements.

DR. ASTILL (Eastman Kodak Company): You mention this concept of a dual value - a warning value and a medical intervention value. To what extent is this being accepted by NIOSH in these proposals that are being formulated?

DR. STOKINGER: I have no idea. I have had an opportunity to review the seven criteria documents on these biologic standards and I get the impression that perhaps the concept is falling on deaf ears. We'll hear more of this from Mr. Linch and maybe he can reconstruct the way in which we can get it across to have a dual limit.

DR. STARA (National Air Pollution Control Administration): It would appear to me that there are work areas where good air sampling is not possible, and in these areas, biologic samples would be even more important than in an industrial plant. For example take airport workers or take people in gasoline stations. Wouldn't you think that the biologic limit values would be very important in cases like that?

DR. STOKINGER: Yes, that's right. Absolutely. Where you have intermittent exposures it's the only way to do it. You can't get any true estimate of the exposure under such conditions with gasoline, service station or airport operations.

AMRL-TR-72-130

PAPER NO. 2

BREATH ANALYSIS IN BIOLOGIC THRESHOLD LIMIT DETERMINATION

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INTRODUCTION

A decade of experience with the use of breath analysis to detect toxic gases and volatile organic compounds in exposed individuals indicates the method possesses the precision for determining whether the Biologic Threshold Limit has been exceeded once these biologic norms have been defined. Breath analysis is sufficiently simple, rapid, specific and inexpensive, that it should be a part of the diagnostic armamentarium of each occupational physician, industrial hygienist, and clinical toxicologist.

The rationale for the use of expired breath analysis for the determination of the magnitude of chemical exposure is based on the fact that all absorbed gases and volatile compounds which circulate in the blood stream are excreted in part via the lungs. The breath concentration of such a compound is directly related to its blood stream concentration which in turn is a reflection of the amount absorbed and the elapsed time following exposure.

Physicians first used breath analysis to determine whether exposure to a compound capable of producing the signs and symptoms of the patient's illness had occurred. This proved to be particularly useful in the initial evaluation of patients manifesting signs of central nervous system depression which could have been produced by overexposure to a toxic gas or volatile organic solvent. Rapid infrared spectrographic or gas chromatographic techniques quickly ruled in or out the possibility of exposure to these chemical agents, permitting the physician either to commence the definitive treatment for the chemical poisoning or to promptly proceed to the next most likely cause for the illness (Stewart, 1963, 1965).

With the exception of carbon monoxide and ethanol, there were insufficient data available in the 1950's with which to relate the concentration of a compound detected in an individual's breath to the magnitude of his exposure. Therefore, the acquisition of postexposure breath data became a high priority item for physicians wishing to extend the diagnostic usefulness of this method. Breath data were collected from attempted suicides, industrial accidents, workmen routinely exposed to known concentrations of gases and solvents, and from experimental human exposures (Stewart, 1961, 1963, 1964, 1965, 1966, 1968, 1969, 1970; Peterson, 1970; Baretta, 1969). These efforts have resulted in the accumulation of sufficient toxicological data to make breath analysis a useful technique not only for identifying the compound to which exposure had occurred, but in those cases when sufficient data exist to determine whether the Biologic Threshold Limit has been exceeded.

TECHNIQUE OF BREATH ANALYSIS

Collection of the Sample

When the identity of the gas or volatile compound is unknown, a breath sample is collected in a 5-liter Saran® or Mylar® plastic bag in an uncontaminated area. Quantitative analysis requires that the alveolar portion of the breath be analyzed. This portion can be collected by having the subject forcefully expire his residual breath into the bag after he has completed a normal expiration. An anesthetic mask coupled to a one-way valve can be used to collect breath samples from comatose individuals (Stewart, 1963, 1965).

The bagged breath sample is then evacuated directly into a long-path-length gas cell of an infrared spectrometer, following which the infrared spectrum is automatically recorded in 5 to 20 minutes. Breath samples should not be stored in these plastic collection bags for periods longer than 24 hours before analysis because of the slow loss of the contained gas and vapors through the walls of the bag. The rate of this loss varies with the solubility of the compounds in the plastic bag, the ambient temperature, and the time delay prior to analysis.

Medical centers lacking infrared equipment can mail breath samples to appropriate infrared laboratories for analysis. To collect a breath sample which is to be mailed, silica-gel is placed in the plastic bag before breath inflation (Stewart, 1963). Following collection of the breath, the silica-gel is agitated within the bag which allows the compound to be adsorbed on the surface of the silica-gel. The bag containing the silica-gel is then deflated and then shipped to an analytical laboratory where the compound is desorbed from the silica-gel and identified.

When the identity of the toxic gas or vapor is known, breath collection and analysis become extremely simple because gas chromatographic techniques, which require very small aliquots of breath, can be used. An alveolar breath sample is trapped in a 30-50 ml glass pipette (figures 1-4). These pipettes are constructed so that the needle of a gas-tight syringe may be introduced into the pipette's gas chamber to obtain an aliquot for gas chromatographic analysis (Stewart, 1965).

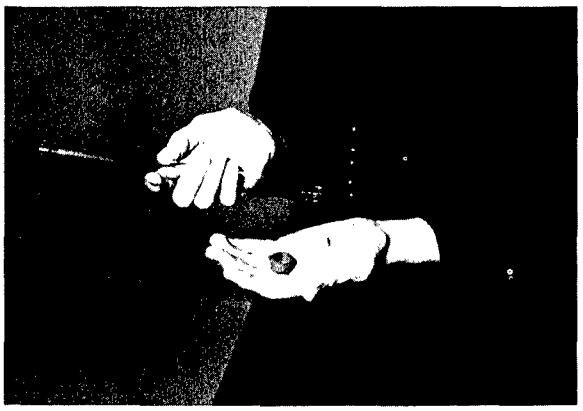

Figure 1. THE BREATH PIPETTE IS A 50 ml CYLINDER FITTED WITH A PLASTIC SCREW CAP. EACH CAP CONTAINS FOUR SARAN DISCS WHICH SERVE AS GAS BARRIERS.

Figure 3. AFTER THE DISTAL CAP IS SECURE, THE PROXIMAL END OF THE PIPETTE IS REMOVED FROM THE MOUTH AND QUICKLY SEALED WITH A FINGER TIP.

Figure 2. THE BREATH SAMPLE IS "COLLECTED" BY CAPPING THE DISTAL END OF THE PIPETTE WHILE FLUSHING IT WITH ALVEOLAR BREATH.

Figure 4. THE BREATH SAMPLING IS COMPLETED BY SECURING THE PROXIMAL CAP.

To obtain a breath sample in a glass pipette the subject first flushes the pipette three times with his expired breath. Then after a normal inspiration the subject holds his breath for 30 seconds and exhales through the pipette collecting the end-tidal or alveolar portion. To insure that the collection is obtained in an uncontaminated area, a background air sample is collected in a second pipette.

Instrumentation of Breath Analysis

Commercially available infrared spectrometers equipped with long-path-length gas cells currently offer the most practical means for the qualitative analysis of unknown compounds in the breath. The two major advantages of the infrared method are the speed with which the analysis can be performed and the specificity of identification. The breath sample is introduced directly into the evacuated gas cell from the plastic collection bag and the contents are scanned for the presence of toxic gases and volatile compounds. Each organic compound possesses its own specific infrared spectrum or "fingerprint." This provides a means with which to make an unequivocal diagnosis or exposure. A single infrared scan will detect the presence of multiple gases or volatile compounds, greatly simplifying the analytical approach in cases involving exposure to several toxic agents.

The qualitative analysis of breath by the gas chromatographic method is much more tedious than is the case with the infrared method. Identification of an unknown compound usually requires multiple determinations on two or more different columns. However, once the identity of the toxic agent is known, gas chromatographic analysis is as practical as infrared analysis and is often more sensitive. With the employment of the proper column and instrument parameters, only 0.1 to 1.0 ml breath aliquots are required, and analytical time varies from a few seconds to 10 minutes.

The gas chromatograph possesses exquisite sensitivity of detection. Those equipped with hydrogen flame detectors can detect as little as 0.1 to 0.2 ppm of most volatile compounds in the breath. The helium ionization detector and electron capture detector further extend this sensitivity into the parts per billion range for most gases and many solvents. This provides a means for establishing a diagnosis of exposure long into the postexposure periods for those compounds slowly excreted from the body. For example, it has been possible to detect carbon tetrachloride in expired breath samples four months following an attempted suicide by ingestion of the solvent.

DIAGNOSIS OF EXPOSURE

The specific identification of a gas or volatile compound in the expired breath in a concentration exceeding that which could result from endogenous metabolic production establishes the diagnosis of exogenous exposure to that compound. The diagnosis of excessive exposure to the compound is entertained only when breath

concentrations are shown to exceed those values found in individuals maximally exposed to no-effect concentrations of the compound in question.

Listed in table I are those gases and volatile compounds which can be readily detected in the expired breath. The gas chromatographic sensitivities listed are the minimum amounts detectable under current operating conditions in this laboratory. These are practical limits of detection and can be improved when necessary. The infrared sensitivities are the minimum concentrations of the compound which will give an absorbance of at least 0.01 in a 10-meter path-length cell. Five to ten times this amount usually must be present to permit a positive identification of the compound.

TABLE I
ANALYSIS OF GASES AND VAPORS IN EXPIRED AIR

Compound	Gas Chromatographic Sensitivity (ppm)	Infrared Sensitivity (ppm)
Acetone*	0.1	5
Acrylonitrile	3.0	5
Amyl acetate*	2.0	1
Benzene*	0.1	20
Bromobenzene	2.0	10
Tertiary-butyl alcohol	0.1	5
Carbon dioxide*	100	5
Carbon monoxide*	0.01	5
Carbon tetrachloride*	0.001	0.5
Chloroform*	0.01	1
Chloropicrin	0.5	2
Cyclohexane	0.1	40
Dichlorodifluoromethane* (Freon 12)	0.5	1
Dioxane	0.1	2
Ethyl alcohol*	0.1	5
Ethyl ether*	0.1	5
Ethylene oxide	0.1	5
Trichlorofluoromethane*	0.010	1
Gasoline	5.0 μg/liter	5**
Methyl alcohol*	0.2	5
Methyl bromide	6	50
Methyl chloride*	0.2	30
Methyl chloroform*	0.01	2
Methylene chloride*	0.1	2
Nitrous oxide*		25
Perchloroethylene*	0.01	2
Phosgene		1
Sulfur dioxide		15
Toluene	0.3	5
Trichloroethylene*	0.01	2
Turpentine	0.4	10**
Vinyl chloride*	0.1	10
Vinylidene chloride*	0.1	5
o-Xylene	0.1	10
m-Xylene	0.1	10
p-Xylene	0.1	10

* Human data available

** Aliphatic hydrocarbons can be detected at 3.40 μ, but cannot usually be identified specifically at low concentration.

ESTIMATION OF THE MAGNITUDE OF EXPOSURE

The total amount of a gas or volatile compound absorbed by the body is dependent upon the concentration of the compound in the breathing zone, the duration of the exposure, and the rate of absorption. The amount of the compound in the breath in the postexposure period is related to the total amount absorbed, the solubility in various body tissues, the rate of metabolism and excretion, and the intervals since the last exposure. These factors determine the compound's concentration in the blood reaching the lung and, hence, the alveolar breath. Under carefully controlled conditions, each gas or volatile compound possesses a characteristic and predictable breath excretion or decay curve (figure 5). Therefore, when it is desirable to estimate the magnitude of a given exposure, it is necessary to obtain a sufficient number of breath analyses to accurately define the excretion curve (figure 6). Those compounds for which sufficient human data are available to make such an estimation are so designated in the table.

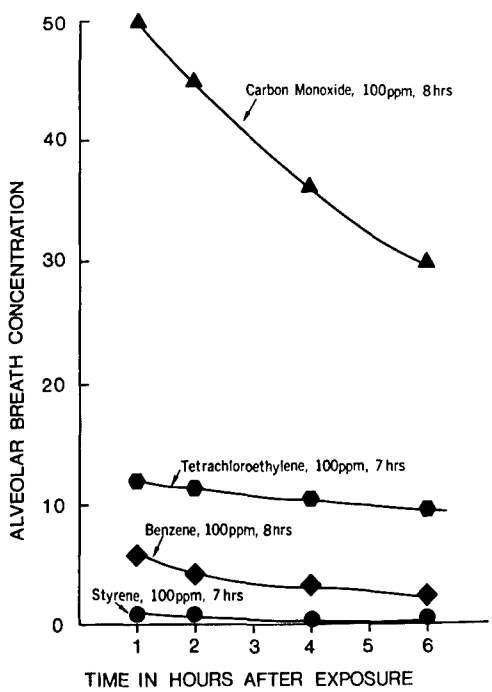

Figure 5. EACH GAS AND VOLATILE COMPOUND HAS A DISTINCTIVE BREATH DECAY CURVE.

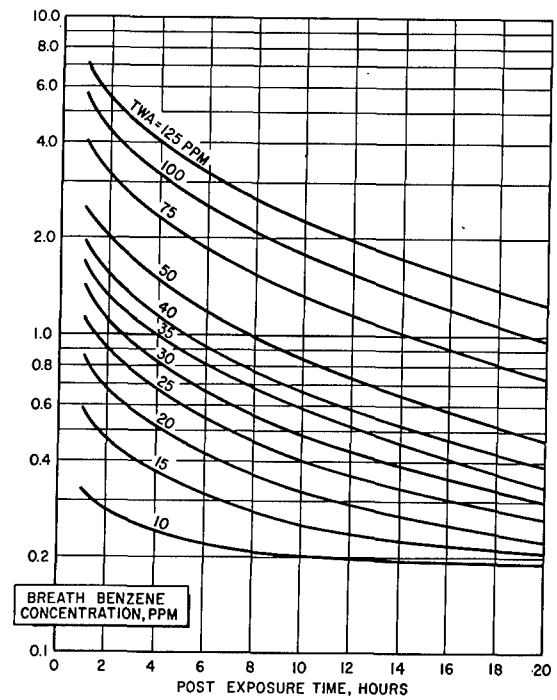

Figure 6. THE SERIES OF BENZENE BREATH DECAY CURVES. TWA STANDS FOR THE TIME-WEIGHTED AVERAGE OF BENZENE EXPOSURE OVER A $7\frac{1}{2}$ HOUR INTERVAL.

ILLUSTRATIVE CASE REPORT

A 27-year-old white mechanic (Case D in figure 7) reported late for work with the excuse that he had been "overexposed" to methyl chloroform on the previous working day. He stated that on the preceding day he had worked in the vicinity of a man who was cleaning engine parts with the solvent. He had been aware of the solvent's odor throughout the working day, but at no time had he become dizzy or experienced nausea. That evening, two hours after work, he experienced a sudden onset of nausea, vomiting, explosive diarrhea, and dizziness. His illness had lasted for six hours, during which time he had been visited by his family physician who had advised him to check with the plant physician to learn whether his solvent exposure could have been responsible for his illness.

A physical examination by the plant physician the next morning was completely normal.

Breath analysis 17 hours after exposure detected the presence of methyl chloroform, 3.5 ppm (figure 7). When this value was compared to experimentally obtained breath decay curves from subjects exposed to known concentrations of the solvent, it became immediately apparent that the mechanic's time-weighted vapor exposure had not exceeded the Threshold Limit Value for methyl chloroform,

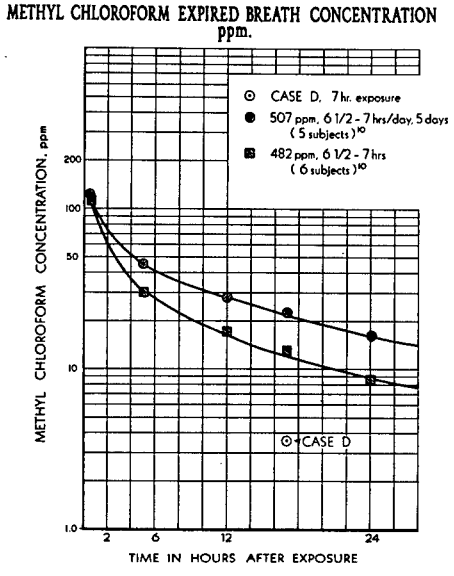

Figure 7. THE MAGNITUDE OF A SEVEN-HOUR EXPOSURE TO METHYL CHLOROFORM CAN BE ESTIMATED BY COMPARING THE POSTEXPOSURE METHYL CHLOROFORM BREATH CONCENTRATION TO BREATH CONCENTRATIONS OF EXPERIMENTAL SUBJECTS WHO WERE EXPOSED TO KNOWN CONCENTRATIONS OF THE SOLVENT.

COMMENT

The case of the mechanic beautifully illustrates the utility of breath analysis as a screening device to determine whether or not exposure to a gas or volatile compound has occurred. In addition, a properly timed breath analysis sample can yield sufficient data to allow an accurate estimation of the magnitude of the most recent exposure. When information regarding the peak concentration of a given exposure is desired, serial breath analyses can be used to provide this information.

Ideally, each work environment in which exposure to a toxic gas or volatile agent occurs should have breathing-zone, air monitoring equipment which features an alarm system. In this situation proper air monitoring would preclude inadvertent overexposure while yielding valuable exposure data. Unfortunately, the majority of industries cannot afford these expensive systems for monitoring even a single agent. In this situation, breath analysis becomes an inexpensive screening tool and a valuable supplement to the periodic industrial hygiene survey techniques now in vogue.

Breath analysis is capable of yielding human toxicology information which continuous air monitoring cannot: (1) it monitors skin absorption which can occur as a result of contact with a toxic liquid; (2) it can detect the amount of a volatile compound absorbed as a result of accidental or deliberate ingestion; (3) preexposure breath analysis can determine the magnitude of exposure to an industrial compound occurring as a result of "moonlighting" activities; (4) breath analysis can accurately reflect strikingly different tissue concentrations of a compound in two identically exposed individuals as a result of enzymatic deficiency or drug-induced overactivity.

Finally, the following comments should assist the physician in his evaluation of a workman's exposure: (1) All breath samples should be collected in an uncontaminated area. Ideally, a control air sample should be obtained simultaneously with breath sample collection and analyzed for background contamination. Failure to take this precautionary measure can result in an erroneous conclusion. (2) The concentration of a compound in a breath sample collected within the first 15 minutes following exposure is a good indication of the most recently encountered breathing zone concentration. It may not accurately reflect the total amount of the compound which has been absorbed. (3) The concentration of a compound in serial breath samples collected over a 24-hour period will generally allow the estimation of the time-weighted mean exposure to that compound. It will not provide information regarding the peak concentrations to which an individual has been exposed.

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DISCUSSION

DR. ROWE (The Dow Chemical Company): In your comment about the persistence of carbon tetrachloride as compared with perchloroethylene. I am trying to remember - I guess I hadn't seen data on that. Is that an experimentally determined fact or not?

DR. STEWART (Medical College of Wisconsin): No, those data came from individuals who actually, in attempted suicides, had drunk the compound and were followed. We have followed one individual attempting suicide with carbon tetrachloride who actually absorbed, to my recollection, about an ounce and a half of carbon tetrachloride. That individual had measurable amounts of carbon tetrachloride in the expired breath for as long as six months after the ingestion. The perchloroethylene has been monitored in individuals for as long as 2-1/2 months with estimates that one could follow them for at least 4 to 6 months, and we have followed 1, 1, 1-trichloroethane which has been used in several attempts at suicide by psychotic type individuals, the most recent one being 4 days ago. The young 21 year old male drank Liquid Gold which is used for refinishing furniture, but the solvent there is 1, 1, 1-trichloroethane, and we estimate that he totally absorbed something like 6 ounces. So we have been watching the individual for evidence of liver and renal involvement and to date the individual surprisingly and this was a massive intoxication, has not shown any. We estimate at the present level of excretion that we'll be able to follow this individual for about 8 months.

COMPARATIVE METHODS OF BIOLOGIC MONITORING OF BENZENE EXPOSURES

[One Man's Elimination of Benzene (C_6H_6)]

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INTRODUCTION

In a paper describing improved techniques for monitoring occupational exposure to benzene, Sherwood and Carter (1970) reported results from a single experimental exposure made to calibrate the system. Since then, industrial experience with the method has been described (Sherwood 1971a and 1972a), and monitoring systems reviewed (Sherwood 1972b and to be published a). Proposals have also been made for a new approach to an acceptable risk limit for benzene in air (Jones and Brief, 1971, Sherwood, 1971b), and this has also been related to other criteria (Sherwood, to be published, b).

Reasonably good agreement has been found between the experimental exposure results and those from industrial exposure, but some fundamental questions required answering.

The objective of the experiments described here was to answer these:

1. Does energy expenditure at work have a marked effect on the quantity of benzene absorbed?

2. How does absorption and elimination differ between long and short periods of exposure?

3. Does the presence of other hydrocarbons affect the absorption and elimination of benzene?

These questions have not been completely answered in this paper, but another factor which may be as at least important is described.

INSTRUMENTATION

The techniques and instruments used in these experiments are those developed for evaluating industrial exposures and comprise:

1. Personal air samplers (Sherwood and Greenhalgh, 1960), modified to be intrinsically safe against explosion risk. These collect benzene vapor on silica gel traps for subsequent elution and gas-chromatographic assay.

2. Breath sample tubes for spot checks of benzene in exhaled breath of workers after exposure, and a more definitive breath sampling respirator in which exhaled air is passed through a silica gel cartridge for benzene evaluation.

3. Urine samples preserved with toluene and analyzed by perchloric acid hydrolysis, followed by gas chromatographic determination of phenol.

The only changes made to the published techniques were the use of two-stage extraction of benzene from the respirator cartridge with iso-octane and water, and, in later experiments, the use of 30-minute air samples to reduce the analytical load (penetration through the gel over 30 minute periods was determined to be less than 0.3%).

All urine excreted was collected to determine the rate of elimination of phenol as well as its concentration.

The exposure equipment (figure 1) was constructed to two principal criteria: components had to be readily available and the whole equipment had to be self-service, that is, the experimental subject had himself to be able to check and adjust exposure and sampling conditions during the tests, and change air samples without need for an attendant.

The chamber itself was constructed from the components of a self-pressurized air blouse (Sherwood and Greenhalgh, 1965); the fan and battery unit was fitted to a trolley with the other equipment and 100 liters/minute of air delivered to the top of the headpiece by flexible hose. Steady concentrations of benzene vapor in air were established by blending in a measured and controlled flow of dry air which had passed through two bubblers in series containing benzene or a hydrocarbon mixture. The bubblers were immersed in a constant temperature water bath, and the short connection to the main air flow was electrically traced.

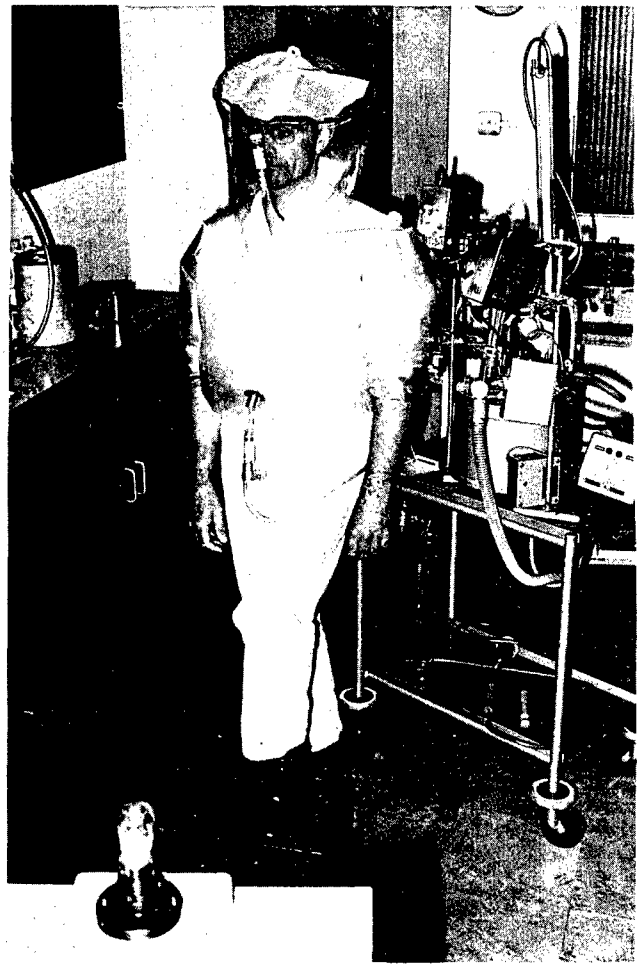

Figure 1. Experimental Exposure Rig

To confirm the vapor concentration, and to estimate the uptake of benzene by the subject, air samples were taken for 15 or 30 minute periods throughout the exposure from points near the inlet to the blouse and at its outlet.

During the tests, the 70 kg subject was either seated, standing, or working at a constant energy expenditure task. The latter was established by exercising the subject on a 20 cm step, the step rate being controlled to 10 times per minute by a flashing lamp. Energy expenditure was assessed by gas chromatographic measurement of carbon dioxide in spot samples of outlet air. The respiratory quotient was assumed to be 0.9.

The opportunity was also taken to determine likely errors in the field sampling techniques and these are reported in the Appendix.

Elimination of Benzene in Exhaled Breath

The first experiment reported here was undertaken in 1969, before field work had commenced. A better understanding of elimination has justified reappraisal of the data previously reported. Originally, two distinct phases of elimination could be seen, the first typified by a half life of about 1 hour, the second by a half life of the order of 1 day. This pattern was also observed (Sherwood 1972b) after occupational exposure of the same subject.

More precise evaluation of the data as part of the experimental program reported here permits identification of three phases of elimination which may be due to release from different compartments of the body. These may correspond to the groups - viscera, lean tissue and fat - proposed by Mapleson (1963).

Calculation of Elimination Curves and of the Quantity of Benzene Eliminated in Breath

Each phase of elimination is characterised by a given half-life. That is, the concentration in breath is a negative exponential function of time after exposure, each of 2 or 3 phases being distinguished by particular elimination constants.

Concentrations in exhaled breath measured by breath sample tubes represent end-tidal air, and are about 1.5 times those measured by respirator. Accordingly, all observed results from the tubes have been reduced by a factor of 1.5 before plotting in the elimination curves. It must be stressed that the values quoted represent mean concentrations in the whole exhaled breath and have been calculated from an assumed cartridge weight gain of 25 mg/liter from the moisture in breath.

To construct the elimination curves shown in figures 2 and 3, visual plots have first been made to determine the periods over which each value of the elimination constant is valid. Regression equations have then been calculated for each phase from the breath concentrations measured by respirator, and from these the elimination curves have been plotted.

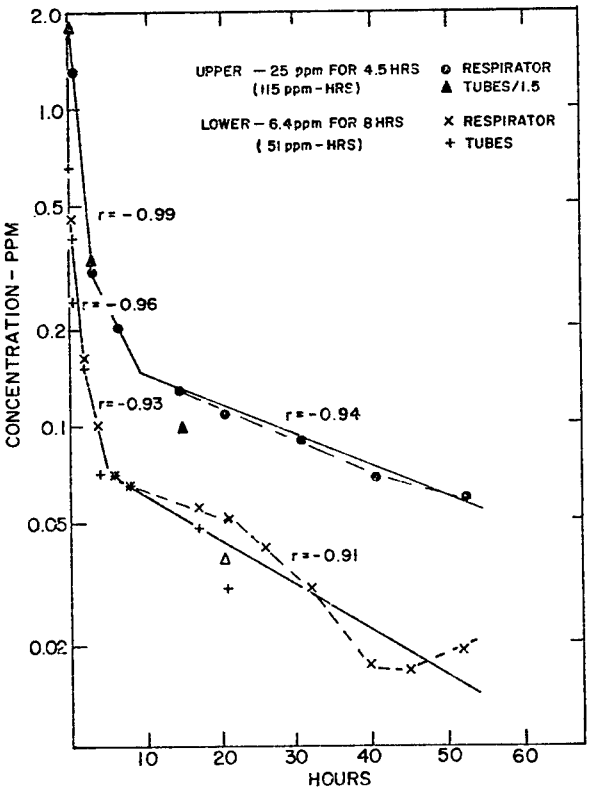

Figure 2. ELIMINATION OF BENZENE IN BREATH-1

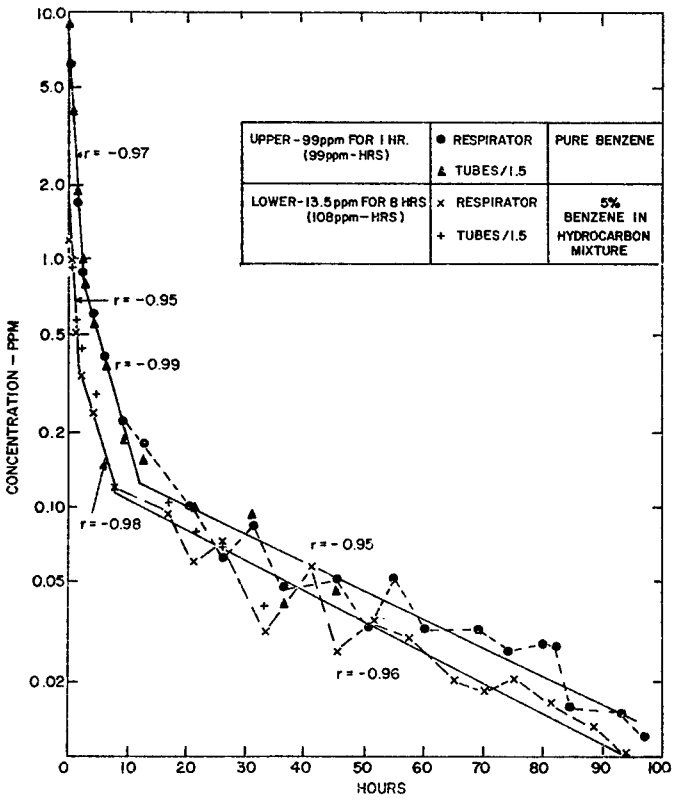

Figure 3. ELIMINATION OF BENZENE IN BREATH-2

The quantity of benzene eliminated in breath has been calculated thus:

Let the total quantity of benzene eliminated in breath after exposure = Q (mg)

This equals the sum of the quantities eliminated in each phase

$$Q = Q_1 + Q_2 + Q_3$$

Now the elimination rate in each phase is an exponential function:

$$\frac{dQ}{dt} = -\lambda Q$$

where λ = elimination constant
= fraction exhaled in unit time.

$\frac{dQ}{dt}$ = rate of elimination in breath

Call this R (mg/hour)

Let R_1 be initial elimination rate.
R_2 be rate at end of 1st phase and beginning of second.
R_3 be rate at end of 2nd phase and beginning of final phase.

Then quantity eliminated in each phase is:

$$Q_1 = \frac{R_1}{\lambda_1} - \frac{R_2}{\lambda_1}$$

$$Q_2 = \frac{R_2}{\lambda_2} - \frac{R_3}{\lambda_2}$$

$$Q_3 = \frac{R_3}{\lambda_3}$$

So total elimination in breath:

$$Q = \frac{R_1 - R_2}{\lambda_1} + \frac{R_2 - R_3}{\lambda_2} + \frac{R_3}{\lambda_3}$$

Where elimination is described by two phases only, the third component has been deleted:

$$Q = \frac{R_1 - R_2}{\lambda_1} + \frac{R_2}{\lambda_2}$$

Elimination rates (mg/h) have been calculated from the concentration (ppm) by multiplying by a factor of 1.86. For convenience in visualization, half-lives rather than elimination constants have been quoted -

$$T\,\tfrac{1}{2} = \frac{0.693}{\lambda}$$

Experimental Results

The excretion curve for the original sedentary exposure is shown in the upper curve in figure 2. Elimination is at first very rapid, but after about 3 hours, a less rapid period lasting about 7 hours is evident. This is followed by a slow but steady decline during a period of over 50 hours.

This pattern is also observed (lower curve, figure 2) following 8 hour exposure at an energy expenditure of about 200 J/sec (about 3Kcal/min). It will be observed that the initial period of rapid excretion is a smaller fraction of the total elimination than in the original experiment (upper curve). This could be expected as the exposure was for 8 hours, whereas the original exposure was 4.5 hours, so a larger fraction of the absorbed benzene could be expected to reach the organs from which release is slow.

With the probable exception of the first phase of elimination, the concentration in breath can be expressed as a negative exponential function of time. The first phase probably represents release from several compartments including the lung and blood; measurements taken 3 and 13 minutes after exposure have generally indicated a half-life of about 10 minutes, whereas over the whole typical first period of 2 hours, half-lives from 0.7 to 1.7 hours have been observed.

The second period is generally marked by a half-life of 3 to 4 hours, and the third period by a half-life of 20 to 30 hours.

Benzene in Mixtures

Figure 3 shows the elimination pattern for benzene in breath following 8 hour inhalation of a hydrocarbon mixture containing about 5% benzene by volume. Measurements covered a period of 100 hours after the exposure and the pattern is evidently similar to that following inhalation of pure benzene shown in figure 2.

Short-Period Exposure

Also shown in figure 3 is elimination following a 1-hour exposure to a higher concentration. While the general pattern is still followed, it will be observed that, when compared with longer exposures, a larger proportion of the total quantity eliminated appears in the first two phases.

This would appear to confirm that, in short-period exposure, a smaller proportion of the initial intake of benzene reaches organs from which it is only slowly released.

Variations in Concentration on Long-Term Elimination

Figure 3 shows apparently random variation in respirator samples during the long-term elimination phase. If, however, the two curves are plotted to a common time base (figure 4) it will be seen that the patterns coincide. This may be a diurnal rhythm or originate from the time when the slow excretion becomes predominant - the time base for the cycle of high and low values is nearer 12 than 24 hours.

In figure 5 regression curves for elimination following the four exposures have been replotted to a common proportion of the concentration to which the subject was exposed. This indicates that the concentration in exhaled breath is initially closely related to exposure concentration. The amount of benzene in rapidly responding compartments is thus related to the concentration present in the inhaled air.

In figure 6 the curves are transposed to a common base of exposure dose (that is, the multiple of time and concentration, ppm-hours), and it is evident that longer term elimination (probably from fatty tissue) is much more closely related to exposure dose than to concentration.

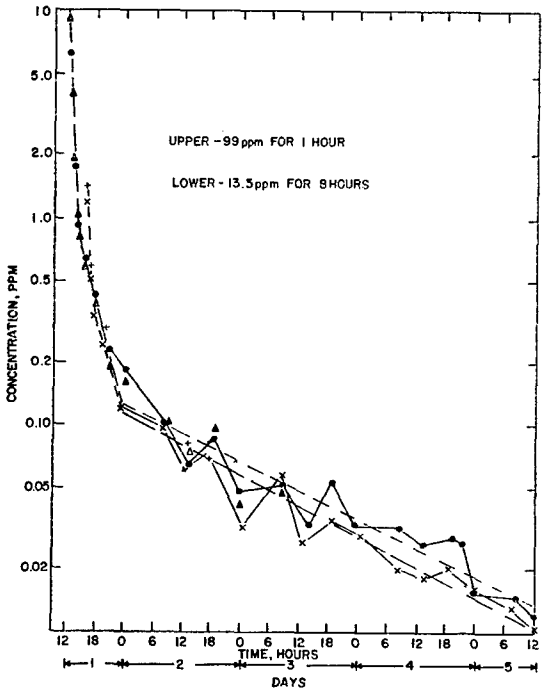

Figure 4. ELIMINATION OF BENZENE IN BREATH-COMPARISON OF FLUCTUATIONS WITH TIME.

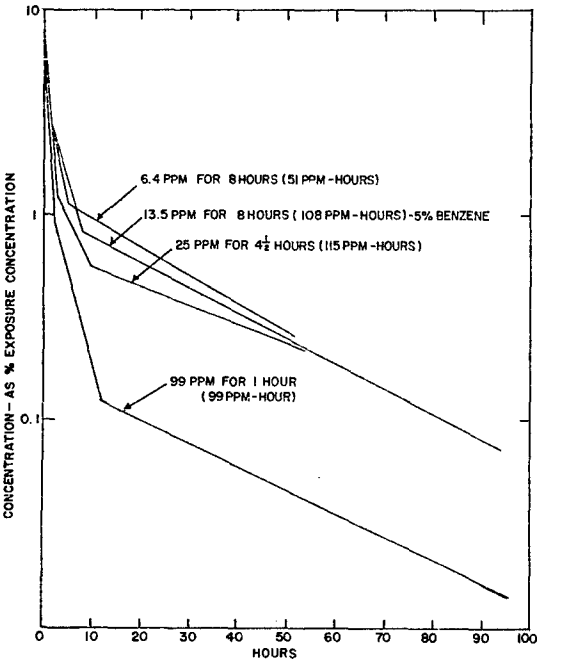

Figure 5. ELIMINATION OF BENZENE IN BREATH-CONCENTRATION AS PROPORTION OF EXPOSURE CONCENTRATION.

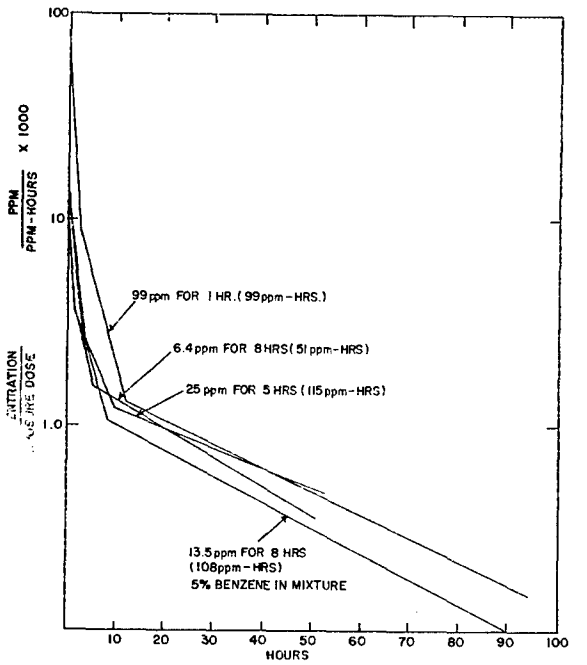

Figure 6. ELIMINATION OF BENZENE IN BREATH-RELATED TO EXPOSURE DOSE.

Quantities of Benzene Eliminated in Breath

Table I confirms that the quantity of benzene exhaled in the first two hours is closely related to the exposure concentration (ppm) but that the amount eliminated in the long-term phase is more closely related to the exposure dose (ppm-hours). (The first entry in the table is from earlier work during which long-term elimination was not expected and therefore not precisely measured).

Table I

Quantities of Exhaled Benzene Related to Exposure Concentration and to Exposure Dose

Exposure	Exposure Dose ppm-hours	Quantity of Exhaled Benzene Related to:- Concentration (mg/ppm) Phase 1	Phase 2	Phase 3	Total	Exposure Dose (mg/ppm hour) Phase 1	Phase 2	Phase 3	Total	Activity
25 ppm for 4.5 hours	115	0.19 (24)	0.11 (14)	0.49 (62)	0.78 (100)	0.040	0.024	0.110	0.170	Sedentary
6.4 ppm for 8 hours	51	0.17 (19)	0.14 (15)	0.59 (66)	0.90 (100)	0.020	0.017	0.074	0.113	Moderate (220 J/sec)
99 ppm for 1 hour	99	0.11 (40)	0.076 (28)	0.088 (32)	0.27 (100)	0.107	0.076	0.088	0.271	Moderate (220 J/sec)
13.5 ppm for 8 hours (5% benzene in mixture)	108	0.18 (20)	0.18 (20)	0.54 (60)	0.90 (100)	0.023	0.023	0.067	0.113	Moderate (220 J/sec)
11 ppm for 8 hours (alcohol 3 hours after end of exposure)	90	0.19 (28)	<— 0.49 —> (72)		0.68 (100)	0.022	<— 0.060 —>		0.083	Light (106 J/sec)
8.2 ppm for 8 hours (alcohol 27 hours after end of exposure)	65	0.19 (22)	0.36 (41)	0.34 (37)	0.89 (100)	0.024	0.046	0.042	0.112	Moderate (220 J/sec)
		0 - 2	2 - 10	10 - 60		0 - 2	2 - 10	10 - 60		
		approximate time after exposure (hours)								

(Bracketed values represent percentage of total exhaled)

It is to be noted that short-term exposure to a high concentration leads to markedly increased concentrations in organs from which the benzene is rapidly released, but to relatively slight increase in that which is retained for a long period.

These estimates are only approximations as all respirator measurements of inhaled breath after exposure have been made on a sedentary subject. During sleep the elimination rate is likely to be less than that measured, while during physical activity it will be greater. Some measure of this effect has been obtained by respirator measurements during step exercise. When the subject exercised at 220 J/sec, the rate of excretion (μg/min) increased by a factor of 1.7 and 2.0 at two different levels of concentration, while the concentration in breath (ppm) increased by a factor of 1.3. As the quantities released have been calculated from breath concentrations, it is considered that the error in estimating the total benzene exhaled is unlikely to exceed 30%.

Patterns of Phenol Excretion in Urine

The work already cited reported an apparent excretion half-life for phenol in urine of about six hours in one subject, following either experimental or occupational exposure, and compared this with other reported values. In the more closely defined conditions of later experimental exposures it has been possible to distinguish two distinct phases of elimination which approximate to the 2nd and 3rd phases of benzene elimination in breath.

Unfortunately, the ability to detect the long-term component above the variable natural background was not recognized until late in the current series of exposures, so full data are only available for the final two tests.

As urinary excretion is essentially a batch process, phenol concentrations are shown as histograms in figure 7. The concentration of phenol in a sample depends both on the time of voiding and on the time since previous voiding.

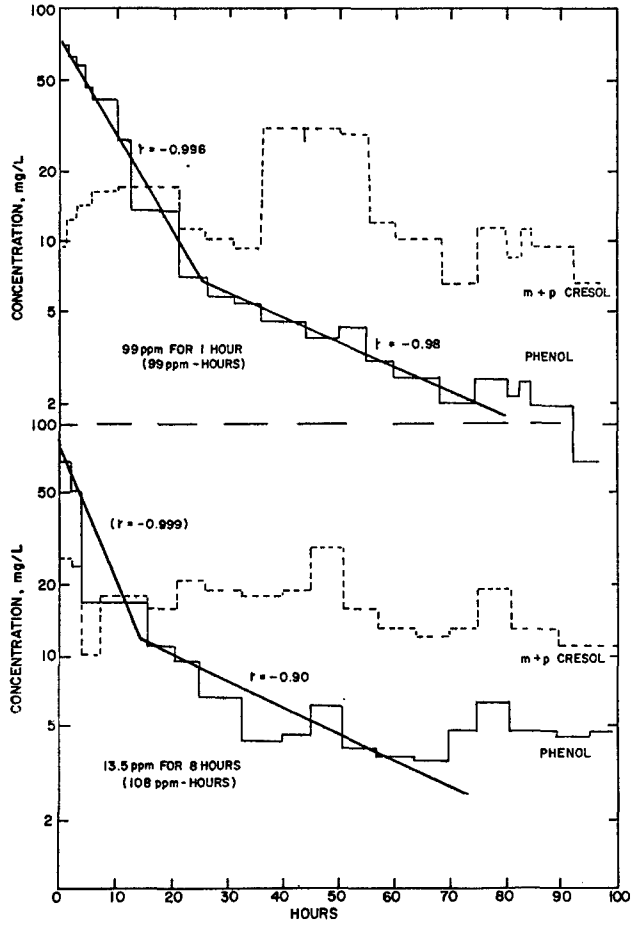

Figure 7. EXCRETION OF PHENOL IN URINE

The curves also shown have been calculated from the regression equations which have been based on the excretion at mid-time of each sample, rather than the time of voiding. All results have been normalized for specific gravity as this correlated better with time than the observed values or the calculated excretion rates. The mean specific gravity for each set of results has been taken as a standard.

Calculations of the total phenol excreted have been based on two phase equations similar to those for benzene in breath, and 1 mg/liter has been taken to correspond to 0.09 mg/hour from the observed values. For these estimates a natural background level of 3.0 mg/liter has been assumed, based on measurements taken before each exposure. The relative proportions of the phenol eliminated in each phase, shown in table II, confirm the impression from figure 7 - that shorter period exposure produces a larger proportion of phenol in the first phase.

Table II

Quantities of Phenol Excreted in Urine Related to Exposure Concentration and to Exposure Dose

Exposure	Exposure Dose ppm-hours	Quantity of Phenol Excreted Related to:- Concentration (mg/ppm)			Exposure Dose (mg/ppm hour)			Activity
		Phase 1	Phase 2	Total	Phase 1	Phase 2	Total	
25 ppm for 4.5 hours	115	1.7	-	1.7 +	0.36	-	0.36 +	Sedentary
6.4 ppm for 8 hours	51	2.6	-	2.6 +	0.33	-	0.33 +	Moderate (220 J/sec)
99 ppm for 1 hour	99	0.61 (92)	0.05 (8)	0.66 (100)	0.61	0.05	0.66	Moderate (220 J/sec)
13.5 ppm for 8 hours (5% benzene in mixture)	108	2.9 (69)	1.3 (31)	4.2 (100)	0.37	0.15	0.52	Moderate (220 J/sec)
11 ppm for 8 hours (alcohol 3 hours after end of exposure)	90	3.6 (86)	0.57 (14)	4.2 (100)	0.44 (86)	0.07 (14)	0.51 (100)	Light (106 J/sec)
8.2 ppm for 8 hours (alcohol 27 hours after end of exposure)	65	4.4 (80)	1.1 (20)	5.5 (100)	0.55 (81)	0.13 (19)	0.68 (100)	Moderate (220 J/sec)
		0 - 20	20 - 60	0 - 60	0 - 20	20 - 60	0 - 60	
		approximate times (hours)						

(Bracketed values represent percentage of total excreted)

As some tests were made at weekly intervals there is evidence of slight increase of backgrounds and, following the final exposure, concentrations remained at about 5 mg/liter more than 5 days after exposure.

Characteristic of all excretion curves has been an elevation of both phenol and meta- or para-cresol in the period 40 to 50 hours after exposure. The cresols do not appear to be a direct metabolite of the benzene, as the amounts absorbed during the experiments correspond to total eliminated in breath or excreted as phenol in urine.

Influence of Ethyl Alcohol

The first exposure tests with the new equipment revealed patterns of elimination quite unlike those previously observed. By chance, these tests coincided with a period marked by social consumption of ethyl alcohol, and the anomalies are now attributed to that cause.

Figure 8 shows elimination in breath and urine when alcohol was absorbed about three hours and about 27 hours after exposure; in both cases total consumption was about 50-70g.

The figures indicate no long-term elimination of benzene in breath when alcohol is absorbed soon after benzene exposure, and an increased proportion of phenol in the rapid phase of urinary elimination (tables I and II).

An apparently anomalous rise in phenol excretion after the inhalation experiment originally reported can now be attributed to alcohol consumption two days after exposure, as the pattern of excretion very closely follows that reported here for alcohol consumption 1 day after exposure. The phenol concentration rose by 5-10 mg/liter above expected values following alcohol intake.

A single test was made to determine the possible effect of alcohol on the absorption of benzene in fat by determining the benzene vapor concentration over olive oil containing benzene with the addition of ethyl alcohol; when the concentration of alcohol was five times that of the benzene, the concentration of benzene vapor was found to be no more than 10% higher than when alcohol was absent, so the effect is not considered significant, but tests at higher alcohol concentrations need to be undertaken.

Although the use of ethyl alcohol as an agent to reduce toxicity of both methanol and ethylene glycol has been discussed (Leaf and Zatman, 1952; Wacker et al., 1965), possible protective effects against benzene must be a matter of speculation. It appears that alcohol could accelerate elimination and a rewarding series of tests can be envisaged.

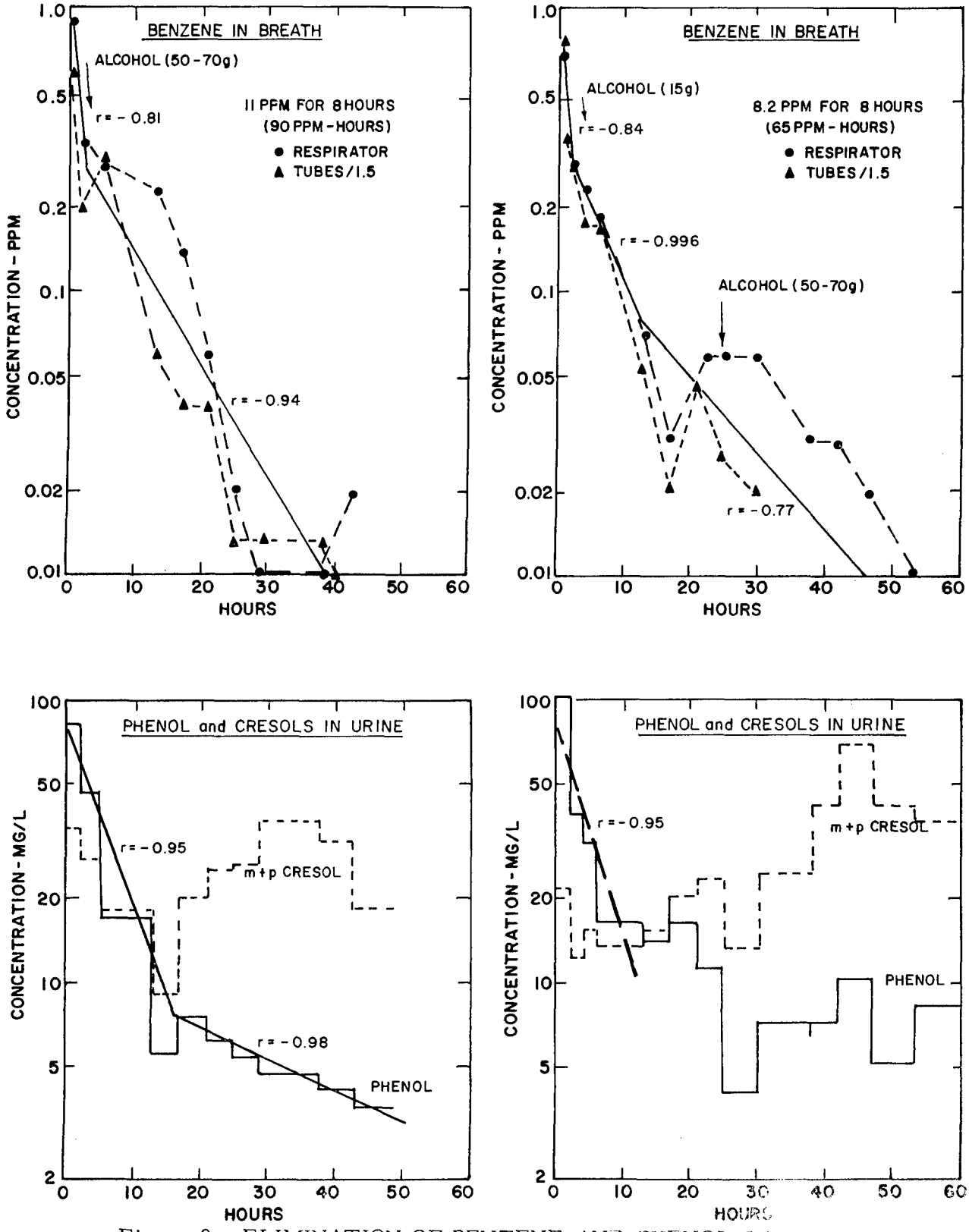

Figure 8. ELIMINATION OF BENZENE AND PHENOL IN PRESENCE OF ETHYL ALCOHOL.

Programs of routine urine monitoring have shown enhanced excretion of phenol following the therapeutic consumption of acetyl salicylic acid; this increase may have been due to earlier alcohol ingestion which necessitated the drug treatment.

Doses of both alcohol and acetyl salicylic acid were taken about 80 hours after two exposures, but specific effects could not be identified with certainty as the elimination levels were low.

Comparison of Breath and Urine Results

The comparison of the elimination half-lives in breath and urine in table III shows similar rates for elimination in the period commencing about 20 hours after exposure. During the earlier period, urinary excretion changes less rapidly than does the benzene concentration in the breath, though there is a similarity with the middle phase of breath elimination.

Table III

Comparison of Elimination Half-lives of Benzene in Breath and of Phenol in Urine

	$T\frac{1}{2}$ Breath (hours)			$T\frac{1}{2}$ Urine (hours)	
Exposure	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2
25 ppm for 4.5 hours	1.3	6.4	30	6.9	-
6.4 ppm for 8 hours	1.1	3.0	21	6.7	-
99 ppm for 1 hour	0.7	3.9	27	7.0	28
13.5 ppm for 8 hours (5% benzene in mixture)	1.1	3.3	24	5.2	27
11 ppm for 8 hours (alcohol 3 hours after end of exposure)	1.4	<— 7.7 —>		4.8	28
8.2 ppm for 8 hours (alcohol 27 hours after end of exposure)	1.7	6.5	11	3.5	-

The effect of alcohol on the long-term elimination in breath is evident, but the effect on the pattern of phenol excretion is complex. Alcohol immediately after exposure appears to prolong the first phase of phenol excretion; if taken some time after exposure, the effect is less clear.

The relative quantities of benzene eliminated in breath and as phenol in urine can be estimated from Tables I and II, 1 mg of phenol being equivalent to 0.83 mg of benzene. Following an exposure of 8 hours, 80% of the benzene is eliminated as phenol in urine; following 1 hour exposure, slightly less than 70% is by the urinary route. Where complete elimination data have been obtained, estimates of total excretion are in close agreement (5%) with the total retention which has been calculated from the difference between inlet and outlet concentrations during exposure.

When alcohol was absorbed shortly after exposure, not all the probable intake of benzene appeared to be eliminated in breath and as phenol in urine. 11% of the total eliminated was in the long term phase. After 1 hour exposure, long term elimination (phase 3 in breath; phase 2 in urine) represented 17% of the total eliminated; after 8 hour exposure to a mixture, it represented 35%.

Correlation of phenol in urine against benzene in breath for the period after 20 hours shows that it is approximately described by the equation:

phenol in urine (mg/liter) = 100 x benzene in breath measured by respirator (ppm)

Correlation coefficients varied from 0.75 to 0.94.

Between 2 and 20 hours after exposure this relationship is less accurate, and it is not valid for the first 2 hours.

Application to Monitoring

From the regression equations the concentrations in breath at likely sampling times have been calculated and related to exposure concentration and to exposure dose. These are shown in Table IV.

Table IV

Benzene Concentration Measured by Breath Sampling Respirator

Exposure	Exposure Dose ppm-hours	Time after Exposure (hours) 00	0.05	0.5	1	7	16	70	00	0.05	0.5	1	7	16	70
		Benzene concentration in exhaled breath % exposure concentration							Benzene concentration in exhaled breath related to exposure - dose (ppm) x 10^3 (ppm hours)						
25 ppm for 4.5 hours	115	7	6	5	4	-	0.44	0.15	15	14	11	8	-	1.0	0.3
6.4 ppm for 8 hours	51	8	7	6	4	-	0.8	(0.13)	10	9	7	5	-	1.0	(0.16)
99 ppm for 1 hour	99	7	6	4	2	0.4	0.11	0.03	68	64	40	24	3.5	0.9	0.28
13.5 ppm for 8 hours (5% benzene in mixture)	108	9	9	7	4	-	0.6	0.14	11	11	8	6	-	0.8	0.18
11 ppm for 8 hours (alcohol 3 hours after end of exposure)	90	8	8	6	5	-	0.7	-	10	9	7	6	-	0.8	-
8.2 ppm for 8 hours (alcohol 27 hours after end of exposure)	65	8	7	6	5	-	0.8	-	10	9	8	6	-	1.0	-

Values most applicable to routine monitoring are underlined.

It must be stressed that these are mean concentrations in exhaled breath over 10-minute periods, and that the concentration in end-tidal air is likely to be 1.5 times greater. Soon after exposure the concentration, which is seen to be related to exposure concentration, is in accord with the criterion already proposed; the concentration after 16 hours, which is more closely related to exposure dose, is rather lower than the earlier proposal; 100 ppm - hours produces about 0.1 ppm by respirator rather than 0.2 ppm as first suggested (see table VI).

Table V

Phenol Concentration in Urine

(Normalized to Mean Specific Gravity of Each Series)

Exposure	Exposure Dose ppm-hours	Time after Exposure to Mid-Time of Urine Secretion (hours) 00	1	6	11	00	1	6	11
		Related to exposure conc. mg/liter per ppm				Related to exposure dose mg/liter per ppm - hour			
25 ppm for 4.5 hours	115	2.4	2.1	1.3	0.8	0.52	0.47	0.29	0.17
6.4 ppm for 8 hours	51	5	4	2.9	1.4	0.58	0.52	0.36	0.17
99 ppm for 1 hour	99	0.8	0.7	0.4	0.3	0.81	0.73	0.43	0.27
13.5 ppm for 8 hours (5% benzene in mixture)	108	6	5	2.6	1.3	0.70	0.60	0.32	0.17
11 ppm for 8 hours (alcohol 3 hours after end of exposure)	90	7	6	3.4	1.3	0.86	0.75	0.43	0.17
8.2 ppm for 8 hours (alcohol 27 hours after end of exposure)	65	11	10	2.5	1.6	1.4	1.2	0.31	0.20

Table VI

Modified Benzene Exposure Criteria

$\frac{1}{2}$ Hour after Exposure to	Benzene in Breath (by respirator) ppm	Phenol in Urine mg/liter
25 ppm in air	1.5	-
100 ppm - hour	-	50
Morning (16 hours) after exposure to 100 ppm - hour	0.1	20

Attention should again be drawn to the fact that rapid reduction in the immediate post-exposure period implies that end-of-shift samples, though sensitive, have doubtful quantitative value. For example, the concentration 1 hour after 1 hour exposure is 2% of the exposure concentration, whereas after 7 hours it is 0.4%. End of shift samples could well be used as a screening method with further samples being required the following day for quantitative assay only if high concentrations have been detected.

Table V shows the concentrations of phenol in urine in samples at corresponding times. The information shows that, throughout the period of excretion, the phenol concentration (normalized for specific gravity) is a better measure of exposure dose than of exposure concentration. The criteria proposed earlier should probably be modified, as shown in table VI, if the one subject tested is assumed to be representative of all workers. Further field data are required before definitive standards can be set.

Benzene and Phenol in Venous Blood

A single blood sample taken 1 hour after exposure showed that the concentration in the vapor space over blood corresponded to that measured in exhaled breath.

Extrapolation of measurements of partition coefficient indicate that the concentration in blood was about 0.1 μg/ml, so that the total benzene content of blood was likely to be less than 1 mg at that time, and probably less than 5% of the original amount absorbed by the body.

The phenol concentration in the same sample was about 2 mg/liter, whereas urine at that time contained about 70 mg/liter.

CONCLUSION

It must be emphasized that all the reported results are from a single experimental subject.

They suggest that alcohol may have marked effects on the retention and elimination of benzene from the body. They show that short exposure may lead to relatively high short-term intakes, but that long-term retention differs little from that caused by long-term exposure. No significant difference was detected between inhaling pure benzene or as 5% of a hydrocarbon mixture. The effects of work rate during exposure have not yet been resolved.

It is to be hoped that laboratories better able to undertake fundamental research will develop this type of study to obtain more definitive data on a representative group of homo sapiens.

ACKNOWLEDGEMENT

The analytical techniques for this program were developed by Mr. F. W. G. Carter of Esso Research Centre, Abingdon, Berkshire, England. The author wishes to thank him for this and to acknowledge that he provided all the analytical measurements in this program.

APPENDIX

Supplementary Data on Evaluation Techniques

1. Reliability of Breath Sampling Methods

Field experience has indicated that the breath sample tubes are more liable to misrepresent exhaled breath concentrations than the respirator.

Identifiable sources of error are the presence of benzene in ambient air at the place of test, incomplete sealing of tubes, - which is particularly critical when samples have to be stored or be transported by air, and failure to obtain a sample of end-tidal air. The first error can be identified (but may not be controlled) by collecting a sample of ambient air in a tube. The second by rigorous checking of the lips of the tubes, and of seals, liners and caps. Transportation of samples by air was simulated by holding two prepared samples in a vacuum vessel at 500 torr (equivalent to about 3,000m altitude) for one hour. Comparison with two untreated samples showed a loss of 18% which is of little biological significance.

Errors due to failure to obtain end-tidal air samples were investigated by preparing deliberately unsatisfactory samples. A sample collected by a very short exhalation indicated a loss of 15% compared with properly collected samples, and deep inhalation followed by short and rapid exhalation indicated a loss of 35%.

The first error is not significant in field monitoring, but considerable care was taken in the experimental exposures to obtain representative samples. To check dependability, pairs of breath tube samples were always collected. Over the whole series of about 100 samples the correlation coefficient between pairs of samples was 0.987. (See also figure A1).

The breath sampling respirator has been assumed to give more dependable results than breath tubes, and this is confirmed by a correlation coefficient of 0.996 between respirator results and the mean value of each pair of breath tubes. (See also figure A2). Direct calculations from respirator results indicate the rate of benzene elimination in breath (that is, μg/min.). All reported values of concentration in these tests have been derived from the assumption that the cartridge weight gain due to moisture in breath is 25 mg/liter (Sherwood and Carter, 1970).

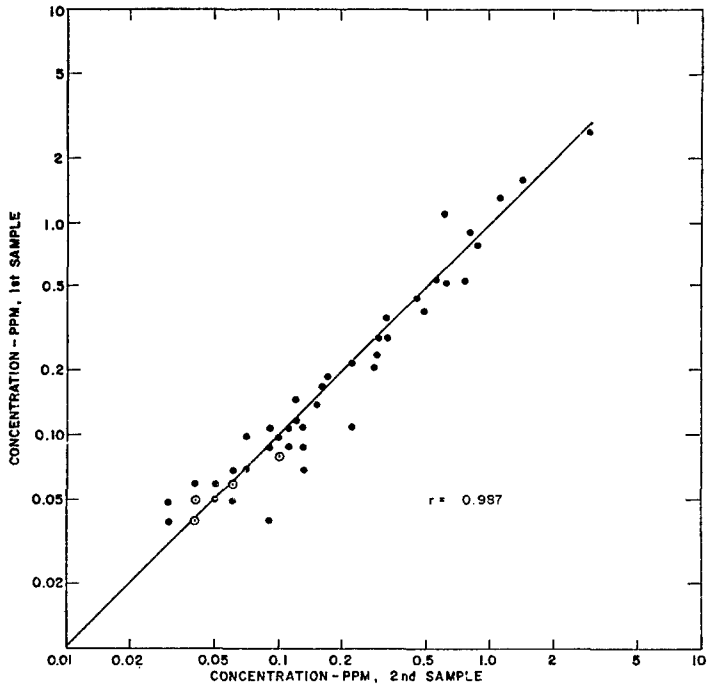

Figure A1. CORRELATION OF BREATH TUBE PAIRS.

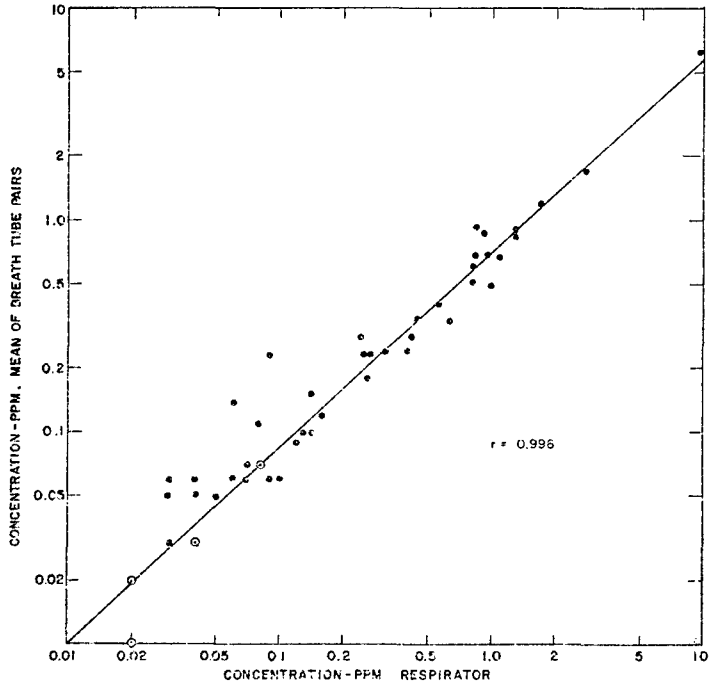

Figure A2. CORRELATION OF MEAN VALUES OF BREATH TUBE PAIRS AND RESPIRATOR.

As would be expected (and has been found in field work), respirator results are generally lower than breath tubes, as the mean, rather than the maximum, concentration in breath is determined. Over the whole series of trials, a mean ratio of 1.475 was determined; for mathematical convenience in expressing results from these trials, all breath tube results have been reduced by a factor of 1.5 to give a mean rather than maximum concentration in breath, and to enable results to be combined with those from the respirator for evaluation.

2. Interpretation of Phenol in Urine Results

The collection of 24-hour urine samples is not a popular routine among industrial workers, and the initially rapid changes in level that occur shortly after exposure make long-period samples insensitive as low levels of phenol are naturally present. For the studies reported here, it has been essential to collect frequent samples so as to observe the pattern of elimination.

The volume and specific gravity of each sample has been determined, and the observed concentration, the concentration normalized for specific gravity, and the excretion rate (μg/min) have been recorded. Specific gravity normalization has been made by multiplying each observed concentration by the mean specific gravity for each set of results minus unity, and then dividing by the specific gravity of the particular sample minus unity.

In table AI are shown the correlation coefficients for the logarithm of phenol concentration or rate against time. As concentrations normalized for specific gravity show the highest correlation, these values have been reported throughout these studies.

Table AI

Correlation Coefficients between Logarithm of Phenol Excretion and Time

	Concentration		
Elimination Phase	Observed	Normalized	Excretion Rate
Early (0-25 hours)	-0.90	-0.996	-0.92
Late (25-70 hours)	-0.76	-0.98	-0.93

In this work, normalization has been restricted to a maximum factor of two. Interlaboratory comparisons have shown discrepancies in results for samples having specific gravity less than 1.010, and in routine monitoring programs such samples should be rejected and further samples requested.

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DISCUSSION

DR. ASTILL (Eastman Kodak Company): I was wondering, Dr. Sherwood, if you considered the possible effect of smoking on the elimination of benzene. As you probably know, smoking raises the carboxyhemoglobin level and there seems to be some possibility that carbon monoxide may, therefore, tie up say cytochrome P-450 and interfere with drug metabolism. I guess the question is - do you smoke?

MR. SHERWOOD (Esso Europe Inc.): The subject who was exposed is a nonsmoker. His experimental rig is such that it is difficult to design a smoking experiment.

DR. ASTILL: But you could do it before you did the experiment.

MR. SHERWOOD: I think the subject would object, sir. No, I'm sorry. This is really a toxicological question which I hope someone here can answer. I, as a mechanical engineer, can not.

AMRL-TR-72-130

PAPER NO. 4

SIMULATION OF UPTAKE, DISTRIBUTION, METABOLISM, AND EXCRETION OF LIPID SOLUBLE SOLVENTS IN MAN

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In order to properly evaluate exposure tests, it is necessary to know the dynamics of the uptake, distribution, metabolism, and excretion of noxious substances.

There are three main factors determining the fate of inhaled lipid soluble solvents in the body:

(1) The transport rate of compounds from lung to tissue,
(2) The capacity of body tissue to absorb the compound,
(3) Elimination routes, such as metabolism or renal or intestinal excretion.

The capacities (C) of tissues to absorb the solvent are determined by their volume (V) and by the solubility of noxious gas in tissue. The solubility can be calculated as the product of the concentration of noxious gas in inspired air (c) and the partition coefficient of the noxious gas between tissue and air, (λ tis/air), $C = V \cdot c \cdot \lambda$ tis/air.

The rate of transport of the noxious gas to the tissues (F_{tis}) depends on the blood flow through the tissue (F) and on the concentration of the noxious gas in the blood. The concentration in the blood is determined by the concentration in inspired air, and by the partition coefficient between blood and air, (λ bl/air), (Papper and Kitz, 1963), $F_{tis} = F \cdot c \cdot \lambda$ bl/air.

The ratio of the capacity of the tissue to absorb the solvent to the rate of transport of the solvent to the tissue is the time constant,

$$T = \frac{C}{F_{tis}} = \frac{V \cdot \lambda \text{ tis/bl}}{F}.$$

The time constant in an open system such as the body determines the time necessary to saturate the compartment to 63% of its capacity. The time constant can be predicted from the physiological constants (tissue volumes and tissue perfusion rate), and from the partition coefficient of the solvent between tissue and blood.

The reciprocal of the time constant is the rate constant, 'k',

$$k = \frac{1}{T} = \frac{F}{V \cdot \lambda \text{ tis/bl}}$$

which can be substituted in the equation for the uptake curve, $A = C(1 - e^{-kt})$ where 'A' is the amount of noxious gas in the tissue at a given time, 't'.

In order to calculate the half-life, the time constant is multiplied by the natural logarithm of 2, or the natural logarithm of 2 is divided by the rate constant,

$$t_{\frac{1}{2}} = 0.693\,T = \frac{0.693}{k}.$$

The capacity of the tissue to absorb noxious gas varies, as does the blood flow through the tissue. The whole body uptake (A_B) is described as the sum of the uptake curves of numerous physiological compartments,

$$A_B = C_1(1 - e^{-k1t}) + C_2(1 - e^{-k2t}) + \ldots + C_n(1 - e^{-knt}).$$

If we analyze uptake curves (or desaturation curves), usually only three exponential functions can be distinguished. The reason for this is that the rate constant of one tissue must be at least ten times greater than the rate constant of another tissue to make noticeably different slopes in a semi-logarithmic plot.

Table I lists volumes and blood flows of various tissues, as well as their ratios (Mapleson, 1963). The problem can be simplified by considering the body as having two pharmacokinetically different compartments: (1) tissues with a ratio less than five minutes (called the vessel rich group, VRG), and (2) tissues with a ratio greater than five minutes (called the vessel poor group, VPG).

In addition to the tissue volume and blood flow, the partition coefficient between tissue and blood determines the time constant and the rate constant. The tissue/blood partition coefficient of lipid soluble compounds is usually between one and five, except for fat/blood partition coefficients which are between fifty and two hundred (table II). The tissue/blood partition coefficient influences the time constants or the rate constants for fat more than ten times as much as other tissues. For this reason, adipose tissue and bone marrow have to be considered as another pharmacokinetic compartment in dealing with lipid soluble compounds.

TABLE I

Volumes and Blood Supplies of Different Body Regions for a Standard Man, 70 kg, 1.73 m^2, Cardiac Output 6 liters/min.

Tissue	Volume, liters	Blood flow, liters/min.	$T = \frac{V}{F}$, min.
Heart	0.3	0.24	1.2
Liver	3.9	1.58	2.5
Gray Matter	0.75	0.60	1.2
White Matter	0.75	0.16	4.7
Kidney	0.3	1.24	0.2
Muscle	30	1.0	30
Skin	7.8	0.2	39
Fat	10	0.2	50
Fatty Marrow	2.2	0.06	37

TABLE II

Partition Coefficients

Solvent	Tissue/Blood	Fat/Blood	Fat/Tissue	Reference
CS_2	4.4	60	14	Teisinger and Soucek, 1948
Chloroform	0.8	67	84	Lowe and Hagler, 1969
Trichloroethylene	1.7	100	49	Lowe and Hagler, 1969
Benzene	1.2	57	48	Kozakova, 1955
Styrene	7.3	129	18	Van Rees, 1964

Eger (1963) has estimated volumes and blood flows for three pharmacokinetic compartments, the vessel rich group (VRG), muscle group (MG) and fat group (FG), (table III). Their VRG is made up of heart, brain, hepatoportal system, kidney, endocrine glands, and spinal cord. Their MG includes muscle and skin, and their FG is made up of adipose tissue and bone marrow. Their model is for a standard man (weight seventy kilos, surface area 1.73 square meters, cardiac output six liters per minute).

TABLE III

Pharmacokinetic Compartments for a Standard Man, 70 kg, 1.73 m^2, cardiac output 6 liters/min.

		Compartment		
	Unit	VRG	MG	FG
Volume	liters	6	33	14.5
Blood Perfusion	liters/min	4.5	1.1	0.3
Volume/Blood Flow	min	1.3	30	48

VRG = Liver, Heart, Kidney, Brain, Endocrine Glands and Spinal Cord
MG = Muscle, Skin
FG = Fat, Fatty Marrow

The tissue capacity, rate constant, time constant, and half-life for three pharmacokinetic compartments determined for benzene are shown in table IV. The half-lives demonstrate that the viscera are saturated after approximately five minutes of exposure, and muscles after two hours. However, almost one week of continuous exposure would be required to saturate the adipose tissue.

TABLE IV

Constants Determining Uptake of Benzene by a Standard Man Ventilation = 7 liters/min.

Compartment	Tissue Capacity (liters)	Time Constant	Half-life	Rate Constant min^{-1}
VRG	42	1.6 min	1 min	0.64
MG	231	36 min	25 min	0.028
FG	5800	50 hrs	35 hrs	0.00033

One way to understand the uptake, distribution and excretion of volatile compounds is to simulate the body by a group of connected vessels. The vapors in alveoli exert a partial pressure which is simulated by the first vessel in figure 1a. The gas flow to the lungs is regulated by pulmonary ventilation which is pictured as a tube with a stop valve. Alveolar air is equilibrated with arterial blood in the lungs and distributed to different compartments, represented by vessels VRG, MG and FG. In figure 1, tube diameters are proportional to blood flow, and the size of the vessels is proportional to the capacities of compartments to absorb noxious gas. The first to be filled will be the small VRG compartment connected by the large tube, and the last to be filled will be the large FG compartment connected by the

capillary tube. Simulating the end of exposure, the source is disconnected by removing the first vessel (figure 1b, 1c). If this happens when only the VRG vessel has been filled (figure 1b), the vessels labelled VRG and BL immediately start draining, but the vessels labelled FG and MG continue filling until the pressures in all vessels are in equilibrium (figure 1c). Thereafter, all vessels are draining, with the FG vessel draining at the slowest rate. A similar situation exists in the body and is known in pharmacology as drug redistribution.

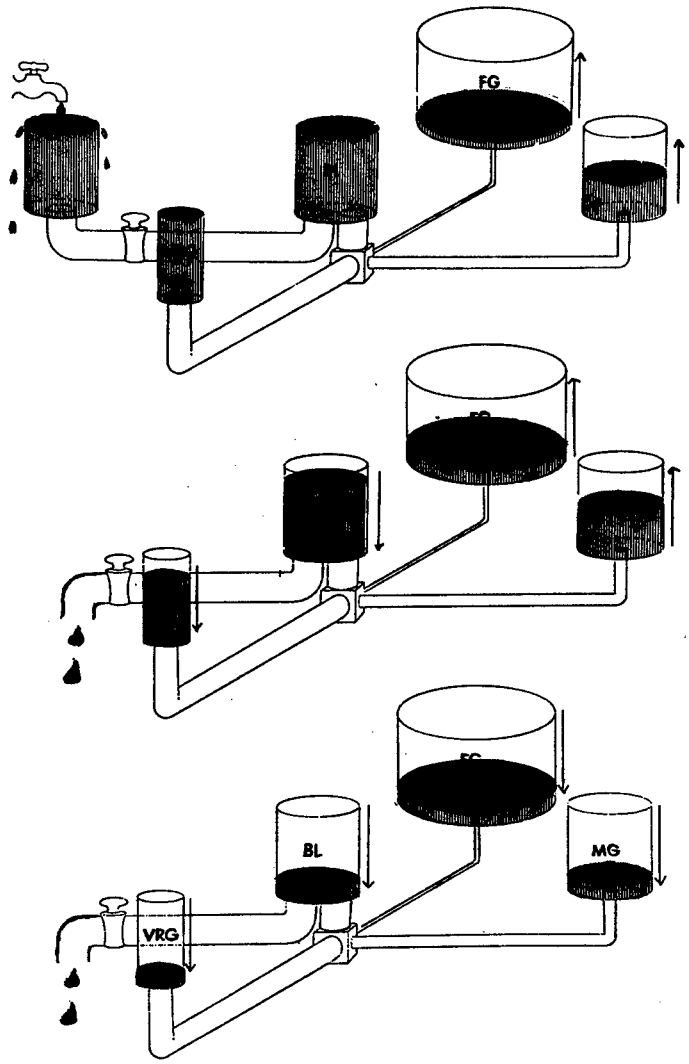


Figure 1. SIMULATION OF UPTAKE, DISTRIBUTION AND EXHALATION OF LIPID SOLUBLE SOLVENTS BY CONNECTED VESSELS. NO METABOLISM.

Sherwood (1972) and Stewart (1972) demonstrated that the concentration of benzene in breath samples collected immediately after the end of a work shift correlates with the concentration in the inhaled air, but the samples collected the next morning correlate with the exposure dose. This is exactly what this model shows. After source cut-off, the first VRG vessel drained. The pressure of this vessel was equilibrated with the input pressure, so that the outflow is related to the input pressure, which represents the concentration in inhaled air. The next day the VRG and MG compartments were equilibrated with the FG compartment. With regard to the slow transport rate to the FG compartment, the time of exposure is the determining factor of the solvent level in the FG compartment. Therefore, the outflow depends on both the original input pressure and the duration of the uptake or exposure dose.

Benzene, as well as most organic compounds, has an important elimination route in metabolism (Teisinger, Fiserova-Bergerova, 1955 and Sherwood, 1972). The metabolism of noxious compounds is mainly an irreversible process requiring microsomal enzyme systems.

Drug metabolism takes place mainly in liver microsomes. However, five to twenty percent of metabolic activity may be extrahepatic. Cytochrome P-450 has been found in liver, lung, kidney, intestine, brain, and skin (Conney, 1972).

Drug metabolizing enzyme activity in skin is very low, and so far it has been reported only in connection with benzopyrene hydroxylase. The rest of the metabolizing tissue belongs to well-perfused organs of the VRG pharmacokinetic compartment.

In order to simulate the uptake, distribution and excretion of volatile lipid soluble compounds, including their metabolic turnover, we inserted holes in the VRG vessel (figure 2). In this way we removed from circulation that part of the noxious compound which was metabolized. The new output, of course, accelerates the excretion and influences the redistribution of solvent in the body.

The kinetics of the metabolic turnover is governed by the Michaelis-Menten law. The rate constant estimated from the fraction of metabolized drug in the body characterizes the metabolic output. Teisinger and Soucek (1952) introduced the metabolic coefficient (KM), which is the difference between the measured uptake rate and the calculated uptake rate (assuming no metabolism), divided by the concentration of the noxious gas in inhaled air,

$$KM = \frac{a_{meas} - a_{cal}}{C} .$$

In table V are the metabolic coefficients for four solvents, as calculated by Teisinger and Soucek (1952).

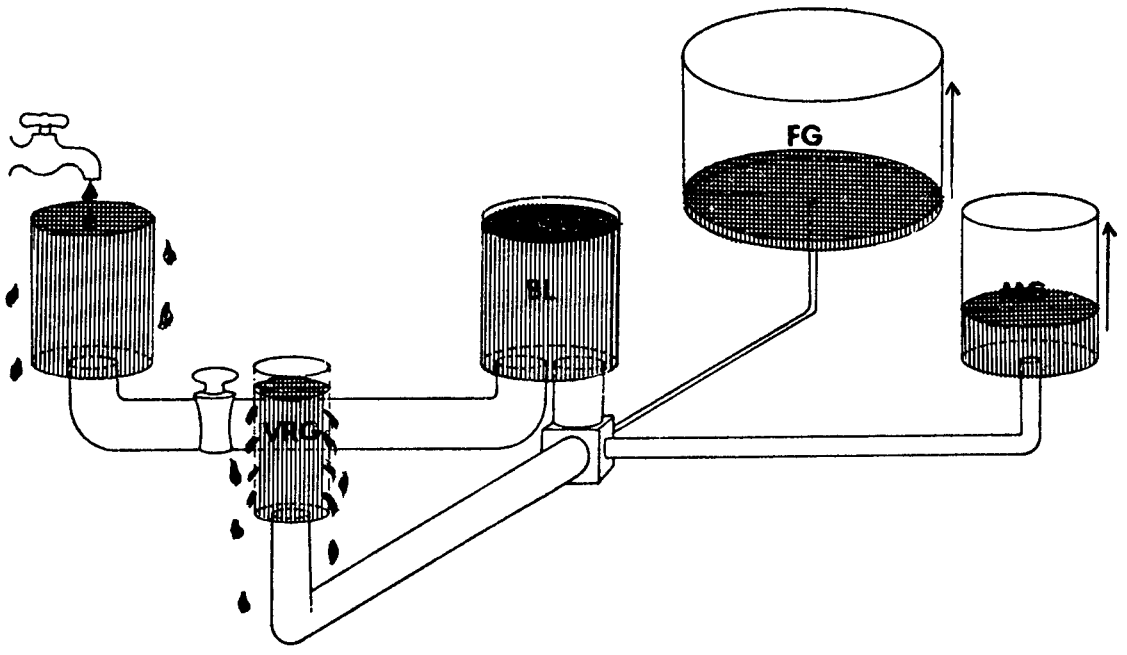


Figure 2. SIMULATION OF UPTAKE, DISTRIBUTION, METABOLISM AND EXHALATION OF INHALED ORGANIC SOLVENTS BY CONNECTED VESSELS.

TABLE V

Metabolic Coefficients (L/min)

Solvent	KM
CS_2	2.4
Benzene	3.2
Toluene	5.4
Trichloroethylene	5.4

A very useful way to simulate the uptake and excretion is by an electrical network composed of capacitors and resistors (Mapleson, 1963; Severinghaus, 1963; Fiserova-Bergerova and Cettl, 1972; Fiserova-Bergerova, Vlach, and Kishore, In Preparation). The same differential equations which govern the electric analog govern the uptake and distribution, and can be solved by analog computer (Fiserova-Bergerova and Cettl, 1972) or by straight integration by digital computer (Fiserova-Bergerova, Vlach and Kishore, In Preparation). The programs for the solution of the equations are usually available.

The analogy between the elements in an electric network and pharmacokinetic compartments is evident from figure 3. The battery stands for the source of noxious gas. The resistor (R_L) represents pulmonary ventilation. The capacitors are analogous to the capacities of the pharmacokinetic compartments to absorb noxious gas. The resistors (resistance being the reciprocal of conductivity) simulate the flow of noxious gas to the tissues through the blood. When the battery is disconnected, the capacitors are discharged. This simulates desaturation. The current in the network is proportional to the uptake (or washout) rate. The voltage in the capacitors is proportional to the levels of noxious gas in the pharmacokinetic compartments during uptake or desaturation.

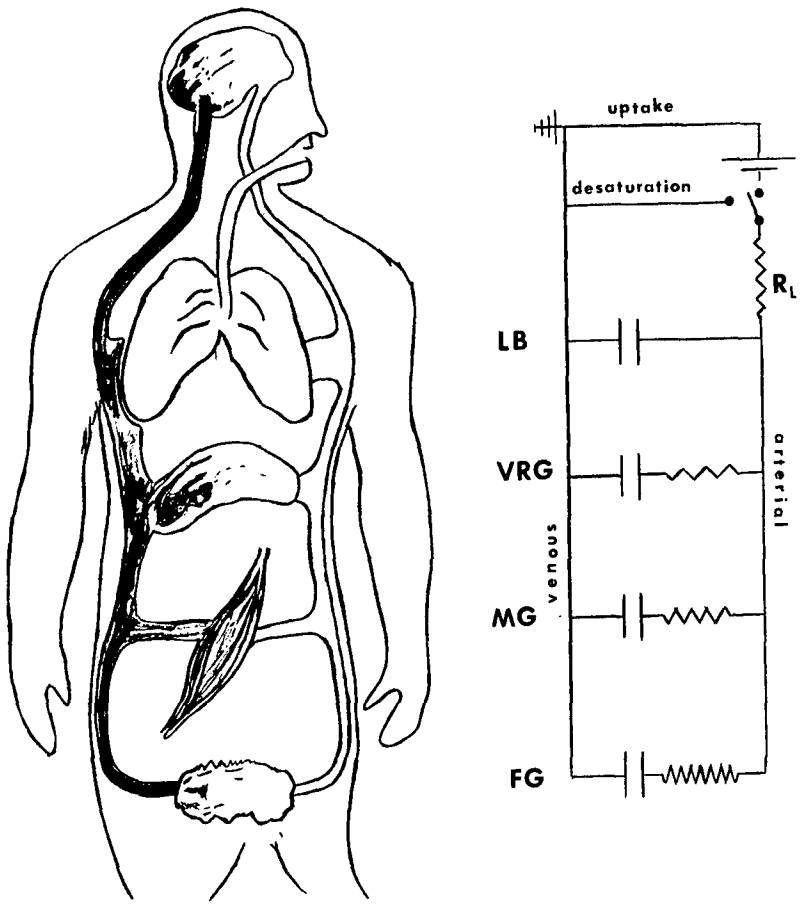


Figure 3. ANALOGY BETWEEN PHARMACOKINETIC COMPARTMENTS AND ELECTRIC NETWORK SIMULATING UPTAKE, DISTRIBUTION, AND EXHALATION OF LIPID SOLUBLE SOLVENTS.

The difference between the electric analogs for inert compounds presented by Mapleson (1963) and for metabolized compounds (Fiserova-Bergerova and Cettl, 1972) is evident from figure 4. The sum of the values of the resistors connected to the capacitor VRG is the reciprocal of the metabolic coefficient. In this simplified model, the resistor is grounded to illustrate the irreversibility of the metabolic pathway.

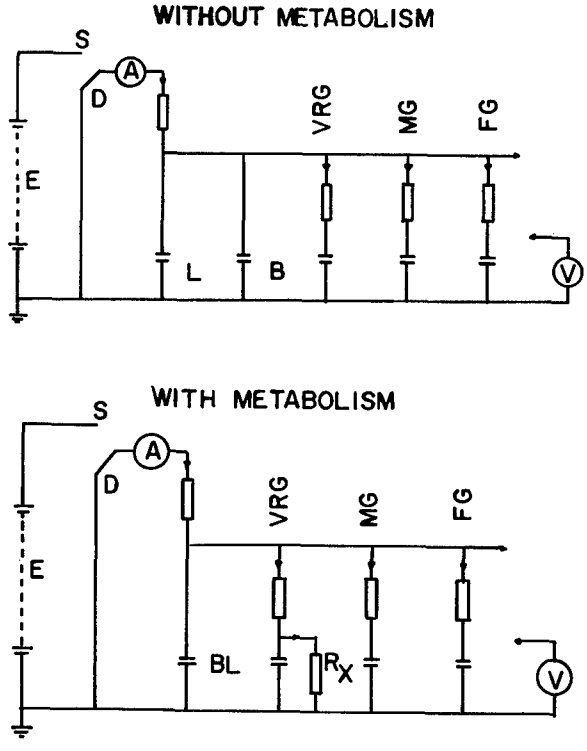
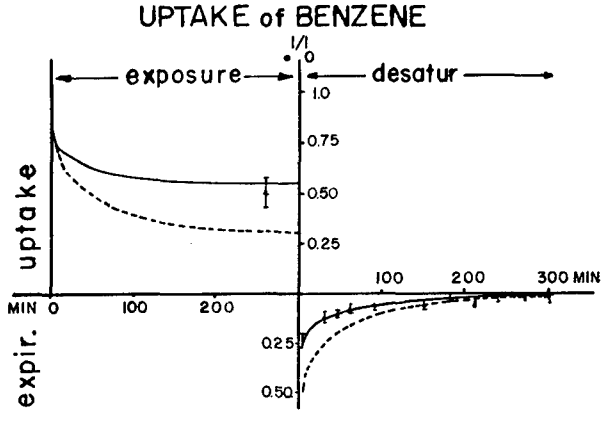


Figure 4. ELECTRIC ANALOGS FOR INERT AND METABOLIZED VOLATILE LIPID SOLUBLE DRUGS.

Prediction of Uptake and Distribution of Benzene and Verification by Experimental Data

A group of volunteers was exposed in an exposure chamber to a constant concentration of benzene vapor for a period of five hours (Teisinger and Fiserova-Bergerova, 1955; Teisinger et al., 1952). Inhaled and exhaled air was analyzed for benzene. This experiment was simulated on an electric analog (Fiserova-Bergerova and Cettl, 1972; Fiserova-Bergerova et al., In Preparation). The values of the resistors and capacitors were derived from the partition coefficients for benzene (Kozakova, 1955) and from the physiological constants of a standard man (table IV) (Eger, 1963). Pulmonary ventilation was eight liters per minute. The metabolic coefficient was 3.2 L/minute (Teisinger and Soucek, 1952).

The uptake and desaturation rates were calculated for a model with metabolism and for a model without metabolism (Fiserova-Bergerova and Cettl, 1972; Fiserova-Bergerova et al., In Preparation). The results are in figure 5. The uptake and desaturation curves registered for the analog (which includes metabolic turnover) follow the experimental points closely.



The lines represent the model for a standard man (70 kg., 1.73 m^3) including metabolism (solid line) and presumably without metabolism (broken line). The points represent the means and standard deviations measured in 14 volunteers. On the abscissa is the time from the start of exposure or desaturation, respectively. On the ordinate is the ratio of uptake or of desaturation, respectively, to the amount of benzene inhaled per minute during exposure. An analog computer was used for the solution.

Figure 5. UPTAKE AND DESATURATION OF BENZENE IN 5-HOUR EXPOSURE.

Sherwood (1972) presented a figure for exhaled air in a similar experiment on volunteers. In figure 6, the curves drawn by Sherwood were removed, leaving only the experimental points. A new curve was drawn according to the computer. This curve runs through the original experimental points.

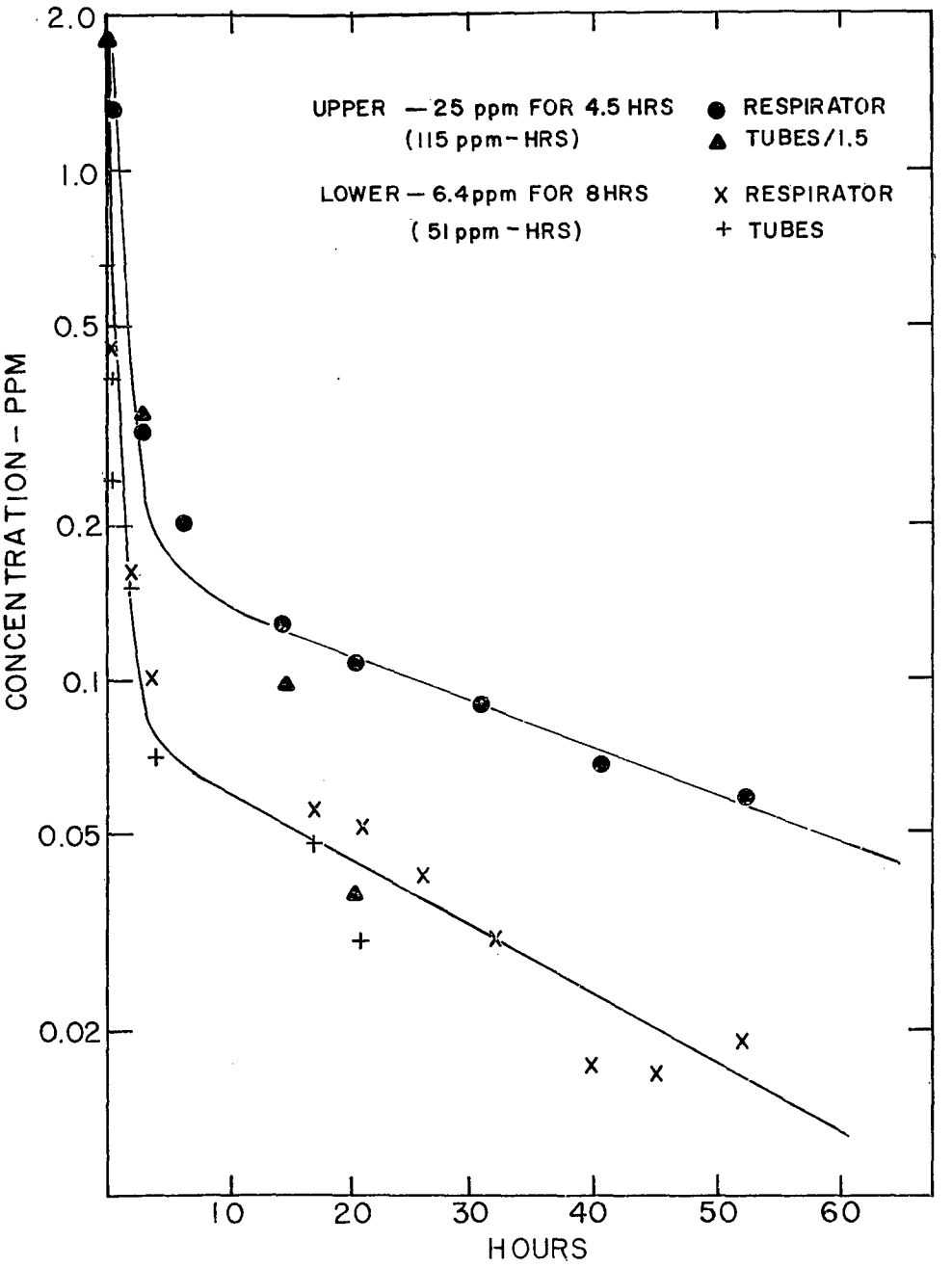


Figure 6. ELIMINATION OF BENZENE IN BREATH-1.

Uptake and desaturation rates by individual compartments are demonstrated in figure 7. Without metabolism, uptake by VRG is negligible after 15 minutes. Metabolism increases the total uptake, with the VRG compartment contributing most to the increase. Metabolism influences the uptake by MG very little, but slows down uptake by FG.

The desaturation curve shows benzene redistribution. When exposure terminates, the body desaturates, but the FG groups continue uptake for one and one-half hours, until the vapor pressure of benzene in FG is equilibrated with other tissues.

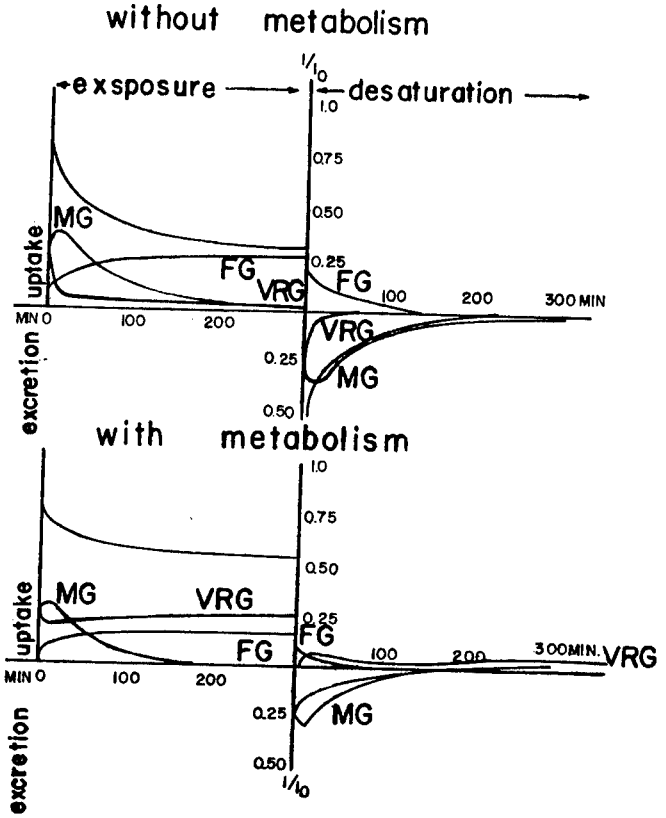


Figure 7. UPTAKE OF BENZENE BY COMPARTMENTS.

Metabolism in general depresses the pulmonary desaturation as well as the amount exhaled. The most striking difference appears in the VRG compartment. Its washout would be very fast if metabolism did not occur. With metabolism, the VRG compartment continues with uptake of benzene from blood throughout the entire desaturation.

The percentage of saturation of tissues during the saturation and desaturation period is apparent from figure 8. The upper part of the figure shows the situation which would develop if no metabolism occurred. The VRG and MG compartments would be saturated to seventy percent and the FG compartment to ten percent. After exposure terminates, the concentrations of benzene in MG and VRG drop fast, but the benzene level in FG does not change noticeably during five hours of desaturation. Metabolism causes the benzene level in all tissues to drop almost one-half, but the pattern of desaturation does not change.

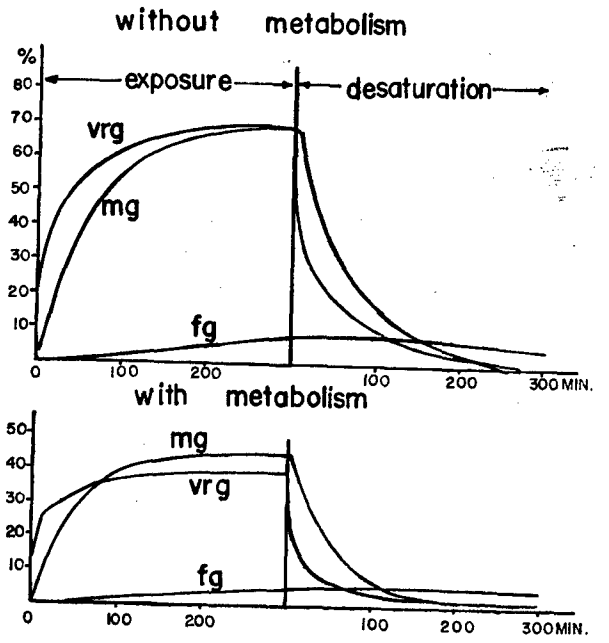
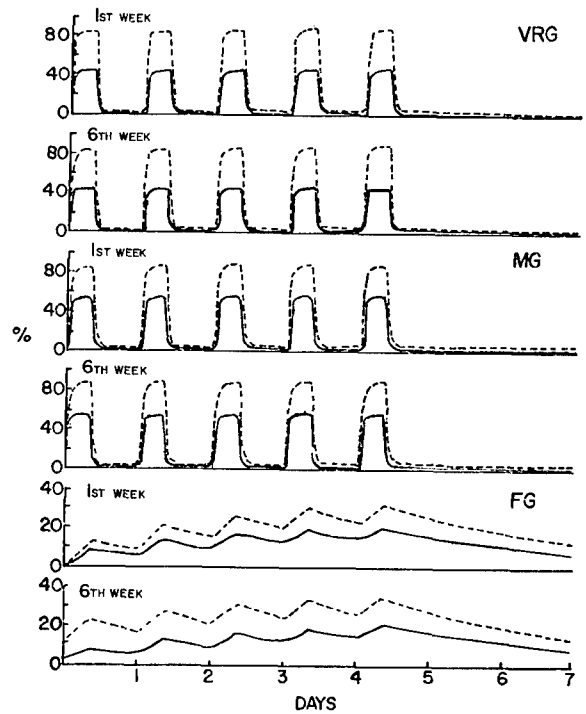


Figure 8. SATURATION OF COMPARTMENTS BY BENZENE.

The accumulation of industrial solvents in the body during chronic exposure is of great concern to industrial hygienists. Figure 9 demonstrates chronic exposure during the work week. The exposure period was eight hours, corresponding to an eight-hour shift.

Starting on Monday morning, the level of benzene in VRG tissues rises very fast, but drops rapidly when exposure terminates. Three hours after the end of exposure, only one percent of saturation remained, and the saturation dropped only a little during the night. The residue raised the level only slightly higher on Tuesday morning, when exposure began again. The same thing occurred on the following days. On Saturday morning the saturation level was one and one-half percent. During the weekend the level dropped to one-half percent. The lower figure shows the levels for the fifth week of exposure. The pattern is more or less the same, but on Monday morning the level began at one-half percent of saturation.



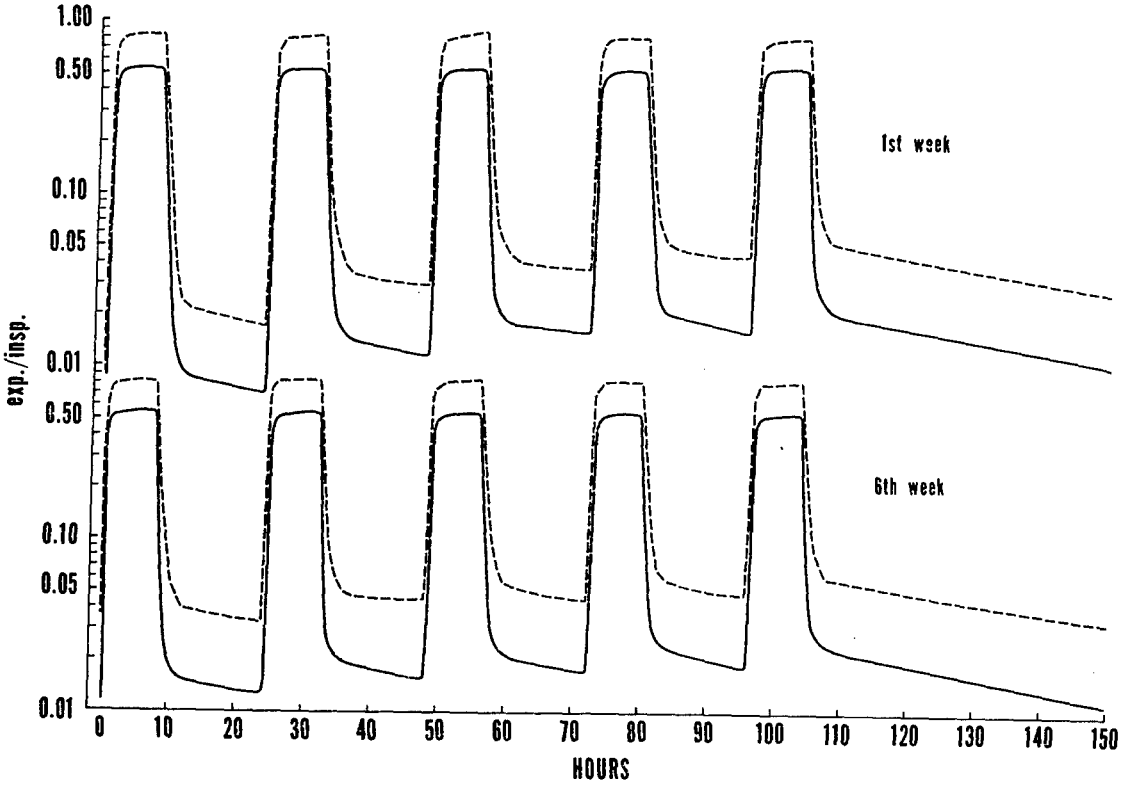
Simulation of work week conditions. Cumulation is expressed as a percentage of tissue saturation (ordinate). Recorded for the first week of exposure, and for the sixth week of exposure, when the steady state is reached.

Figure 9. CUMULATION OF BENZENE IN VRG, MG, AND FG COMPARTMENTS DURING CHRONIC EXPOSURE.

The figure for muscles is similar to that for the VRG compartment, but the uptake as well as the depletion in FG is very slow. At the end of the weekend, four and one-half percent of FG capacity was still taken by benzene. The lower figure shows a similar pattern for the sixth week of exposure.

At the end of the shift, the predicted total uptake is about 58 percent of the inhaled concentration, and is higher at the beginning of the week than at the end. VRG uptake, including metabolism, contributes 45 percent, FG uptake only 12 percent, and MG uptake less than 1 percent. Over the weekend the pulmonary desaturation takes place, but the fraction of benzene released from the FG compartment is also transported to the metabolizing organs.

The effect of the accumulation of benzene in the body on the concentration of benzene in exhaled air is apparent from figure 10. The lowest concentrations are after single exposure.



Simulation of work week condition. Benzene concentration in exhaled air is expressed as a percentage of benzene concentration inhaled during exposure. The unbroken line includes metabolism. The broken line presumes zero metabolism.

Figure 10. BENZENE CONCENTRATION IN EXHALED AIR DURING CHRONIC EXPOSURE.

At the end of the week, the concentrations are doubled in the late phases of desaturation. If the subject is regularly exposed to benzene over a period of five weeks, the steady state is reached. The concentration of benzene in exhaled air is higher than it was during the first week of exposure, especially at the beginning of the week.

The simulation of the fate of inhaled vapors of lipid soluble solvents contributes to the understanding of the pharmacokinetics of these kinds of drugs. The accumulation of noxious compounds during chronic exposure can be predicted, as well as uptake, distribution, and excretion of new industrial products. The agreement between theory and experimental data for benzene was demonstrated. The agreement between the computed curve and the experimental data for other volatile lipid soluble compounds, such as carbon disulfide, fluroxene and methoxyflurane support the validity of the theory.

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PAPER NO. 5

BIOLOGICAL MONITORING FOR INDUSTRIAL CHEMICAL EXPOSURE CONTROL: TETRAALKYL LEAD AND CYANOGENIC AROMATIC NITRO AND AMINO COMPOUNDS

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Biological Monitoring for Cyanosis Control

Of the techniques proposed for occupational health surveillance, man himself must be considered the best sampler of his work place. Regardless of the degree of sophistication applied to environmental and personnel monitoring, the ultimate answer to the question - how much hazardous substance was absorbed and how did the exposure effect the workman? - must be derived from direct quantitative analysis of expired air, body fluids, or tissue for the presence of the hazardous substance or its metabolites and by indirect determination of the magnitude of its effect on the functioning of the target organ or tissue. With few exceptions even the most hazardous materials have a no-effect level below which exposure can be tolerated by most workers for a life-time without incurring any significant physiological impairment.

The response of test animals, usually rodents, indicates only relative toxicity and possibly the target organ or tissue and cannot be applied directly to human experience.

The extreme variability in human response to any given stress must always be a controlling factor in the establishment of reasonable threshold limit values (Stokinger, 1971). Therefore, evaluation of the response to chemical stress must be carried out on relatively large groups. When the tolerance level has been established, then biological monitoring can be applied to control exposure below that limit.

The first consideration in the formation of an exposure control program must be evaluation of the relative magnitude of absorption through each of the three "portals of entry" into the human body:

1. By mouth
2. By inhalation
3. By skin contact.

Air analysis based on fixed station or personnel monitor collection cannot provide by itself a surveillance program adequate for health conservation when significant absorption occurs through the skin. Furthermore, the portal of entry frequently determines the toxicity potential. For example, para-chloroaniline exhibits a very low oral toxicity but by the skin absorption route it is one of the most potent of the cyanogenic agents. At normal ambient temperatures, para-chloroaniline's vapor pressure is below the inhalation hazard level. In other cases, both routes contribute (Stokinger, 1971). This factor was taken into account in the establishment of TLV's for airborne chemical exposure by the notation "skin" where applicable (Stokinger, 1971).

The medical surveillance program which has been applied to the control of exposure to cyanogenic aromatic nitro and amino compounds on Du Pont's Chambers Works serves as a good example of completely successful biological monitoring. This choice also illustrates both the acute and chronic phases of industrial exposure problems. Acute exposure usually produces cyanosis with or without a significant loss of hemoglobin, whereas the chronic exposure from prolonged subacute absorption may produce a reversible anemia. The overall effect is referred to as the cyanosis-anemia syndrome.

Cyanosis is a sign of tissue oxygen deficiency and occurs when the oxyhemoglobin (HbO_2) level falls below the critical oxygen demand level. Chemical anoxia may be produced by (a) reactive gases and vapors absorbed through the lungs (CO, H_2S, HCN, nitrobenzene and aniline); (b) ingestion of compounds such as nitrates, nitrites, sulfides, and some medicinals - "sulfa" drugs, acetanilide, etc.; (c) the fat soluble liquids and solids absorbed directly through the intact skin. Cyanosis was a not uncommon industrial illness, and a number of deaths from contact with nitro and amino compounds have been reported (Von Oettingen, 1941). The subacute hemoglobin (Hb) depressing effect, anemia, produced by these compounds received little attention, however.

Most of the aromatic nitro and amino compounds are not in themselves cyanogenic, but oxidation-reduction enzyme systems promote metabolism to known active derivatives which arise from either oxidation of the amine or reduction of the nitro group (figure 1). The conjugation products of these metabolites with Hb are loosely classified as methemoglobin (MHb) in which the iron has been oxidized to the ferric state (Jackson and Thompson, 1954). In addition to the displacement of oxygen from the iron atom of Hb by these metabolites, inhibition of the enzyme system which maintains the Hb iron in the ferrous state also occurs (Steere, 1971).

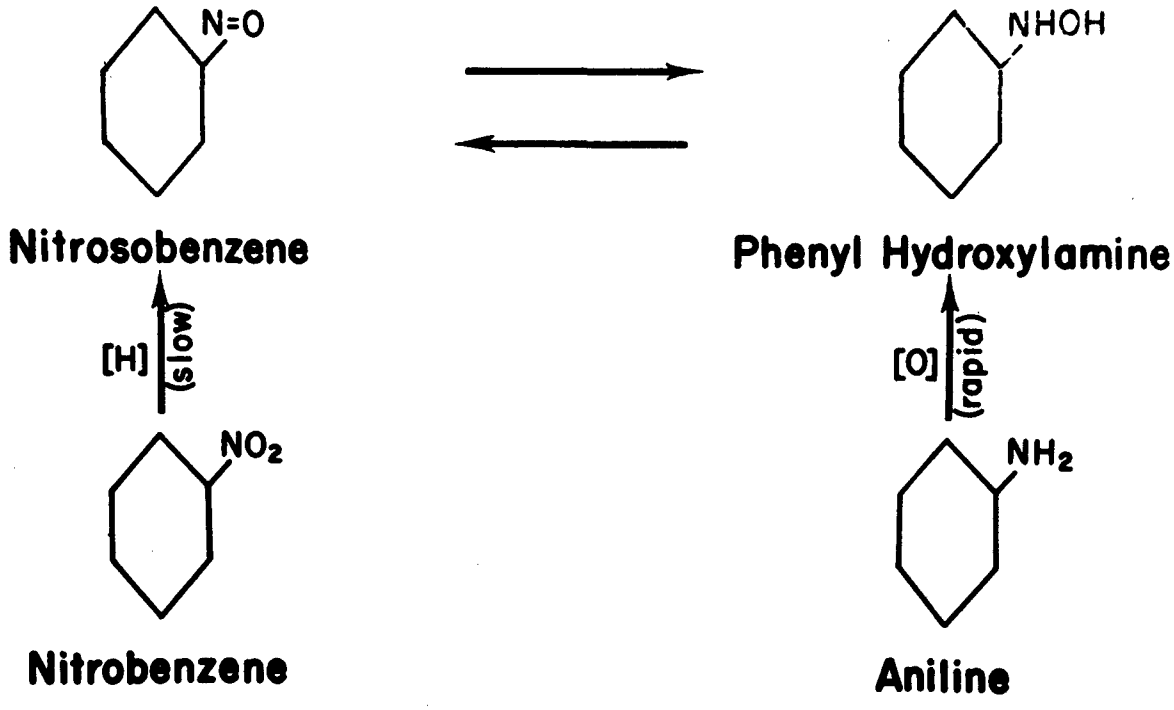

Figure 1. CHEMICAL CYANOSIS MECHANISM

Improvements and modifications in the micro-procedures for the rapid determination of methemoglobin (MHb), oxyhemoglobin, and Hb were developed to not only diagnose cyanosis severity, but also to provide the information necessary for effective prevention (Steere, 1971). Precursors to cyanosis were detected by the difference between the acid hematin and oxidized Hb methods for total Hb and recorded as percent Hb complexes (%HbC) (Steere, 1971). Total oxygenatable Hb in some cases, notably after exposure to the nitrochlorobenzenes, is less than would be expected from MHb analysis. Therefore, a direct determination of HbO_2 either gasometrically or by reflectance spectroscopy should be included in the analysis (Steere, 1971).

Blood analysis detects the effect of the cyanogenic agent after absorption. These laboratory results provide a secondary control technique and diagnostic information. The primary control program is based on urine analysis which provides early warning of conditions which might lead to Hb alterations. Urine collected from crews assigned to areas where aniline and its derivatives are present is first acid hydrolyzed to convert the N-acetyl conjugates to free amines which are then diazotized, and coupled to produce a blue-red azo dye which is quantitated spectrophotometrically. Aromatic nitro compounds are first reduced under alkaline conditions to the corresponding aniline derivative without hydrolyzing the acetylated amines, and then analyzed by the aromatic amines method (Koniecki and Linch, 1958). This combination of analyses differentiates amino from nitro compounds in locations where both are encountered.

Although considerable information relative to small animal response to cyanogenic agents was available (Hamblin, 1967), little if any of it could be applied directly to our health conservation problem. Therefore, our exposure control program was based on clinical and laboratory evaluation of 187 cyanosis cases during the ten-year period following 1956. Since 26% of this group exhibited no clinical signs and only 37% presented symptoms, diagnosis was based exclusively on laboratory findings. (table I)

Table I (2)

Biological Threshold Limit Values (BTLV)

[Routine Frequency (1) - 60-Days (coincides with urine analysis)]

	Blood Component					
Action	Methemoglobin %	Hemoglobin g/100 ml	% HbC (ref. 1)	Oxyhemoglobin %	Hemoglobin Loss Rate %/24 hr.	COHb %
Warning	5-9	Below 14.0	10 or above	Below 90	13.0	10
Medical Intervention	10 and over	13.0 and lower	15 or above	Below 85	-	20

NOTE: (1) Also applied when medical intervention is necessary when urinary excretion exceeds TLV, or post unusual exposure incidents.

(2) Revised October 25, 1971.

The relationship of the frequency of abnormal specimens and the probability of the occurrence of a cyanosis case followed a typical "S" shaped probability curve. If less than 12% of the blood specimens from a given work crew are abnormal, cyanosis would not be expected to develop. The 20% control limit will detect more than 70% of potential cyanosis cases. [figure 2 (Steere, 1971)] The cases which over the past five years have occurred in a population presenting less than 5% abnormal specimens have been the sequela of unpredictable accidental product releases. The efficiency of this control basis became apparent during the first three years of application (figure 3). Not only is the relationship of abnormal frequency rate for blood specimens to frequency of cyanosis cases disclosed, but the trend line confirms the improvement in exposure control attained through improved operating procedures, better mechanical maintenance, and revised engineering design.

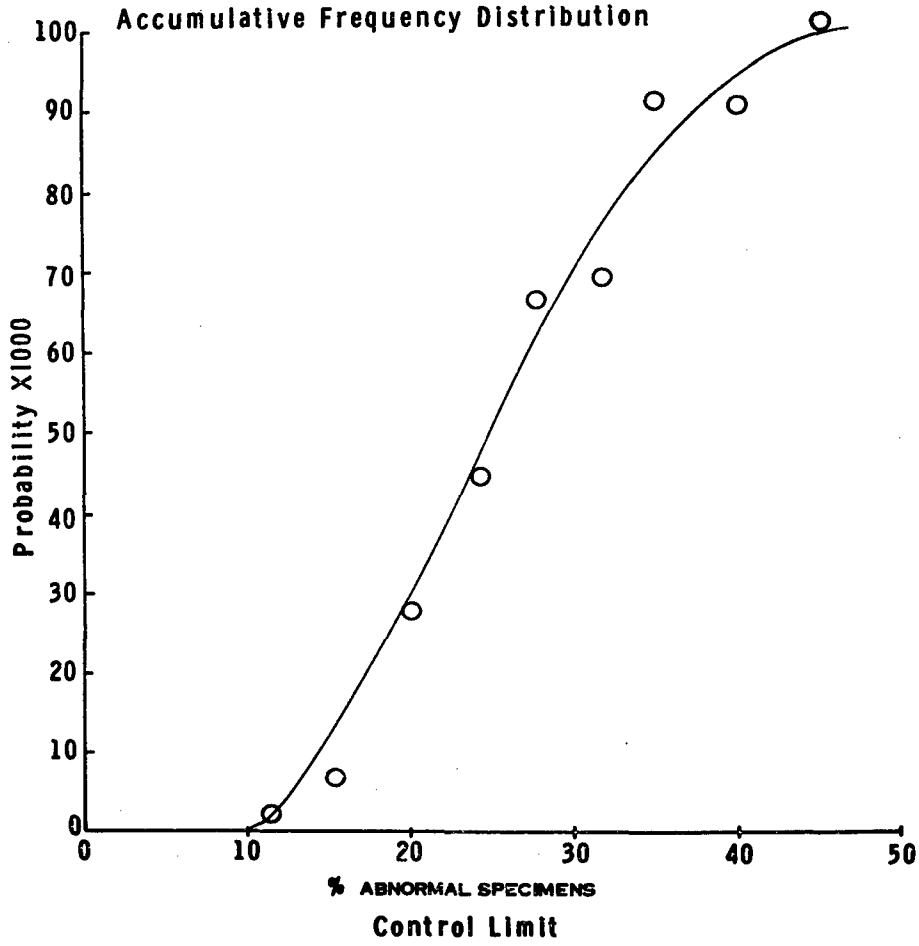

Figure 2. ESTIMATED PROBABILITY OF CYANOSIS OCCURRENCE.

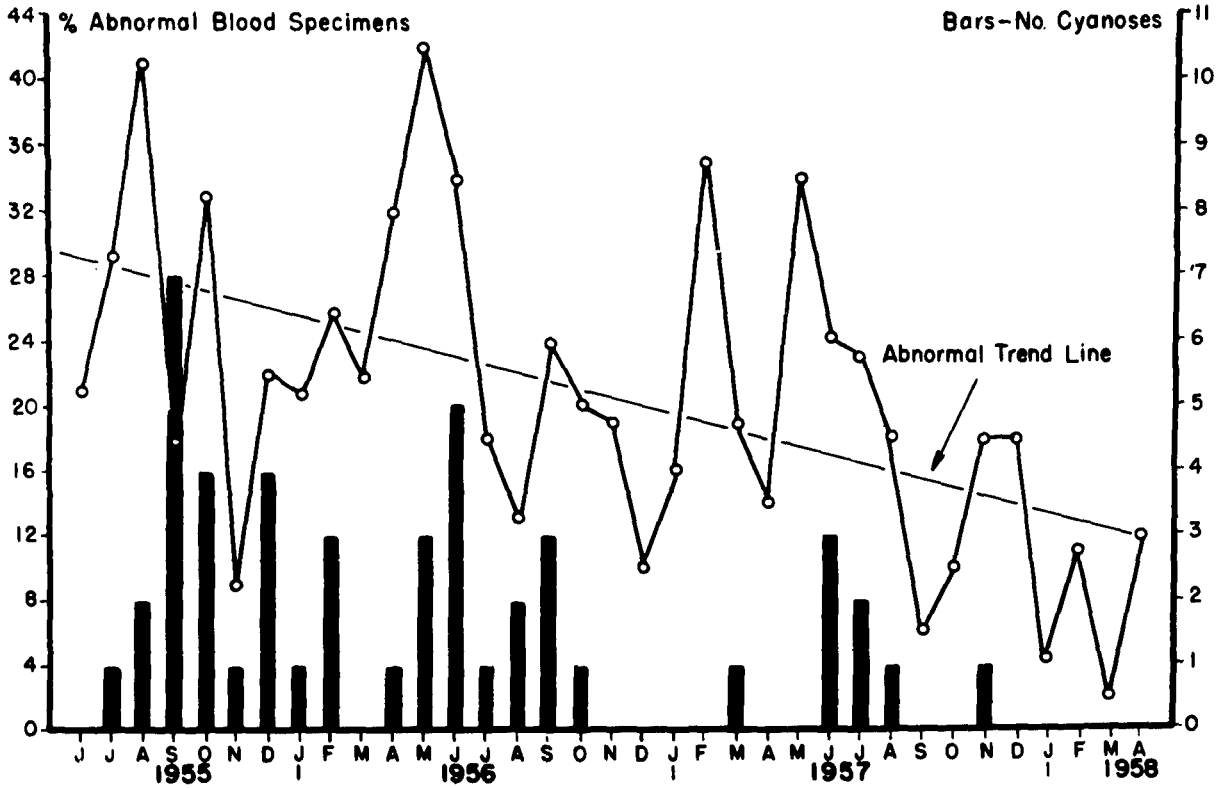

Figure 3. RELATION OF ABNORMAL BLOOD SPECIMENS TO CYANOSIS INCIDENTS.

The data from the ten-year study furnished additional information relative to:

1. Relationship between causative agent structure and biochemical potential (table II).

2. Effect of temperature on cyanosis occurrence (figure 4).

3. Susceptibility or predisposition to cyanosis.

Table II

The Chemical Cyanosis Anemia Syndrome
Relationship Between Causative Agent Structure and Biochemical Potential

Ranked in descending order of relative hazard (No. 1 most, No. 13 least potent).

Rank	Cyanogenic Potential			Anemiagenic Potential			Over-all Potential		
	Initials	Product Name	Score	Rank	Initials	Score	Rank	Initials	Score
1	OCA	ortho-chloroaniline	-	1	NB	2.0	1	DNB	3.7
	MCA	meta-chloroaniline	-	2	MNCB, PNCB	4.0	2	NB	3.9
	PCA	para-chloroaniline	-	3	MNT	4.5	3	CAM	4.2
	CAM	mixed-chloroaniline	2.3	4	PT	5.0	4	PT	4.6
2	DNB	dinitrobenzene	2.7	5	DNB	5.5	5	MNCB; PNCB	4.9
3	MNA	meta-nitroaniline	-	6	DCA	6.5	6	NA	5.5
	PNA	para-nitroaniline	-	7	NA	6.5	7	MNT	5.7
	NA	nitroanilines	4.4	8	OT	7.5	8	MT	6.0
4	PT	para-toluidine	4.6	9	CAM	8.5	9	OT	6.3
5	NB	nitrobenzene	4.7	10	AN	9.5	10	AN	6.7
6	MT	meta-toluidine	4.7	11	Polyac	9.5	11	Polyac	6.8
7	ONCB	ortho-nitrochlorobenzene		12	MT	10.0	12	DCA	6.8
	PNCB	para-nitrochlorobenzene		13	NN	-	13	NN	7.0
	MNCB	mixed - nitrochlorobenzene	5.3						
8	AN	aniline	5.4						
9	-	"Polyac"=para-dinitroso-benzene	5.5						
10	OT	ortho-toluidine	5.8						
11	ONT	ortho-nitrotoluene	-						
	PNT	para-nitrotoluene	-						
	MNT	mixed - nitrotoluene	-						
	DNT	dinitrotoluenes	6.1						
12	NN	nitronaphthalene	6.7						
13	DCA	dichloroaniline (2, 5 or 3, 4)	7.4						
gmb	2-3-64								

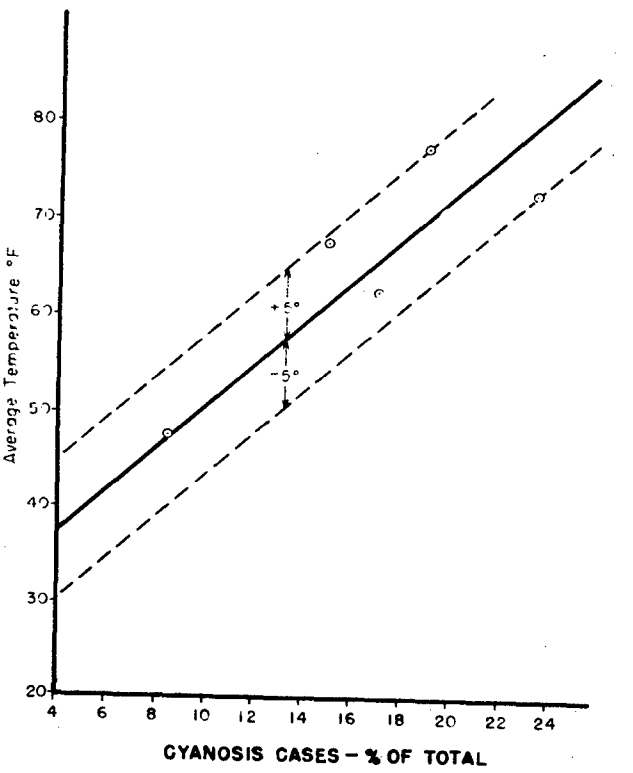

Figure 4. EFFECT OF TEMPERATURE ON CYANOSIS OCCURRENCE

In the study group of 187 cyanosis cases, we found that 30 (21%) of the 143 employees involved contributed 74 cases (40%). Eight were classified as chronic repeaters (30 cases) and removed permanently from areas of potential exposure to cyanogenic compounds. A simplified MHb reduction test is now available as a pre-employment screening test to detect these individuals. (Brewer and Tarlov, 1962)

4. A base from which a relationship between urinary excretion of the compounds and their metabolites listed in table II could be established for primary exposure control. Using a safety factor of two, biological TLV's (BTLV's) were derived from urinary excretion data collected during the cyanosis episodes and confirmed by subsequent cyanosis-free control periods (table III).

Table III

Biological Threshold Limit Values (BTLV)

[Routine Analysis Frequency - 60 Days (1)]

Compound	TLV, mg/liter		TLV for Air 1972	Suggested Revision
	Warning	Medical Intervention		
Heavy Metals				
Inorganic Lead	0.100	0.150	0.15 mg/m^3 (2)	
Tetra Methyl Lead	0.100	0.150	0.15 mg/m^3	
Tetra Ethyl Lead	0.100	0.150	0.100 mg/m^3	
Arsenic	0.30	0.60	0.50 mg/m^3	
Mercury, Inorganic	0.050	0.100	0.05 mg/m^3	
Mercury, Alkyl	0.050	0.100	0.01 mg/m^3	
Cyanogenic Chemicals				
Aniline	10	20	5 ppm	-
Chloroanilines	10	20	-	1 ppm
o-Toluidine	25	50	5 ppm	10 ppm
m, p-Toluidines	10	20	-	1 ppm
Nitroanilines (dust)	10	20	1 ppm	5.6 mg/m^3
Dichloroanilines (2,5- & 3,4-)	10	20	-	5 ppm
Chloroaminotoluenes	10	20	-	5 ppm
Anisidines (Vapor)	25	50	0.5 mg/m^3	10 ppm
Mixtures	10	20	- (3)	- (Calc.
Nitrobenzene	25	50	1 ppm	5 ppm
Nitrochlorobenzenes (Vapor)	25	50	1 mg/m^3	5 ppm
o-Nitrotoluene	25	50	5 ppm	10 ppm
m, p-Nitrotoluenes	10	20	5 ppm	-
Dichloronitrobenzenes (2,5- & 3,4-)	10	20	-	5 ppm
Dinitrotoluene (Vapor)	25	50	1.5 mg/m^3	10 ppm
Chloronitrotoluenes	10	20	-	5 ppm
m-Dinitrobenzene (Vapor)	1[illegible]	20	-	1 ppm
Nitronaphthalene	[illegible]0	20	-	1 ppm
Mixtures	[illegible]0	20	- (4)	- (Calc.

(1) Also pre- and postwork specimens are collected whenever unusual exposure conditions are encountered and to monitor "Chem-Proof Air Suit" (completely air-conditioned body Protection[3]) use.

(2) Tentative value.

(3) Calculated as aniline.

(4) Calculated as nitrobenzene.

Each abnormal blood or urine analysis is reported immediately to the employee's immediate supervisor for corrective action to reduce further exposure. Whenever more than 20% of the specimens submitted by a work crew exceed the BTLV, the supervisor of that area is warned that exposure conditions are out of control and require remedial action. This system not only provided an overall quality control program for the evaluation of exposure control performance, but also provided information relative to (1) location of those pieces of equipment or routine operations that repeatedly produce excessive exposure (2) work habits of either individual workmen or shift crews (3) detection of individuals unusually susceptible to hemoglobin damage and (4) estimation of the relative cyanogenic potential of new aniline and nitrobenzene derivatives.

Routine medical examination was carried out on a 60-day schedule for each employee assigned to the cyanogenic chemicals areas. At this time blood and urine specimens were taken and his records reviewed. This routine is repeated after each known or suspected exposure incident. During the past five years the quality of the control program has reduced exposure experience to a level where the frequency of routine visits could be reduced to three times annually and the blood analysis to the determination of Hb and HbO_2. Concurrently, urine collection frequency was increased to a monthly basis.

With the exception of the parent aniline and nitrobenzene, the cyanogenic aromatic nitro and amino compounds do not contribute an airborne hazard under normal ambient temperature conditions. Steam-borne mists do, however, require special attention. The results collected from an extensive investigation of the hazards associated with the manufacture of methylene-ortho-chloroaniline illustrates this condition (Linch et al., 1971).

Results from personnel monitoring (Linch, Wiest and Carter, 1970) indicated methylene-ortho-chloroaniline in air concentrations were only slightly above the threshold of detection (0.01 mg/m^3) on only a few occasions. During this period urinary excretion levels for the four operators involved varied from less than 0.04 to 3.8 mg/liter. Based on the estimated volume of air inhaled, complete pulmonary absorption, and 90% metabolism to unidentified metabolites, the amount of amine absorbed by each man would be six times as great as the highest personnel monitor concentration recorded (0.02 mg/m^3). Therefore, assignment of a TLV for methylene-ortho-chloroaniline in air alone would not be appropriate for health control. This study also revealed wide differences in metabolism between individual workmen as disclosed by urinary excretion of unchanged amine (Linch et al., 1971).

The effectiveness of a sound exposure control program based on biological monitoring for medical surveillance and quality control is summarized in figure 5.

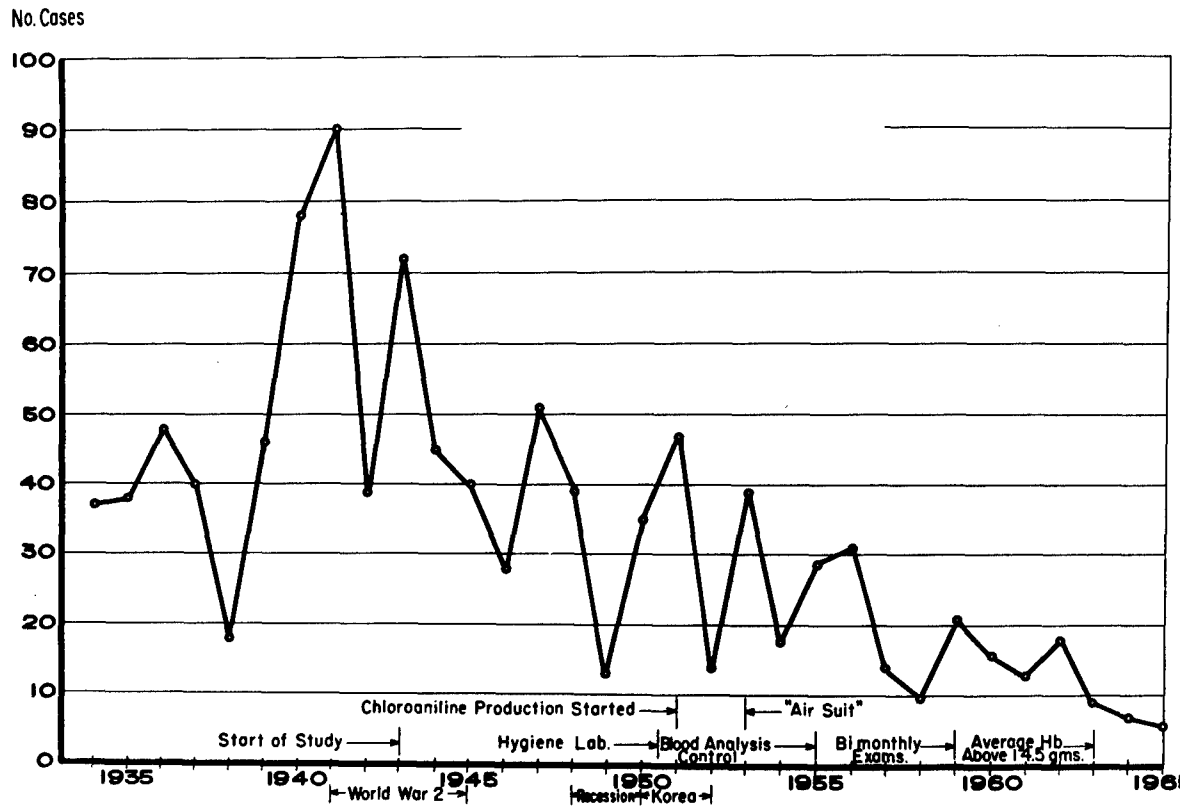

Figure 5. CYANOSIS SUMMARY ANNUAL BASIS (Chambers Works)

Improvement in chronic exposure control was seen in the increase of average Hb level from 14 gms to 15 gms Hb/100 ml during the 1964 to 1969 period. Complete descriptions of the laboratory methods, medical diagnosis, treatment, recovery, susceptibility, control and prevention are available (Steere, 1971) The suggested additions and revisions for the TLV Table for airborne nitro and amino compounds were based on the assumption that the value of 5 ppm was correct for aniline, and that the BTLV's could be used as a basis for comparison.

Validation and Revision of TLV's: Tetraalkyl Lead Study

The following discussion illustrates the use of personnel monitoring together with biological monitoring to justify an upward revision of the TLV's (Linch, Wiest and Carter, 1970).

A Notice of Intent from the American Conference of Governmental Industrial Hygienists (ACGIH) (Stokinger, 1971) received in early December, 1967, relative to a possible downward revision in the Threshold Limit Values (TLV) for tetraethyl and tetramethyl lead prompted an evaluation of the airborne lead exposure control program in the manufacturing area.

Examination of the results obtained from 77 fixed-station monitors (FSM) indicated that for the period August 14 to November 13, 1967, the TLV had been exceeded on numerous occasions. Weekly averages were above the 0.075 mg/m^3 limit in the TML area 77% of the time and in other areas primarily involved with TEL for 22% to 34% of the time. The average organic lead concentrations were 0.30 and 0.16 μg/m^3 respectively. However, based on routine 60-day physical examinations and urinary lead excretion data, the health record was excellent during this period. Less than 10% of the employees excreted more than 0.1 mg lead/liter. In a crew of over 450 employees, only nine men exceeded a urinary excretion level of 0.15 mg lead/liter and none reached or exceeded 0.2 mg lead/liter. Medical histories of employees in either the TEL or TML operations disclosed no significant differences from employees in other Chambers Works areas.

One of the possible sources of discrepancy between the observed atmospheric lead levels and urinary excretion may be found in the improvement of the procedure for detection of organic lead in air. On the premise that the average collection efficiency was 50%, adoption of the aqueous iodine monochloride reagent (Hamblin, 1967) would double the apparent lead concentrations on which the TLV's were based.

Du Pont's highly successful lead exposure control program is based on urine analysis rather than air analysis which has major process control applications but only a minor secondary role in health control. The obvious conclusion that the fixed-station monitors (FSM) may not disclose the true inhaled air concentrations of lead in a highly variable ambient work atmosphere appeared to be valid.

After a review of our survey the Threshold Limit Committee raised the TLV for TEL from 0.075 to 0.100 mg/m^3 and TML from 0.075 to 0.150 mg/m^3, with an appended footnote, "For control of general room air; biological monitoring is essential for personnel control" (Stokinger, 1971).

The personnel monitor is shown assembled in figure 6, and the component parts in figure 7.

The inorganic (particulate) lead was collected on filter membranes and analyzed by the standard dithizone method. The organic lead vapor was collected in 0.1M iodine monochloride and analyzed by a modified dithizone procedure.

The component parts were assembled as shown in figures 6 and 7. The pump was attached to the operator's belt in a position which would least interfere with the wearer's work assignments. The collection assembly then was attached to the wearer's shirt as close as practical to the employee's breathing zone.

Sampling was started when the employee left the changehouse and continued until he returned for lunch break. After lunch, the monitor was replaced and sampling continued to shift end. Six and seven monitors were operated simultaneously on each day shift in work areas selected to include the widest possible range of activities. Each operator selected was monitored for five days on his day shift rotation. This rotation presented a maximum of individual differences in lead metabolism.

Urine specimens were submitted 24 hours before start of monitoring and then at the end of each shift.

The fixed-station monitors were operated where personnel monitors were being worn.

Linear and multiple regression analysis of daily individual results disclosed no correlation between fixed station and personnel monitors. Regression analysis also disclosed no correlation between individual paired urine and monitor results on a daily basis. With few exceptions, personnel monitor results were significantly higher than corresponding fixed-station results. Displacement of the urine results by 24 or 48-hour intervals to account for the lag in urinary excretion after exposure likewise produced no correlation.

An approximate linear relationship was found by converting the weekly averaged personnel monitor TML and inorganic lead results to TLV coefficients (Brewer and Tarlov, 1962). The sum of the coefficients (TLVC's) then was related to the averaged urine analysis for the operator who wore the monitor (figure 8).

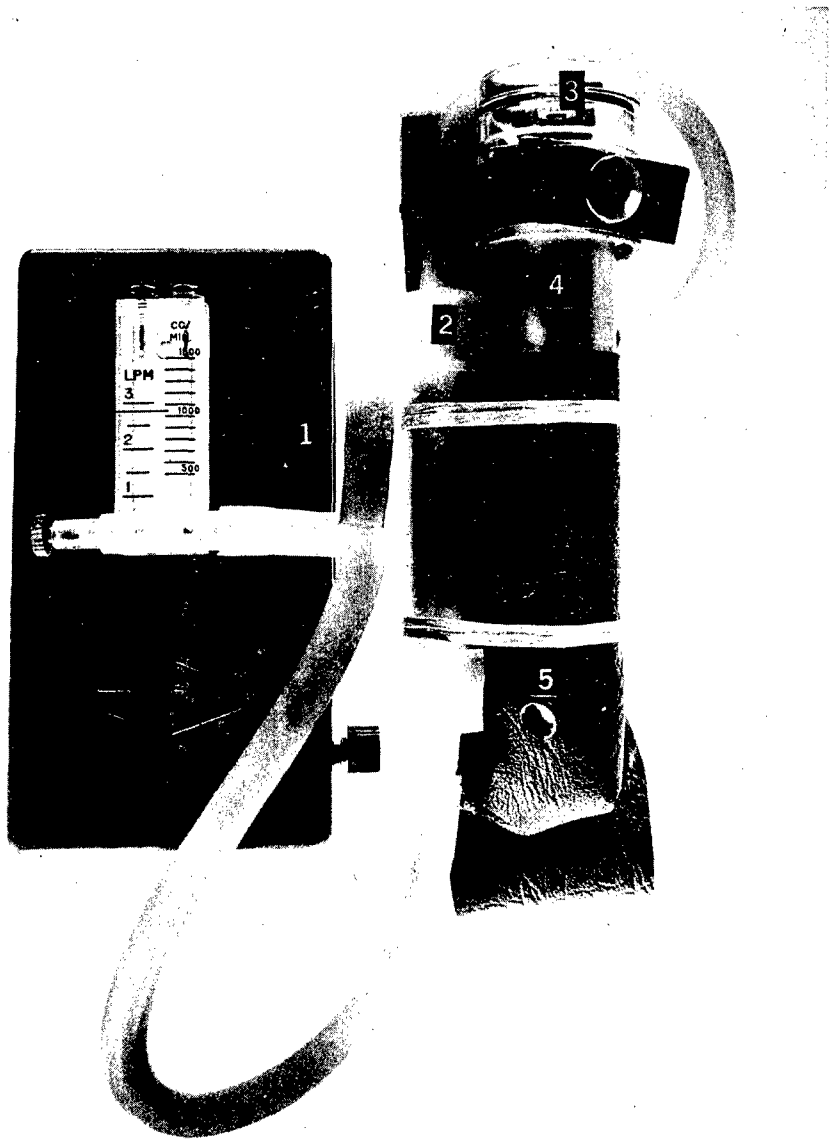

Figure 6

1. By-pass capillary for "Unico Mighty-Mite" Air Sampler

2. Activated carbon trap protection for the Air Sampler

3. "Millipore" Monitor - 0.8 mμ pore-size filter (Cat. No. AAWPO3700).

4. Aluminum shield containing the "Spill-Proof" Microimpinger - sight port at 5.

5. Coated fabric carrying case - attached top and bottom to wearer's shirt by "safety pins."

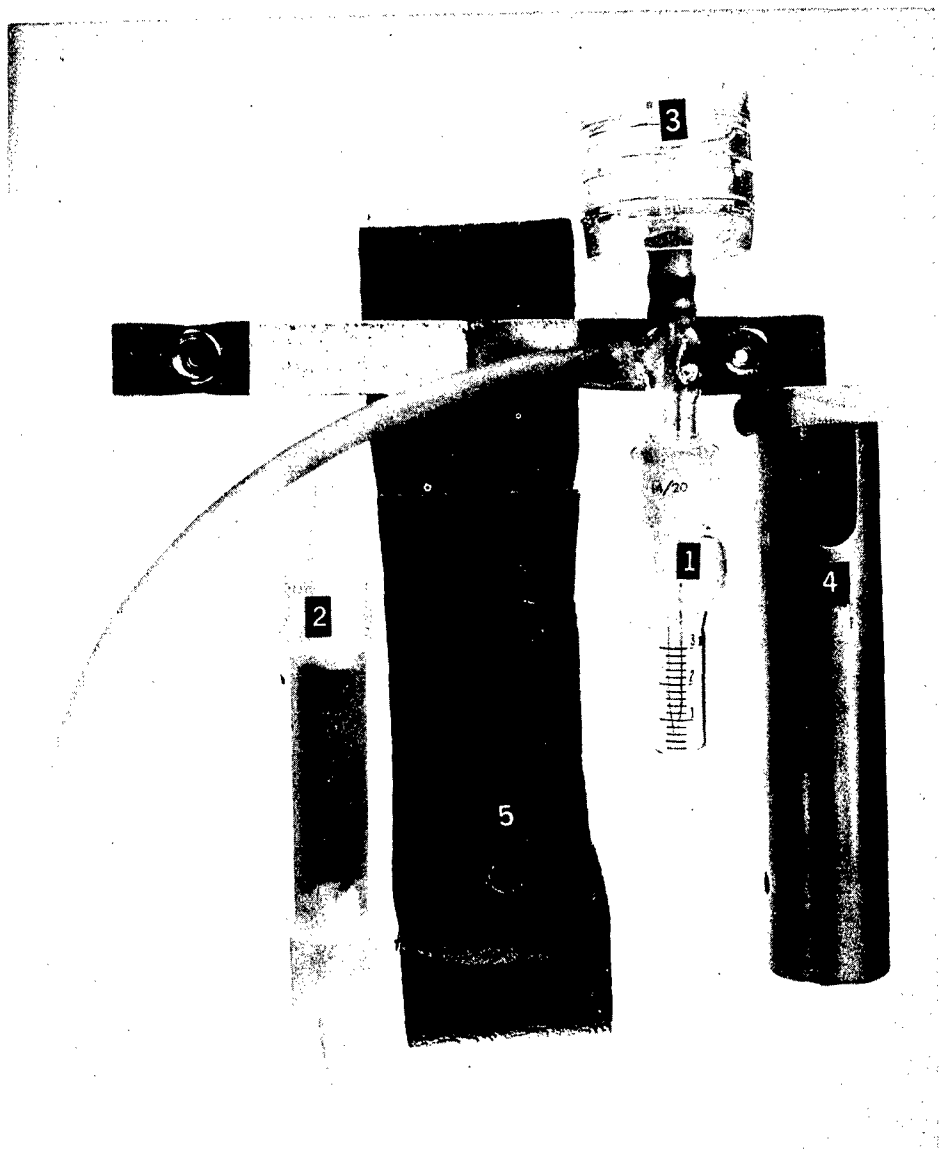

Figure 7

1. "Spill-Proof" Microimpinger (AIHA Journal, 28:497).
2. Activated carbon trap - polypropylene.
3. "Millipore" Monitor - butt seal to inlet tube of the microimpinger.
4. Aluminum shield - rubber band retainer at the top.
5. Coated fabric carrying case.

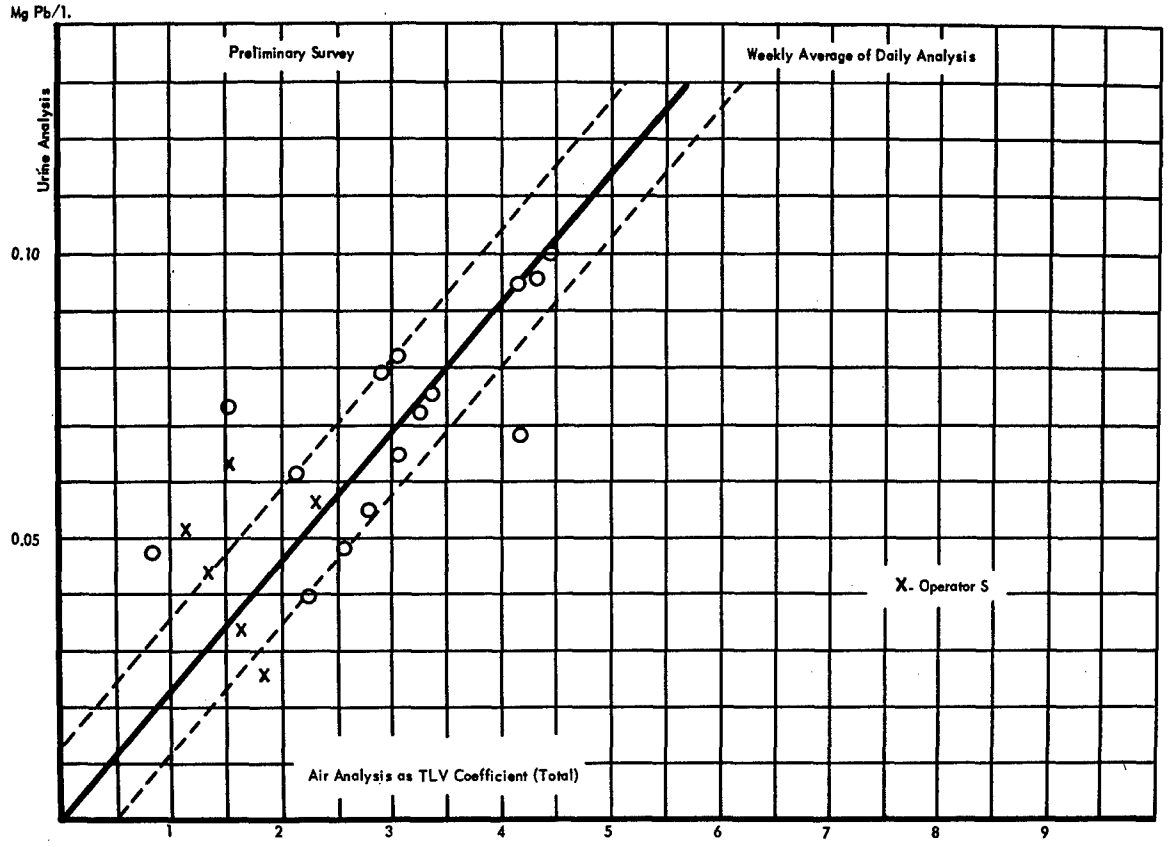

Figure 8. TML - RELATIONSHIP OF TLV COEFFICIENT TO URINARY EXCRETION.

Seventy-two percent of the points were included between $\pm$ 0.01 mg/liter limits established for the urine analysis deviation range (95% confidence limits) as indicated by the dashed lines on each side of the curve. The TLVC may vary $\pm$ 0.25 without significantly affecting the absorption of lead.

Excursions of the time-weighted average lead-in-air concentrations exceeded four times the combined TLV before the average urinary level reached 0.10 mg/liter (figure 8).

On an overall basis in the TML operation, the average organic lead-in-air analysis was more than twice the current TLV, but the average urinary lead concentration was not significantly elevated above high normal. Ten percent of 115 urine specimens gave results above 0.10 mg/liter, but none above 0.15 mg/liter.

Airborne lead levels for the TEL operation were 0.120 mg per cubic meter (1.5 times TLV). Again the average urinary lead was not significantly elevated above normal. Only 3.5% of the urine specimens contained more than 0.10 mg lead/liter and none exceeded 0.15 mg/liter.

The three-month preliminary survey was terminated at the end of April, 1968. Routine monitoring was installed the first week of October, 1968. Daily results obtained from urine and air analysis were averaged on a monthly basis through the month of March, 1969, to obtain sufficient number of urine analyses for comparison with personnel monitor results. The groups under study averaged 40 operators, each of whom submitted a urine specimen every 60 days at the time of their routine medical examination. Seven personnel monitors were in continuous use. Since the operations are carried out in a horizontal profile, the men and monitors were assigned by floors within the manufacturing buildings.

The averaged organic and inorganic lead results from the monitors were converted to coefficients and the sum (TLVC) used for statistical analysis. The revised TLV's were used in the calculation of the coefficients to determine whether further relaxation of the limits might be justified.

If the urinary excretion averages are plotted versus the previous month's TLVC's a reasonably good linear correlation is obtained (figure 9).

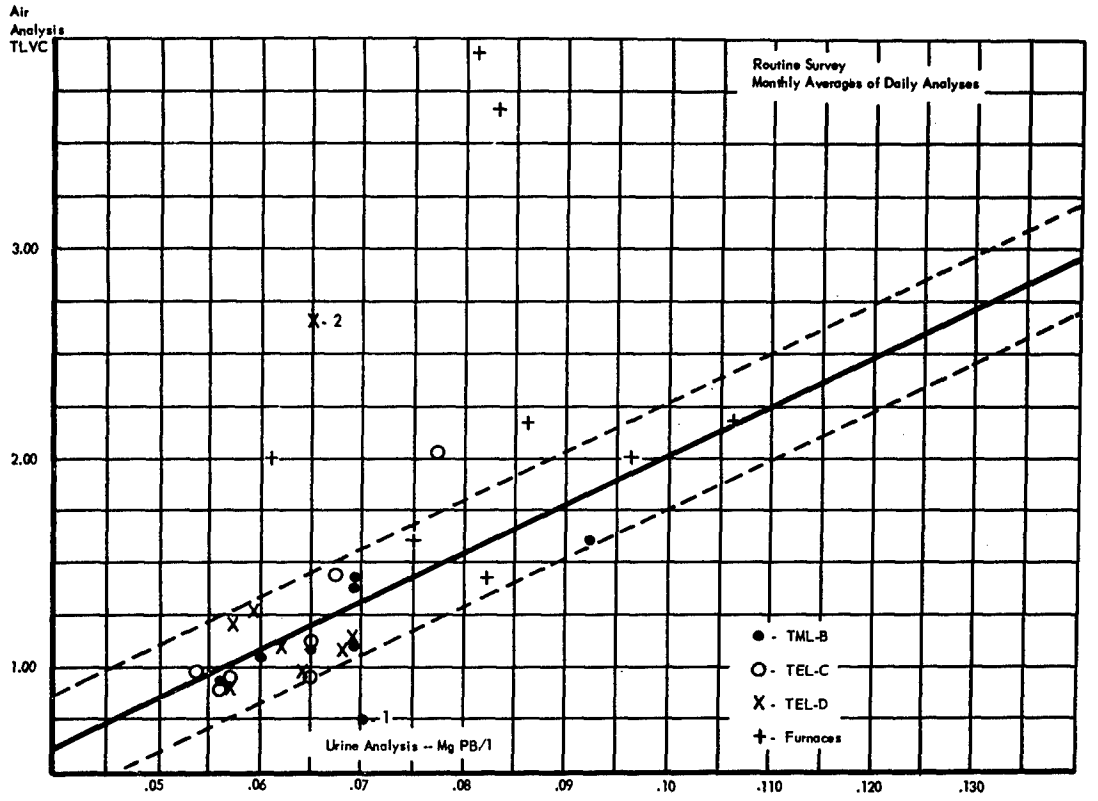

Figure 9. RELATIONSHIP OF PREVIOUS MONTH TLV COEFFICIENT TO URINARY LEAD EXCRETION

If the furnace crew was deleted, "conformance" of the plotted points increased from 78% to 86%. These results confirm the safety factor incorporated in the revised TLV's and indicate that even these new limits can be exceeded by a factor of two before the average urinary excretion rate reaches 0.10 mg lead/liter.

The current exposure control program based on urine analysis and medical history provides satisfactory health protection for employees engaged in the production of tetraalkyl lead compounds.

Fixed-station air monitoring does not provide valid results required for organic lead exposure control based on air analysis.

Although personnel monitoring established an approximate relationship between airborne alkyl lead concentrations and urinary lead excretion, such a program is costly to administer and the equipment is a definite source of annoyance to operating personnel.

The revised TLV's can be exceeded by a factor of two before the urinary excretion control point is exceeded. Since this 0.10 ± 0.01 mg/liter urinary lead level is one-half of the usually accepted hazard level of greater than 0.20 mg lead/liter, further upward revision of the atmospheric TLV would be justified.

From the foregoing discussion one inescapable conclusion has been reached. Biological monitoring of man himself is the basis upon which any other procedure must be based. Not until personnel monitoring is correlated with absorption rate, absorbed dose related to degree of physiological response and epidemiological data evaluated can a valid TLV for airborne toxic substances be established. In many cases as we have demonstrated, air analysis alone, even on a personnel monitor basis, is not enough to guarantee freedom from occupational illness.

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OPEN FORUM

DR. FRIESS (National Naval Medical Center): We'll declare the Open Forum session open now. Questions, comments, diatribes from the floor, and potential answers and cross speech from the panel up here. May I ask that each of you preface your remarks with name and affiliation and use the traveling mike which will be handed to you so that your comments are recorded on tape. The session is open - questions from the floor?

CDR. LAWTON (Department of the Navy): I'd like to ask Dr. Stewart to give us some examples of the case where the venous blood doesn't give as good a reading as the exhaled breath sample in a toxic situation.

DR. FRIESS: That's a good question, but Dr. Stewart disappeared shortly after his talk and isn't available to answer; but I heard the comment that he made and I know of no such phenomenon. Is there any information on it from the audience or the podium?

MR. SHERWOOD (Esso Europe Inc.): I can perhaps quote one example from benzene, sir, where we measured the benzene concentration and the vapor phase over venous blood and found it to be identical to that in exhaled breath.

DR. FRIESS: You spoke of the variability which you get in venous blood for the same subject at various times.

DR. VERA THOMAS (University of Miami School of Medicine): I think the correlation with the alveolar air should be completely perfect. It means if you take the last trace of the exhaled air that is supposed to represent alveolar air, it should be perfect, and it always is, and so maybe it depends on how the samples of air are collected.

DR. AZAR (Haskell Laboratory, E. I. duPont): I'm not sure of the reference on this but I believe what Dr. Stewart was referring to was that like certain of the fluorinated anesthetic agents, the alveolar breath sample is more a reflection of the arterial blood concentration. If you've got an individual with a real fat extremity, the arterial blood may leave the anesthetic there in the fat. It's very fat-soluble. Then if you take a venous sample, it won't really be representative of the true alveolar breath sample. That's been shown with some of the fluorinated anesthetic compounds.

MR. WANDS (National Academy of Sciences): I wanted to ask Mr. Sherwood if I understood him correctly this morning when he spoke of the benzene mixtures with other hydrocarbons. It acted essentially as free, pure benzene - no interaction

with hydrocarbons? What kinds of hydrocarbons were these? Same volatility range essentially as gasoline?

MR. SHERWOOD: It was gasoline, but of course the ingredients were pretty mixed and the volatility range was not by any means the same. Quite a lot of it was made up of toluene which is appreciably heavier. It was perhaps of interest to note that in the inhalation system, though the benzene was 5% of the liquid gasoline by volume, it was 10% by volume in the air bubbled through the gasoline.

MR. WANDS: That follows normal vapor pressure laws certainly. I wonder then if our anesthesiologist would care to comment on any interactions between mixed anesthetics. I'm concerned about this business of mixtures and the buildup in the depots - this sort of thing. Do we have any knowledge here?

DR. VERA THOMAS: No doubt drug interaction experiments are very difficult things to do. When I saw the influence of the other drug (the other hydrocarbon) that Mr. Sherwood used, I would consider the effect can be in two places. First a gas effect, when the drug is absorbed more air enters the alveolus because you depress the volume of the gas in the air by the amount that you absorb. The other effect I would consider then is the effect of the activity of the microsomal enzyme. The induction of microsomal enzymes by a lot of drugs has been observed. Mr. Sherwood gave an example of ethanol, and talking about anesthetics, I know that ethanol can have two effects. Some people say that it can be a microsomal enzyme inducer. I couldn't prove this, but I could prove the inhibitory effect. I did an experiment where we administered fluorinated hydrocarbons as an anesthetic dose to mice - control mice and mice which I made drunk by intravenous injection or by stomach administration of ethanol - and then I had a control group which was raised on 5% ethanol in drinking water. Since these hydrocarbons were metabolically defluorinated, and since approximately 50% of the fluorine goes to the bone, we determined the fluoride concentration of the mice. The animals which were pretreated with the chronic exposure to ethanol didn't show any changes from the controls in the increase of anesthetic fluoride, but the ones which got anesthetic while drunk from ethanol showed much lower increases of fluoride in the bone. We assume this was an inhibitory effect of ethanol on the microsomal enzyme. I don't think either mechanism should be neglected and I think this is one of the most difficult things to predict.

DR. FASSETT (Eastman Kodak Company): I was intrigued with Dr. Thomas' pictorial representation of the compartments of the body in relation to solvents and so forth. I had one question about the space between the lung and the container called blood. There is a rather important pump there called the heart and I'm wondering if this shouldn't be considered in this pictorial attempt to view this phenomenon, because certainly if you have a compound with a low blood-air distribution coefficient, it's going to reach saturation very rapidly; then the thing that determines the level is the cardiac output. We know that cardiac output of 6 liters/minute is all right for resting people. But, if you have somebody in trouble diving

into a machine or other emergency, it could go up as much as 5 or 10 times sometimes. So I would think that this would be considered. I suppose that in your actual mathematical formulation you had a figure for blood flow, but in the acute phase certainly the cardiac output is of major importance.

DR. VERA THOMAS: Yes. I didn't demonstrate it because I don't yet have enough data for it, but it's one thing I'm now working on. This pulmonary cardiac index - it means if you take cardiac output and you divide it by the ventilation, the factor is, at least according to physiological references, 1.25; and if you assume that the excess blood flow then is to supply mainly the muscle compartment, so it should modify the curve. I just recently finished a program which will be able to handle changes in ventilation, at least I hope, and I have to verify it again by experimental data. And I think one of the good sources of experimental data will be Mr. Sherwood's paper, and perhaps also Dr. Stewart's data. Because I don't now have access to occupational solvent data any more. Then as well, as I said, to consider different physiology - if a person is rather obese or a skinny person, and so on. I finished this program just about two days before I came here. When I get back, I will be anxious to test it. I know that there are people who will help me to get the experimental data from their experiments. Mr. Sherwood will be testing this too and maybe by the next meeting I will be able to report something about it.

DR. FASSETT: I think it would be very interesting to apply this to the case of the things that might be anesthetic to the point of interrupting cardiac output going to the brain.

DR. VERA THOMAS: This is what I learned from anesthesiologists - that the brain belongs to the vessel-rich group. But once you raise cardiac output, I think that you who are physiologists will agree with me that it goes mainly to the muscle compartment.

DR. SMITH (Federal Aviation Administration): I can see how excretion levels and circulating blood levels of the contaminant can be a good indicator of exposure, but it disturbs me a little bit when we consider the use of circulating enzymes as an indicator of exposure levels because of their normal variability from one person to another. If you're following an individual through the opportunity for exposure, then I can see how LDH or cholinesterase or some other function can be used as a gauge of exposure. But if you survey a human subject after opportunity for exposure, how can you use this enzyme activity as a valid indicator? You must work on the basis of human population means which leaves a great room for an individual response that does not fit this pattern.

DR. STOKINGER (U. S. Public Health Service): Yes, you're quite right. These things function best when you have a baseline prior to exposure, but this is not very often obtainable so you just have to resort to an average or a norm of the population of the same age and general background. Occasionally, of

course, you can do this if you have a good medical department and that's what we're advocating - to do more of this type of work in the preplacement job examination. If you have a bunch of mercury miners, it would behoove you to make these tests on them and get their individual baselines prior to working in the mines - the way we're advocating doing these hypersusceptibility tests prior to job placement, you see. You're quite right - this isn't done as often as it should be and I hope this will be a thing for the future.

DR. DREW (National Institute for Environmental Health Services): Dr. Thomas, do you have any data regarding the metabolism of benzene? You mentioned, as I recollect, that approximately 80% of the benzene was metabolized in the liver and I presume that the remaining 20% would be metabolized by extra hepatic microsomes. Do you have data to support this or is this a hypothesis?

DR. VERA THOMAS: No, I have certain data to support this, and this is the work which I did in vitro where we minced organs and added benzene to them under aerobic conditions and looked for the places where the phenol is created. We did this work in Prague. I think I never published it - with my own moving between the United States and Czechoslovakia. But I have a paper, I think I even have a slide here, which I can show you. Would you like to see slides, anyone?

DR. DREW: Well, we can discuss it later, but I'm very interested in benzene metabolism.

DR. VERA THOMAS: We also found significant activity in the kidney, for example.

DR. BACK (Aerospace Medical Research Laboratory); Dr. Stokinger, I'd like you to address your thoughts to what is the best test to be used for a single compound if we have 10 parameters we can look at. Which biological parameters are you going to pick? Let's say we have a compound which affects methemoglobin formation, we can also find out it may decrease total cholinesterase, we also may find that it works on 6GPD, we also may find that it depresses microsomal enzymes of one kind or another. There are many things that a compound may do and you're going to set a biologic threshold based on which one of those - the one that is the most easily obtained, the one that has the most significant physiologic function? A case in point, hydrazine and unsymmetrical dimethylhydrazine, which we're working on right now, cause effects on diamine and monamine oxidases. One of the ways of finding that out is to give an animal putrescine, and look at how putrescine is then handled. The amount necessary to deplete the metabolism of putrescine to zero is below any lower threshold limit value that we can envision, and yet it is an effect. Now, whether it has any physiological function is of grave doubt. On the monomine oxidase side, we can give an animal methylamine and then use a very small amount of hydrazine and stop the metabolism of methylamine. All other parameters that we look at under the same dose effect gets us nothing. Now, is that the answer we're looking for?

DR. STOKINGER: Well, I would think we have a possible precedent in an answer to your question in the slide I showed you of the five biologic responses to lead in man. There we had, if you noticed in the last line, the delta-amino levulinic acid dehydratase, which is probably the most sensitive indicator or response to lead, yet it turns out in using it for evaluating overexposure to lead in industries to be far too sensitive and we go to delta-amino acid in the urine as the better indicator of more true response. It's too sensitive. This, of course, comes about as a result of a great deal of extra work. You have indicated through your putrescine business that you have to know some other facts. The facts in this case of not using the dehydratase for industrial overexposure to lead came about in the following way from duPont workers. They showed a couple of years ago that when they gave animals a dose of lead that would result in complete inhibition of this delta-amino levulinic acid dehydratase, and then bled these animals with appropriate controls to 50% of their blood volume, and then watched the return of the blood cells to normal levels, there was absolutely no difference in the animals that had the completely inhibited dehydratase from the controls that had no lead and, therefore, they said on the basis of these experiments that they didn't feel this was a good method - it was a more sensitive one - but that doesn't mean it had any real physiologic significance in measuring overexposure to lead. You'll have to work out details in individual cases, I think. That's the best I can answer off the top of my head.

MR. LINCH (Consultant - Environmental Health, Safety Engineering): I'd like to supplement Dr. Stokinger's remarks on this relative to the practical application in our manufacturing areas. We were asked to look at delta-amino levulinic acid in urine as an indicator of excessive lead level and we found that the delta-amino levulinic acid content was not significantly elevated until the urinary excretion level got into the medical surveillance range at 0. 2 mg/liter, which we don't permit. So by using our warning level of lead in urine, we were able to maintain the lead exposure below the concentration that caused any significant elevation of the delta-amino levulinic acid, so we dropped that test.

DR. FRIESS: Dr. Back, I'd like to enter this one on the issue of the rejection of a biological indicator because it's too sensitive. That rubs me a bit raw at the moment, and I would like to ask whether one could use the theme that you accept the most sensitive biologic indicator as long as the tissue lesion which it produces is reversible - the most sensitive one associated with a reversible lesion. Would this be operational in your view?

DR. BACK: No, I think it's still much too sensitive.

DR. FRIESS: What is your idea then on the level at which you reject the test as being too sensitive?

DR. BACK: That's what I was asking you folks up there.

DR. FRIESS: You got a partial answer.

DR. BACK: Well, I can tell you this. This dose level that we're talking about, let's take hydrazine, for instance, or take monomethylhydrazine. Monomethylhydrazine at .04 millimoles per kilogram, in a single dose now, produces about 100% decrease in the ability of an animal to metabolize putrescine to CO_2. CO_2 comes off as radioactive CO_2. You're using radiorespirometry as your indicator. Well, .04 millimoles per kilogram of MMH is way below the threshold limit value for an 8-hour workday, 5-day workweek, and we've already human tested 900 ppm-minutes, finding as our most sensitive indicator, Heinz body formation, if you will. Now, as a Committee, we're going to have to set limits based on known facts. Well, we know two facts. One of them is that we can find an effect way below the present threshold limit value which is for an 8-hour workday, yet we're trying to set the limit for 10 minutes. Now, what do we use for man rating? We've already man rated it at the request of members of the Academy at 900 ppm-minutes. They weren't worried too much to begin with about small effects that one could notice, but whether a man could really breathe 90 ppm for 10 minutes or whether it was too irritating for self-help, etc. So we do have an end point there. A man can indeed breathe 90 ppm for 10 minutes with no great untoward effects except that he will have some Heinz body formation which is reversible. Well now, both monoamineoxidase inhibitions and Heinz body formation are reversible. One of them is some thousand times more sensitive than the other. It's almost like setting a threshold on a zero value. Our tests are getting more and more sensitive.

DR. STOKINGER: The way I would like to handle it would be to do two things. One, you consider your earliest reversible indicator as your warning limit but then you must do a second experiment to find out the biologic significance of each of these responses. Now, if there's no physiologic significance to 10% or 25% cholinesterase reduction, or your putrescine changes, then you set your medical intervention level at a higher level than these indicators would indicate. You use them as warning levels and then you've got to find out what are your indicators that give an undesirable physiologic response on repeated exposure. You have to do a longer term exposure.

DR. ANTHONY THOMAS (Aerospace Medical Research Laboratory): You must remember that this work has been primarily for DOD and NASA use. All we are trying to avoid is irreparable damage or serious decrement of performance where an individual couldn't handle a missile system or something like that. And I don't think the concept of biological TLV should apply at all to emergency exposure limits because the whole philosophy of emergency exposure limits is that it happens very rarely in the lifetime of an individual. With TLV's, you are trying to protect the worker who is going to work at his job for 30 years or more. I don't think in an emergency exposure situation we can look at the most sensitive indicator. You've got to look at organ functions.

DR. FRIESS: And there the concept of reversibility is important, Dr. Thomas. The exposure might be once in a lifetime, but you accept no damage which is irreversible.

DR. BACK: Well, one thing just to clear it up. The limits I was talking about - half of it was for the military but the other half was for people outside the fence. These were PEL's that we're setting for EPA and other people, so it involves total population, not just the Air Force population. But they are for a short term. Now, we've brought time into the situation, and Dr. Thomas recognizes that time is of great importance here because we're not saturating anything, we hope, for these short periods of time. Therefore, the absorption curve is a great deal different from that which you see with long-term, continuous exposures or 8-hour exposures.

DR. CULVER (University of California): I'd like to take half a minute and set forth a concern I have about biological threshold limits and their application to the real world of protecting people in the work place, if I may. I categorize the strategies for the protection of workers roughly into three areas. The first is isolation, and this is whether it is putting the isolating device on the individual with respirators, protective clothing, or whether it's using glove boxes or other means of isolating the hazard. The second broad category of protective measures is environmental control, and the third one seems to be the one we're talking about today, which is biological indicators and medical management. If you look at these three categories in terms of how they're applied to protecting people in the real world and if we draw a scale from no effect to death, and (this is getting close to some of the things that Dr. Back is talking about) place reversible and irreversible symptoms on that scale where you have physiological change at a low level, and we look at where we are trying to protect people with these various strategies of protection, we find that the isolation measures are really trying to protect them down in that level. The environmental control and TLV's are really working more someplace just below physiological change up through symptomatic changes, hopefully below reversible change, with some few exceptions. As I listen to the discussion today on biological indicators and medical management, we're starting at physiological change probably, or maybe just a bit below if we're measuring the accumulation of a toxic material in the body prior to the time that it causes this physiological change and we're going up and sort of titrating ourselves close to irreversible change. It seems to me that as we try to develop more and more sophistication in the monitoring of workers and their health, we're also trying to push their exposure limits up and up. I get that sense a little bit. I would hope that is really not the true case. In any event, if we are going to use biological indicators, if they are going to come into practice, then they have to come into practice concommitantly with much greater, closer control of the worker. Now, in this country, someplace between 60 and 80% of our workers are occupied in small plants where there is no professional control in terms of people observing people. Hopefully, there is environmental control in terms of slot exhaust and things of this sort. But, we need to make it very clear, I think, to people, if they do accept biological TLV's that these can really only be applied in your large plants - your duPonts, your Eastman Kodaks, places that have good, sophisticated industrial hygienists, physicians, and the like. When you get out where there is

less possibility for the control of people, then we have to continue to emphasize these methods of control, isolation of man from the environment or means of protecting the man through control of the environment. One last point, then I'll give up the microphone. As we approach a threshold between reversible and irreversible change, and I hope our toxicologists are good enough to be able to allow us to pinpoint that boundary, and if we control workers' exposures to that level, then we are allowing less and less opportunity for accident to occur in the work place. And, believe me, accidents occur abundantly in the small plant work place. By accidents I mean unexpected spills, a worker who makes a mistake and turns the wrong valve, that sort of thing. I think we need to have a cushion between the level that we control people at and the level that is going to produce symptoms or damage, to allow for the real world operating conditions that we find out in industry. Thank you.

DR. FRIESS: All right, can we take a crack at that?

DR. HENDERSON (Olin Corporation): I'll take the other side because this has been treated as an either/or situation, and I don't think we operate on that basis. We don't operate either isolation or environmental control or biological monitoring. We operate with all three of these. Biological monitoring, as Dr. Stokinger has pointed out, is our ultimate backup to be sure that these other systems are working. There are many cases where we haven't gotten good enough environmental monitoring to be absolutely sure of our control, so we want this as a backup. So I think that we have to look at this not as an either this, or this, or this, but as complementary systems that give us greater assurance that all of our systems are working. Now, I agree wholeheartedly that we want a cushion on this. We cannot push our people right up here. We can't, if we're working with organic phosphate esters, push them on their cholinesterase where they've got no reserve. We have got to monitor them and be sure that we're keeping them with a reserve and pull them out of exposure before they use up their reserve. This is the advantage of biological monitoring because it tells us that we're getting a breakdown somewhere. So look at this as a total system, not as isolated packages.

DR. STOKINGER: I'd just like to make a little correction. I think the point was missed in my paper this morning that the biologic threshold limits are the counterpart of the threshold limits for air. That's why the similar nomenclature. So there is a cushion with a safety factor proportional to the magnitude of the seriousness of the response. We're not pushing anything up to the dangerous limit by setting biological threshold limits. That's not the intent. The way Dick Stewart is doing it for us is that we're exposing individuals, taking breath analyses after biologic threshold limits, and at two other concentrations that bracket this limit, the threshold limit value for air, so that the responses we get are pegged to the industrial air standards.

DR. CULVER: Is that true of lead, Dr. Stokinger, if you put a man in 0.15 mg/m^3 of lead and dust? Are you going to expect to see 80 micrograms of lead in the blood?

DR. STOKINGER: Yes, sir. And 150 micrograms per liter of urine.

DR. FRIESS: There's one point of confusion that the chair has at this point in the biological sector that you showed as your last bar. I took our conversation of the past few minutes to mean to depress that upper limit down as far as possible, but find a realistic point beyond which you shouldn't depress it further because your measure was then a really nonimportant physiological measure. Well, I took it to mean that we're attempting to press that bar down to very low levels of detection of a change as long as the change is still of physiological significance. I didn't see it rising as high as you have it.

DR. CULVER: I think that your attempt is a good one. I'm concerned that as industrial medicine is practiced out there in the hinterlands, the physicians and others who have not perhaps the level of sophistication of the committee at the table are going to be using those levels as a means of titrating the man up to as high a level as he can without getting into trouble. I've seen that happen.

DR. FRIESS: It should be avoided, don't you think?

DR. CULVER: It certainly should be avoided and I think there should be something put into the biological threshold levels if they are set forth by government agencies to discourage that practice.

MR. LINCH: I would like to interject the voice of industry at this point. Number one, I think that perhaps you missed one of the points in the paper I read this morning that we use the biological threshold limits for an entirely different purpose and that is as a quality control program as the statistician defines quality control. It's an attempt to determine how effective these preventive measures are and we set our TLV's at one-half the level which is ordinarily expected to show some physiological response. The second point I would like to make - I think you are going to have to depend more upon industry than perhaps in the past to determine a practical threshold limit value. For example, going back to lead again, with urinary excretion versus delta-amino levulinic acid. We are perfectly happy and can live with this limit of 100 micrograms per liter of urine. It's not causing any particular financial difficulty and yet that's a level below that at which delta-amino levulinic acid is elevated. Now, another point I'd like to make on the isolation. This may be the practice in some plants but it's not the practice in duPont's plant. We use protective clothing to send men into lethal environments. We use our chemproof airsuits, have done so for 20 years without injuring anyone. So we do use protective garments to go into environments where engineering control isn't possible or has broken down.

MR. WANDS: I'd like to throw a slightly different question at both Mr. Linch and Dr. Stokinger. These gentlemen have suggested the use of the biological values, enzymatic changes, and various other parameters as preplacement or perhaps even preemployment screening devices. Do you anticipate any legal difficulties or any union difficulties with this technique? We're beginning to see some problems with the sickling phenomenon and the social and legalistic and all the other problems we're running into here with the use of preemployment, preplacement screening for the sickling phenomenon.

DR. STOKINGER: There is one difference in the legal implications and the population at large with the sickle cell anemia trait. And that is I don't think you have to, as an industrial physician or whoever takes the sample and evaluates it, define or tell the person the reasons why you're giving him a different placement in this job. Whereas the point of the matter is in the population at large, they know very well why you're doing this and all the implications of it. But I don't see any reason for informing the individual why you gave him the job you did.

MR. LINCH: Well, speaking from 20 years' experience with duPont, we have each year tightened up our specifications on Class I employees, and now include limitations on where a man can work depending upon his human physiology. As I had mentioned before, in our cyanogenic areas we put a man on a conditional transfer when he goes into the area and he is very closely followed by medical to determine whether in the new work areas, the cyanogenic areas, he shows any unusual response. Now, as far as the union difficulties are concerned, the reaction has been in the other direction. If we fail to apply these tests, we will have the union steward coming into medical and asking why. We attempted, for example, to desist in our urine sampling program in an area where we were finding no positive results, and the union wouldn't hold still for it. They said you reinstate the urine sampling program regardless of whether you find positive results, and we did. We still find negative results but the union still insists we continue the program of surveillance. They are all for it.

DR. MC NAMARA (Department of the Army): I'd like to ask Mr. Linch what are the abnormal blood changes you mentioned in regard to your cyanogenic program?

MR. LINCH: The abnormal changes apparently are the beginnings of a cyanosis cycle. In other words, there is a certain amount of reaction of the hemoglobin to these intermediates produced, as I indicated in the slide, before methemoglobin forms. We have seen these complex concentrations go as high as 20%. There would be a 20% difference between these two methods for determining hemoglobin. And if corrective action wasn't taken within a period of an hour or so, then this would revert to methemoglobin and we'd have a true cyanosis case on our hands. By applying the methylene blue therapy that is standard in our medical department, we could abort these periods so that we did not develop a frank methemoglobinemia. There's some beautiful work done by Jackson and Thompson in Great

Britain which elucidates at least eight derivatives of methemoglobin in this cycle. You'll find reference to it in the Second Edition of the Chemical Rubber Company's Handbook of Laboratory Safety where our entire cyanosis control program is written out in detail. This was done with radioactive tracer techniques.

DR. BUCHWALD (Department of National Health and Welfare, Ottawa, Canada): I think I'll reserve some of the comments I have for later on this afternoon, but I think apropos the discussion of a few minutes ago, the concept of the two limits, the warning limit and the medical intervention limit, seems to be highly appropriate. I think it's worth discussing further. I think there certainly seems to be a need for something like this.

MR. LINCH: Well, it's been a program that's been highly successful in our plant and it has not been expensive. It's a program that can be administered with a minimum of expense.

DR. BUCHWALD: I'm surprised that this hasn't really been taken more notice of in the past, but I think Harvey Elkins mentioned it in his Cummings Memorial lecture in 1967, and he ascribed it to Don Cummings of many years earlier. There is much merit in this and I think we know exactly where we stand. I wanted to comment on the enzyme levels, on their applicability, and a particular example is cholinesterase levels. It's most important that you have a preexposure baseline on this. Our own experience was that about 1 in 12 individuals had an extremely low activity. So low that if you measured the level in this individual at some stage during his work, you could be extremely scared and think that he had lost 50% of the activity, and immediately hospitalize him. He would be put out of work and the workmen's compensation board would be looking at his claims. Whereas, if you had actually measured his baseline levels, you may have discovered that his normal activity was 50% of that of the normal population. In terms of applying this, this has been done quite successfully in the province of Alberta in terms of the pesticide applicators, where, as part of the licensing program, pesticide applicators are required to have a blood cholinesterase level done before their exposure to the pesticide 3, these organo-phosphorus pesticides. Again, coming down to the real life situation in terms of looking for a biologic threshold limit, the case of lead is a very good one. Here we have 5 parameters which can be measured - which one do you take? And for the average industrial organization, the small company that has 3 or 4 employees making batteries - what do you do for them? You can't go through this battery of tests for each one of their employees. Yet these particular tests are by far the best way of monitoring the individuals that are employed there. In this case, you have to set your site either at a blood level or a urinary level, things that we know most about and which fit most appropriately the picture which you are looking at. And lastly, I want to come back to the original discussion in terms of the breath analyses. I haven't very much experience in doing breath analyses but it seemed to me from looking at the calibration curves, or looking at the blood level of carboxyhemoglobin and the amount of

carbon monoxide in the breath, that there was quite a scatter for individuals away from the calibration curve. In industry we are dealing with individuals in many cases and not necessarily the group. It seems to me that unless you're very careful and have had a lot of experience in taking breath samples, the results could be way off the mark. I would like to have some comments from the people who are expert in doing breath analyses as to how difficult this procedure is, how repeatable the results are, particularly when the samples are being taken with the help of inexperienced medical personnel and so on.

MR. SHERWOOD: I wonder if I might tackle that one. I also have the data available, which I could use the blackboard for, showing the results of tests on workers using the data from my exposure experiments. But first of all, answering the question on breath sampling, one does have the simple problem of trying to explain to a man how to give a breath sample. In the case of a respirator, it's quite straightforward. In a breath pipette or breath sampling tube, it is more difficult and I have done some trials on this. And the worse situation is where a man takes a deep breath, blows just shortly and then if I can sort of copy the situation, I happen to have a breath sampling tube here, one is supposed to blow steadily, but if he's drunk and if he's Japanese and you're trying to explain in basic English what to do, instead of giving you an end expiration sample, he is more likely to give you a sample of short inspiration and expiration. If that happens and he gives that short blow, then you may be in error to the extent of about 45%. Now, you could say in terms of doing your research work, that's enormous. But in terms of interpreting the man's exposure, when you don't know whether he had it 8 hours ago or half an hour ago, it's hardly significant. And I think if you take careful breath samples this way, you can be within 10%. Doing them in the laboratory, you can get very much better than that and I've got correlation coefficients which are in my paper and you'll find that one does get better than .99 as a regression between pairs of samples. Does that answer that aspect?

The man with the most exposure to benzene was working on the rack. For breath, we reached 0.7 ppm and that showed 0.6. And I would say that he was considerably more adipose than my experimental subject so it could be that fat isn't a significant factor. In urine, he was showing 17 mg/l and we showed 8 mg/l in the experimental subject. Sixteen hours later, the next morning, in the breath sample we expected 0.12 and got 0.13. And in urine we expected 0.53 mg/l and saw 0.55. These results were much too good. At the lowest exposure, we found the worst error was in urine the following morning. It was much higher than expected. And you could then say that this was accumulated from previous exposure.

DR. BUCHWALD: That answers my question partially. I admit it's a very useful procedure for monitoring your control, but we have been dealing with biologic threshold limits which are recommended criteria and these limits may be taken by legislative agencies and put into legislation. Something which has a potential error

of 20 to 50% has got no place being written into legislation. And these are the things I think we are faced with. We'll have to be very careful in terms of the levels that one legislates. Am I thinking the right way here?

MR. SHERWOOD: May I comment, sir, that in the United Kingdom now, the select committee report on the future of occupational health and safety has said that the present legislation is hampering the progress of health and safety in the United Kingdom and recommends scrapping the lot. Now I think some of you people in the United States might be glad to hear that. But this is a very real problem. We have a question, I think, of interpretation of extremely complex biological parameters by people who may be a little more than clerks and I think this is a problem that one is faced with in putting a tolerance on both the standard and its interpretation. To my mind, there must be an automatic appeal mechanism by which, before any serious action is taken, there is appeal to someone who is competent to make an interpretation.

MR. LINCH: Well, if I may change hats and speak as an analytical research chemist, I'd like to point out one thing that perhaps I didn't emphasize in the chart I prepared relating urinary excretion to the personnel monitor analysis, and that is to enter on your curves the limits of your analytical methods deviation. Remember, I said I had plus or minus 0.01 milligrams per liter as an analytical deviation, but that didn't include sampling. So that on those curves that relate carbon monoxide in the breath to carboxyhemoglobin in the blood, there should be included as dash lines on each side the limits of sampling and analytical deviation. So, I think you'll find those points all fall within standard deviation. This is a concept that you want to keep in mind when you're interpreting any results.

DR. STOKINGER: Just one little suggestion about Dr. Buchwald's concern about what we do in small plants and this highly sophisticated biologic threshold limit value. Don't you think that as the future rolls in upon us there'll be such a thing as mobile industrial hygiene units that can, for a small fee in a group program, do these very tests that we are worrying about?

MR. LINCH: Dr. Stokinger, to answer your question, yes. The duPont Company has just set up a new department in selling education and applied technology. And they are looking into, actively now with field surveys, the possibility of supplying industrial hygiene service to small companies with 10, 15, 20, and 40 employees at a fixed fee for a certain sophistication for a job.

DR. FRIESS: Well, I should like to thank the audience and our distinguished panel of the morning, and now we'll move on to the afternoon talks.

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PAPER NO. 6

ANALYSES FOR TOTAL, IONIC, AND ELEMENTAL MERCURY IN URINE AS A BASIS FOR A BIOLOGIC STANDARD

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Threshold Limit Values for concentrations of materials in air and Biologic Threshold Limits are tools used in control of exposure to potential hazards in work environments. As originally conceived, they were to be used as guides by professionally qualified personnel. As long as they were guides and the professionally qualified personnel using them recognized the limitations of these guides, the potential for harm to large numbers of persons or for excessive costs to industry from misuse was small. Now that these Threshold Limit Values and Biologic Threshold Limits are being used as legal limits there is a tendency to be conservative in setting limits in order to comply with the requirement of the Occupational Safety and Health Act of 1970 that the standard set shall be the one "which most adequately assures, to the extent feasible, on the basis of the best available evidence, that no employee will suffer material impairment of health or functional capacity even if such employee has regular exposure to the hazard dealt with by such standard for the period of his working life." If an unnecessarily conservative approach is used in setting limits that become legal standards, then industry can be burdened with excessive costs in meeting such standards.

The values for specific biological material being measured for a Biologic Threshold Limit may not necessarily correlate with values for extent of exposure based on analyses of work environment air. Stokinger (1972) presented the following factors as a rationale for the use of Biologic Threshold Limits:
1. Air concentrations do not represent the only source of body burden when there is a potential for skin contact and the material can be absorbed through the skin.
2. Intermittent short term peaks of exposure may not be detectable by air measurements and may contribute a disproportionate fraction of the total body burden.
3. Sampling of air in the general work environment may not provide a true picture of an individual's exposure, particularly if there is a mixed exposure.

4. Individual work habits and individual personal hygiene can influence the extent of absorption of a material. 5. A second job ("moonlighting") can be a source of exposure not evaluated by air measurements in the primary work environment. Any one of the foregoing five factors is sufficient to make one not expect a correlation between levels of a biological material chosen for establishment of a Biologic Threshold Limit.

Prior to 1972 the Threshold Limit Value for elemental mercury and inorganic salts of mercury published by the American Conference of Governmental Industrial Hygienists carried a notation indicating these materials could be absorbed through the skin. Smith, Vorwald, Patil, and Mooney (1970, p. 692) in a discussion of sources of absorption of mercury by workers in mercury cell chlor-alkali plants stated, "Finally, some mercury may be absorbed through the skin, as has been reported with some frequency in the literature, but an international committee convened in 1968 to consider threshold limit values concluded that, 'As the rate of penetration is slow, the practical importance of skin absorption is uncertain. Contamination of skin or work clothes with mercury compounds, however, could cause heavy exposure to mercury vapor by inhalation.'" Smith et al. (1969).

Although Smith et al. (1970) mentioned the possibility of absorption of mercury through the skin and of heavy exposure by inhalation of mercury vaporizing from contaminated skin and clothing, they did not evaluate the fraction of the body burden contributed by these sources. Their estimates of time-weighted average exposures of workers in mercury cell chlor-alkali plants were based on measurements made in the air of the general work environment. They did not use sampling devices on personnel to see whether the microenvironment surrounding a worker with contaminated skin and clothing was different from the general work environment.

The data in table I show the concentrations of mercury vapor found in the general environment of lunch rooms, in the breathing zones of individuals and in air close to hands contaminated with mercury. It can be seen from these data that the mercury vapor concentrations in the microenvironment near a person whose hands and clothing have become contaminated with mercury may be an order of magnitude higher than the mercury vapor concentrations in the general environment.

Items 16 and 17 in table I illustrate the difficulty of removing mercury from hands that have been contaminated by contact with liquid mercury. Even when a barrier cream is applied before hands come in contact with mercury, not all of the mercury can be washed off the hands and the hands can continue to be a source of mercury vapor.

Table I

Mercury Vapor Concentrations at Operators' Breathing Zones During Lunch Period

	Hg (mg/m^3)
Plant A	
1. Near lunch table (background)	0.03
2. Operator A's breathing zone during lunch	0.25
3. Operator B's breathing zone (had not been working with mercury)	0.03
4. Operator C's breathing zone	0.25
5. Operator D's breathing zone	0.05
Plant B	
6. In center of plant cafeteria during lunch period	0.04
7. Operator E's breathing zone during lunch	0.49
8. Operator F's breathing zone	0.07
Plant C	
9. Operator F's breathing zone during lunch period	0.60
10. Operator H's breathing zone	0.16
11. Operator I's breathing zone (different day from G and H)	0.09
12. Operator J's breathing zone (same time as sample I)	0.06

Tests with Hands Cupped Loosely Over End of Sampling Tube	
13. At Operator C's (above) hand during lunch	1.8
14. At Operator K's hand during lunch (had not worked with liquid mercury since previous day)	0.2
15. Industrial Hygienist's hand (contaminated from touching mercury-contaminated equipment)	
a. before washing	0.8
b. after thorough washing with soap and water	0.14
16. Lab tests, Industrial Hygienist's hands	
a. after rubbing hands for several minutes with small droplet of mercury	>2.0
b. after thorough washing	0.18
17. Lab tests, Industrial Hygienist's hands	
a. after applying various types of barrier creams to hands, then rubbing with droplet of mercury	>1.5
b. after removing barrier creams by thorough washing with soap and water	0.06-0.09

The data in table II show the concentrations of mercury vapor found near clothing and hands in a different industry than the one from which the data in table I were obtained. Here again, it is obvious that the microenvironment around a person whose clothes and hands are contaminated with mercury had a much higher concentration of mercury vapor than the general environment. Air changes in the general environment can remove mercury vapor and lower the concentration; unless a fan is blowing air directly on a person air changes in the general environment have little effect on the microenvironment around a person.

Table II

Mercury Vapor Concentrations in Air
Near Contaminated Clothing and Skin

Locker Room	mg Mercury/ Cubic Meter of Air
General Room Atmosphere	0.03 - 0.04
Air Near	
1. Outer clothing furnished by company and laundered daily; worn one shift before measurements	0.1 - 0.2
2. Gloves	0.08 - 0.2
3. Hands (before washing)	0.5 - 0.6
4. Clean Hands (washed)	0.04 - 0.08
5. Sweater (employee in mercury recovery area)	0.2 - 0.5
6. Rubber Coated Shoes (inside) (outside)	.02 - .05 .10 - .5
7. Cotton undershirt worn approximately 6 hours in cell room. Person had no known contact of outer clothing with liquid mercury nor salts of mercury.	0.01

Vostal (1972) has reported finding pools of mercury in the washing machine in the home of a person employed in work that resulted in contamination of skin and clothing with liquid mercury. From the data in tables I and II, and from the report of Vostal (1972), contamination of skin and clothing serves as a source of heavy exposure to mercury vapor during the 8-hour work shift and can also lead to a continuing exposure during the 16 hours away from the work shift. This continuing exposure may be compared to exposure from "moonlighting" mentioned by Stokinger (1972).

Exposure to elemental mercury vapor appears to involve all of the factors listed by Stokinger (1972) as a rationale for Biologic Threshold Limits. All of the factors are such that one would not logically expect estimated time-weighted average exposures to mercury vapor based on measurements in the general work environment to correlate with urinary mercury concentrations; the mercury in the general work environment air may be only a small fraction of the total sources of mercury vapor to which an individual is exposed. Personal hygiene and work habits can influence the extent of total exposure.

Smith et al. (1970, p. 694, figures 3, 4, and 5) show a relationship between their estimated time-weighted average exposures and group average urinary mercury concentrations, uncorrected and corrected to specific gravity of 1.018 and 1.024. A group average estimated time-weighted average 8-hour exposure to 0.05 mg mercury vapor per cubic meter of air was shown to correlate with a group average urinary mercury concentration of approximately 0.15 mg/liter. The failure to obtain correlation between exposure levels and urinary mercury levels on an individual basis was attributed primarily to biological variation; the data presented in tables I and II indicate that errors in estimation of exposure could be a more important factor than biological variation as a reason for failure of correlation on an individual basis.

The work of Smith et al. (1970) was a major factor in the proposal to lower the Threshold Limit Value for elemental mercury vapor and inorganic salts of mercury from 0.1 mg/m^3 to 0.05 mg/m^3. The basis for the recommendation was to provide a larger margin of safety and not because there were adverse effects detected in persons exposed to an estimated time-weighted average exposure of 0.1 mg/m^3 during 8-hour work shifts. As indicated by the data in tables I and II, the actual total exposures to mercury were probably far greater than the estimated time-weighted average 8-hour work shift exposures.

On the basis of the correlation of the estimated group average time-weighted average exposure to 0.05 mg mercury per cubic meter of air with a group average urinary mercury concentration of approximately 0.15 mg/liter, Smith (1971a), Elkins (1971) and Stokinger (1972) have proposed 0.15 mg of mercury per liter of urine as the Biologic Threshold Limit for exposures to elemental mercury and inorganic salts of mercury. Smith et al. (1970) showed that for 188 employees having an estimated time-weighted average 8-hour work day exposure to 0.01 to 0.05 mg mercury per cubic meter of air, 24.5% of the group had urinary mercury values in the range of 0.11 to 0.30 mg/liter and 2.7% had urinary mercury values in the range of 0.31 to 0.60 mg/liter. If the 188 persons were divided into four groups with exposure ranges of 0.01 to 0.02, 0.02 to 0.03, 0.03 to 0.04 and 0.04 to 0.05 mg/m^3, the lowest exposure group would be expected to have the most persons with urinary mercury values in the range of 0.11 to 0.15 mg/liter and the highest exposure group would be expected to have the most persons with urinary mercury values in the range of 0.26 to 0.30 mg/liter; at least 25% of persons exposed to a time-weighted average 0.04 to 0.05 mg/m^3 of mercury can logically be expected to have urinary mercury values in the range of 0.26 to 0.30 mg/liter. A value of 0.15 mg/liter of mercury in urine as a Biologic Threshold Limit may give a false indication of overexposure in approximately 25% of the cases.

This proposed Biologic Threshold Limit of 0.15 mg/liter does not appear to be well founded; it is based on a group average and it is based on a correlation between air concentrations and urine concentrations whereas the rationale proposed by Stokinger (1972) and the available information on sources of body burden when working with mercury would lead one to expect there should not necessarily be a good correlation between available estimates of exposure and urinary mercury concentrations.

A better approach to a Biologic Threshold Limit for elemental mercury and inorganic salts of mercury might be some measure that evaluates the potential hazard of the exposure. Magos (1967), Viola and Casano (1968), Rabinovitz (1972), and others have reported that the brain takes up 10 times as much mercury when the mercury is administered as elemental mercury as when the mercury is administered as an inorganic salt; this tenfold difference is found when the doses are administered by injection or by inhalation. Although elemental mercury is quickly oxidized to Hg^{2+} in the body, if the rate of absorption of elemental mercury into the body exceeds the rate of oxidation, elemental mercury can penetrate into the brain and accumulate in the brain. If elemental mercury is oxidized in the blood before it reaches the brain, the amount accumulating in the brain may be essentially zero.

Smith (1971b) reported that the mercury content in the cerebellum of control monkeys was 0.4 μg/g (dry weight basis) compared with mercury contents of 0.6, 11, and 64 μg/g for monkeys exposed to 0.1, 0.5, and 1.0 mg of mercury vapor per cubic meter of air for 6 hours per day, 5 days per week for 5 years for the 0.1 and 0.5 mg/m^3 groups and three and one-half years for the 1.0 mg/m^3 group. The almost twentyfold increase in mercury concentration in the cerebellum with only a fivefold increase in extent of exposure suggests a protective system capable of preventing accumulation of mercury in the brain at the lower exposure. Similar differences in mercury concentrations with the different exposures were found in the medulla, occipital and frontal portions of the brain. The available data on differences in accumulation of mercury in the brain with different dose rates and with different forms of mercury suggested that there might also be differences in the form of excretion of mercury in urine.

The cold flameless atomic absorption procedure for the determination of mercury in urine and other aqueous systems is easily adapted for determination of elemental, stannous chloride-reducible and total mercury in urine. Gage and Warren (1970), and Magos (1971) have described sampling and pretreatment procedures for determination of inorganic and organic mercury in biological samples.

Urine samples from employees having potential exposure to mercury in mercury cell chlor-alkali plants have been analyzed for elemental, stannous chloride-reducible, and total mercury. The final step in the three determinations is the same; elemental mercury is swept from the diluted, pretreated urine sample into a gas stream and the amount of mercury in the gas stream is measured by cold flameless atomic absorption. The pretreatment of the aliquots for determination of elemental, stannous chloride-reducible, and total mercury provides the method of distinguishing the three forms in urine.

Elemental mercury is determined by diluting a suitable aliquot of urine, one or two ml depending on concentration of elemental mercury, with fifty ml of water and sweeping any elemental mercury present out of the liquid into the gas stream for measurement. Stannous chloride-reducible mercury is determined by diluting a suitable aliquot of urine with 50 ml of water, adding 1 ml of a 20% stannous chloride solution, and then sweeping the mercury into the gas stream for measurement. The stannous chloride solution can be added to the diluted urine aliquot that has been analyzed for elemental mercury or can be added to a separate diluted aliquot; if added to a separate aliquot, elemental plus stannous chloride-reducible mercury will be measured together. Total mercury is determined by adding 5 ml of concentrated nitric acid to a 1 ml aliquot of urine, allowing to digest for 1 minute, diluting with 50 ml of water, adding 1 ml of 20% stannous chloride solution, and sweeping the mercury into the gas stream for measurement. Details of the glassware used,

preparation of solutions and standards for the determination of total mercury in urine have been described by Krause, Henderson, Shotwell and Culp (1971). The method easily detects 5 nanograms of mercury in a 1 ml urine aliquot (0.005 μg/ml, 0.005 mg/liter).

Table III shows total, stannous chloride-reducible, and elemental mercury concentrations in urine samples from a welder employed in a chlor-alkali plant. The data indicate that elemental mercury can be found in urine after a short-term elevated exposure to elemental mercury vapor. Unfortunately, we were just starting our program of analyses for total, stannous chloride-reducible and elemental mercury in urine when the elevated exposure of this welder occurred so we did not do all of the analyses that would have been desirable. We have, however, analyzed several hundred urine samples by the triple analysis approach and the results show a definite pattern.

Table II. Total, Stannous Chloride Reducible, and Elemental Mercury in Urine from a Welder Before and After Exposure to Elemental Mercury While Welding.

Date of Sample	Total Mercury (mg/L)	Stannous Chloride-Reducible plus Elemental Mercury (mg/L)	Elemental Mercury (mg/L)
2/9/72	.70	.06	.04
2/11/72*	1.50	.37	.34
2/17/72	.73	---	---
2/18/72	.58	---	---
2/22/72	.94	.33	---
2/23/72	.64	.26	---
2/24/72	.44	.22	---
2/25/72	.51	.22	---
3/9/72	.47	.01	.00
3/10/72	.61	.01	.00
3/11/72	.52	< .005	< .005
3/12/72	.46	< .005	< .005
3/13/72	.78	< .005	< .005
3/14/72	.59	.01	< .005
3/15/72	.44	.01	< .005
3/16/72	.52	.01	< .005
3/17/72	.53	.01	< .005
3/18/72	.47	.01	< .005
3/19/72	.57	.01	< .005
3/20/72	.70	.01	< .005

*Sample collected at end of shift after exposure while welding.

We seldom find more than a barely detectable amount of elemental mercury in urine samples. This is consistent with the conclusion of Clarkson, Gatzy and Dalton (1961) that elemental mercury is quickly oxidized to Hg^{2+} once it reaches the blood. One would expect to find elemental mercury in blood or urine for only a short time after an elevated exposure to elemental mercury vapor. Not all of the Hg^{2+} may become rapidly bound in some other form so Hg^{2+} might be expected to persist in blood and urine for some time after elevated exposure. Figure 1 is a plot of total mercury versus stannous chloride-reducible plus elemental mercury in urine samples. Data are available to extend this curve below 0.3 mg total mercury per liter of urine but the curve is essentially flat below 0.3 mg as it is between 0.3 and 0.5 mg/liter. These data indicate that at rates of exposure to elemental mercury vapor leading to urinary excretion of total mercury of less than approximately 0.6 mg/liter the body is able to bind the mercury in a form not reducible by stannous chloride; this binding may represent the body detoxication mechanism. When the rate of exposure results in a total urinary mercury above approximately 0.6 mg/liter, then the amount of stannous chloride-reducible mercury increases; this may represent the rate at which adverse effects from exposure may occur if the level of exposure continues. If the rate of exposure to elemental mercury vapor is elevated above some as yet undetermined rate, as in the case of the welder (table III), then elemental mercury as well as stannous chloride-reducible mercury may be found in urine for a short time after exposure.

Our experience in the operation of six mercury cell-chlor alkali plants has shown that no "material impairment of health or functional capacity" has been noted in our employees even though total urinary mercury concentrations in some workers have exceeded 0.5 mg/liter. On the basis of our experience with analyses of urine for total, stannous chloride-reducible and elemental mercury, it is suggested that a Biologic Threshold Limit for exposure to elemental mercury could be 0.5 mg/liter total mercury plus 0.02 mg/liter stannous chloride-reducible plus elemental mercury. The Biologic Threshold Limit for exposure to inorganic salts of mercury and to aryl mercury compounds may be even higher but there is a paucity of information correlating urinary mercury concentrations resulting from exposure to such mercury compounds with observations on signs of adverse effects.

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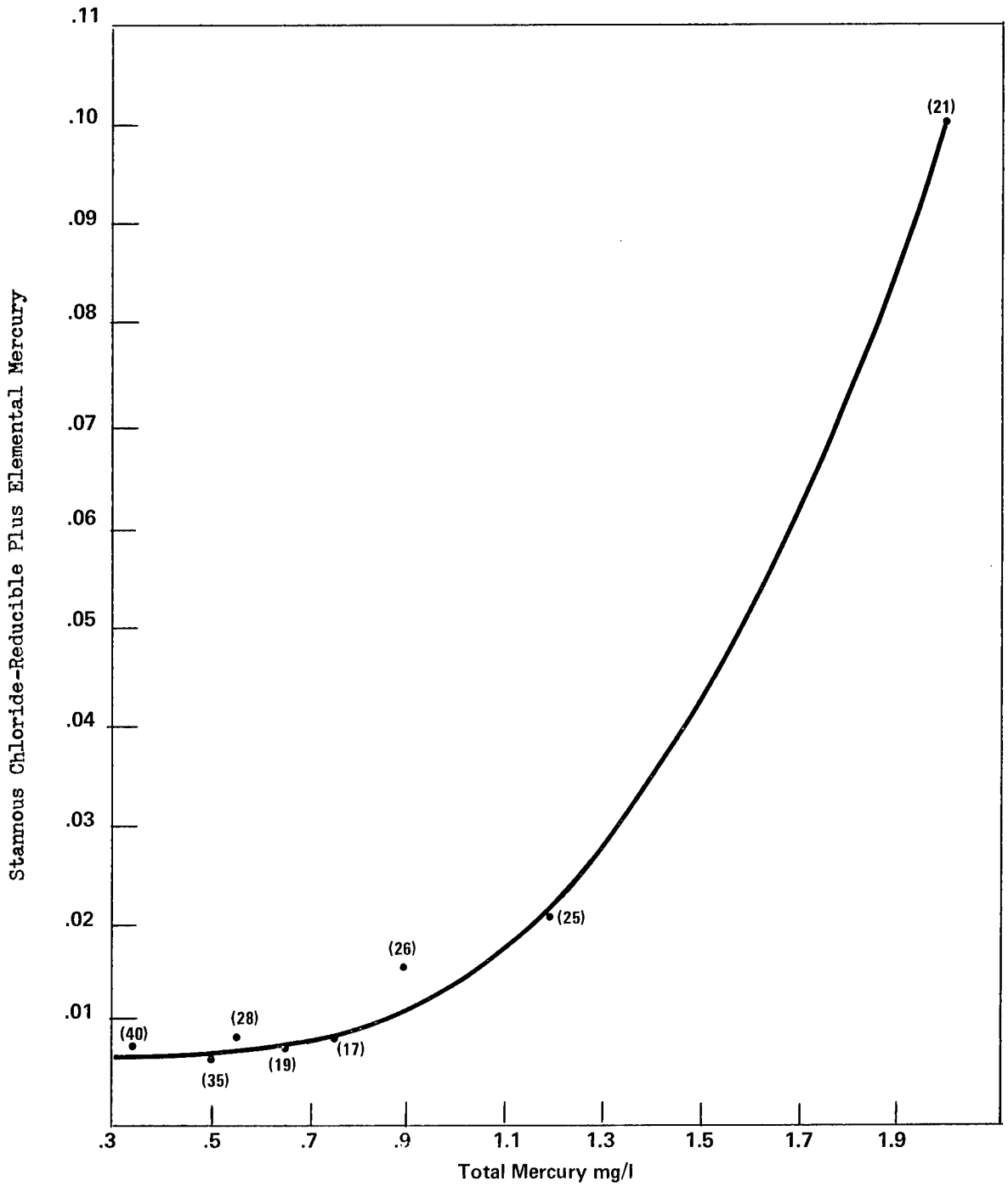

Figure 1. Total Versus Stannous Chloride-Reducible Plus Elemental Mercury in Urine, Group Averages (Number of Samples Per Group in Parentheses).

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PAPER NO. 7

SOME FACTORS INVOLVED IN ESTABLISHING AND USING BIOLOGICAL THRESHOLD LIMIT VALUES (BTLV'S)

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INTRODUCTION

It is proposed in this paper to review and discuss some of the factors which are involved, both in establishing biological threshold limit values, and in interpreting the results of biological monitoring carried out to see whether established or suggested standards are being met.

When the individual is monitored to determine his exposure, rather than the environment itself being used as a criterion of exposure, variables arise from the material to which the individual is exposed, from the biochemical behavior of the material within the individual, and from a variety of extraneous factors. Any distinction between them appears to be arbitrary, but the emphasis in this presentation will be on the biochemical and extraneous variables.

Some of the factors arising from the substance being monitored for include the physicochemical properties of the substance, its stability in the environment and the actual conditions under which it is used or generated. As far as the individual is concerned (table I), the routes by which a substance enters the organism, the extent of absorption from whatever site by which it gains entry, the relation between physiological activity and absorption from the site of entry, the location and distribution of the substance after absorption, the routes of elimination and any metabolic processes which occur will have a considerable bearing on the selection and interpretation of the type of measurement made to establish a BTLV.

Table I

Biochemical Factors in Biological Monitoring

Route of Entry to the Organism
- Lung (Vapor, Aerosol, Particles)
- Oral (Swallowing Dusts, Aerosols)
- Skin

Absorption from Entry Site
(Particles may be inert)

Relation between Route of Absorption and Physiological Activity

Fate and Distribution after Absorption

Route of Elimination

Relation between Elimination and Exposure

The substances for which BTLV's are now being proposed, and which have been discussed in other papers at this symposium, have illustrated many of these points. More particular attention should be paid to those which arise from the exposed individual, his behavior during exposure, his habits and his environment. These are listed in table II.

Table II. Extraneous Factors in Biological Monitoring.

- Activity
- Habituation
- Health of Exposed Individual
  - Obesity
  - Pulmonary Function
  - Cardiac Output
- Metabolic Variation
- Induction or Inhibition of Metabolic Processes by
  - Dietary Constituents
  - Drugs
  - Other Environmental or Industrial Exposures
- Physiological Effects of Concomitant Exposures
- Intake from Nonoccupational Sources
- Normal Endogenous Levels
- Duration of Exposure
- Frequency of Exposure
- Possibility of Excursions
- Period of Time between Exposures
- Time of Measurement with Respect to Exposure
  - During
  - Subsequent to

Clearly one of the most important of these, bearing directly on the final analytical measurement, is that due to nonoccupational exposures. The effects of this are seen, for instance, in background lead levels, which may vary because of other environmental exposures or from such unsuspected causes as the use of poorly glazed pottery. Elevated urinary fluoride levels, close to those resulting from industrial exposure, have been reported in certain far western states of the United States. Elevated carboxyhemoglobin levels occur with cigarette smoking, which provide a high background for carbon monoxide monitoring. Elevated urinary selenium occurs also with cigarette smoking, and a variety of phenols arise from the diet to provide a background in nonspecific urinary monitoring of phenols for benzene exposures.

In addition, there are normal endogenous levels for some substances which may or may not be of some significance. Examples of these are urinary phenols arising from catecholamine metabolism, the background sulfate level which presented problems with some of the earlier methods of detecting benzene exposures, and urinary hippuric acid levels in toluene and benzyl alcohol monitoring. The enhanced elimination of acetone by the diabetic could be included in this context.

Duration of exposure is clearly of importance in dealing with substances which do not rapidly reach equilibrium in the organism, and where short high level excursions may produce excretory levels similar to those resulting from much longer exposures. The frequency of exposures, such as the number of successive eight hour day exposures, and the period of time between each exposure may influence the value obtained on monitoring. Such measurements may depend both on variations between individuals and on the pharmacokinetics of the substance being measured. The time at which a measurement is made with respect to an exposure may be of crucial importance, since with many vapors and gases the removal of an individual from an exposure may have a marked effect on the equilibrium which has been reached. In cases where exposed operators are also performing tasks calling for varying degrees of activity the effects of these should be considered on excretion levels. It is also known that under certain circumstances foreign chemicals within the mammalian organism can stimulate or induce changes in their metabolism pathway. In consequence, habituated employees may show elimination levels for substances which have a significant metabolism contribution, quite different from those of the naive individual. These differences may arise both from the speed of elimination and differences in metabolic pattern, but the degree of habituation is important in evaluating the results of monitoring the individual. It is perhaps worth pointing out that acclimation in many cases represents protection for an individual and should be considered before setting BTLV's well below what experience has shown to be safe for experienced workers.

The health and life style of an exposed individual may have a very clear bearing on both his biological and biochemical responses to exposures. Thus the uptake and elimination of gaseous materials is likely to be affected by factors such as obesity, cardiac output and pulmonary function. Obese individuals may store lipophilic solvents more readily than nonobese individuals, and elimination values may reflect mobilization of stored materials. Measurements of body burden may also reflect metabolic variations of genetic origin, and they also may reflect unsuspected pathological conditions.

To this group of factors which may affect biological monitoring values should be added those which may exert an effect on metabolic processes. The phenomenon of induction of microsomal oxidase activity is by now quite well known. Its importance in biological monitoring can arise from the effect of other environmental constituents or industrial chemicals, dietary habits such as the use of alcohol and vitamins, and therapeutic regimens such as the use of barbiturates and anticoagulants. The range of foreign compounds which are known inducers of microsomal oxidase activity is steadily increasing. While the exact implications for humans are as yet unclear, there is enough evidence to indicate that such factors as those indicated may affect the disposition and elimination of many of the substances on the Threshold Limit Value list. It is clearly important to establish whether such factors are important in biological monitoring.

In addition to effects on metabolic fate, the altered physiological activity arising from the use of alcohol or drugs may, for example, affect both uptake and elimination of volatile solvents. It seems likely that there is much less information on this latter point than is desirable.

Some evidence for the importance of several of these factors will be reviewed in this paper, based on studies carried out in our laboratories with volatile solvents, specifically with acetone and methylene chloride. The data presented have been derived from both human and animal studies. Data from earlier studies on acetone by Haggard, Greenburg and Turner (1944) will also be included for consideration in this light.

FACTORS WHICH AFFECT ACETONE MONITORING

Theoretical Considerations

In turning to acetone as an illustration for many of the factors discussed above, we are dealing with a solvent of long standing use in a wide range of industrial applications, of a very low order of toxicity. It is a minor metabolite of lipids, normally present in humans at levels of 0.12 ± 0.63 µg/liter in expired air, and less than 1 mg/100 g in blood. These values can reach 30 ppm in the expired air of an uncontrolled diabetic. The industrial use of acetone may lead to prolonged breathing of the vapor, but our experience with thousands of operators observed over many years shows long term repeated 4 hour exposures at low to moderate atmospheric levels, and short excursions well over the TLV to be without any undesirable effect, and we are of the opinion that the current TLV of 1000 ppm for an 8-hour working day is reasonable (Raleigh and McGee, 1972).

Acetone is readily absorbed into the bloodstream after inhalation, and studies carried out in 1944 by Haggard et al. have established the various features of acetone uptake, elimination, distribution and metabolism from the atmosphere; the equation in figure 1 provides a useful picture of the various factors which should be considered in biological monitoring for acetone, and presumably for any solvent which shows similar uptake and elimination properties.

$$\text{Acetone Blood Concn. in Time T} \qquad D = \frac{\text{Blood Conc.}}{\text{Air}} = 334$$

$$C_T = \frac{[\text{Resp. Vol. x Atmos. Concn. x T}] - [\text{Metabolism Rate x T}]}{0.83 \times [\text{Resp. Vol.} \times \frac{1}{2D} \times T] + [\text{Urine Secrn. Rate} \times \frac{1.35}{2} \times T]}$$

Tissue Distribution Coeff.

Av. Loss in Breath

Av. Renal Elimination

Figure 1. Acetone Blood Concentration and Atmospheric Exposures (Haggard, Greenburg and Turner, 1944)

Blood concentration is clearly affected by the duration of exposure, by respiratory volume and metabolism rate. Respiratory volume is sensitive to exercise and activity, and in consequence we would expect to see both increased uptake and increased elimination in breath with exercise. Since metabolism rate is also sensitive to exercise, activity will tend to speed elimination during and postexposure. Since atmospheric exposures to acetone occur at levels far below those needed to produce saturation of the blood, the blood level is sensitive to varying periods of exposure, and also to excursions. The equation also indicates that fasting, dehydration and increased air concentration will lead to increased blood levels.

Biological monitoring frequently employs postexposure measurements, and the equation indicates that respiratory volume, metabolism rate, and urinary secretion rate are also important in acetone clearance.

An estimate of the metabolism contributions is of importance, not only in evaluating parameters for biological monitoring but in evaluating the effect of factors which may affect metabolic rates and pathways. While the precise course of acetone metabolism is unclear, two major pathways exist in mammals, one maintaining the 3-carbon skeleton intact presumably involving an intermediate such as pyruvate, and the other involving fission to 1- and 2-carbon fragments (Browning, 1965). The end result appears to be incorporation into the intermediary metabolism. In the absence of identifiable intermediates or enzymes we are left with acetone in blood, breath and urine as the only indicators of exposure. The proportion of the acetone intake accounted for by metabolism diminishes with increasing intake, leading to extensive elimination of unchanged acetone with prolonged or large intakes. However, in the range of exposure of interest in biological monitoring the metabolism rate in humans increases with intake, reaching a plateau of about 2 mg/kg/hr (table III). A rough calculation indicates that after 2 hours at 1000 ppm, almost 2/3 of the intake would be metabolized postexposure. The importance of the metabolism contribution is also indicated by the estimated blood concentration of acetone at the TLV, which for a resting individual at 8 hours is about 90 mg per liter. At this concentration Haggard (1944) has shown that almost half of the intake will be metabolized after exposure ceases. The proportion metabolized during exposure would, of course, drop as the exposure was prolonged.

Table III

Variation of Metabolism Rate with Exposure Level in Humans (Estimated)

Blood Acetone[1] Concentration mg/liter	Metabolism[1] Rate mg/kg/hr	Metabolized in[1] 4 Hours after Intake %	Approximate Air Concentration to Produce Blood Level ppm	Hrs.
2 - 8	1.1	93	100 - 500	2[2]
21 - 33	1.9	80	200 - 400	8[1]
42 - 57	2.1	69	1000	2[1]

[1] Haggard, Greenburg and Turner, 1944; after oral intake of 60-80 mg/kg doses
[2] DiVincenzo, Yanno and Astill, 1972

Experimental Results

With these considerations in mind it would be appropriate at this juncture to examine the experimental results for the influence which the above factors have on the handling of acetone by humans and animals.

While much of the early work with acetone was carried out at relatively high exposure levels, our studies (DiVincenzo, Yanno and Astill, 1972a) have been directed towards a somewhat larger group of human subjects in controlled atmospheres at 100 or 500 ppm. In addition, dogs were exposed to controlled atmospheres to determine if they sufficiently resembled humans in their elimination pattern for use in studies of the effect of other environmental factors. Nine male volunteers were fasted, and singly exposed in a test room to the appropriate acetone concentration for 2 to 4 hours, with a work regimen added as required. Atmospheric and breath samples were collected in Saran ® bags and analyzed by gas chromatography. Venous blood and urine were analyzed by gas chromatography of head space vapors obtained by heating the sample in a closed system. Similar procedures were used with dogs, except that they were fitted postexposure with latex masks to allow breath sampling during the late phase of expiration.

The effect of magnitude of exposure on blood concentration during exposure in humans is presented in figure 2. Since saturation has not been reached, the blood level cannot be related to the exposure without knowing its duration. Table IV indicates further the difficulties inherent in interpreting blood concentrations after atmospheric exposures. Activity, prolongation of exposures, changes in exposure level, and repeated exposures are included. It is obvious that even moderate exercise has a striking effect on the blood concentration. Repeated daily exposures evidently establish a steady state, but this in turn is subject to the effect of activity. It is worth noting that moderate exercise at an exposure of less than half the TLV produces a blood concentration which approaches that for the resting TLV. A similar picture is found with experimental animals (table V). A simple correlation exists between size of intake and the blood level at the end of exposure for dogs and rats. In addition, it is of interest that regular periodic interruption of exposures in rats produces a marked drop in blood levels in comparison with uninterrupted exposures at the same level.

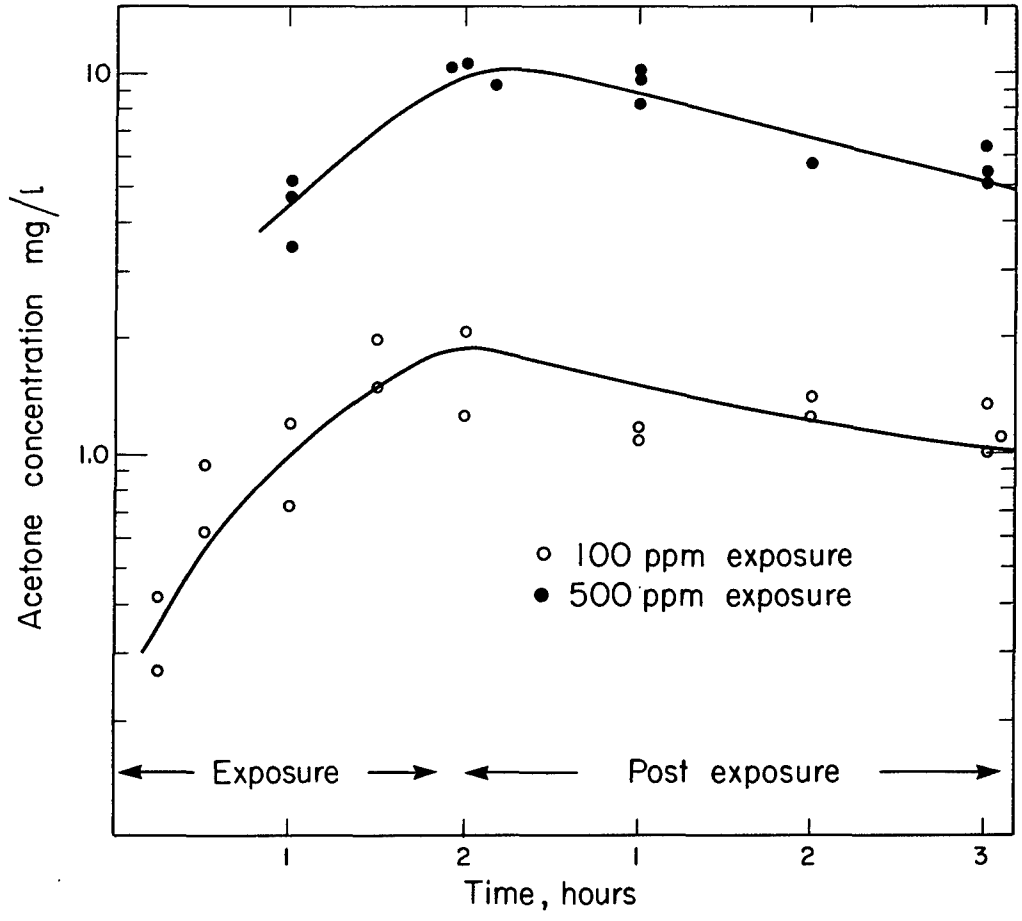

Figure 2. Acetone blood values obtained from 8 subjects experimentally exposed to solvent vapor for 2 hours at 100 and 500 ppm respectively. Each value represents a single blood determination.

Table IV

Atmospheric Exposures to Acetone and Blood Levels in Humans

(Haggard et al., 1944, DiVincenzo et al., 1972)

Exposure[1]		Av. Blood Level at End of Exposure in mg/liter	
ppm	Hours	Resting	Active
100 (4)	2	1.8	
211 (1)	8	30	
422 (2)	8	25, 30	62, 62
500 (4)	2	10	
1266 (1)	8	99	
2110 (1)	8[2]	200	400

[1] ( ) = no. of subjects, [2] = repeated daily

Table V

Atmospheric Exposures to Acetone and Blood Levels in Rats and Dogs

(Haggard et al., 1944, DiVincenzo et al., 1972)

	Exposure		Blood Level at or Immediately After End of Exposure
	ppm	hours	mg/liter
Dogs (5)[1]	100	2	3 - 5
	500	2	10 - 15
	1000	2	20 - 25
Rats	2110	8 (1)[2]	400
		24 (9)[2]	1020 - 1050
	4300	8 (12)[2]	1310 - 1930
		24 (12)[2]	2420 - 2500

[1] ( ) = One dog, separate exposures,
[2] ( ) = Consecutive days.

Similar considerations apply to alveolar air concentrations during atmospheric exposures (table VI). Under resting conditions in both dogs and humans, a simple proportionality exists between the intake level and alveolar air concentration. Under conditions of typical operator activity in actual industrial exposures at the TLV, expired air concentrations show a very wide range of values. There is also a marked dependence of breath acetone level on time of sampling during the exposure, the higher values being found late in the working day.

Table VI

Atmospheric Exposures to Acetone: Alveolar Air Concentrations in Humans and Dogs

(DiVincenzo et al., 1972; Raleigh and McGee, 1972)

	Exposure		Alveolar Air Conc. (during or immediately after exposure)
	ppm	hours	ppm
Dogs (5)[1]	100	2	1.4 - 2.5
	500	2	7 - 8.8
	1000	2	12 - 17
Humans (4)[2]	100	2 (at rest)	18 - 28
	500	2 (at rest)	80 - 120
Humans (9)[3]	950 - 1060	8 (working)	1 - 64 a.m.
			68 - 420 p.m.

[1] ( ) = One dog, five separate exposures
[2] ( ) = Volunteers in controlled atmosphere
[3] ( ) = Operators, sampled in adjacent area.

Two factors of some importance in breath sampling during exposure are worth indicating at this point. The alveolar air concentration during exposure to a blood soluble gas is less than the atmospheric concentration, because of factors such as the distribution, metabolism and urinary elimination. It is thus essential that alveolar air is not contaminated with dead space air in the sampling process, otherwise falsely elevated values will result. On the other hand, unless breath sampling is done in the area of exposure, or in an adjacent area of similar atmospheric concentration, the alveolar air concentration will be depleted by losses to the blood and atmosphere, producing anomalously low values.

Continuous monitoring of 2 groups of human subjects exposed in a test room for breath excretion during and after leaving the test room are presented in figure 3. A steady state appears to be reached during exposure for subjects at rest, contrasting with operator exposures on the previous slide. On ending the exposure, a dramatic fall in alveolar air concentration occurs. Within about 30 minutes elimination in the breath follows approximately exponential kinetics, closely paralleling the situation in blood, as would be expected. Postexposure expired air levels reflect the initial differing levels of atmospheric exposure and as will be shown subsequently do so in dogs also. However, this measurement again cannot be directly related to the magnitude of exposure without further understanding of the exposure conditions (figure 4). The postexposure values are clearly enhanced by prolonging the exposure from 2 to 4 hours, and also by exercise during exposure.

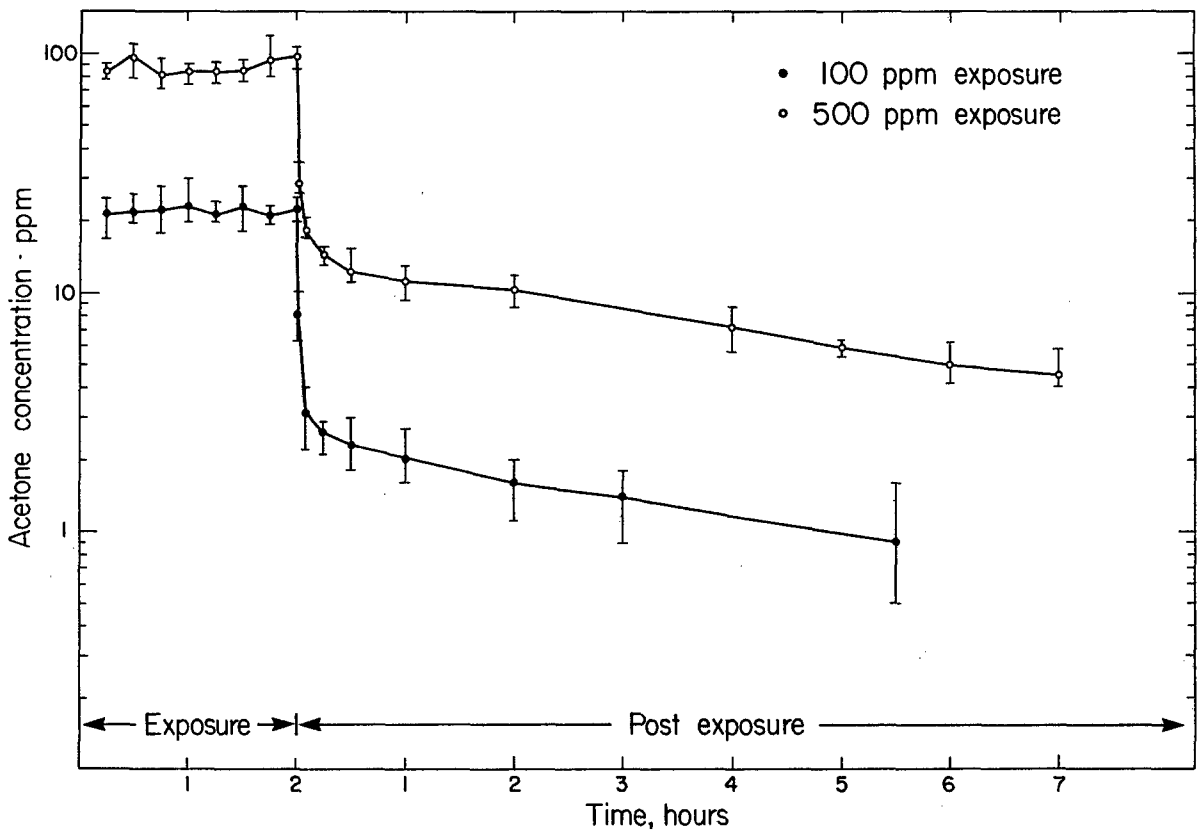

Figure 3. Human serial breath excretion curves from subjects exposed experimentally for 2 hours to acetone vapor at 100 and 500 ppm respectively. Each value represents the mean and the range of 4 exposures.

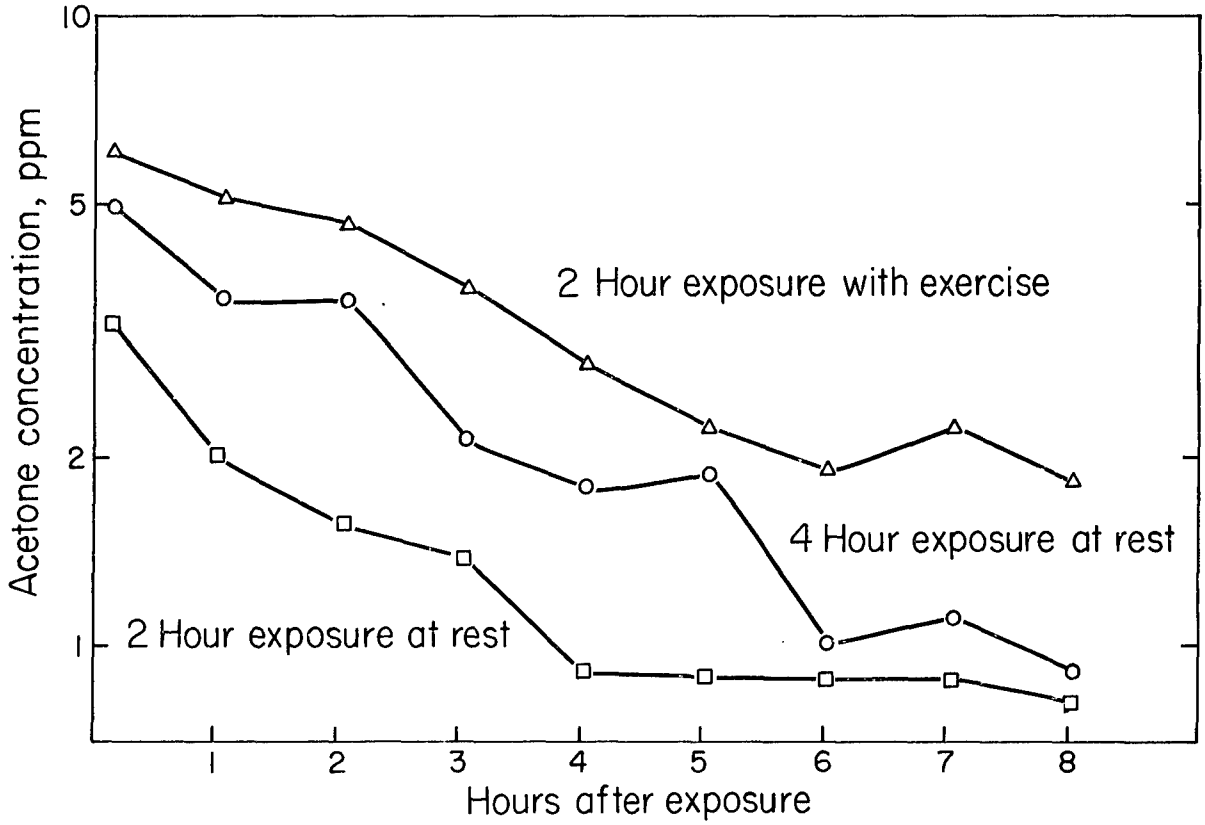

Figure 4. Effect of duration of exposure and exercise on postexposure breath concentrations of acetone (means) from humans exposed to 100 ppm acetone.

Thus, under carefully controlled conditions proportionality exists in the expired air and blood with regard to the exposure level. However, these values are clearly sensitive to variations introduced by activity, time of sampling during or postexposure, prolongation of exposure time, and by repeated daily exposures. Haggard (1944) has estimated that an increment of about 20% of the single exposure value will be added to postexposure levels for an 8-hour daily exposure of 400 ppm, repeated long enough to establish a steady state (about 3-4 days). Biological monitoring of solvents of this type thus should clearly indicate the time during or after exposure for the measurement, breath sampling procedures should be rigorously defined, and the work status of the individual should be defined both during and subsequent to the exposure. The duration, frequency and length of periods of nonexposure should be considered. Periodic monitoring during exposure for substances of this type has the advantage that the sensitivity of both alveolar air and blood concentrations to changes in the exposure level will permit detection of excursions.

SOME FACTORS IN METHYLENE CHLORIDE MONITORING

An interesting comparison can be drawn between acetone and methylene chloride from the point of view of several of the factors discussed. Methylene chloride is an inert solvent of relatively low toxicity used extensively for industrial purposes. It shows a much lower blood-air distribution coefficient than acetone, and a ready initial absorption from the lung is followed by saturation of the blood. Further uptake by the blood depends on distribution to lipophilic tissues in the body, removal from the blood by elimination (urine or skin), and a limited metabolism. Breath excretion curves are presented in figure 5 for 100 and 200 ppm human exposures to methylene chloride (DiVincenzo, Yanno, and Astill, 1972b). These were performed similarly to the acetone exposures discussed, alveolar air levels being measured by gas chromatography of samples collected in Saran® bags. Proportionality is seen between atmospheric concentration and breath concentrations both during and postexposure. The postexposure curve is biphasic and reflects the initial release of methylene chloride from the blood to the alveolar air space and the slower mobilization of methylene chloride from the tissues. Blood levels are given in figure 6, and show both a much faster postexposure die-away curve than for acetone (acetone has a half life of about 3 hours for a 100 ppm exposure, whereas that for methylene chloride is 45 minutes), and a correlation between postexposure blood levels and atmospheric exposure levels.

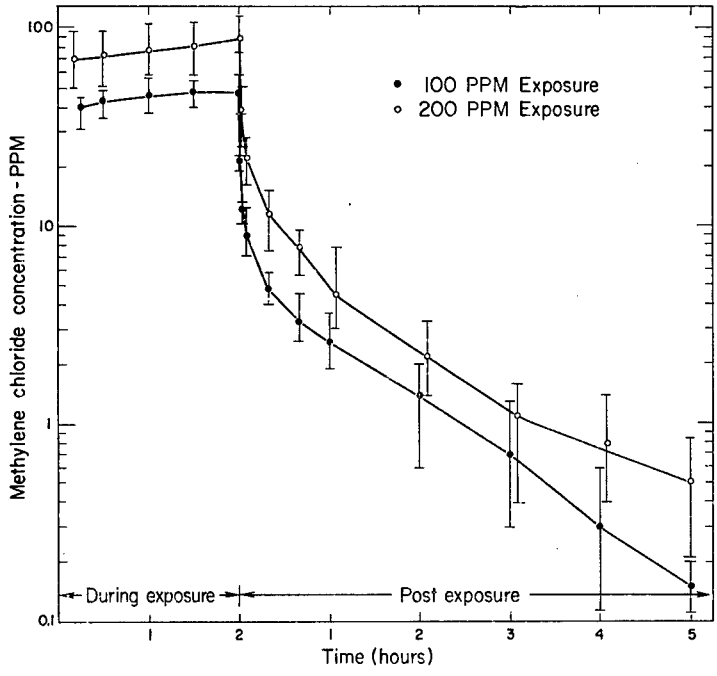

Figure 5. Human serial breath excretion curves for 2 hour exposures to 100 and 200 ppm respectively. Each value represents the mean and the range of 5 exposures at 100 ppm and 7 exposures at 200 ppm.

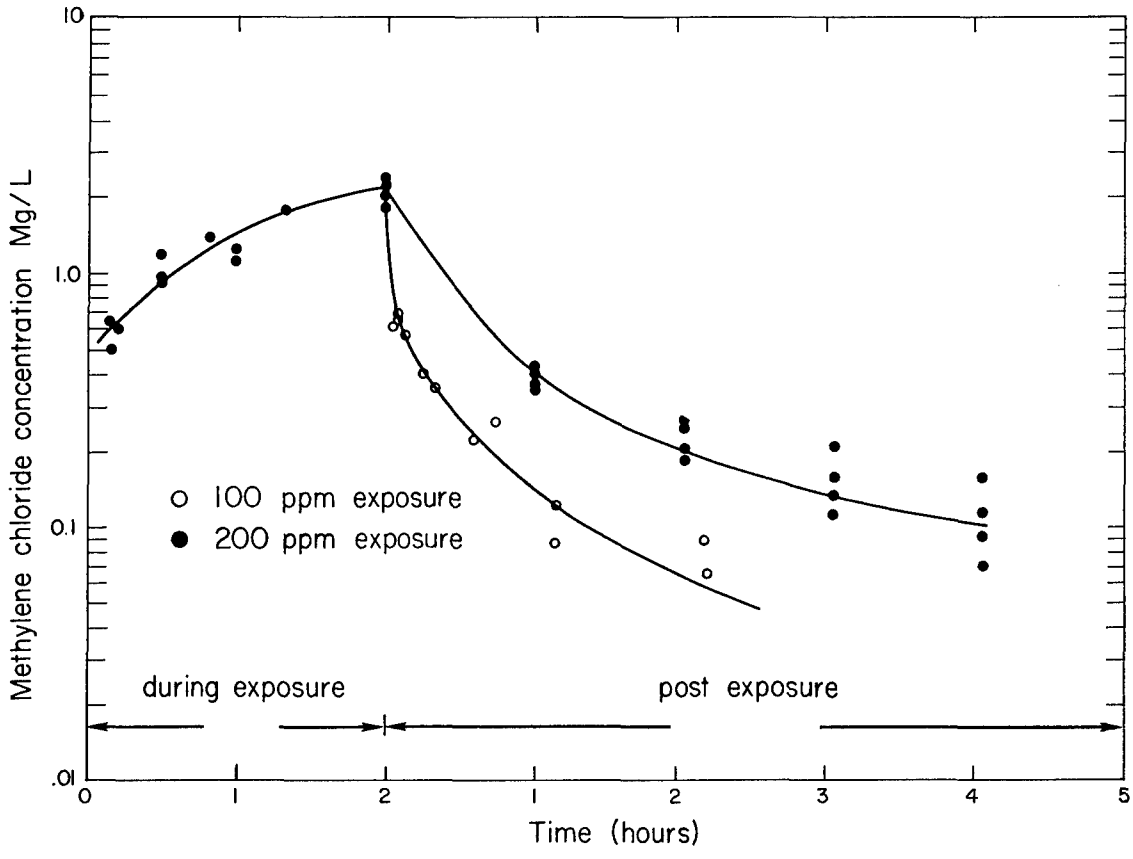

Figure 6. Methylene chloride blood concentrations obtained from 11 subjects experimentally exposed to solvent vapor. Several blood samples were procured from each subject at different times. Each point represents a single blood determination.

The effects of prolonging exposure (figure 7) and of activity differ somewhat from acetone, and arise from the fact that equilibrium with a sparingly soluble gas is reached fairly rapidly once the blood approaches saturation. Duration of exposure for a given concentration is thus without any immediate effect on postexposure breath concentration. Activity by exposed subjects has a limited effect (figure 8) compared with acetone, again arising from the saturation of the blood. Any increased uptake which occurs must arise from increased cardiac output which will increase the extent of distribution of methylene chloride to the tissues; the increased respiration rate due to exercise will only maintain saturation as methylene chloride is taken up by the tissues. Activity is thus unlikely to drastically alter the uptake of inert solvents.

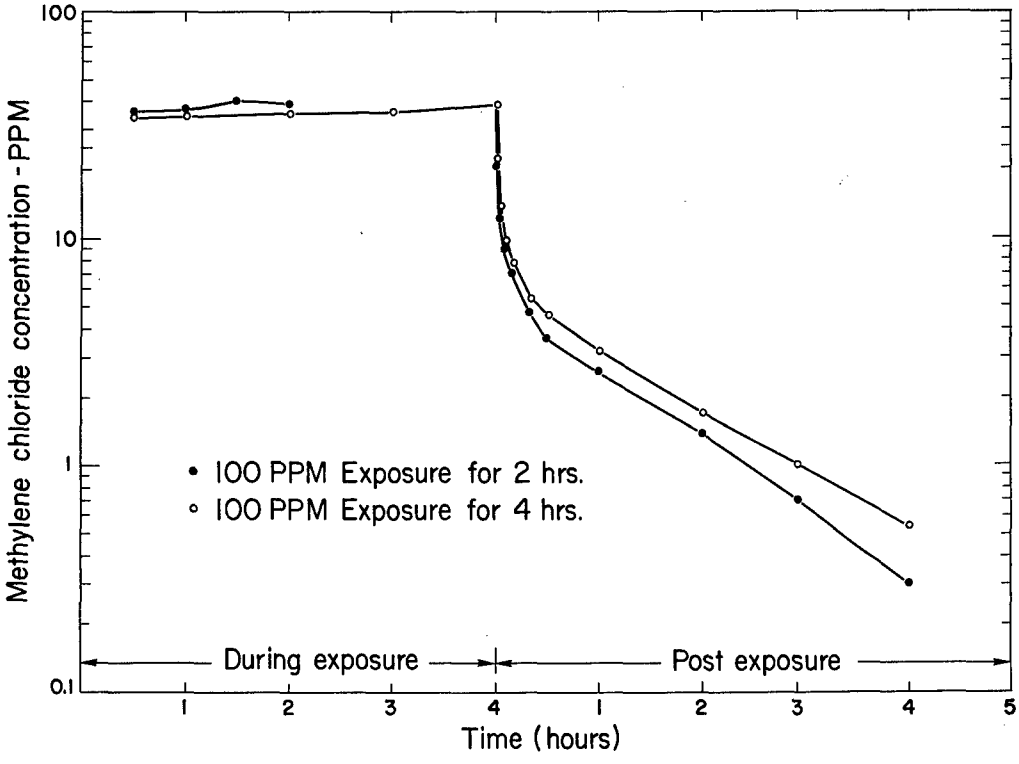

Figure 7. Serial breath excretion curves from subjects exposed to 100 ppm of methylene chloride vapor for 2 or 4 hours. Each value represents the mean of 5 exposures.

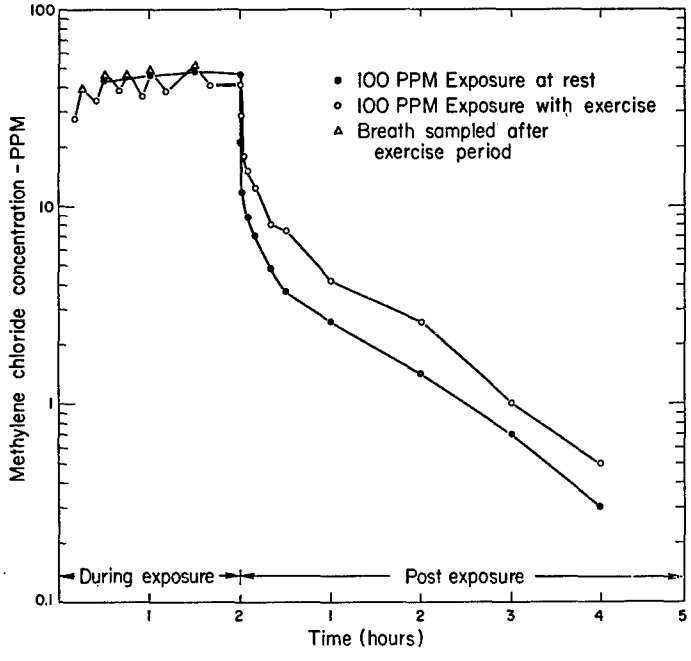

Figure 8. Serial breath excretion curves from subjects exposed for 2 hours to 100 ppm of methylene chloride vapor. During the exposure the subjects remained either at rest or participated in an exercise program. Each value represents the mean of 5 exposures at rest and 4 exposures for the exercise experiment.

OTHER EXTRANEOUS FACTORS AND ACETONE EXPOSURES

A considerable volume of literature has accumulated, particularly with reference to experimental animals, concerning the effects of foreign compounds on their own metabolism and on that of other foreign compounds. These effects on metabolism, associated with the drug processing enzymes in the microsomal fraction of the hepatic parenchymal cell, range from the inhibition of activity to induction. With the exception of a few substances such as chlorinated hydrocarbons and pesticides, fairly large levels of intake are generally needed for inhibition or induction to occur. The extensive human use of alcohol and barbiturates, however, and the large range of concomitant environmental exposures indicate that there is an urgent need to understand and identify what effects these factors have on the parameters used in biological monitoring, particularly those involving metabolic processes.

In addition to the factors discussed, we would like to present some preliminary studies of our own in which the effects of ethanol or of phenobarbital, administered immediately before an acetone exposure, are studied in experimental animals.

There are few reported interactions between acetone and other substances in biological systems, although the use of acetone as a medium in toxicity studies suggests that a careful examination of the literature might reveal more. Acetone reportedly shows only a simple additive effect with phenol on the olfactory and reflex action thresholds in humans (Pogosyan, 1966). Of more interest is the reported potentiation of barbiturate narcosis in mice by acetone (Postolache, Safta, Cuparencu and Steiner, 1969), and an apparently contradictory report of the in vitro stimulation of aniline hydroxylase and of other hydroxylases in the microsomal fraction of rat, mouse, rabbit and dog liver cells (Anders, 1968). The hydroxylase stimulation apparently occurs in concentrations of 0.45 - 1.8M acetone per 0.5 ml of "enzyme" suspension; this appears to represent an acetone concentration of greater than 100 mg of acetone per gram of liver. Potentiation of barbiturate narcosis requires intraperitoneal doses of about 200 mg/kg and above of acetone, for 70 mg/kg IP injections of phenobarbital. These acetone doses correspond roughly to 5000 ppm 8-hour exposures in humans.

We have previously established the suitability of the dog for studies with acetone which can be extrapolated to humans (DiVincenzo et al., 1972a). Post-exposure blood and breath curves show a relation to exposure level fairly similar to that for humans, and the half life of acetone in blood is about the same in both species (3 hours, resting). Control studies were set up for dogs exposed to 1000 ppm of acetone for 2 hours, receiving 1 ml/kg of ethanol, and 20 mg/kg IP doses of phenobarbital. Ethanol was measured in serum by the head space vapor analysis method, and in breath by gas chromatography. Blood phenobarbital was measured by gas chromatography also.

In the ethanol interaction study, each of three dogs received intravenous injections of ethanol as a 30% solution in isotonic saline at a level of intake of 1 ml/kg of body weight. This was followed immediately by atmospheric exposure to acetone. Values are given in figure 9 for acetone levels in breath and blood up to 5 hours postexposure. It is clear that the acetone levels in breath and blood are elevated above those in the absence of ethanol. Whether this represents an effect on respiration rate caused by the narcotic action of ethanol, or is a slowing down of the metabolism rate of acetone is unclear, although the enhanced blood levels suggest some contribution from the latter. The ethanol blood curve is presented in figure 10, and indicates a very perceptible increase in blood alcohol concentration with a simultaneous acetone exposure. This is almost tenfold between 2 and 4 hours after exposure, with an increase in ethanol half life from about 30 minutes to 50 minutes. However, there appears to be little, if any, effect of acetone on breath elimination of ethanol. Acetone and ethanol thus appear to exert a mutual reinforcing of the slowing down of their elimination from the blood.

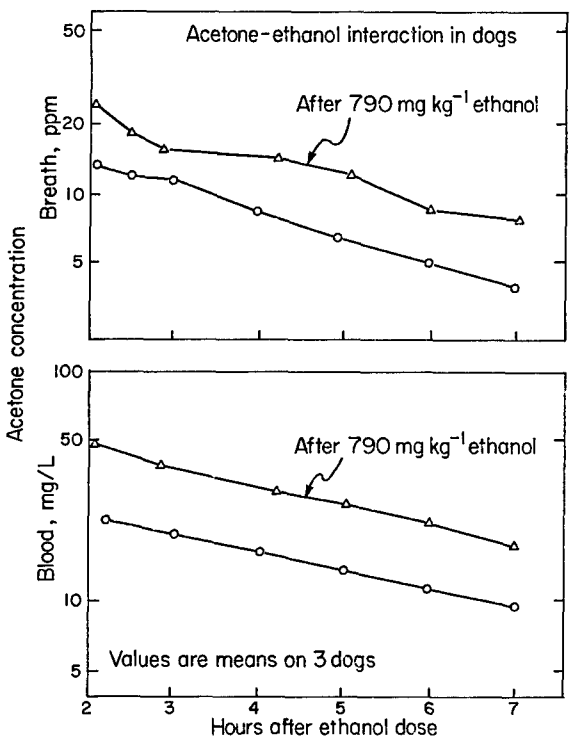

Figure 9. Acetone-ethanol interaction in dogs.

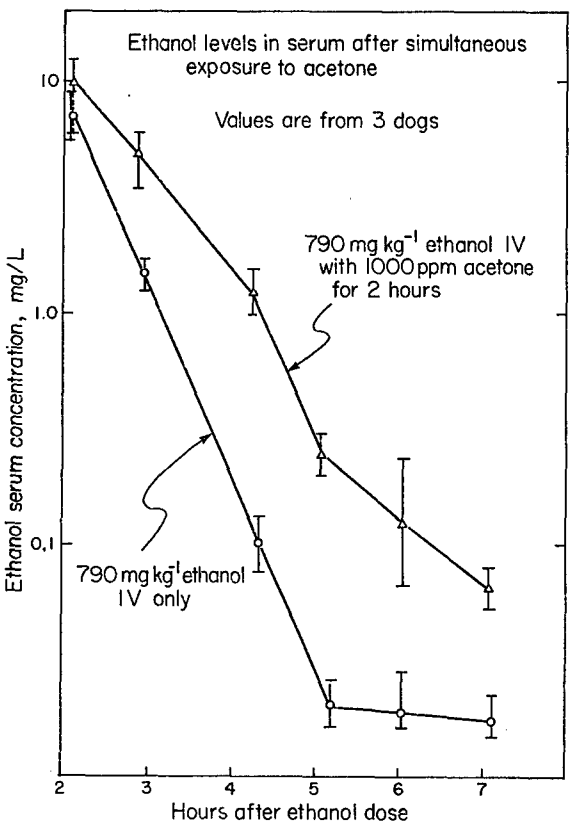

Figure 10. Ethanol levels in serum after simultaneous exposure to acetone.

Effects of phenobarbital on acetone blood levels are shown in figure 11, where it appears that IV injection of 20 mg/kg of sodium phenobarbital before a 2 hour exposure of acetone at 1000 ppm has essentially no effect on the acetone blood time concentration curve. A similar situation occurs with regard to breath elimination of acetone following the administration of phenobarbital (figure 12). It would appear, therefore, that the use of barbiturates is without effect on biological monitoring for acetone based on postexposure blood or expired air levels. However, when we turn to the effect of acetone on the elimination of phenobarbital (figure 13), we find that both the concentration of phenobarbital in blood is elevated, and the shape of the blood time-concentration curve is significantly altered. This is in agreement with the observation of Postolache (1969) that acetone prolongs sleeping time for barbiturates.

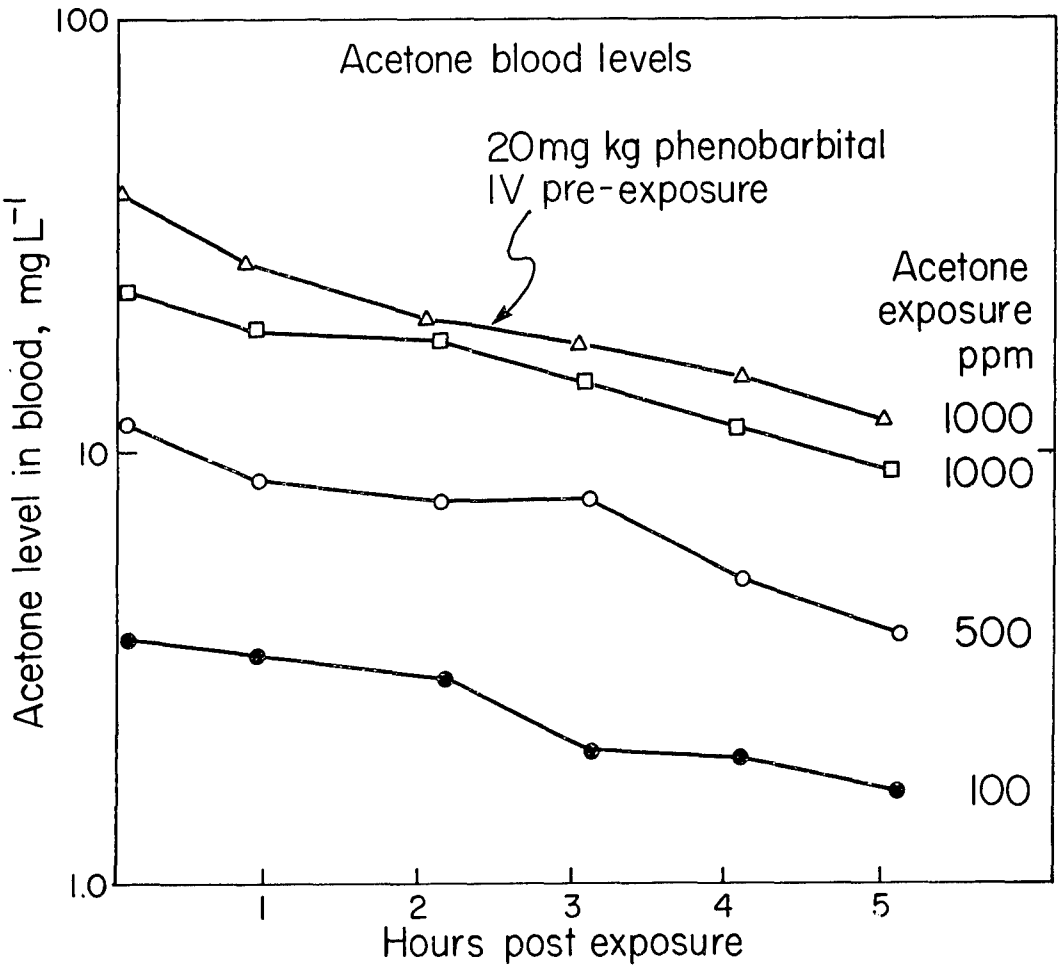

Figure 11

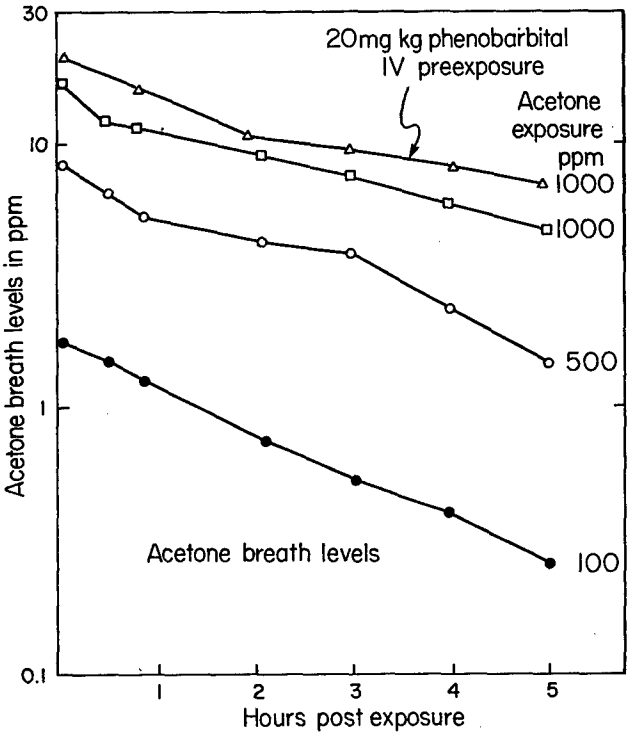

Figure 12

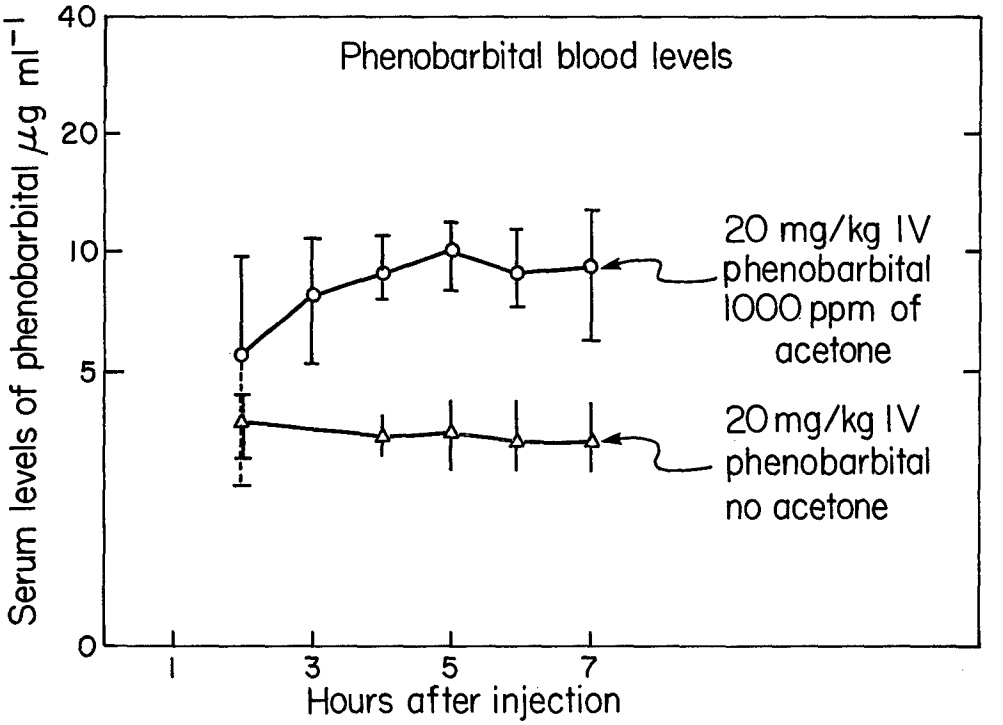

Figure 13

These effects are intrinsically interesting; ethanol has been identified as a competitive inhibitor of microsomal enzyme activity in acute dosages, and also as an inducer of microsomal mixed function oxidases (Rubin and Lieber, 1971). It is also oxidized by the same system to the extent of about 20% of the intake. It combines with cytochrome P450 to give a modified type 2 spectrum, and its interactions are principally with substances which give a type 2 spectrum. Thus, acute doses of ethanol will competitively inhibit phenobarbital metabolism if the two substances are given simultaneously, but after the acute phase, induction of the same enzyme system will lead to enhanced phenobarbital metabolism.

The results with phenobarbital suggest that acetone exerts an acute inhibitory effect on microsomal oxidase activity. The in vitro results with acetone indicate that acetone also exerts a stimulatory effect. Acetone may thus behave similarly to alcohol in its interaction with barbiturates, i.e., acutely it inhibits barbiturate metabolism, and chronically it stimulates. The slowing down of acetone elimination by ethanol is less readily accounted for in terms of enzymatic effects, and may well arise from the pharmacological effects of acetone.

Clearly the effect of factors of this type on the handling of common solvents calls for much more extensive examination. The implications for biological monitoring and the values thereby obtained are probably much more important than for the actual exposure situation, since we have always had situations which involve multiple exposures, many of which are unavoidable. In addition, occupational exposures do not usually involve operators who have acute levels of drugs or alcohol in their bloodstreams; it is more common for such levels to be residual. Nevertheless the limited experience given here indicates that the levels of substances in blood or expired air which may be used as criteria of exposures can be subject to variations caused by other substances.

CONCLUSIONS

In this paper we have tried to identify the factors which may exert some influence on the setting of biological threshold limit values and may be of importance in interpreting the results of biological monitoring. We have attempted to present experimental evidence, where possible, for the effects of some of these factors. There is no doubt that biological monitoring will have an important part to play in maintaining a safe and healthy working environment, but for a large number of substances, particularly solvents, we still lack sufficient data on meaningful biochemical and biological parameters which relate to exposures. In evaluating what data are available, and in acquiring data to establish biological monitoring, the various factors discussed should play an important role.

ACKNOWLEDGEMENT

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PAPER NO. 8

SOME PRACTICAL PROBLEMS ASSOCIATED WITH BIOLOGIC THRESHOLD LIMITS

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The analysis of biological specimens for toxic chemicals and products of pathological processes is an indispensable step in any toxicological investigation. Extension of this activity to human specimens and environmental toxicology is logical, particularly the search for sensitive indicators to herald the onset of adverse reactions. The proceedings of the Prague Symposium on Maximum Allowable Concentrations (I. U. P. A. C., 1961) firmly established the concept of Maximum Biological Concentrations for toxic substances used in industry. Vigliani's contribution (I. U. P. A. C., 1961, p. 285-8) set out clearly the advantages and limitations of using biological indicators, but due to a number of inherent difficulties, ranging from sampling problems to wide individual variability, little progress has been made in the establishment of generally acceptable Biologic Threshold Limits (BTL's). Nevertheless, it is evident from numerous publications (e. g. Zielhuis, 1971) and locally accepted guidelines (e. g. Alberta Department of Health, 1969) that BTL's are widely used, the selected levels being based on the experience and judgement of individual authorities. During the past few years, the more common and potentially harmful industrial (and environmental) contaminants have been the focus of intensive toxicological investigation, and sufficient reliable data appear to be available for the establishment of acceptable BTL's for a number of selected toxicants. However, before agreement on such BTL's can be reached, the influence of a number of practical problems will require consideration.

CHOICE OF BIOLOGICAL SPECIMEN FOR STUDY

Figure 1 shows the basic pathways by which toxic substances are absorbed, distributed and excreted in the human system. For application of a BTL the toxic substance must be predictably absorbed, distributed in the system, possibly metabolized and then excreted. Elkins (1967) showed how a number of selected inorganic substances could be systematically separated into those for which blood, urinary and exhaled air Threshold Limits (TL's) would be applicable. The selection was restricted to a number of airborne contaminants for which appropriate

Threshold Limit Values (TLV's) were already in existence. Elkins also chose to classify the TL's into Biologic (e. g. blood) or Excretory (e. g. urine, breath) according to the nature of the specimens analyzed.

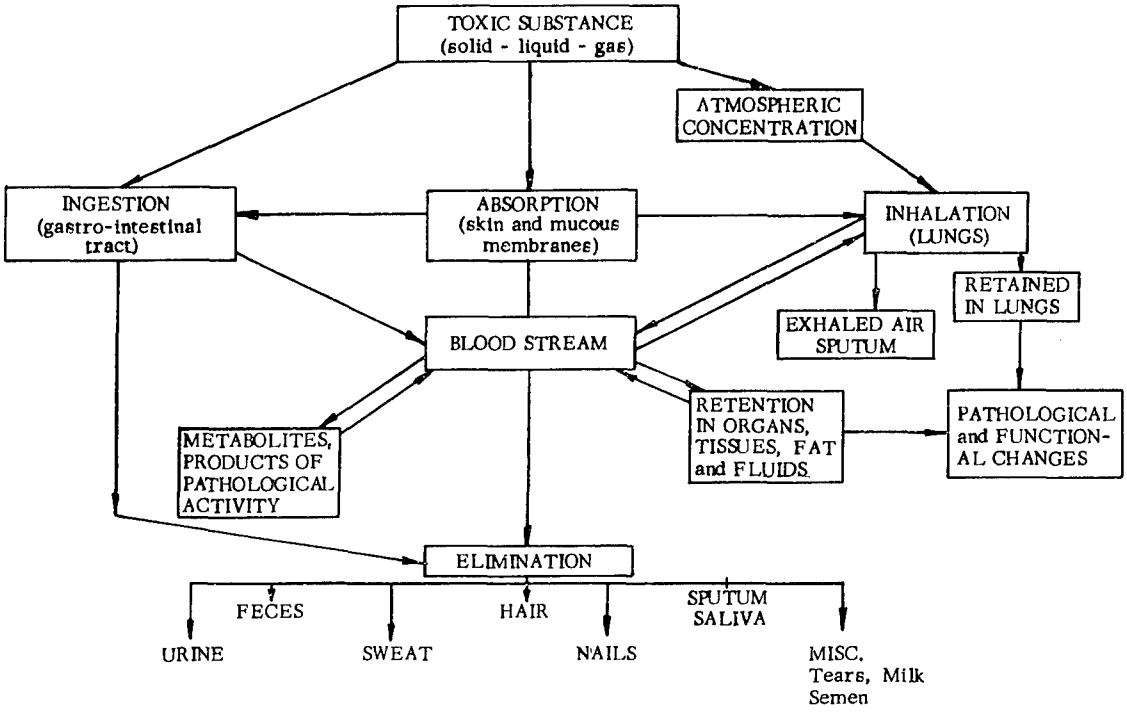

Figure 1. ABSORPTION AND EXCRETION OF TOXIC SUBSTANCES.

The choice of the specimens to be considered for BTL's will depend on the manner in which the toxic substance being studied relates to the absorption and excretion pattern shown in figure 1, and also on the circumstances which surround each specific case. Carbon monoxide (CO) presents an almost ideal model for considering with figure 1 and application of a BTL. CO is absorbed and excreted almost entirely through the lungs. The majority of CO (>80%) reacts reversibly with hemoglobin in red blood cells and the adverse effects can be correlated directly with the fraction of hemoglobin bound by CO (i. e., unavailable for oxygen transport) as carboxyhemoglobin - COHb (USPHS, 1970). Small amounts of CO are produced endogenously or broken down metabolically, the quantities being insignificant

relative to those normally absorbed and excreted through the lung during and after exposure. Thus the BTL can be based on measurement of COHb in the blood, the limit to be chosen depending on the degrees of protection required, which differ markedly for public and occupational exposures. There are, however, different approaches to measuring COHb in blood: Buchwald (1969 a, b) analyzed blood from a finger prick and gave reasons for the choice of method, while Breysse and co-workers (1966, 1969) were able to justify the indirect approach of determining CO in exhaled air. Each method is valid providing that the variables are recognized and the limits of confidence are clearly stated.

The problem of lead absorption presents an interesting case for discussion. In the occupational environment, lead is absorbed by inhalation of particulate matter (dust, fume) and through the gastro-intestinal tract. Because inhalation appears to be the most important mode of absorption, the gastro-intestinal route is often overlooked. The TLV of 0.2 mg/m^3 is applicable only if gastro-intestinal absorption is negligible; thus sampling of the working atmosphere for airborne lead is of limited value in the overall assessment of the occupational environment. Analyses of blood and urine in exposed persons are the most useful means for monitoring individual response to the contaminated environment. The question is, which of the biochemical responses should be monitored? Zielhuis (1971) critically reviewed the interrelationship between five of the seven most widely determined biochemical responses to lead, those not dealt with being the inhibition of delta-aminolevulinic acid (ALA) dehydrase in blood and the appearance of punctate basophils among the red cells. He concluded that lead in the blood is the best index of the "active" body burden of lead. A group of very widely experienced industrial health specialists (Lane et al., 1968) published a statement which specified the magnitude of four biochemical tests consistent with four categories of lead absorption (normal, acceptable, excessive and dangerous). They pointedly stated that "a diagnosis of lead poisoning should be based on clinical findings and supported by biochemical evidence of unusual exposure."

A common pitfall has been the use of biochemical responses to lead, by inexperienced individuals, to predict the onset of poisoning or to establish relationships with atmospheric concentrations. Attempts have been made to establish relationships when it is obvious from biochemical considerations that no direct relationship is possible. Consider the inhibition of ALA dehydrase, which catalyzes the formation of porphobilinogen from ALA, an essential step in heme synthesis: this results in the accumulation of ALA and the consequent increase in the rate of excretion of this acid in the urine. There appears to be a reasonably good relationship between the inhibition of ALA dehydrase activity in red blood cells and the concentration of lead in blood (Hernberg et al., 1970), with individual variations within predictable limits. Since the excretion of ALA in urine is an indirect response (i.e., ALA must pass from blood to urine via the renal barrier), the resulting relationship between ALA excretion and blood lead concentration is subject to many deviations, particularly when all the individual variabilities are taken into account (Hernberg et al., 1970). In this situation it would be ridiculous to attempt

a correlation between atmospheric lead concentration and the excretion of ALA in urine. Confirmation of this is shown in the work of Williams et al. (1969), Selander and Cramer (1970) and de Bruin and Holboom (1967), who studied the correlations between lead exposure and some of the biochemical tests used as indicators of lead absorption. The poor correlation coefficients and wide individual variations which were observed in relationships such as lead in air against urinary ALA concentration, hemoglobin concentration and punctate basophil count, could well have been predicted from the basis of biochemical knowledge.

As expected, for any individual there are wide diurnal variations in urinary excretion of lead, coproporphyrins and ALA (Kehoe, 1960; Ellis, 1966; Williams et al., 1969) but the blood lead concentration remains fairly constant (Kehoe, 1960). Because of wide individual variability, Blumenthal et al. (1972) and Specter et al. (1970) found urinary ALA determinations unsuitable as screening tests for lead "poisoning" in children. There is little doubt that blood lead is the only biochemical parameter which can be used unequivocally to determine the amount of lead absorbed and to relate this to the risk of intoxication. There have been two major objections to the use of blood lead analyses as routine tests for lead absorption in the occupational environment. The first relates to analytical problems, in terms of technique, reliability and expense, but with recent improvements in methodology and instrumentation this objection is no longer valid. The second objection results from the human problem of reluctance, on the workers' part, to provide blood specimens on a regular basis. There is little objection to the donation of blood up to three times per year or in emergency situations, but requests for more frequent samples can result in complaints and grievances. The use of more sensitive analytical techniques has resulted in a reduction of the quantity of blood required to one milliliter or less, yet there still appears to be resistance to the frequent demand for specimens. On the other hand, most workers are amenable to the provision of urine specimens during working hours. Consequently, it is easier to base a lead absorption monitoring program on regular urinary lead and coproporphyrin (or ALA) analyses. Such a monitoring program can yield much useful information when the results are considered on a group basis and when continuing records are kept for individuals (Ellis, 1966). In the detection of pediatric lead absorption, it has been stated that "... obtaining venous blood specimens from infants and young children can be difficult and must be done by experienced personnel," (Specter et al., 1971). On the other hand, Blumenthal et al. (1972) observed "While it appears easier to get a urine sample than a blood sample, this advantage may not be as great as speculated. Some children do not produce urine on demand ..."

When there is a choice of specimens (blood, urine, exhaled air, etc.) which may effectively be used as biologic indicators, it is important not to be dogmatic about the one which is most suitable. The best choice is the one which can be used most reliably and effectively under the circumstances which prevail in each individual situation.

SAMPLING PROBLEMS

Under this heading it is possible to examine a number of operations and variables where problems may be encountered. It will be evident that for surveys, research studies and routine monitoring, the variables require standardization, and attention to detail is necessary if meaningful results are to be obtained.

Obtaining Blood Samples

Small samples of capillary blood (up to 0. 5 ml) can be obtained after puncturing the skin with a lancet. The favorite sites for such punctures are the fingers and the ear lobes. The procedure for making a good puncture and obtaining the sample quickly, dextrously and painlessly requires practice, and technicians should be properly trained in the art of doing this. Future cooperation from donors can only be assured by the use of skilled techniques and by the adoption of a confident and courteous manner. With workmen, particularly those engaged in manual tasks the fingers may be dirty and calloused. The hands should be well washed before a finger is punctured, and it seems obvious that the finger selected should be one which is rarely used for manipulative tasks; for example the ring finger of the left hand in a right-handed individual. Where the finger prick is likely to interfere with work or if there is a possibility of infection, it is better to sample from the ear lobe. In some cases it is difficult to stop the bleeding from an ear lobe puncture and specific instructions should be given to donors about the application of direct pressure. The inclusion of a styptic pencil in the sampling kit is an advantage.

In considering donor cooperation, it is easier to obtain small samples of capillary blood on a regular basis than larger volumes by venipuncture. Thus the choice of analytical procedure should be guided, in part, by an appraisal of sampling problems.

Blood samples should be taken in accordance with recommended techniques (e. g. Levinson and MacFate, 1969). Donors should be interviewed on their own in a separate room or office and without onlookers. This prevents the spread of undue alarm about the sampling procedure and allows for confidential conversation with the donor.

Obtaining Urine Specimens

A variety of different specimens may be collected. These include early morning, spot, cumulative and 24-hour samples.

Spot samples are fairly easy to obtain in most working environments. It is best to provide containers which are large enough to accommodate the whole voiding and have an opening sufficiently wide for easy filling.

Cumulative samples are the composite from two or more voidings and can be readily collected during the course of a working day by providing each person with a container large enough to accommodate the desired sample.

Early morning (or late evening) samples are more difficult to obtain due to reluctance in taking the containers home. The provision of opaque bags or boxes in which to carry the containers is helpful, but there is always some uncertainty concerning the time at which the specimen was voided.

Genuine 24-hour samples are virtually impossible to obtain in the work-home environment because the donors may find it inconvenient or forget to include all voidings in the containers provided. Some persons, particularly beer drinkers, can pass very large volumes of urine - in several cases the donors completely filled 2.5 liter bottles in a 15-hour period and would have provided more if additional containers had been available.

Women may have difficulty in filling sample bottles; if suitable wide-mouth containers are not available, then they should be provided with individual clean and sterile funnels or beakers. Many women are reluctant to provide specimens during menstruation and it may be necessary to wait until the period is over before a sample can be obtained. Some women are very reserved regarding personal matters and tact must be exercised to solicit their cooperation.

It is evident that a successful monitoring program requires the cooperation of all donors and takes into account the convenience and personal inhibitions of individuals.

Time of Sampling

The time at which samples are taken requires careful consideration since the patterns of absorption, metabolism and excretion vary so widely from one substance to another.

With substances or metabolites which tend to accumulate in the system because the rate of absorption exceeds the rate of excretion, or because excretion is very slow, a relatively steady state is reached with respect to the tissue and blood concentrations, so there is usually a fairly broad time interval during which samples may be taken. Thus, for workers regularly absorbing inorganic lead, the blood lead concentration reaches a plateau which remains steady within the limits of analytical variability (Kehoe, 1960; Buchwald, unpublished data), and blood samples may be taken at any time of the day on any day during the working week. With urine specimens, the situation is quite complicated due to the wide diurnal variations in urinary lead and ALA excretion (vide supra). Ellis (1966) referring to lead excretion, stated forthrightly "... that the results of a single voiding of urine are in themselves meaningless, regardless of whether or not the specimen is collected at some specified time, expressed as a rate of excretion or

adjusted to either a constant specific gravity or creatinine concentration." Since these diurnal variations generally fluctuate above and below a mean value (Kehoe, 1961; Ellis, 1966), less variable results should be attainable by analyzing composite samples from several voidings.

The situation is similar with respect to other heavy metals although none have been so extensively studied as lead. Consequently, there is much less certainty about the biological specimen which should be taken, and about the concentrations of toxic substances or metabolites which reflect harmful levels. Absorption of elemental mercury or inorganic mercury compounds, for example, can be monitored by analysis of blood, urine, saliva, and hair for mercury. In the past, urine analyses have been the most popular because the older analytical techniques were insensitive and inaccurate at the low concentrations of mercury generally found in blood. In general, wide diurnal and individual variations have been observed in the mercury content of urine and blood of persons working in similar exposure conditions (Molyneux, 1966; International Committee, 1969). Mercury is one of the most elusive elements on which to perform reliable trace analyses, and many of the observed variables may be the result of unsuspected problems in analytical technique. However, with the recent introduction of the highly sensitive flameless atomic absorption techniques for the analysis of mercury, more data should be available in the near future regarding the reliability of blood and urine analyses as indicators of mercury absorption. The International Committee (1969) pointed out that no correlation exists between either blood or urine mercury concentrations and the occurrence and severity of the symptoms of "mercury poisoning," although they are very valuable as diagnostic aids. Similarly it is impossible to correlate exposure with urinary and blood mercury levels for individuals, but a positive correlation can be made on a group basis (International Committee, 1969). When a group investigation is possible the blood or urine specimens should all be taken on the same day. Urine specimens should be a composite of the day's voidings, including the early morning and evening samples, if practicable. Where individuals regularly exposed to mercury are being investigated, it will be found that blood and urine samples taken on three days, each one week apart, will tend to yield much more reliable information than a single sampling. Composite urine samples are necessary to minimize the wide hour to hour variations in mercury levels which occur in spot samples.

Analysis of carbon monoxide in blood presents a case where timing is of the utmost importance. On cessation of exposure, elimination is fairly rapid, the rate of elimination being exponential with a half life of about 5 hours in sedentary males (Peterson and Stewart, 1970). The rate of elimination increases with physical activity, and for persons undertaking light physical activity half of the CO is eliminated in 2-3 hours. Thus the time of sampling relative to exposure periods must be known if computations of CO dose are to be made. The importance of this has been discussed elsewhere (Buchwald, 1969a) but is well illustrated by the data shown in figure 2.

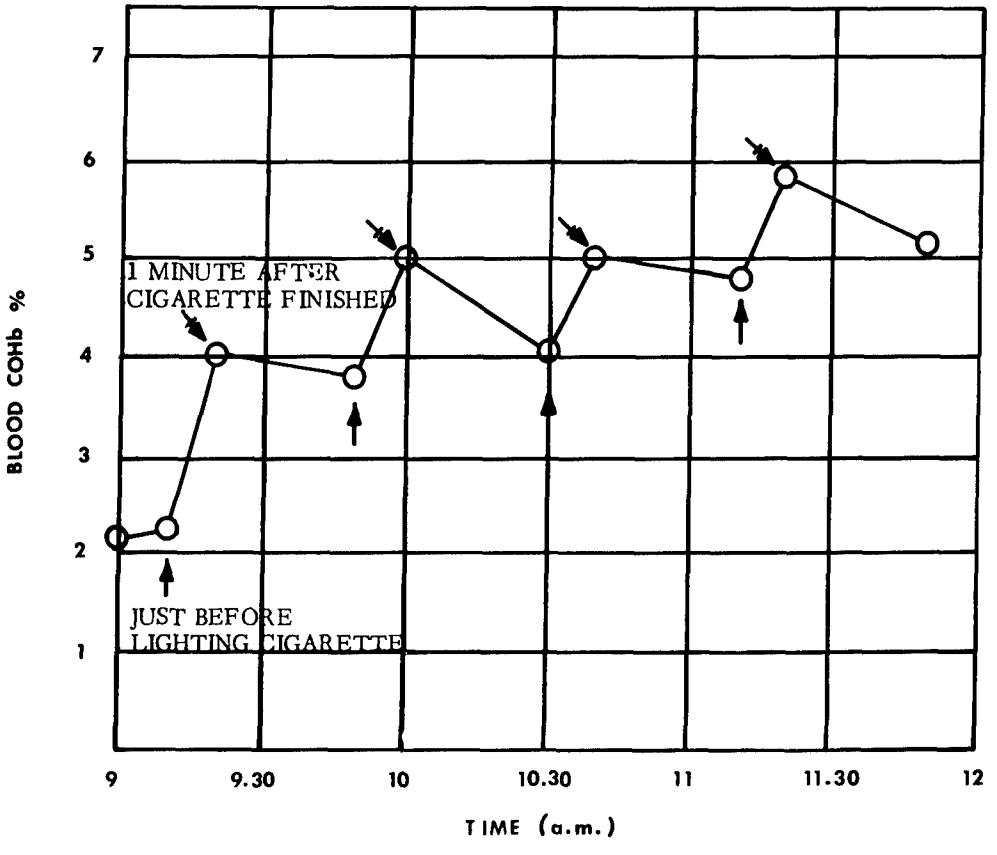

Figure 2. RISE OF COHb CONCENTRATION AFTER SMOKING SEVERAL CIGARETTES.

Industrial exposure to trichloroethylene (TRI) is frequently monitored by analyses of urine for trichloroacetic acid (TCA) and occasionally by blood and urine analyses for trichloroethanol (TCE). Ahlmark and Forssman (1951) found after a single exposure to TRI that the excretion of TCA did not begin until 2-4 hours had elapsed and attained a maximum after 20-40 hours. Similar observations were made by Soucek and Vlachova (1960) who found that the urinary TCA reached a maximum 25-35 hours after exposure ceased, while the TCE concentration reached a maximum within about 10 hours. Both groups of researchers observed wide variations of TCA concentrations in spot samples but noted that daily average and 24-hour concentrations were much more consistent. These observations have been confirmed recently by Stewart et al. (1970), Nomiyame and Nomiyame (1971) and Ertle et al. (1972). For five regular 6-hour daily exposures to TRI, Ertle et al. (1972) found that the blood level of TCE reached a peak at the end of the exposure period and then dropped sharply until the next period started. The daily excretion of TCE in the urine reached a maximum within 3 to 4 days but the TCA excretion only peaked on the last (5th) day of

exposure. Thus, in monitoring regular exposure to TRI with urine analyses for TCA, the samples (composite specimens) should be taken at the end of the working week. Following a single exposure to TRI, the samples for TCA analysis should be obtained between 36 and 60 hours after cessation of exposure (Nomiyama and Nomiyama, 1971).

Measurements of benzene in expired air and phenol are widely used in monitoring exposure to benzene (Sherwood, 1972). Analysis of benzene in exhaled air simply indicates the rate at which benzene is being directly eliminated from the body. The results can be meaningful, bearing in mind the limitations set out by Sherwood, 1972; for this purpose the best time to take the sample is within 30 minutes of cessation of exposure. Up to 40% of the absorbed benzene is metabolically oxidized to phenol which is excreted in the urine (R. T. Williams as quoted by Docter and Zielhuis, 1967). Analysis of phenol in urine is the most widely used method for determining the amount of benzene metabolized by an individual, and it seems to be a better index of potential hazard than atmospheric or exhaled air analyses. After exposing humans to benzene (80 gm/m^3) for 8 hours, the maximum urinary excretion of phenol occurred at the end of the exposure period, the concentration dropping rapidly thereafter ($t\frac{1}{2} \approx 4$ hours) (Teisinger and co-workers, 1955, as quoted by Docter and Zielhuis, 1967). The rapid excretion of phenol in urine has been observed by several other workers, all of whom found the half-life to be between 3 and 7 hours (Barodej et al., 1962, quoted by Docter and Zielhuis, 1967; Piotrowski, 1971 and Sherwood, 1972). It seems that relatively short exposures (<2 hours) might best be monitored by exhaled air analyses shortly after the exposure period. Regular exposures for longer periods should be monitored by urinary phenol analyses on spot samples taken at the end of the working shift (or not less than 6 hours after exposure began, or more than 4 hours after exposure ceased). A summary of the key points which should be observed during the routine assay of phenol in urine was presented by Sherwood (1972).

Contamination and Adulteration

Accidental contamination of urine specimens can be a major problem, particularly when dealing with trace substances such as cadmium, lead, mercury, fluoride and selenium. Contamination may occur at the time of sampling or can result from materials in or on the sample container.

When trace elements such as cadmium, lead and mercury are being sought at concentrations in the region of 0.1 mg/liter (0.1 ppm) only a few micrograms of contaminant are required to invalidate any results obtained. Thus, it is necessary to ensure that all containers and ancillary equipment used for sampling have been decontaminated. Washing with warm 1:1 nitric acid followed by rinsing with distilled or deionized water is generally effective for such decontamination. It is also necessary to check that any preservatives or reagents used in containers are free from the element being sought. This applies equally to all sampling equipment, for blood, urine and other biological specimens.

Accidental contamination of urine specimens is always possible when sampling is done at the place of work. For example, serious contamination has resulted from dust or particles containing lead falling into the sample bottle from dirty hands and coveralls. This type of contamination has been noted especially in lead oxide production and battery manufacturing where contamination of the hands and coveralls are prevalent. The donors must be instructed to remove their coveralls and wash their hands before taking the sample, and containers must be stored in a clean room or locker. This procedure may involve some loss of time if a composite sample is being collected. In a typical example, employees were asked to fill containers when they arrived before a shift, at lunch breaks, and after cleaning up to go home. An extra 5 to 10 minutes away from the job were allowed to ensure cooperation.

The author has experienced a few instances of deliberate adulteration of urine specimens. In one instance a man working with mercury metal diluted his urine with tap water - he was worried by the possibility of dismissal in the event that he was absorbing too much mercury. This dilution was discovered because the routine specific gravity check on all unsupervised sample collections gave values less than 1.008, while all samples collected under supervision had specific gravities in excess of 1.018. In another case a workman had a compensation claim pending with respect to lead poisoning and thought the evidence would be augmented by the addition of a little lead oxide to his specimens. In this case, the lead concentrations were inconsistent and impossibly high, such that adulteration was suspected; blood lead and urinary ALA analyses were easy alternatives not subject to adulteration.

Blood samples are rarely contaminated and are generally not available for adulteration. However, contamination is possible from rubber or plastic stoppers on sample tubes and from the introduction of preservatives or anticoagulants. Continued checks are necessary to ensure that such contamination does not occur. Special "lead-free" Vacutainer®tubes are available for blood lead samples; even with these, it is worthwhile checking one tube from each batch for the possibility of contamination.

TRANSPORTATION AND STORAGE OF SPECIMENS

In many hospitals, medical clinics, research organizations and larger industries, facilities exist for quick transportation and processing of biological specimens. However, if such facilities are not available then there are a number of problems associated with the transportation and storage of specimens. For example, in the province of Alberta, the Industrial Health Laboratory was located at Edmonton and specimens were sent there from remote locations up to 400 miles away. The specimens were transported by air freight, bus,automobile and in the mail. They were subjected to mechanical shock and vibration, extremes of temperature (from -40 to +120 F) and delays of several days. In the laboratory there could be further delays until staff and equipment were available to carry out analyses.

Among the many methods presented for the analyses of biological specimens it is remarkable how few take into account their integrity between the times of sampling and analysis. The USPHS method for determining lead in biological materials (Keenan et al., 1963) is an exception; methods for cleaning and decontaminating equipment are given in detail. The use of formaldehyde or EDTA were advocated for preserving urine and directions were given for dealing with sediments deposited in older samples. The importance of precipitates and sediments in urine specimens and methods for dealing with them are matters which are conveniently ignored by many authors. Deposited matter is particularly prevalent in urine specimens having high specific gravities (1.024) or high phosphate content, and in samples which have been chilled or frozen. The sediment deposited from urine may contain up to 80% of heavy metals (such as lead and mercury) present in the sample but very little of the organic compounds such as phenol or TCA.

Before any method is accepted for widespread use, the stability of the sample following transportation and storage should be thoroughly evaluated. In one procedure for estimating blood levels of COHb (Buchwald, 1969a) the stability of the sample was checked under a variety of conditions, and the accuracy, repeatability and reproducibility of the analytical method were checked after various time intervals up to one week. In an addendum to their paper on the determination of urinary fluoride, Rowley and Farrah (1962) commented on the stability of the specimens following various methods of preservation. Sherwood (1972) showed that urinary phenol concentrations remained constant for one year in a specimen preserved with a few drops of toluene and stored at normal refrigerator temperature (5 C). The importance of these checks is typified in the report by Magos et al. (1964) who showed that up to 50% of the mercury could be volatilized from urine by bacterial action within 40 hours of voiding. These authors stated "Indeed, volatilization of mercury from infected urine samples may be one of the causes of the wide variability in concentrations of mercury in urine samples from equally exposed workers." Addition of a few drops of toluene to the specimen prevented this loss. Loss of mercury from neutral water and urine specimens has been observed, this occurring from both glass and polyethylene containers, but it was not known whether this was due to volatilization or adsorption on the container surface. Acidification of the specimen with nitric acid (1 ml concentrated acid to 100 ml liquid) or the addition of EDTA (ethylenediaminetetraacetic acid, sodium salt, 0.2 g for each 100 ml liquid) prevented this loss. It is noted that Kopp and Keenan (1963) recommended the use of formalin (for glass bottles) and EDTA (for polyethylene bottles) as preservatives in urine samples collected for mercury analysis. A number of clinical chemistry textbooks give useful instructions for the sampling and preservation of urine and blood (e.g. Levinson and MacFate, 1969, pages 80-86, and Harrison, 1949).

PROCESSING AND ANALYSIS OF SPECIMENS

The processing and analysis of samples either in the field or when they reach the laboratory are the most important operations related to BTL's. These will not be discussed in detail since problems and sources of error associated with them are

well recognized, although not necessarily heeded. Anyone familiar with inter-laboratory trials will understand the frustrating and sometimes inexplicably wide divergence of analytical results on a given sample. The American Industrial Hygiene Association's blood lead study (Keppler et al., 1970 and comments by Weil, 1971) is a typical example. In a recent study of mercury in human tissues (Kevorkian et al., 1972), the results were completely invalidated by analytical variability and lack of attention to detail in sampling and presentation of results.

Major factors contributing to analytical dependability can be listed as follows:

homogeneity of samples
contamination from equipment and reagents
contamination and losses during transfer and manipulation
choice of analytical method
quality control
ability and experience of analyst and technician.

The only way of reducing the errors and wide divergence of results caused by any combination of the above factors is to introduce standard methods for the analysis of biological materials for specific substances. These methods must spell out in detail the equipment, reagents, conditions and procedures for each step in the analytical procedure, including the checks to be made for contamination, losses, accuracy, repeatability and reproducibility. The final step should include mandatory participation of the laboratory in inter-laboratory trials and certification of the laboratory and designated staff for performance of the specified analyses. Certification should be renewable only after periodic inspection and participation in inter-laboratory trials. The above comments refer to an idealized situation, one which would have to be implemented if BTL's are specified as health standards by regulatory authorities.

DOCUMENTATION AND REPORTING OF RESULTS

This is a catchpot for a number of practical details and other points which may be frustrating or annoying if not rectified, particularly for persons not experienced in sampling and analysis of biological specimens.

Packing, Labelling and Documenting of Samples

When samples are to be sent by mail, freight or over rough roads, leaking and breakage of containers can be encountered. Glass containers should be strong and packed into individual slots in a case capable of withstanding shocks, drops and compression. Cap seals and lids should be leakproof and secure, particularly when the containers are upside down or on their sides and subjected to temperature changes.

Labels or other identification marks should be checked for legibility and permanence. Certain types of adhesive labels readily peel from glass or polyethylene surfaces. There is nothing more frustrating than receiving a batch of samples from which the labels have dropped off, or in which a container has broken or leaked thereby smudging or erasing the label marks on neighboring containers. In most cases it is well worthwhile assigning an intelligent technician to devise a foolproof system for the packing and labelling of samples.

A package containing several samples should include a packing note which states clearly where the samples are from, how many samples there are and why they have been sent. In laboratories processing many samples each week, much time can be wasted in tracking down the source and requirements of a box of samples in which the receiver finds an enlightening note stating "letter and details following in the mail!" Most analysts are perceptive individuals and may engage in research studies relating to the samples they receive. It is soul-destroying merely to receive blood or urine samples for analysis identified only by a name or a code number. With most samples it is helpful to have such information as name, age, sex, date of sampling and reason for taking sample. In many cases additional information is required, for example in making an assessment of CO exposure from blood samples analyzed for COHb (Buchwald, 1969a) it is necessary to know the time at which the sample was taken relative to exposure, the period of exposure, the number of cigarettes (pipes or cigars) smoked on the day of sampling and the time elapsed since the last cigarette was smoked. This information had to be provided on a questionnaire card which accompanied each sample.

On receipt at the laboratory the details pertaining to each sample should be permanently recorded and most laboratories prefer to assign their own code numbers. This record should include the time of receipt and remarks on the condition of each sample; for example, unusual color and sediments in urine specimens are worth noting. In the author's laboratory all urine specimens less than 48 hours old were routinely tested for specific gravity and screened for pH, proteins, ketones, glucose and hemoglobin. With these tests it was possible to detect several instances of sample adulteration and a few cases requiring further study by their personal physicians.

Reporting of Results

There appears to be considerable confusion regarding the units in which the results of analyses are expressed. Blood analyses for specific substances are generally expressed in mg or μg per ml of whole blood, but the units of mg per 100 ml or mg percent are frequently used in clinical chemistry. There are trends to introduce complicating factors in expressing certain blood analysis results; for example, because blood lead is concentrated in the erythrocytes there is a move to apply a correction based on hematocrit to all whole blood lead concentrations.

Attention has already been drawn to the many different ways the results of urine analyses may be reported (Buchwald, 1964). In the many reports dealing with the urinary excretion of TCA, the samples analyzed included spot, cumulative, 12-hour, 24-hour and early morning specimens, while the concentration has been expressed as mg per 100 ml, mg per liter, mg per liter corrected for urine concentration, mg per 12 hours and mg per 24 hours. Under the circumstances, it is not surprising that such widely differing results have been obtained in attempts to correlate TCA excretion with exposure to TRI. The use of a concentration correction is advocated by some workers (Buchwald, 1964; Elkins and Pagnotto, 1969), and can be very useful for comparison of results where the excretion pattern is proportional to the total concentration of dissolved matter in the urine. If the total concentration of solute in the urine is outside normal limits (low and high) it is advisable to collect another specimen and repeat the analysis.

When results of analyses are reported, it would be most helpful to the receiver if the limits of detection and accuracy were also noted. For example, with a given procedure using 1 ml of blood, the limit of detection for lead is 0.03 μg per ml and the estimated accuracy is $\pm$ 0.03 μg per ml in the concentration range 0.03-0.50 μg per ml (accuracy may be defined as the standard deviation of repeated analyses on the same specimen, alternatively some analysts prefer to use 95% confidence limits). The term "trace" should not be used in reporting unless the limits for this can be quantitatively defined; similarly the figure 0 or the term "nil" require quantitative definition, i. e. $<$0.03 μg per ml in the lead example cited above. Every laboratory report should present all the quantitative parameters which may be important to the interpretation of the results.

Significance of Results and Follow-up

Many laboratories interpret the results of analyses on biological specimens and comment on their significance. If adequate guidelines for interpretation are not available, considerable divergence may be found in the recommendations made by different laboratories. The Alberta Department of Health (1969) and Lane et al. (1968) clearly set out ranges of significance (normal, acceptable, excessive, dangerous, etc.) for the results of blood lead, urinary lead and ALA analyses. However, it is important to recognize that biochemical analyses are merely indicators of absorption, body burden and excretion of toxic substances, they are not necessarily the sole criteria for establishing that poisoning has taken place. A diagnosis of poisoning should only be made on the basis of a complete clinical picture based on signs, symptoms and evidence of pathologic changes. Thus Lane et al. (1968) stated quite clearly that a diagnosis of lead poisoning should be based on clinical findings and supported by biochemical evidence of excessive lead absorption. This view was supported by a special committee (under the chairmanship of Sir Brian Windeyer) set up to inquire into lead poisoning at an Avonmouth (U. K.) plant (Editorial, 1972). In this committee's view, the use of the term lead poisoning, with its emotive and legal overtones, is of doubtful value, and it was suggested that emphasis be put on lead absorption, measured by blood-lead levels, as the criterion for corrective action. It has always been difficult to decide when absorption ends and poisoning

begins; it is clearly incorrect to make a clinical diagnosis of lead poisoning if the only abnormality is a raised blood lead concentration (Editorial, 1972).

Correctional or legislative action based on BTL's must be cognizant of the practical problems likely to be encountered and the resulting uncertainty of individual results. When excessive absorption of a toxic substance is indicated by a biochemical analysis, then confirmatory analyses associated with clinical and environmental investigations are necessary.

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PAPER NO. 9

BIOLOGIC THRESHOLD VALUES: A STEP BEYOND TLV'S

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INTRODUCTION

The primary goal of an occupational health program is to protect employees from illnesses arising from the work environment. A secondary goal is a total health program that will permit employees to be productive for their entire span of working life. In the U. S. the pioneering efforts of the American Conference of Governmental Industrial Hygienists under the leadership in the last several years of Dr. Stokinger in developing the concept and use of Threshold Limit Values (TLV's) for individual compounds has done much to reduce disease from the occupational environment in the United States. There are, as has been said earlier today, many circumstances where TLV's are not entirely adequate and it is time now to move forward into a more highly refined means of protecting the health of the worker. This advancement should be built upon the solid lessons of our many years of successful experience with TLV's.

PAST LESSONS

We have learned that the main route of exposure for workers is by inhalation but that skin or eye contact with some materials can produce significant local injury and with a few can cause systemic poisoning. Ingestion has not been considered a frequent route of industrial exposure. However, the subcommittee on the toxicology of metals of the Permanent Commission and International Association on Occupational Health expressed concern last month at their triennial conference that ingestion of inhaled particles can be a significant route for adding to the body burden (Friberg, 1972). We have learned that an employee's exposure during a work period is usually to more than one material and at varying concentrations for each material. We have also found that the effects of such differences in exposure are enhanced by the great diversity among individuals. These concepts have been well recognized by industrial hygienists and occupational physicians with the result that many procedures have

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* The opinions expressed herein are those of the author and do not necessarily reflect those of the Academy, its Committees, or its contracting agencies.

been developed to observe the individual's occupational exposure directly through analysis of body fluids or tissues. In moving forward into the new era of greater biological monitoring for employee health protection, it is important to remember all of the lessons of the past.

OFF-SITE BODY BURDENS

One of the most important lessons from our more than 25 years experience with TLV's is that each employee brings to the workplace a body burden of toxic substances acquired from food and water, recreation, personal habits, living conditions, general air pollution, and similar nonoccupational circumstances. These body burdens of toxic materials represent a baseline for each individual upon which are superimposed the daily occupational loads as well as any residual burden from previous work exposures.

It logically follows that any single measurement of an individual's body burden is not proof of either the presence or absence of occupational injury or even of overexposure in the industrial environment. Even repeated analyses exceeding some established limit do not necessarily indicate dangerous working conditions. For example, employees in a factory producing leaded glass were examined periodically for lead by means of urine analyses. Room air samples were also collected at the same time. One employee was found to have an excessive concentration of urinary lead. This was discounted on the basis that none of the other employees had high urinary lead values and the air sample was well below the TLV, which at that time was 0.15 mg/m^3. It was suggested that the sample may have been contaminated by dust during sampling. However, in the next two weeks this employee's urinary lead rose to much higher levels, still with no change in the air levels or in the body burdens of his fellow workers. A close observation of his work habits gave no explanation for the high levels. It was finally determined that the man operated a small farm and his tractor had an exhaust pipe directly in front of his face and it used conventional gasoline fortified with tetraethyl lead. The problem was easily corrected by helping the employee modify the exhaust system on his tractor. His urinary lead concentration returned to normal and an effective well-trained employee was kept healthy and on the job. Similar examples have been found in the automotive industry where work may be done privately after hours on body repairs, but without the protective exhaust ventilation. Periodic blood lead determinations soon show the effects of those nonindustrial activities.

In both of these situations simple dependence upon the TLV and an air sampling program would not have protected these employees from lead poisoning because the sampling would not have been related to their total exposure. It may be argued that

if the after-hours working environments, that is the farming and home automobile body repair shop, had been subject to air monitoring the TLV might have been adequate to protect these employees. What then of the welder employed by the demolition and salvage company to cut up structural steel? His cutting torch produces lead fumes as it burns away the red lead paint. Proper air monitoring and control methods should protect him from excessive exposure in the industry. However, consider the very real possibility that the welder is an alcoholic addicted to "moonshine" whiskey which is notoriously high in lead. The only thing that will protect that worker from lead poisoning and his employer from losing his services at the expense of a compensation claim is a biologic monitoring for body burden of lead.

The preceding are examples of how an employee can bring to the workplace a body burden of the same toxic substance to which he is exposed during working hours. Another unusual example of this was described by Stewart and his coworkers (1972) at the Medical College of Wisconsin. They discovered that the use of dichloromethane as a paint and varnish remover produced high levels of carboxyhemoglobin. These body burdens of carbon monoxide apparently were the result of metabolism of the solvent. The elevated blood levels reached a peak some time after exposure to the chlorinated hydrocarbon was terminated. The concentration slowly decreased and became normal within 24 hours although there was still a significant amount of carboxyhemoglobin at 18 hours.

For the preceding circumstances, in which the same toxic material arises from exposures at both the workplace and in nonoccupational exposures, the biologic monitoring might most easily consist of measuring the contaminant concentration directly in the body.

OFF-SITE INTERACTING SUBSTANCES

There are nonoccupational exposures that alter the effect of materials encountered during work. For these situations biological monitoring is the only method that can be used and it should consist of measurements of some biological function or performance. Air sampling measurements have no bearing on this problem. Some examples are given below.

The nature and severity of the effects of chlorinated hydrocarbons upon employees is directly affected by the individual worker's ability to metabolize these toxicants. Much of this metabolism is carried out by adaptive microsomal enzymes of the liver. Anything in the nonoccupational life of the person that modifies microsomal enzyme activities will in turn affect the toxic action of the chlorinated hydrocarbon on that employee. For example, Rogers, Alcantara and Fouts (1963) have shown that the antituberculosis drugs derived from para-aminosalicylic acid inhibit the activity of microsomal enzymes. Employees

receiving PAS therapy could be more susceptible to chlorinated solvents encountered at work. For another similar example, Peters and Fouts (1969) showed that Aureomycin (chlortetracycline) also inhibited liver enzymes. Conversely, Platt and Cockrill (1967) have reported the increase of liver enzyme activities by barbiturates, DDT and carbon tetrachloride. They also studied the interactions among these and it seems likely that the normal effects of these chlorinated hydrocarbons would be modified in employees receiving barbiturates. Individuals seem to differ from each other in their level of microsomal activity (Hammer et al., 1969).

The observations cited above on metabolism by microsomal enzymes refer to animal experiments and usually involved larger doses than are normally administered therapeutically to man. Their implication to biologic monitoring is clear but there are few data relating environmental contaminants, generalized or occupational, with the low doses of medication used for humans.

There are data on the beneficial interaction in humans of large doses of two toxic materials. The metabolism of methanol to toxic products is inhibited by ethyl alcohol. This was reviewed by Röe (1955). Maintaining a fairly high blood concentration of ethyl alcohol permits the methanol to be excreted unchanged through the lungs and kidneys, thus avoiding the acidosis problem. Methanol poisoning by vapors has occurred industrially and although it hardly seems wise to suggest that employees should be given ethyl alcohol regularly for prophylaxis, the interaction should be kept in mind when evaluating methanol exposures.

GENETIC VARIATIONS

Perhaps the greatest reason for the necessity of progressing toward the use of Biologic Threshold Limits is the wide variability between individuals. Take a look at each other - go ahead, the other person isn't really that much different from ourselves - or is he? Remember we each have a different image of ourselves than other people have of us. Is this surprising when we look at the almost unlimited opportunities for genetic variations in the human species? On top of that there is our entire past personal experience, pre- and postnatal that affect not just our appearance but our metabolism as well.

An article in the October 1972 issue of Scientific American discusses the genetic differences among various nationality groups (sic) for the simple hydrolytic enzyme lactase. The lack of this enzyme and the associated intolerance to milk is widely prevalent except among those whose early ancestors were herdsmen. Some anthropologists suggest that this is an example of Darwinian selection and evolution in man.

You are all familiar with the sickle cell problem which seems to affect a worker's performance on tasks that produce a high oxygen demand by the tissues. It is hardly necessary to mention the other examples such as the serum antitrypsin deficiency associated with familial pulmonary emphysema.

Our past methods using TLV's for monitoring the worker's environment have tended to ignore these differences or to allow for them indirectly by a wide safety margin. The first sentence in the preface to the TLV's says they, "... represent conditions under which it is believed nearly all workers may be repeatedly exposed day after day without adverse effect." The preface also points out that TLV's are based on the concept of time-weighted averages of air concentrations. Thus, we see that the TLV's have been developed essentially around measurements of "average air" to which the "average worker" is exposed.

Claude Bernard spoke out in 1865 against the misleading use of statistical averages which creates an illusion that has no relationship to reality for an individual. To show his point he described the work of a fellow scientist who collected all the urine from the toilets of a major European railway station. The mixture was analyzed in the belief that this would provide an accurate descriptor of the "average European."

There is no such thing as an "average worker"!

BEHAVIORAL VARIATIONS

Dr. Samuel Corson (1970) at Ohio State University has shown that dogs react in a highly individualistic manner to classic Pavlovian stress. The animals were given an electric stimulus to which they would normally react by agression or avoidance. However, the animals were not permitted either of these reactions but were trained to whine and lift the paw receiving the shock. He found that some of the dogs also had "visceral responses" to this frustration consisting of an increased respiratory rate, increased salivation, increased heart rate and similar reactions. On the other hand some of the dogs showed none of these visceral responses. These represent the extremes of Pavlov's typology, sanguine or very excitatory and melancholic or very inhibitory.

Dr. Corson cites some indirect evidence that the two patterns of behavior which he observed in response to the experimental frustration are established early in the life of the animal, either by genetic factors, prenatal influences, or immediate postnatal experiences.

Once again we see that there is no "average individual." Our past technique of dealing with occupational exposures as averages is on its way out just as fast as or perhaps faster than we can collect the necessary data and understanding of how to monitor the individual for any or all of the 550 compounds now included in the TLV list. The Occupational Safety and Health Act requires that every worker - not just 'nearly all' - must be protected. This could be interpreted as implying individual monitoring. In my own opinion, the best way of providing protection for an employee's health is by the traditional medical practice of looking at the individual.

The National Institute of Occupational Safety and Health is now moving in this direction. It has already prepared in early draft form "Recommended Biologic Standards" for the following topics: anticholinesterase compounds, benzene, toluene, carbon monoxide, methyl alcohol, arsenic and its compounds, arsine, and lead. At this time there is no way of predicting when these will be ready for transmittal to the Secretary of Health, Education and Welfare for his own recommendation to the Secretary of Labor that they be adopted. The important point is that we are already on our way forward from the TLV concept toward biologic monitoring of occupationally exposed individuals.

SUMMARY

In summary, the expanded use of biological monitoring methods offers a major advance toward the goal of protecting every worker from toxic hazards in the occupational environment. It is recognized that the effects of the exposures being monitored can be increased or decreased by exposures outside of the working hours and by the variability of the individual. Accordingly, the use of biologic observations for this purpose necessitates a consideration of the individual worker's nonoccupational exposures and idiosyncrasies. It thereby provides an opportunity to become a cooperator in the total health maintenance for employees under the cognizance of occupational health personnel. The biologic monitoring can consist of direct analysis of body fluids or tissues for a contaminant, for a metabolite of the contaminant, or for a change in performance or organ function. The net effect can be a healthier, happier, and more productive employee.

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OPEN FORUM

COL. KITTILSTAD (Air Force Surgeon General's Office): Mr. Wands started to say some words that I thought I was going to agree with but I had to disagree with him as he went along. I think many of us are concerned that the TLV's are really beginning to be misused as far as OSHA is concerned. I think the fear that many of us have who are not so deeply involved in the establishment of biologic threshold limits is that the same thing is going to happen with the biologic threshold limits as happened with the air limits, and that is the fact that they have become law, that the people who are going to be enforcing this law not only are not trained to interpret these things but are instructed that they cannot do anything other than to interpret the law as it is on a piece of paper. I'm particularly concerned, and having listened today I think what has been said today has confirmed more than I suspected all along, that the means of establishing the biological threshold limits are no more valid than the ones that we have had to establish the air limits, not that those are bad. The point being that the biological limits are no better than the air limits. I think we are talking about representativeness of samples. I think we are talking about errors that can come in the collection of the samples, the processing of the samples, and these are the samples, and these are the same problems that we've talked about for years concerning air samples. I understand that most of the people here today are extremely interested in toxicology. I'm not a toxicologist myself. I'm an industrial hygienist so probably the tenor of the meeting as it is going on has probably scared me a little bit more than the people of NIOSH who have established the standards. I get a feeling there is a push to bring biological standards into play before we know that much about biological standards. I'm extremely reluctant to go along with the type of scheme where we are pushing something that we really don't have that much of a handle on. This is a problem that bothers me and, from some of the conversations that I've had, I think it bothers some of the other people around here.

MR. WANDS (National Academy of Sciences): You are quite right. You should be scared. I am greatly disturbed myself, not just at the fixing of TLV's to finite, precise points of law. I am equally concerned about the fixing of precise numerical values for all environmental standards, not just occupational environment but the general public environment. I am concerned anytime that we establish a hard and fast number for anything which is biologic. The infinite variability betwixt me and thee not only in our real responses to these exposures but in our personal abilities to analyze for them. You've seen these round robin studies. You've seen the variations of the LD_{50}'s from one laboratory to another. You've seen the variations of the skin and eye irritations indices using the standard FDA required test procedures that every laboratory is supposed to be able to duplicate

all across the country. Somewhere along the line, there does have to be a description of acceptable effects that can be agreed upon. We got into that earlier this morning when we had these graphs over here that Dwight Culver showed and the points that he raised. We have to determine that there is an acceptable level of, let's say, lead in the urine, or let's say benzene in the breath, that may not necessarily belong there. Lord Almighty didn't put it there when he created us. It is not a metabolic function. It is not an essential material and yet it's there. And over the generations, perhaps, we have learned to live with these, or we have developed defense mechanisms that permit us to live with some finite body burden. And I wish that Herb Stokinger this morning had developed more this concept of the word "threshold" as it appears in threshold limit values, as it appears in biologic threshold limits, this concept of a tolerable amount of foreign material that we are able to carry in our bodies without any detectable adverse effects. And this concept of acceptable level of impurity is different in our approach towards occupational health as well as our approach towards environmental health. As differentiated, for example, from the Russian concept where even the most minute change of any kind whatsoever by the most sensitive test method is undesirable. This may just be a simple defense mechanism where one develops a callous on your hand after you've raked the lawn several times this fall, you finally end up not getting blisters but you get a callous that is a defense mechanism. Now, is it good or bad to have callouses on your hands? These kinds of changes are acceptable by and large. This is the concept we have to crank into these standards as we set them. I think the idea of having some legalized standards where agencies like the Department of Labor can bring a compliance action out is necessary. Somebody said today, 60 to 80% of our employees are in small industries where they're not even given any kinds of protection. These are the ones that are being injured. The people in Dick Henderson's shop that have high urinary mercury once in a while are not being damaged because they've got a good medical program to pick these people up before they get injured, but it's the other people. And we do need laws like this but they do have to be written and they have to be administered in some reasonable fashion, and you're quite right to be scared that they cannot necessarily be administered that way as they are being written.

DR. STARA (Environmental Pollution Agency): I am glad that Mr. Wands raised this question, because I thought the Conference title was Environmental Toxicology not Industrial Toxicology. I am interested in comments from you gentlemen as to how exactly such a thing can be solved. How can we set standards for general population that are based on biological monitoring? Also, is there a way that we can ever get to the point where we can set standards for a combination of pollutants instead of single pollutants as I think practically and realistically we have to right now in this country? Also, the industrial population is a captive population; therefore, the biological monitoring is physical and I think it's very

important. I think the general population which covers all segments from the embryo all the way to the aged, the pulmonary cripple, and the cardiac cripple, and so on, is the most difficult issue and I don't think that toxicologists or epidemiologists are yet ready to solve it. I wonder if you had any great comments that can solve some of these problems, at least in our minds.

DR. ASTILL (Eastman Kodak Company): Well, I think there is a possibility that if we carry through some of the programs that we have talked about the industrial environment may become a much safer place in which to exist than the environment outside industry. Certainly, with biological threshold limit values, we are going to have a very close look at the effects of a large number of substances on a large number of people. And, indirectly, we are thereby going to be able to police, you might say, the nonoccupational type of exposure. I think in developing adequate programs for biological monitoring, we're also going to find out a lot of information about combinations of pollutants and things of this kind. I'd like indirectly in touching on this question to revert to this problem which the previous question raised. One of the answers, I think, in a satisfactory setting of BLTV's is this dual level concept that we've touched on earlier today. This is to say that you have a warning level. This takes into consideration, if you have a warning level which you can set with some degree of competence, a lot of the variability that we've been talking about, genetic interferences, particularly the variability in sampling, and also the variability which comes around in analytical measurements. And I think that those of us who have done any analytical measurements in this field are well aware of the great spread. If this concept takes hold, then it's possible for adequate monitoring to be set up for a fairly reasonable understanding of what the monitoring means, and at the same time vital information can be given in a set of industrial exposures without putting somebody within the scope of the law. Then you have an upper level when you reach or are above that level, then you're out of compliance and necessary legal action can be taken. But many of us think that the dual level concept does at least tend to protect the employer and yet at the same time enables us to make some progress in the field of biological monitoring.

DR. HENDERSON (Olin Corporation): I'd like to comment on this business of thinking that we can completely protect the individual against some of these hazards. We've now put a warning on our cigarette packages and yet I still see a lot of the people here that are smoking cigarettes. Again, as I look around here, I would venture to guess that 75% of this audience is over the recommended weight for their height and age. Now, we've got all of this information on what is a reasonable dietary pattern and yet we, ourselves aren't following it. We know the effects of smoking and we aren't following it. I don't think that we are going to be able to legislate a healthy individual. I think we are going to have to do this by education and not by legislation. While I have the floor, I would like to make a

separate comment, however. Since I criticized Dr. Stokinger, I also would like to come to his defense because I think that Mr. Wands was somewhat unjustified in saying that he has not thought of the difference between me and thee, because it is Dr. Stokinger who has been pleading with us to do genetic screening on our people and he has definitely been thinking of it in terms of looking at the individual so that I want to defend him as well as chide him a little bit for what I consider a violation of his own principles.

MR. SHERWOOD (Esso Europe Incorporated): Mr. Chairman, can I follow up Mr. Wands' comment on the word threshold? Can I ask what do we mean by the word threshold? I can see two possible interpretations. One is an acceptable risk and that is we assume, perhaps, that there is some sort of linear relationship between dose and response. If we go on down the line long enough, we get to the point where too few people are affected for us to be concerned. On the other hand, there's another concept of threshold, more of a biological one, and that is that below a certain level there is no response, and I think Dr. Henderson was drawing our attention to that in the case of mercury. I think with benzene there may be something similar too, because in our experience we find that the ordinary worker exposed to benzene excretes his phenol as sulfate. It's only when he gets into an overdose condition, perhaps over 500 mg/liter, that we see the glucuronide activity coming in. Could it be here that there is some threshold mechanism? This is, I think, a very important point that at this stage we try to define what we mean by the word threshold. I might, while I have the floor, also add that I think we should look to the atomic energy industry for some of their experience in the use of biological threshold limit values. I know certainly a number of organizations have always used two levels in their urine results, an investigation level and an action level, which is directly analogous. I should point out that the International Commission on Radiological Protection who developed the standard man did conceive of the man with both ovaries and testes, which I find odd. How standard that is, I don't know, but the atomic energy industry also dreamed up the concept of a maximum credible accident. Might I suggest we consider a maximum credible man?

DR. FRIESS (National Naval Medical Center): There was a discussion of the meaning of threshold this morning in terms of Dr. Back's reaction, you recall, to what is a meaningful effect when you have a variety of effects produced by a given agonist, for example. Which one shall you take to be meaningful in a biologic sense? Did it not emerge from that discussion that essentially one would take the most sensitive indicator which produces an effect at a given level and the effect must be of physiological significance? And then the level which induces that effect shall be termed the threshold level. Was that not the sense of the discussion this morning? Ken, how about it, did that emerge? No? That's all right, this is an appropriate point at which to drop it if it's wrong. But that was what your exchange with Dr. Stokinger seemed to lead to.

DR. BACK (Aerospace Medical Research Laboratory): That's what it turned into. That's not my interpretation, however.

DR. FRIESS: Well, just to get it on the record now, Ken, would you give your interpretation?

DR. BACK: Threshold can mean anything you want it to mean. In one instance, it means that level which you will not exceed in terms of law. Another one is the threshold at which you find effect, so I don't know what Herb's interpretation of biologic threshold is. Maybe he would give it. I think it's different from the threshold limit value.

DR. FRIESS: Well, this is the perfect time to turn to Dr. Stokinger.

DR. STOKINGER (U. S. Public Health Service): Well, it's a long story and there are many facets to it. We have attempted to make a fairly complete answer to it in I think it's the September issue of the Archives of Environmental Health, I think it's the lead article, and some of the title I think was "Concepts of Biologic Threshold Limits and Analysis of the Background to It" and so forth. That was a sequel to a similar article by Dr. Bertram Dinman that appeared in Science under the most peculiar title of "Nonconcept of No Threshold," and I never understood that but it came to the same conclusion. It centered around the fact that there are a lot of people, particularly arising in the fields of radiation biology and cancer oncology, who believe that there are no such things as thresholds in their area. This concept is, we feel, erroneous because it neglects the matter of homeostasis and adaptation which is, of course, a common physiologic fact. All these concepts are brought in and I think if you will read the article carefully you will find a pretty clear view of what the threshold we're talking about is in the field of environmental health. It's too long to go into now. I don't imagine I'd remember the concepts anyway.

MR. WANDS: I'd like to respond also to the other part of Mr. Sherwood's comments. We accept any number of killings and maimings on the highways of our nation and don't get terribly excited about them in spite of the fact that we've got some laws that say thou shalt not speed, thou shalt not drink while driving, all of these things, and they all get violated, get ignored frequently. And yet at the same time, society gets terribly uptight about relatively minor problems and let me bring to your attention the tremendous and in my own personal opinion undue, the tremendous amount of interest and effort that has gone into the development of standards for environmental exposure to beryllium and the number of cases of berylliosis versus the number of highway deaths. There's no relation whatsoever - there's no logic to what kinds of insults society will accept, either for society at large or for the individual in terms of self abuse, such as Dick

spoke of, overeating, overdrinking, oversmoking, all of these things in excess. You cannot legislate these things and there is no rhyme or reason as to what society will accept. At one time, we had the idea that one might be able to get a measure of the kinds of threats that society would live with by looking at the number of accidental deaths from being struck by lightning. Now here is something that you and I don't get terribly concerned about unless we're out on the golf course in the middle of a rainstorm. The sale of lightning arrestors has dropped off tremendously in the last hundred years on homes unless you're out in the middle of a prairie where you're the high spot. People don't get terribly worried about lightning deaths. This might then represent a level of fatalities that society is willing to shrug off without getting excited about. Here then might be a baseline of a socially acceptable threat to health. But that doesn't seem to bear out. There is no way of resolving the question.

DR. BUCHWALD (Department of National Health and Welfare, Ottawa, Ontario): May I add just one little thing to this? I think one of the great problems in our modern society is the news media who give an incredible amount of publicity to something new and different. The problem of lightning is not anything very new. You will see a one-inch column devoted to a death from that. The same applies to the motor accidents. But, you get one case of mesothelioma near an asbestos factory and all hell breaks loose. It's because it's something new and it can be distorted out of all proportion by the news media and it travels right across the whole country and then becomes a political issue, and this is something that we just can't deal with at all.

DR. SCHEEL (National Institute for Occupational Safety and Health): I'd like to turn the attention back to something that Dr. McNamara was concentrating on this afternoon and I think may have missed part of us. He is objecting to the idea of a single number on the basis that this outlaws judgment, and he is speaking to a theme in which he says if we have a dual standard, a warning level, and a no-excess level, we will thereby write into the legislation a mandatory judgment and, on this basis, I think that his idea bears real merit, and it should be seriously considered.

DR. FAIRCHILD (National Institute for Occupational Safety and Health): In Buenos Aires I think I brought up this same point and that was to jar us back to reality in terms of biologic threshold, so I'll bring it up again here today, especially those of us that are concerned in occupational health. The law is written now that the Department of Labor sets standards and we do the research and we recommend these standards. Mr. Wands has done a good job, and Dr. Stokinger and others in pointing this out, but we have to keep this in mind. Right now the law is written so that the Department of Labor inspectors can perform compliance and they are interested not in biologic thresholds because there is no way in this wide, wide world that they are going to use biologic thresholds in carrying out compliance.

So what we really feel that we should recommend is that we plug away, we try to do this in terms of research. NIOSH will use biologic standards wherever possible in their hazard evaluation and in determining potential for injury at the work place. But I think we're fooling ourselves if we think, unless we're all going to change Congress' ideas on this, that the biologic threshold will be used as an enforcement weapon by the Department of Labor. This is not going to happen for a long, long time. It's very good to set up a program in industry to monitor our people and this would go as a recommendation in our criteria documents, but this is not the actual thing that the Department of Labor would use. And I think we have to keep that in focus when we're talking about occupational health. And then, of course, this is different from environmental health, but right now I think we're on this question of occupational health and our responsibilities in the National Institute for Occupational Safety and Health, as the law reads.

DR. CULVER (University of California): My question is directed to Dr. Fairchild. It was my impression that the criteria documents that have been published, such as the one on asbestos, recommend that periodic examinations be performed on asbestos workers. This seems to me to be a step toward a biological threshold. I'm not sure why NIOSH recommends periodic examinations for asbestos workers because there's no possible way that an examination can protect a man from getting asbestosis or mesothelioma. Is it a step in the progression towards enforcement of biological threshold limit values?

DR. FAIRCHILD: Yes, Dr. Culver. This is very true. We want to develop the state of the art and these are the things we're looking for in our criteria documents. But the point I was trying to bring across is that for the actual compliance by the Department of Labor, if they follow our recommendations, and they don't have to, but if they do follow our recommendations, they would probably set up the program whereby the industries themselves have to set this up and prove that these things are being done. But we should not think that, in terms of enforcement, compliance officers are going in and taking blood samples and looking at urines and these kinds of things. But I still go back to the thing in terms of our own research and our hazard evaluation, we will very definitely as a research institute use these and I think that industries who are trying to protect their workers can very well use these in terms of showing that they're been monitoring. My only point I was trying to show is that for the actual compliance of the Department of Labor, I think it's a long way off.

DR. AZAR (Haskell Laboratory, E. I. duPont): I don't thnk we're talking about completely replacing the airborne threshold limit values with biologic threshold limit values but there are some instances where you really have a need to have a biologic threshold limit value which I believe can be enforced by a compliance officer. For example, suppose we've got workers exposed to solvents and these solvents have an airborne threshold limit value of 10 ppm and you can go into that plant and there are alarm systems that go off if the airborne

concentration exceeds 10 ppm and they clear it out and go in and find out what's wrong. But the surprising thing is that both of these solvents are very rapidly absorbed through the skin, so much so that even exposure to just the vapor will go through the skin and adds to the total body burden of lead. Now being a conscientious company, we want to protect the health of the worker. We're not concerned about whether the airborne level is below 10 ppm for some inspector, we're concerned about this worker. So we looked around and we found a metabolite from these solvents that's excreted in the urine. So then we set out to do controlled human exposures to determine what levels of metabolite we would expect in the urine if they were exposed to just the threshold limit value. That data is being prepared now for publication. But how are we going to use it practically? So we went back to the plant physician, and they collect urine samples on these people once a month, and they've established certain bench marks. So if they see the level of this metabolite go up high, they go in and they find out what happened. Where did the isolation break down? We've even had guys in complete chemical suits with an airline respirator and we detect metabolite in their urine. We go back and we find out the man had contaminated shoes and the contaminant was coming through his shoes. Three weeks ago in Amsterdam in an international meeting on lead, they did come out with some recommended biologic threshold limits for the population, but these were considerably below those recommended for industrial populations. For example, group average of blood lead should be less than 25 and individual blood lead should be less than 40. Now this is far below anything that's recommended industrially, so they do have applications in the general environment in regard to this compliance in small plants. It seems to me it would be just as easy for a compliance officer to go in and collect urines and collect bloods, send them to their own lab or an independent laboratory, to NIOSH, and have it examined, then see what's going on. I would probably trust that more than I would some untrained individual going in with a sampling device he doesn't know what he's doing with.

DR. FAIRCHILD: May I just say I think he missed the whole point of my comment. I did not say we don't need biologic thresholds. I'm saying we need them, but I'm also saying they will not be used for compliance. We're a long long way off. I think we should use it for monitoring our people, keeping them well. But as far as compliance is concerned, I just can't see this because you've got to think about quite a few million people. And the compliance office of the Department of Labor doesn't even have physicians in it.

SESSION II

TOXICOLOGY OF HALOGENATED SOLVENTS, AEROSOL PROPELLANTS, AND FIRE EXTINGUISHANTS

Chairman

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AMRL-TR-72-130

PAPER NO. 10

ETIOLOGY OF CARDIAC ARRHYTHMIAS

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There are, as you know, many types of cardiac arrhythmias, such as sino-auricular tachycardia, atrial flutter, arterial fibrillation, bradycardia, A-V block, bigemina, ventricular tachycardia, ventricular fibrillation, and ventricular extrasystoles. Some of these arise as a result of certain types of disease, for example, myocardial infarction, while others can be produced by exposure to a variety of chemicals. It would be presumptuous of me to attempt such a review for I am not a cardiologist nor a clinician. I would, however, like to review some of the factors that appear to be responsible for the occurrence of ventricular extrasystoles following the administration of certain anesthetics, particularly cyclopropane with sympathomimetic agents.

As early as 1895, Oliver and Schäfer noted that the intravenous administration of adrenal extracts in dogs anesthetized with chloroform produced ventricular fibrillation. Levy and Lewis (1911), found that the intravenous administration of epinephrine during chloroform anesthesia produced ventricular tachycardia and ventricular fibrillation in the cat. Heard and Strauss (1918), and Levine (1920) published case reports of arrhythmias during surgery. Lennox et al. (1922) reported 50 cases; Kurtz et al. (1936) reported 109. More recent studies have reported cardiac arrhythmias in hundreds or even thousands of patients. In 1937, Meek et al., developed a standard preparation for study of the interactions of anesthetics and sympathomimetics because they felt that too little attention had been given to determining the accurate concentration of the anesthetic agent, the adequacy of the oxygen supply, the degree of carbon dioxide accumulation, or the exact depth of anesthesia. The basis of their method was to find a dose of epinephrine which produced a reflex inhibition in the normal animal, but under anesthesia might show cardiac irregularities. Stimulation of ecotopic pacemakers was interpreted to mean that the anesthetic agent had sensitized the myocardium.

The term "sensitization" is, in my opinion, completely inaccurate and has probably done more to confuse the issue than to elucidate the mechanisms involved. There are about 40 compounds that have been reported to sensitize the myocardium to either endogenous or exogenous epinephrine and there appears to be no relationship between the chemical structure and biological activity. (table I).

Factors Involved in Chemically Induced Arrhythmias

(1) Exogenous Epinephrine. Orth et al. (1944) studied many sympathomimetic agents in dogs anesthetized with cyclopropane. Epinephrine and norepinephrine regularly produced ventricular tachycardia while propadrine, paradine, and neosynephrine did not. They concluded that catechol nucleus with a primary or secondary amine was required to produce this effect. They also suggested that there were receptors in the mesentery from which nerve impulses traveled by way of the visceral afferent fibers to the celiac plexus and superior mesenteric plexus, from thence to the splanchnics and spinal cord to a brain center above the pons. Efferent impulses then reached the heart by way of the cardiac sympathetic, increasing cardiac irritability. We now know, of course, that the catechol nucleus is not essential since metaraminol (Aramine) produces ventricular arrhythmias.

(2) Endogenous Epinephrine. It has been shown by Katz (1968) that the inhalation of halopropane plus 10% CO_2 produces cardiac arrhythmias in 90% of the animals tested. Transection of the brain between the pons and midbrain did not block the arrhythmias. Transection of the spinal cord at C-1 and removing the pons and medulla blocked the arrhythmia. Pretreatment with reserpine for 7 days, which depletes myocardial and adrenal catecholamine, blocked arrhythmias. It appears, therefore, that the sympathetic nervous system and catecholamine release are involved in the production of arrhythmias. Catecholamines may be released during surgery. Adrenal manipulation during renal surgery may result in the release of epinephrine and the development of arrhythmias. In patients with pheochromocytomas, manipulation and release of epinephrine often produce cardiac irregularities. Ganglionic stimulating agents such as succinylcholine, and DMPP (dimethylphenylpiperazinium iodide) produce ventricular arrhythmia which can be prevented by either pronethalol or hexamethonium. Catecholamines are released during cyclopropane anesthesia.

(3) Electrophysiology of Catecholamines. Before discussing the electrophysiologic effects of these so-called sensitizing agents, I would like to very briefly review a few fundamental principles of electrophysiology. If a microelectrode is placed inside a cardiac cell and another electrode outside, the interior will be negative with respect to the exterior. The magnitude of the transmembrane potential of a quiescent atrial or ventricular muscle fiber is about -90 mv and remains constant until excitation occurs. When the cell is excited there is a rapid decrease in membrane potential with reversal of polarity so that at the end of excitation the inside of the cell is positive with respect to the outside. Depolarization is followed by a period of rapid repolarization, followed by a period of slower repolarization, followed by a period of rapid repolarization, and a return to the resting level.

The rate of firing of an automatic cell depends upon (1) the slope of phase 4 depolarization, (2) the level of the threshold potential, and (3) the maximum level of membrane potential attained at the end of repolarization. Any factor which changes the rate of firing of automatic cells can cause arrhythmia. Excitability is the ability of a cardiac fiber to generate an action potential in response to stimulation. If, during repolarization, a stimulus is applied before the membrane potential has reached the resting level, a response will not occur. Arrhythmias can, therefore, be considered in terms of alterations in automaticity or in conductivity. The electrical phenomena can be explained in terms of ionic movement for the cell membrane separates media of different ionic composition. The concentration of potassium inside the cell is about 150 mEq/liter and above 5.0 mEq/liter outside. The concentration of sodium inside the cell is about 6.5 mEq/liter and 159 mEq/liter outside. Depolarization of the membrane to the critical threshold level increases the permeability of the membrane to sodium and permits an influx of sodium ions. During the upstroke of the action potential there is an absolute increase in sodium conductance and a decrease in potassium conductance. During repolarization there is a decrease in sodium conductance and an increase in potassium conductance. Sodium leaves the cell and potassium enters the cell.

Catecholamines are believed to increase the slope of the spontaneous diastolic depolarization of automatic cells. It is very likely that since automatic cells differ in their sensitivity to catecholamines, there may be a shift in pacemaker site. Therefore, with sufficient catecholamines, automaticity may be so enhanced that multiple pacemakers may develop, resulting in arrhythmias.

The electrophysiologic effects of anesthetics, as well as other agents reported to "sensitize" the myocardium, have not been thoroughly investigated. Studies have been carried out on cyclopropane, halothane, and chloroform to determine the effects on conduction times, refractory periods, and diastolic threshold (excitability) and it is difficult to draw any definite conclusions.

Using isolated canine Purkinje fibers, Davis et al. (1966) observed that cyclopropane increased the rate of repolarization during phase 2, decreased the rate in phase 3, and decreased overall the time required to repolarize. The decrease in time required to repolarize was interpreted as a decrease in functional refractory period and thus might explain in part the appearance of arrhythmias. In a study by Anderson (1967) on the interaction of cyclopropane and epinephrine on sodium transport in the toad bladder, he found that (1) epinephrine increased sodium transport, (2) cyclopropane increased sodium transport, and (3) the combination of epinephrine and cyclopropane exceeded the estimated additive effect of the two agents. Since sodium enters the cell during depolarization, it would, of course, be interesting to know whether cyclopropane increases sodium transport in the heart.

Arterial Pressure

Moe et al. (1948) made a careful study of the role of arterial pressure in the induction of idioventricular rhythms under cyclopropane anesthesia and found that a dose of epinephrine that did not produce arrhythmias at a steady pressure produced arrhythmias if the pressure was elevated. Preventing the rise in arterial pressure permitted eight times the threshold dose of epinephrine to be given without producing arrhythmias. Murphy et al. (1949) showed that epinephrine could produce arrhythmias in dogs anesthetized with cyclopropane in the absence of a rise in arterial pressure. Katz (1970) found that the injection of isoproterenol decreased arterial pressure but was capable of producing arrhythmias. However, when the dose of isoproterenol was insufficient to produce arrhythmias, constriction of the aorta produced arrhythmias. What part the rise in arterial pressure plays in the production of arrhythmias is not clear, but it would appear that it is not absolutely necessary. Stretching the cardiac muscles could increase automaticity, decrease the resting potential, increase the slope of phase 4 of depolarization and produce multi-focal pacemaker activity.

Heart Rate

It has been suggested by Vick (1966) that increased heart rate is important in the development of arrhythmia, since vagal stimulation following the administration of epinephrine during chloroform anesthesia converts the arrhythmia to normal sinus rhythm.

Sympathetic and Central Nervous System

Procedures that have been reported to offer some protection against arrhythmias include bilateral adrenalectomy, stellate ganglionectomy, denervation of the carotid sinus, thoracic sympathectomy and decerebration. It must be remembered, however, that arrhythmias can be produced in animals with denervated hearts and in the heart-lung preparations.

Endotracheal Intubation

Devault et al. (1960) studied the cardiac rhythm and hemodynamics during intubation of patients undergoing surgery. They found consistent increase in heart rate and elevated arterial pressure with arrhythmias. The intravenous administration of 3 mg atropine prior to intubation prevented the increase in heart rate but not the rise in pressure or arrhythmias. The intravenous administration of 5.0 mg of phentolamine prevented the rise in pressure and arrhythmias.

At this point, I would like to review some studies carried out by Dr. Cummings and myself (1956) on a series of adrenergic drugs administered during cyclopropane anesthesia in an attempt to determine the factors responsible for the production of ventricular extrasystoles. Dogs were anesthetized with thiopental sodium, 20 mg/kg in order to allow passage of an endotracheal tube with an inflatable cuff. A Heidbrink anesthetic machine was used to administer both cyclopropane and ether. Electrocardiographic tracings were taken from lead II on a direct writing Sanborne Cardiette. Cyclopropane in a concentration of 16% was usually sufficient to maintain the animals in plane ii or iii anesthesia. Epinephrine was administered in a concentration of 1.0 microgram per kilogram per milliliter, and infused at a rate of 1.0 ml per 20 seconds. This procedure was a modification of the Meek, Hathaway and Orth technique (1937) and was developed by McMillen, Hampton and Drill (1950). It provided an opportunity to plot a distribution curve that would include animals unusually sensitive or refractory to epinephrine and avoided losing animals because of ventricular fibrillation.

Test drugs, including eleven sympathomimetic agents were administered in equipressor doses and in a volume of 2.0 ml. Although many reports have appeared on the cardiovascular effects of adrenergic agents during cyclopropane anesthesia, no systematic study has been made on the relative effectiveness of these drugs under conditions of minimal stress. By using the smallest concentration of cyclopropane to maintain plane iii anesthesia, a minimal amount of epinephrine to elicit changes in cardiac rhythm, and the appearance of multiple extrasystoles, we felt that the data would provide a basis to determine which of the previously proposed factors was primarily involved in the etiology of arrhythmias.

Time does not permit me to discuss in detail the results of each compound tested, so I shall simply summarize the data:

(1) Spontaneous arrhythmias occurred during cyclopropane anesthesia, but not during ether or pentobarbital anesthesia.

(2) The average amount of epinephrine that induced arrhythmias in 36 dogs was 1.98 micrograms per kilogram. Ten times this dose did not produce arrhythmias under ether or pentobarbital anesthesia. (table II)

(3) Successive injections of catecholamines, epinephrine, norepinephrine and isopropylnorepinephrine consistently produced multiple extrasystoles. (table III)

(4) Ephedrine and desoxyephedrine produced arrhythmias on the first injection, but subsequent injections resulted in tachyphylaxis. (table IV)

(5) Mephentermine, Oenethyl and Aranthol produced arrhythmias after two or three injections, but slowly produced tachyphylaxis. (table IV)

(6) Neosynephrine, methoxamine and naphazoline did not produce arrhythmias during cyclopropane anesthesia. (table V)

TABLE I

COMPOUNDS PRODUCING CARDIAC ARRHYTHMIAS

ETHYL CHLORIDE	METHANE	FLUOROCARBONS*
TRICHLOROETHYLENE	ETHANE	DIGITOXIN
CYCLOPROPANE	PROPANE	OUABAIN
CHLOROFORM	n-BUTANE	ACETYLSTROPHANTHIDIN
HALOPROPANE	CYCLOBUTANE	CARBON TETRACHLORIDE
METHOXYFLURANE	CYCLOBUTENE	BENZENE
FLUROXENE	CYCLOPENTANE	XYLENE

*11, 12, 22, 114, 115, 142, 152, 318

TABLE II

EFFECT OF DURATION OF CYCLOPROPANE ON AMOUNT OF EPINEPHRINE REQUIRED TO INDUCE ARRHYTHMIAS

Minutes	EPINEPHRINE, microgm. per kgm.				
	Exp. No. 1	2	3	4	5
0	1.7	1.7	2.2	2.6	1.8
30	1.4	1.5	2.4	2.6	2.0
60	1.4	1.8	1.8	1.8	2.0
90	1.5	1.7	1.8	1.8	2.2
120	1.6	2.1	1.6	1.2	2.0
150	1.8	1.7	1.6	2.6	2.2
180	1.8	1.8	1.8	1.2	2.4
210		2.1		1.6	2.4
240		1.7		1.6	2.6
270				1.6	
300				1.8	

TABLE III

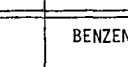
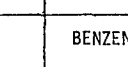
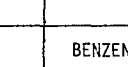
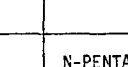
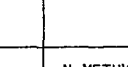
CHEMICAL STRUCTURE AND CARDIOVASCULAR EFFECTS OF THE ADRENERGIC DRUGS ADMINISTERED DURING 16 PER CENT CYCLOPROPANE ANESTHESIA

DRUG	CARBON CYCLE or CHAIN	RESIDUE	DOSE microgm/kg.	TIME OF INJECTION minutes	0	45	90	135	180
EPINEPHRINE (5)#	CATECHOL	$CH(OH)-CH_2-NH-CH_3$	1.6 - 2.4	B.P. mm. Hg. ARRHY. * HEART RATE	+59 + +22	+60 + +22	+55 + +20	+60 + +20	+60 + +20
LEVOPHED (4)	CATECHOL	$CH(OH)-CH_2-NH_2$	1.2 - 2.0	B.P. mm. Hg. ARRHY. RATE	+53 + +10	+65 + +10	+60 + +10		
ISUPREL (5)	CATECHOL	$CH(OH)-CH_2-NH-CH(CH_3)_2$	10	B.P. mm. Hg. ARRHY. RATE	-73 + +146	-70 + +180	-70 + +150		

#( ) = number of experiments; * + = multiple extrasystoles, 0 = absence of ventricular arrhythmias

TABLE IV

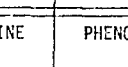
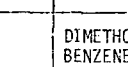
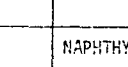
CHEMICAL STRUCTURE AND CARDIOVASCULAR EFFECTS OF THE ADRENERGIC DRUGS ADMINISTERED DURING 16 PER CENT CYCLOPROPANE ANESTHESIA

DRUG	CARBON CYCLE OR CHAIN	RESIDUE	DOSE microgm/kg.	TIME OF INJECTION minutes	0	45	90	135	180
EPHEDRINE (4)	BENZENE		2,000	B.P. mm. Hg. ARRHY HEART RATE	+87 + +45	+3 0 +20	-15 0 +20	0 0 +12	
DESOXYN (4)	BENZENE		1,000	B.P. mm. Hg. ARRHY RATE	+62 + +117	+30 0 +10	-22 0 0	-32 0 0	
WYAMINE (7)	BENZENE		300	B.P. mm. Hg. ARRHY. RATE	+64 + +31	+39 + +20		+15 0 +15	+3 0 +10
OENETHYL (3)	N-PENTANE		500	B.P. mm. Hg. ARRHY RATE	+67 + +93	+55 + +60	+43 0 +60	+27 0 +22	+5 0 +20
ARANTHOL (4)	N-METHYL-PETANOL		7,000	B.P. mm. Hg. ARRHY RATE	+60 + +75	+58 + +60	+51 + +45	+43 + +35	+29 0 +30

#( ) = number of experiments; * + = multiple extrasystoles, 0 = absence of ventricular arrhythmias

TABLE V

CHEMICAL STRUCTURE AND CARDIOVASCULAR EFFECTS OF THE ADRENERGIC DRUGS ADMINISTERED DURING 16 PER CENT CYCLOPROPANE ANESTHESIA

DRUG	CARBON CYCLE OR CHAIN	RESIDUE	DOSE microgm/kg.	TIME OF INJECTION minutes	0	45	90	135	180
NEOSYNEPHRINE (5)	PHENOL		25 - 30	B.P. mm. Hg. ARRHY RATE	+37 0 -8	+37 0 -10	+40 0 -10		
VASOXYL (5)	DIMETHOXY-BENZENE		1,000	B.P. mm. Hg. ARRHY RATE	+50 0 -20	+17 0 -20		+15 0 0	
PRIVINE (3)	NAPHTHYLENE		25	B.P. mm. Hg. ARRHY RATE	+52 0 -20	+17 0 -5	+6 0 0	+4 0 0	

#( ) = number of experiments; * + = multiple extrasystoles, 0 = absence of ventricular arrhythmias

As I previously indicated, a number of factors have been implicated in the etiology of cardiac arrhythmias which develop either spontaneously or following the administration of epinephrine. The results of our studies led us to believe that the two most important factors were positive inotropism and loss of intracellular potassium. If the drugs are divided into class according to their inotropic effects, it is clearly evident that those compounds that increase the contractile force produce ventricular extrasystoles, and those that do not produce ventricular extrasystoles exert no positive inotropic action (table VI). In support of this concept, Goldberg (1953) observed that epinephrine and norepinephrine produced forceful contractions of short duration; epinephrine, desoxyephedrine, mephentermine, Aranthol, and Oenethyl produced similar effects on contractile force, but of longer duration, and that phenylephrine and methoxamine produced only slight inotropic effects. Of particular interest is the compound, Isuprel, which has a marked hypotensive effect but produced cardiac arrhythmias. This led us to test other hypotensive agents such as histamine, acetylcholine, and nitroglycerine. In no instance did any of these compounds produce cardiac arrhythmias. This difference appears to be due to the fact that Isuprel possesses a positive inotropic effect while histamine, acetylcholine, and nitroglycerine do not.

TABLE VI

THE INOTROPIC ACTION OF CLASS I, II and III DRUGS

Drug	Inotropic Response Initial Injection	Subsequent Inotropic Responses
Class I (consistent ventricular arrhythmias; brief tachycardia)		
Epinephrine	marked increase	marked increase
Levarterenol	" "	" "
Isuprel	" "	" "
Nicotine	" "	" "
Acetylcholine (nicotinic dose)	" "	" "
DMPP	" "	" "
Class II (ventricular arrhythmias only after first few injections; long tachycardia)		
Ephedrine	marked increase	decreased effect, eventual depression
Oenethyl	" "	" "
Aranthol	" "	" "
Desoxyephedrine	" "	
Mephentermine	" "	
Class III (neither ventricular arrhythmias nor tachycardia)		
Methoxamine	slight or no increase	slight or no increase
Phenylephrine	" " "	" " "
Naphazoline	" " "	" " "

The second point I should like to discuss is the role of potassium. The transmembrane voltage of the cardiac cell is maintained by a differential distribution of ions inside and outside the cell. During the phase of depolarization, the cardiac cell loses potassium, and during repolarization, potassium reenters the cell. Therefore, it would appear that compounds which "sensitize" the myocardium increase the permeability to potassium. Indirect evidence which supports this concept is that plasma potassium is twice as great when epinephrine is administered in combination with cyclopropane. It is probable that the escape of potassium across the cell membrane is intimately connected with the contractile force of the myocardium. Since cardiac arrhythmias can be prevented by the prior administration of

beta-adrenergic blocking agents, we proposed that compounds which "sensitize" the myocardium increased the permeability of cardiac muscle fiber, and the administration of sympathomimetic agents or the release of endogenous catecholamines causes a marked increase in contractile force which interferes with the exchange of sodium and potassium during depolarization and repolarization (figure 1). It would appear that Class I drugs cause an escape of potassium that is directly proportional to the inotropic effects, and during the repolarization phase, potassium is prevented from entering the cell.

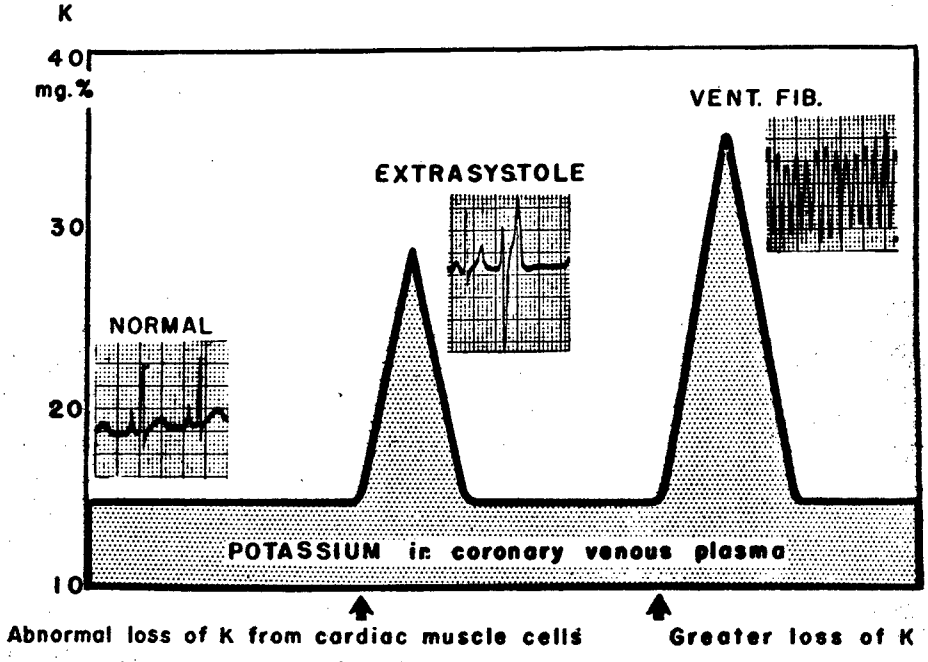


Figure 1. Hypothetical Relationship Between Coronary Venous Plasma Potassium and Cardiac Arrhythmias.

I have emphasized the role of cyclopropane and certain sympathomimetic agents in the induction of cardiac arrhythmias because they have been extensively studied in both animals and man. Whether the hypothesis that I have proposed applies to other compounds known to produce arrhythmias is something that needs investigation. I am inclined to believe that the mechanism is the same, for it has been shown by Taylor et al. (1971), Azar (1971), Van Stee and Back (1972) and Wills et al. (1972) that spontaneous arrhythmias occur during the inhalation of fluoroalkanes and that these arrhythmias, as well as those produced by cyclopropane or chloroform, can be prevented by the administration of beta-adrenergic blocking agents.

There are, of course, species differences in the response to fluoroalkanes, but the cardiovascular response following the administration of epinephrine appears the same. Whether the arrhythmias are mediated through beta-adrenergic receptors, altered conduction with delay in depolarization or repolarization, or changes in permeability to sodium and potassium, is not clear.

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AMRL-TR-72-130

PAPER NO. 11

THE NEGATIVE INOTROPIC EFFECT OF BROMOTRIFLUOROMETHANE EXPOSURE IN THE DOG

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and
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For several years we have been engaged in the systematic investigation of the pharmacological consequences of the inhalation of low-molecular-weight halogenated alkanes (Van Stee and Back, 1969, 1972). The work began with studies of fluorocarbon 1301, $CBrF_3$, and gradually has been expanded to include other related compounds. The recent approval by the Air Force for the use of a $CBrF_3$ fire-extinguishing system in the C5-A, and the development of a hand-held fire-extinguisher containing a mixture of 1301 and 1211, $CBrClF_2$, have underscored the urgency for a complete evaluation of these compounds and has provided the impetus for a continuation of our fluorocarbons research program.

The purpose of this paper is the presentation of what we believe to be a novel method for the determination of the maximum rate of ventricular pressure change or peak dP/dt divided by developed pressure, and an example of the application of the method to the evaluation of the inotropic effect of an unknown, $CBrF_3$. The selection of $CBrF_3$ as the unknown in this set of experiments was not entirely arbitrary since we have published other evidence from time to time in the past suggesting that exposure to $CBrF_3$ has a negative inotropic effect on the heart.

The physiological basis for the selection of peak dP/dt ÷ P as an index of the vigor of myocardial contraction is based on a concept of the interdependence of the dynamics of the isovolumic phase of myocardial contraction and the contractile properties of cardiac muscle (Taylor, 1970).

The myocardial contractile mechanism may be viewed in terms of a 3-component model originally proposed for skeletal muscle by A. V. Hill (1938) and subsequently applied to cardiac muscle by Mommaerts (1959), Sonnenblick (1964) and others. A commonly applied model consists of a contractile element and an elastic element in a series arrangement, with the series parallel to another elastic element.

For purposes of this discussion we may consider that the contractile properties of cardiac muscle are subject to alteration through either of two mechanisms. This is to say that the relation between the velocity of shortening of the contractile element and the force developed, known as the force-velocity relationship (Katz, 1972), may be altered in two fundamentally different ways. Both of these may affect cardiodynamics during the isovolumic phase of contraction. One means of altering of the vigor of myocardial contraction is termed heterometric autoregulation and is described by the familiar Frank-Starling law of the heart (Burton, 1965) - that the vigor of myocardial contraction is proportional to the tension on the myocardial fiber when contraction begins. This tension is referred to as the muscle preload. Another means of altering the vigor of myocardial contraction occurs without a change in fiber length and hence is referred to as homeometric autoregulation. This change results from an alteration of the internal milieu of the myocardial fiber and may be termed contractility. Inotropic state and contractility are to be considered synonymous within this context.

In reference to the previously mentioned concept of the interrelationship between the cardiodynamics of the isovolumic phase and the contractile properties of the cardiac muscle, we may now appreciate that the rate of change of intraventricular pressure (or dP/dt) bears a relationship to the vigor of myocardial contraction (Goodyer, 1962). If, however, one is to perform a measurement of an index of the inotropic state of the myocardium and such an index is susceptible to alteration through the Frank-Starling mechanism, one of two courses must be pursued: the preload (or end-diastolic tension) must be controlled during the measurement or the index of contractility must be modified to become largely independent of the end-diastolic tension if end-diastolic tension cannot be controlled. The ratio of the peak dP/dt and the developed pressure at that point has been shown to vary with contractility quite independently of end-diastolic volume (Taylor, 1970) and for this reason, since end-diastolic volume was not controlled in this study, we elected to measure peak dP/dt ÷ P as an index of a change in inotropic state of the left ventricular myocardium.

Male beagle dogs weighing an average of 10 kg were used in this study. Food was withheld for 18 hours prior to experimentation. They were immobilized with a single intravenous (IV) dose of thiamylal sodium, intubated, and a catheter inserted into a femoral vein. Surgical anesthesia was produced by the continuous IV infusion of a solution containing 10% by volume ethyl alcohol and 60 mg/liter morphine sulfate in 5% glucose in water (Reier, 1971). Bilateral, midcervical vagotomy was performed. Thoracotomy was performed at the 4th or 5th left intercostal space. Left ventricular pressure was obtained through a stainless steel cannula inserted either via the left auricular appendage or the left ventricular free-wall. As an aside, it may be noted that on the average, better quality recordings were obtained following the free-wall approach. The dP/dt was derived electronically by shaping the left ventricular pressure curve with the appropriate R-C circuit. All recordings were performed on an Electronics for Medicine Model DR-12 physiological recorder.

The original method of determining peak dP/dt ÷ P was according to that diagrammed on the left in figure 1 (Taylor, 1970). A vertical line was drawn from the point corresponding to peak dP/dt through the ventricular pressure tracing. The intercept was then determined and the value divided into that for peak dP/dt. The difficulty of obtaining this value derives from the fact that even at higher paper speeds the value of the pressure curve intercept which coincides with the phase of the most rapid rate of pressure change is difficult to measure accurately. Also, the value for peak dP/dt ÷ P cannot be obtained on a beat to beat basis from the oscilloscope screen.

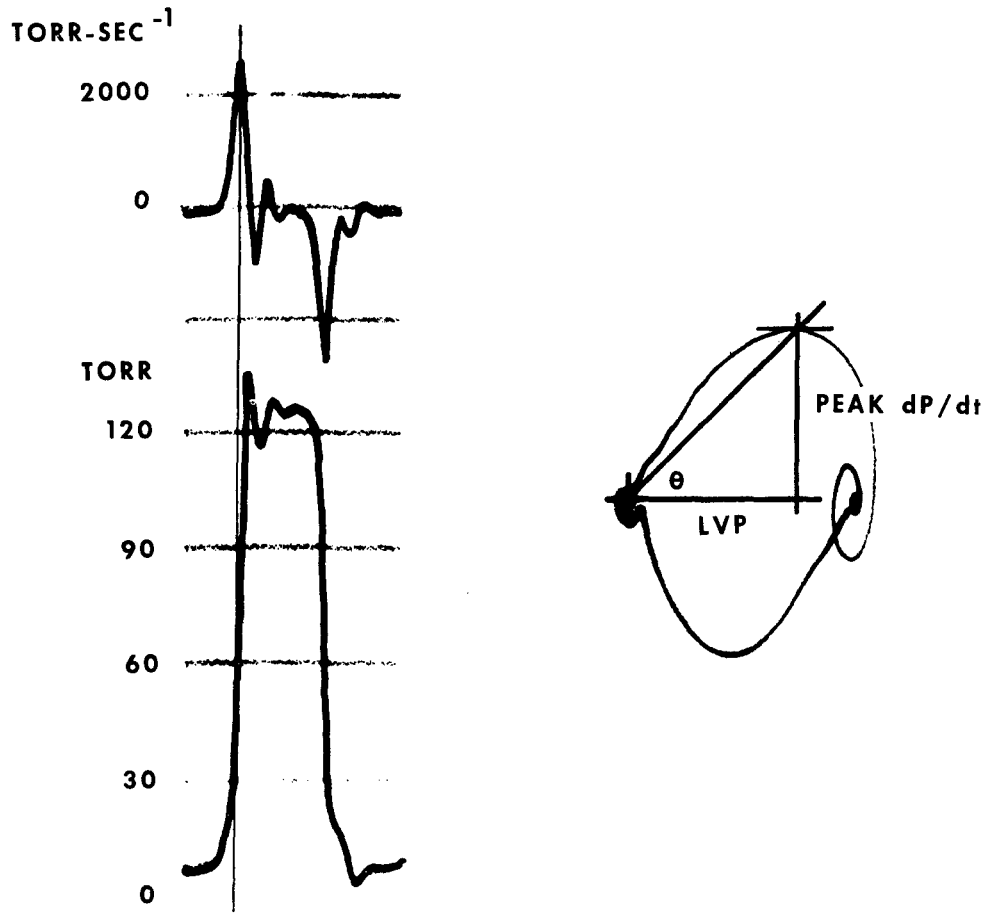

Figure 1.

Both of these difficulties may be circumvented by using an on-line analog computer that continuously divides one function by the other. On the other hand, the same information may be obtained quickly and accurately with a vector oscilloscope in the absence of a computer.

The left ventricular pressure signal is used to control the horizontal deflection and the dP/dt is used to control the vertical deflection of a vector oscilloscope. The Lissajous figure represented in figure 1 is typical of the loops generated by this method.

The zero-points of the axes are easily identified as the intensified spot on the left of the loop. This occurs as a result of the relatively prolonged interval between beats when dP/dt and end diastolic pressure are about zero. A line is drawn along the abscissa, tangent to the top of the loop. This represents peak dP/dt. Another line is drawn connecting the coordinate zero with that point of tangency. Peak dP/dt ÷ P may be read as the tangent of angle θ. Veragut (1965), Sonnenblick (1967), and others have demonstrated that peak dP/dt ÷ P varies as a function of various inotropic interventions and does so independently of ventricular filling.

The pharmacological validation of the loops method of the representation of of peak dP/dt ÷ P was performed using the known inotropic agents isoproterenol and propranolol.

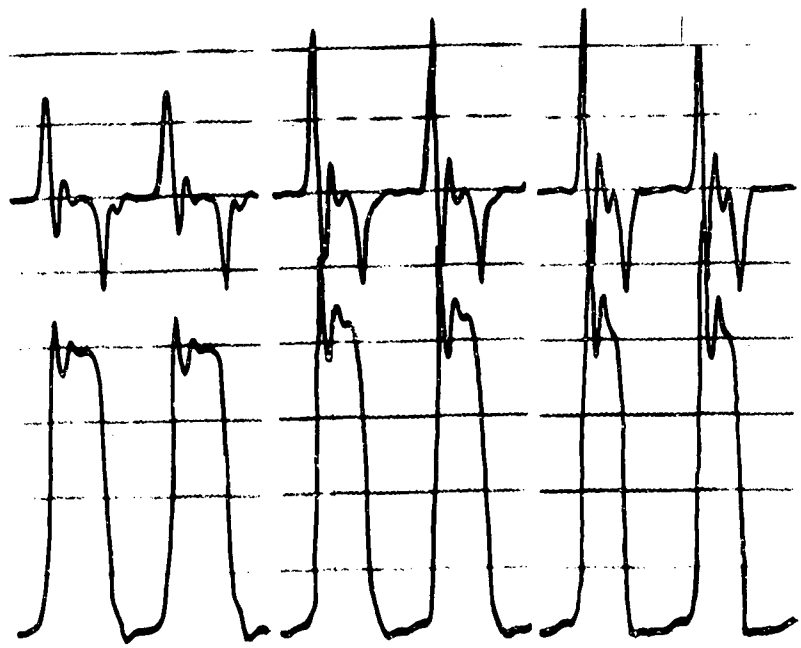

Figure 2.

Figure 2 represents the changes that took place following the injection of isoproterenol, recorded in the conventional manner. The first panel represents the left ventricular pressure curve and dP/dt before the onset of the drug effect. The panel on the right represents the peak drug effect with the center panel being intermediate between the two.

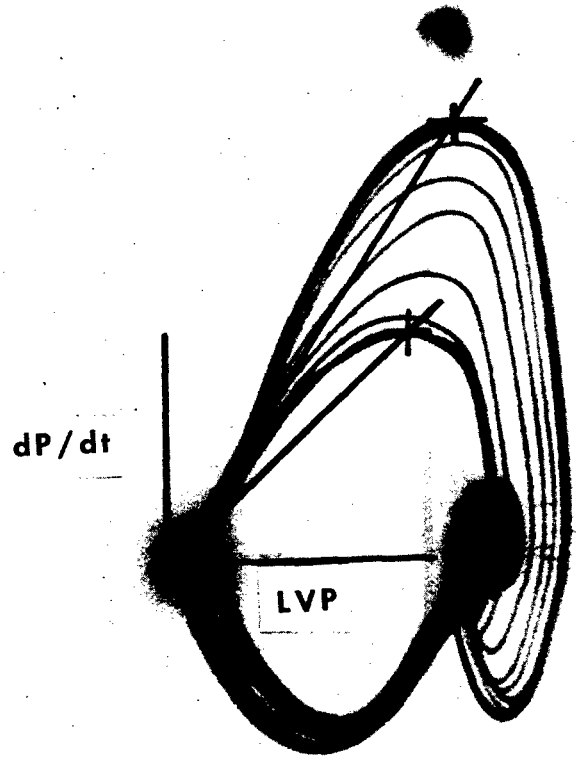

Figure 3.

Figure 3 represents the same information that appeared in figure 2 except that it was recorded by the loops method. The loops representing the recorded extremes were obtained just prior to the onset of the drug effect and at peak effect, respectively. The intermediate loops were recorded as every other cardiac cycle throughout the development to the peak effect. The increase in contractility attributable to the isoproterenol effect is reflected in the marked increase of the slope of the lines that determine the angles θ.

Figure 4 illustrates the results of the pharmacological validation of the loops method of determining peak dP/dt ÷ P. These data were obtained from 4 dogs. Multiple injections of isoproterenol were made on each dog. This was possible because of the very short duration of action of isoproterenol. Propranolol, on the other hand, was given at one dose level, once to each dog because of its relatively prolonged duration of action. Each observation represents several measurements of consecutive cardiac cycles for each effect for which peak dP/dt ÷ P was reported. Numerical and analytical data are included in tabular form in the Appendix. The data clearly illustrate the responses to negative and positive inotropic drugs as well as the dose-response relationship for isoproterenol.

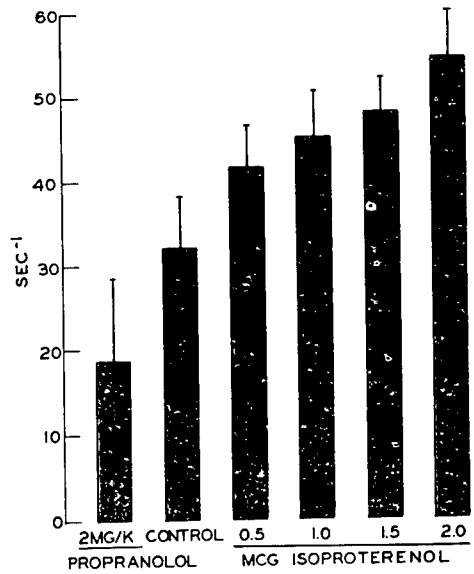

Figure 4. PHARMACOLOGICAL VALIDATION OF THE LOOPS METHOD OF DETERMINING PEAK DP/DT ÷ P AS AN INDEX OF THE VIGOR OF MYOCARDIAL CONTRACTION. INOTROPIC DRUG DOSE VS. MEAN PEAK, DP/DT ÷ P (+ SD).

Having established the validity of the method for detecting relative changes in the inotropic state of the left ventricular myocardium it became possible to use the method to evaluate the inotropic effect of an unknown compound. We had noted in the past that dogs exposed to $CBrF_3$ frequently had an elevated left ventricular end-diastolic pressure. This suggested the possibility that $CBrF_3$ might be a negative inotropic agent.

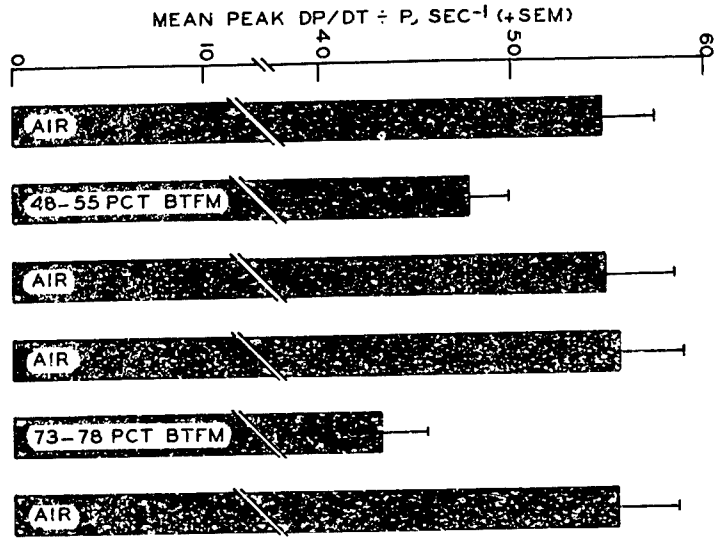

Figure 5.

Figure 5 illustrates the effect of 5-minute exposures of 5 dogs to $CBrF_3$ at 2 different concentrations. Each exposure was accompanied by a set of preexposure measurements and 10-minute postexposure measurements. Although the data are represented with the lower concentration first and the higher concentration second, the exposures were actually performed in that order on only 3 dogs, whereas the order was reversed on the other 2. These data were analyzed using a 2-way analysis of variance. The mean peak dP/dt ÷ P during the $CBrF_3$ exposures differed significantly from those measured during the periods of air breathing.

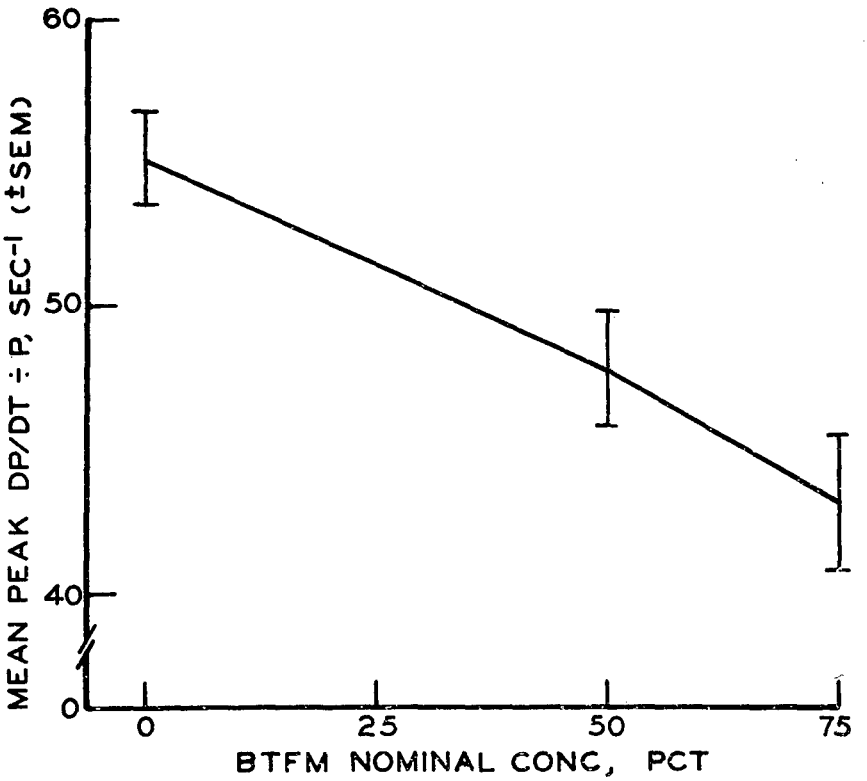

Figure 6. BROMOTRIFLUOROMETHANE (BTFM) CONCENTRATION-RESPONSE RELATIONSHIP.

The data in figure 6 were replotted from the data represented in figure 5. The responses were a parabolic function of the $CBrF_3$ concentration and the slope of the line of regression was significantly different from zero.

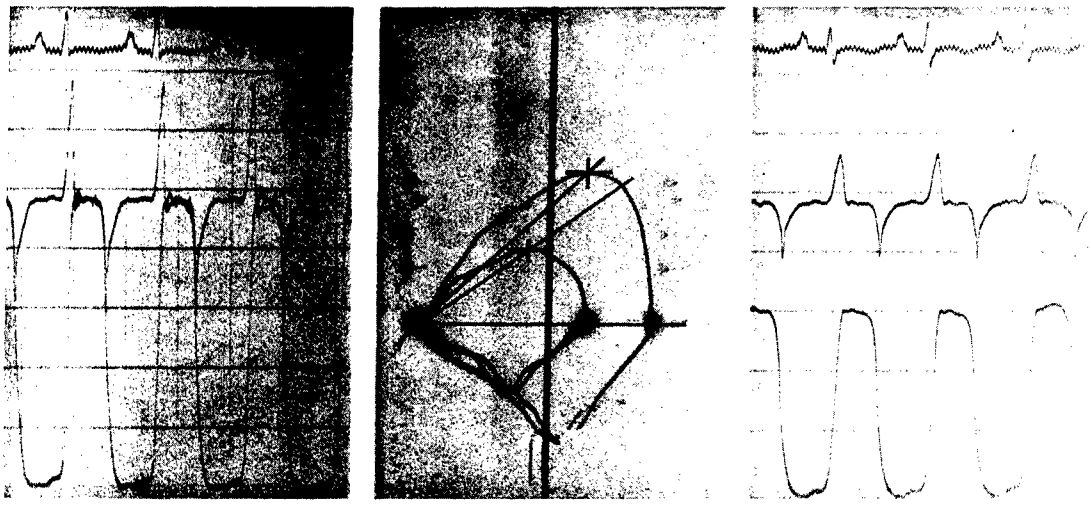

Figure 7.

Figure 7 represents examples of actual recordings made during a $CBrF_3$ exposure. A multitrace recording was performed preexposure and is reproduced on the left. A preexposure loop was then recorded and the exposure begun. After 5 minutes a second loop was superimposed on the first loop. The paper was advanced and another multitrace recording was made. The relationship of the different values for dP/dt and left ventricular pressure between the multitrace and loops recordings is quite apparent.

In conclusion, we offer a simple and elegant means of determining peak dP/dt ÷ P using a vector oscilloscope. This index of myocardial contractility is largely independent of end-diastolic volume and may be obtained easily in any preparation in which left ventricular pressure is determined. Also we have presented the results of an application of the method to the evaluation of the negative inotropic effect of $CBrF_3$ exposure in the dog.

APPENDIX

A. Analysis of the Effect of Propranolol on peak dP/dt ÷ P (figure 4).

Raw Data*

Dog No.	Control	2 mg/kg Propranolol
S92	36	20
S58	24	16
S78	26	8
S56	38	31

*Numbers in body of table represent means of 5 or more loops determinations; sec^{-1}.

Student's t-test for paired observations

$t = 4.406$

$t\ (df = 3,\ p < .05) = 3.182$

t-test: Significant, $p < .05$

B. Analysis of the Effect of Isoproterenol on peak dP/dt ÷ P (figure 4).

Raw Data*

Dog No.	Dose Isoproterenol μg, IV			
	0.5	1.0	1.5	2.0
S92	37	39	45	57
	42	46	46	54
S58	37	43	49	62
	42	49	51	58
				56
				59
				56
S78	35	41	42	42
	39	42	42	46
S56	44	52	50	58
	41	49	54	53
	51		54	
	49			

*sec^{-1}

Parabolic line of regression (Y = peak dP/dt ÷ P, X = μg isoproterenol)

$Y = 40.86 + 0.44X + 3.19X^2$

$r = 0.71$

Analysis of Variance Table for Regression

Source of Variation	SS	df	MS	F	Sig*
Regression	936.97	2	468.48	18.03	$p < .01$
Error	909.24	35	25.98	-	-
Total	1846.21	37	-	-	-

*$F(2, 35\ df) = 5.28$

C. Analysis of Variance of the Effect of $CBrF_3$ on peak dP/dt ÷ P (figure 5).

2-Way Analysis of Variance, Mixed Model, with 7 observations per cell.

Data Layout

Dog No.	A_0(air)	A_1(50%)	A_2(air)	A_3(air)	A_4(75%)	A_5(air)
S26	n = 7 obs/cell					
S66						
S40						
S60						
S78						

Analysis of Variance Table for $CBrF_3$

Source of Variation	SS	df	MS	F	Sig
Column Effects	5543.281	5	1108.656	100.059	$p < .01$
Row Effects	50288.162	4	12572.040	1134.661	$p < .01$
Interaction*	4045.838	20	202.292	11.080**	$p < .01$
Error	3286.286	180	18.257	-	-
Total	63163.567	209	-	-	-

*Since the obs/cell do not represent n separate experimental setups, i.e., not replicated, but rather n obs on the same preparation, there is no test for interaction.

**The value of F for Interaction, MSI/MSE > F (df 20, 180, $p < .01$) and therefore MSI replaces MSE in the denominator for the Col and Row F-tests.

D. Regression Analysis of $CBrF_3$ Data (figure 6).

The values from columns A_0, A_1, A_3, and A_4 (data layout in section C of Appendix) were analyzed by regression for the purpose of graphically representing the concentration response relationship.

Analysis of Variance Table for Regression

Source of Variation	SS	df	MS	F	Sig
Regression	4132.72	2	2066.36	8.72	$p < .01$*
Error	30341.58	128	237.04	-	-
Total	34474.31	130	-	-	-

*F(df 2, 128, $p < .01$) = 4.61

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DISCUSSION

MR. WANDS (National Academy of Sciences): Did you keep your PO_2's constant throughout all of these exposures? You were at a pretty high concentration of $CBrF_3$.

MAJ. VAN STEE (Aerospace Medical Research Laboratory): We did not keep them constant. The animals were not hypoxic. There were times at which the PO_2's were rather high and by that I mean between 100 and 200 torr, but the animals were not hypoxic and this was the important thing.

DR. AZAR (Haskell Laboratory, E. I. duPont): I would just like to caution on that regression curve where you had 2 exposure concentrations, 50 and 75%, and zero. It's a little hazardous to run that regression on 3 points all the way back to zero without filling in that gap between the zero to 50. It implies that there's no threshold, and there may be. I don't know the answer to that. And I would like to ask, what effect does peripheral vascular resistance have on this peak dp/dt?

MAJ. VAN STEE: Peripheral vascular resistance - this, of course, mechanically is referred to as afterload and is represented by the mean arterial pressure. Afterload has been reported from time to time to have one effect or another on contractility. I don't propose to get into a discussion of that. However, I think that we may eliminate this as a consideration here by stating that we have demonstrated unequivocally that variable afterload has no effect on this index and we did this by two means. We increased peripheral vascular resistance using phenylephrine and we found that the slopes of the lines defining angle theta were identical within reasonable blood mean arterial pressure levels and that would be between 60 and 300 torr, so the slope did not change when we gave phenylephrine sufficient to raise the blood pressure. Likewise, toward the end of longer experiments when peripheral resistance tends to decrease in a dog, and consequently, mean arterial pressure is down, we found that there also was no change in the slope of this particular line. Therefore, we don't believe that this index, peak dp/dt over P, is sensitive to changes in afterload, if indeed they do affect contractility.

DR. AZAR: There are some investigators, I believe, that do feel that afterload may be a contributing factor in this and that was why I just wondered if you looked at it.

MAJ. VAN STEE: Yes, we did, most assuredly. And our feeling is that this method is independent of afterload within those ranges of mean blood pressure anyway.

DR. AZAR: In relation to your paper and Dr. Hays', we did check isoproterenol and did not find that it induced cardiac sensitization. Whenever we exposed animals to a known sensitizing compound and epinephrine, we got sensitization. We kept all the variables identical and administered an equimolar amount of isoproterenol which was about 8 or 9 micrograms per kilogram. We did not seen sensitization develop. This has recently also been reported by Clark and Tinston in the Proceedings for Experimental Biology.

AMRL-TR-72-130

PAPER NO. 12

CONTINUOUS ANIMAL EXPOSURE TO LOW LEVELS OF DICHLOROMETHANE

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INTRODUCTION

Dichloromethane, also known as methylene chloride, has been used extensively in the preparation of many spacecraft construction materials because of its desirable solvent properties. The toxicity of dichloromethane (CH_2Cl_2) is considered relatively low and its industrial threshold limit value (TLV) of 500 ppm reflects this opinion. The possibility that large amounts of this chemical might gas-off in the reduced pressure environment of a space cabin, resulting in continuous exposure of astronauts at significantly high concentrations, prompted the need for information on the effects of chronic continuous exposure to dichloromethane.

The effects of intermittent exposure to dichloromethane at 5000 ppm was reported by Heppel et al. (1944). In this study dogs, rabbits, guinea pigs and rats were exposed 7 hours per day, 5 days per week for periods up to 6 months. They found subnormal weight gains, decreased food intake and death of 3 of 8 guinea pigs after 35, 90 and 96 exposures, respectively. Guinea pigs that died showed pneumonia and centrilobular fatty degeneration of the liver. However, none of the other species showed any evidence of toxicity during the course of exposure. Central nervous system (CNS) effects, varying in degree, were produced in the four species mentioned and also in monkeys exposed to 10,000 ppm on a 5 day per week, 4 hour per day schedule. Dogs were removed after 6 exposures because of injuries from

hyperactivity while all other species finished 36-38 exposures. Lehmann and Schmidt-Kehl (1936) observed only drowsiness and slight reduction in body temperature in cats and rabbits exposed 8 hours a day, 6 days a week to concentrations of 1728-2036 ppm for 4 weeks. Little other work has been reported on the chronic toxicity of dichloromethane.

Acute LC_{50} values for mice have been reported by Browning (1965) as 14, 500 ppm for a 2-hour exposure and by Svirbely et al. (1947) as 16, 188 ppm for an 8-hour exposure. Human exposure experience includes one death among four men accidentally exposed to undetermined concentrations as reported by Moskowitz and Shapiro (1952) and the nonfatal exposure of 33 workers to levels of approximately 29-5000 ppm described by Kuzelova and Vlasik (1966).

Continuous exposures of animals to 5000 and 1000 ppm CH_2Cl_2 for periods up to 14 weeks were reported by Thomas (1971), Weinstein et al. (1972), Bullock (1971) and Haun et al. (1971). The results of those studies using dogs, monkeys, rats and mice provided more than sufficient evidence of the harmful, and in many cases fatal, effect of continuous exposure to high level concentrations of CH_2Cl_2. Signs of CNS depression were evident in all species exposed to 5000 ppm and in dogs and monkeys exposed to 1000 ppm. Spontaneous activity measurements showed decreased activity levels in mice exposed to 1000 ppm. All exposed animals either lost weight or showed subnormal body weight increases. Weight changes were dose and time related in most cases. Significant numbers of dogs and mice as well as one monkey died after 3 weeks exposure to 5000 ppm. Because death appeared certain in the remaining animals, except for rats, all but one half of the rat group were sacrificed at 4 weeks. The remaining 10 rats survived the 14-week exposure period. In the 1000 ppm exposure, 6 of 8 dogs had died by 7 weeks. The remaining 2 were sacrificed. Monkeys, rats and all but a few mice survived the 14-week exposure to 1000 ppm. Significant gross and histopathologic lesions were noted in all species that died either from exposure or sacrifice. The primary target organ was the liver, but in many cases the kidney was also affected. Fatty liver changes were severe in dogs. In the hepatotoxicity study of mice exposed continuously to 5000 ppm, there was almost complete reversal of fatty change in the liver indicating adaptation to CH_2Cl_2 exposure. This study showed that CH_2Cl_2 was toxic to all species tested; most to dogs, less so to mice, and least to rats and monkeys.

Additional information on the metabolism of dichloromethane was provided by Stewart et al. (1972) who showed increased carboxyhemoglobin levels in humans after exposure. Exposure of animals in our laboratory to 5000 ppm for 24 hours also showed that CH_2Cl_2 was metabolized to form carbon monoxide as one of its breakdown products. Increased carboxyhemoglobin levels were found in dogs, monkeys and rats.

Having identified the target organs and effects of high level exposures to CH_2Cl_2, experiments were undertaken to define the toxic response of continuous exposure to concentrations which were readily attainable in actual spacecraft flight (25 and 100 ppm).

METHODS

The exposures were conducted in the Thomas Domes at ambient pressure. The air flow, pressure, relative humidity and temperature were automatically controlled. Air flow was maintained at 40 cfm, relative humidity at 50 ± 10%, and temperature at 72 ± 5 F. The absolute pressure within the exposure chambers was slightly negative (725 torr) with respect to ambient to insure a tight seal of the chambers and to prevent contamination of the surrounding laboratory air with CH_2Cl_2 vapor.

Reagent grade CH_2Cl_2 was used as the contaminant in these studies. Liquid CH_2Cl_2 was pumped from a drum by a variable speed peristaltic pump through a flowmeter to a glass evaporating flask to generate the concentrations required for these studies. Concentrations could be adjusted by either changing the pumping speed to deliver more CH_2Cl_2 to the evaporating flask or by increasing or decreasing the air flow through the chamber. The CH_2Cl_2 concentration in each of the experimental domes was monitored continuously throughout the entire study using a flame ionization hydrocarbon analyzer calibrated daily with bag standards of known concentration. Measurement over the entire exposure period showed that contaminant concentrations never varied more than 10% from the desired values.

Each day during the entire course of this study, unused food was discarded and replaced with fresh supplies. Water was provided by animal lick actuated valves, thus eliminating any possible CH_2Cl_2 contamination.

To accommodate the large numbers of mice used in portions of this study, the experiments were conducted in 3 phases. Phase 1 and 2 were hepatotoxicity tests conducted before the start of the 14-week continuous exposure. Both of these experiments were of 2 weeks duration and utilized 200 mice each. One group was exposed to 100 ppm and the other to 25 ppm CH_2Cl_2. The mice were added or removed in groups of 20 to allow for various exposure times. Liver triglyceride levels were measured, and fat stains were made on liver tissue as well as electron microscopic (EM) examinations. During Phase 3, the main portion of the study, the same tests were made on 2 groups of 100 mice exposed to 25 or 100 ppm. Serial sacrifices were made covering exposure times up to approximately 2 months. Dr. Weinstein will discuss the results of these experiments in the next presentation.

The final phase (3) of the study involved the exposure of groups of 170 mice, 20 rats, 4 monkeys and 16 dogs to each CH_2Cl_2 level with a comparable number of controls. Of these numbers, 20 rats, 20 mice, 4 monkeys and 4 dogs per dome completed the full 100 days of exposure. At the end of the exposure period these animals were killed and subjected to gross and histopathologic examination. The balance of the animals were used for special tests during the course of the Phase 3 exposure.

Groups of 20 mice were removed from each dome, fasted for 24 hours then used for determination of hexobarbital induced sleep times at 30-day intervals from onset of exposure. The methods used to determine and interpret sleep time results have been described by Van Stee (1971).

Levels of liver microsomal cytochromes P-450, P-420 and b_5 were determined in groups of 10 mice from each dome after 30-, 60-, and 90-days exposure. These mice were fasted for 24 hours prior to removal from the domes. Immediately upon removal, they were sacrificed and the livers were removed, suspended in 0.5M tris buffer pH 7.5, containing 0.25M sucrose, and frozen to the temperature of dry ice. The frozen livers were then packed in dry ice and sent via Air Express to Dr. F. J. Bullock of Arthur D. Little, Inc. for the cytochrome determinations. The determinations were made in the same manner (Bullock, 1971) as in the CH_2Cl_2 study discussed earlier.

For measurements of spontaneous activity, 10 mice per dome were used. These mice served as their own controls, with daily measurements of their activity over a 2-hour period recorded for 2 weeks preexposure in another dome under the same conditions as in the study, but without CH_2Cl_2. They were housed in specially constructed activity cages which were painted black to produce contrast with the white mice and render their movement more easily recognized by the television camera mounted on the outside of the dome. The cages were placed directly against a dome window so that the TV camera could view most of the cage. The TV camera was connected to a remote activity monitor and recorder. The activity measuring system has been previously described by Thomas (1971) and the results of this test will be discussed by Dr. Thomas in another paper.

The 20 rats in each dome were weighed on a biweekly basis during the study. No other tests were conducted on the rats except for gross and histopathologic examination and organ to body weight ratios at the end of exposure.

The clinical test schedule used for the 14-week exposed dogs and monkeys is shown in table I. Most of the tests were made at biweekly or monthly intervals. The various hematology and clinical chemistry tests are shown in table II.

TABLE I
Clinical Test Schedule for 14-Week CH_2Cl_2
Exposed Dogs and Monkeys

Week of Exposure	0	2	4	6	8	10	12	13	14
Hematology	x	x	x	x	x	x	x	x	x
Carboxyhemoglobin Levels	x	x	x	x	x	x	x	x	x
Blood CH_2Cl_2 Levels				x				x	
Body Weight	x	x	x	x	x	x	x	x	x
SMA-12 Battery	x		x		x		x		x
SGPT	x		x		x		x		x
Pathology									x

TABLE II
Clinical Laboratory Tests Performed on Dichloromethane
Exposed and Control Dogs and Monkeys

Chemistry		Hematology
Sodium	Total Bilirubin	Hematocrit
Potassium	Uric Acid	Hemoglobin
Calcium	BUN	Red Blood Cell
Albumin	Glucose	White Blood Cell
Total Protein	LDH	Reticulocyte
Cholesterol	Alkaline Phosphatase	
Inorganic Phosphorus	Creatinine	
SGPT	Chloride	

In addition to the 100-day exposed dogs, 3 additional groups of 4 dogs each were serially inserted and removed from the 3 domes to allow for exposure periods of 15, 30 and 45 days. The same battery of hematology and clinical chemistry measurements seen in table II were made on these animals before and after exposure. Carboxyhemoglobin (COHb) levels were determined postexposure. Following sacrifice, gross and histopathologic examinations were made. This included fat stains and EM examination of liver tissue.

The concentrations of CH_2Cl_2 in the 25 ppm and 100 ppm domes were remarkably constant throughout all phases of this study. Mean concentrations were determined for each 24-hour period, and averages were calculated over the entire study. The mean concentrations in the exposure domes were 25. 2 ppm and 99. 5 ppm. No fluctuations in CH_2Cl_2 concentration in either dome exceeded 10% of the predetermined level during the course of the study.

At no time during the course of this study did any of the animals show any overt signs of toxic stress. There were a few mouse deaths in both exposure groups and in controls, but no exposure deaths were related in any way to CH_2Cl_2 toxicity. Appetites and weight gains were relatively normal. The rat growth curves are shown in figure 1. There were no statistical differences between exposed and control values at any time period.

Hexobarbital sleep time determinations were made after 30, 60 and 90 days of exposure for each test group of mice and the controls. There were no significant differences between exposure groups although there were differences over time within all groups including controls. Thus, there was no measurable effect of continuous exposure on hexobarbital sleep time.

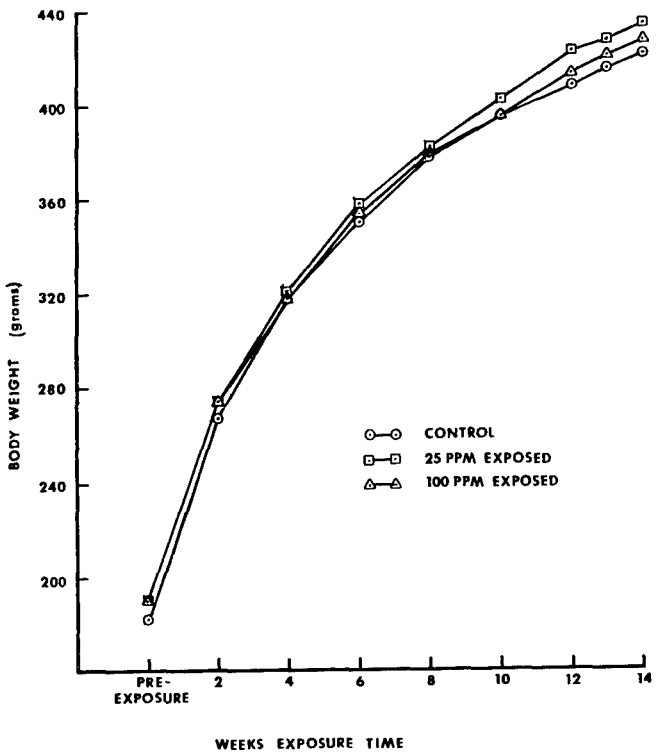

Figure 1. MEAN BODY WEIGHTS FOR 14-WEEK EXPOSED RATS.

Liver microsomal cytochrome determinations were also made according to schedule. Results received from Dr. Bullock are summarized in table III. According to these data, mouse liver cytochromes from animals exposed to 100 ppm CH_2Cl_2 for 30, 60 and 90 days were significantly different from controls. The P-450 determinations were lower than control at all time periods. The b_5 and P-420 values were lower than control at 30 days, but higher at 60 and 90 days. The overall significance of these results will await a final report from Dr. Bullock. No significant difference from control was seen in cytochrome values for mice exposed to 25 ppm CH_2Cl_2 for the same periods.

Mice exposed continuously to 25 ppm CH_2Cl_2 showed no pathologic changes; however, the livers of the 100 ppm exposure group did show positive fat stains. Liver cytoplasmic vacuolization was noted, and Oil-Red-O stains were positive for rats from both continuous exposures. Rat kidneys from both exposure groups showed evidence of nonspecific tubular degenerative and regenerative changes. A careful examination of all rat organ weight data revealed no biologically significant differences when mean organ weights and ratios of exposed rats were compared with those of the control group.

TABLE III
The Effect of Continuous Exposure to 25 ppm and 100 ppm CH_2Cl_2 on Mouse Liver Microsomal Cytochromes

CH_2Cl_2 Conc. (ppm)	P-450	b_5	P-420
	30 Exposure Days***		
Control	0.866	0.860	0.507
25 ppm	0.815	0.780	0.455
100 ppm	0.511**	0.642**	0.227**
	60 Exposure Days***		
Control	0.959	0.990	0.504
25 ppm	0.984	0.981	0.460
100 ppm	0.708*	1.173	0.842
	90 Exposure Days***		
Control	0.848	0.815	0.506
25 ppm	0.867	0.854	0.419
100 ppm	0.653**	0.944**	0.646*

* Different from controls at 0.05 significance level.
** Different from controls at 0.01 significance level.
***Results expressed as mμ moles cytochrome/mg microsomal protein.

An examination of hematology and clinical chemistry results of the 14-week exposed dogs and monkeys and the 15, 30 and 45 day exposed dogs showed no significant differences from control values. Likewise, gross and histopathologic examinations for all of these dogs and monkeys were negative with respect to CH_2Cl_2 induced changes.

Alluded to earlier were the interesting results of COHb and CH_2Cl_2 measurements in the blood of dogs and monkeys exposed continuously to both CH_2Cl_2 concentrations. The COHb measurements in this study were prompted by the work of Stewart et al. (1972). In our study, in comparison to control values, we found significant but nontoxic elevations in exposed dogs and monkeys (figure 2). The 100 ppm exposed monkeys had the highest COHb levels throughout the study, followed in descending order by the 100 ppm dogs and the 25 ppm monkeys. The 25 ppm exposed dog COHb values are no different than controls. Figure 2 shows clearly that monkeys had higher COHb values than the dogs with respect to the CH_2Cl_2 exposure concentrations. This is exactly reversed from the blood CH_2Cl_2 levels shown in table IV and indicates some connection between the two. The 25 and 100 ppm exposed monkeys

had lower CH_2Cl_2 blood levels than the dogs at the same exposure levels. These data suggest strongly that the monkeys metabolic processes were more effective in the conversion of CH_2Cl_2 to CO.

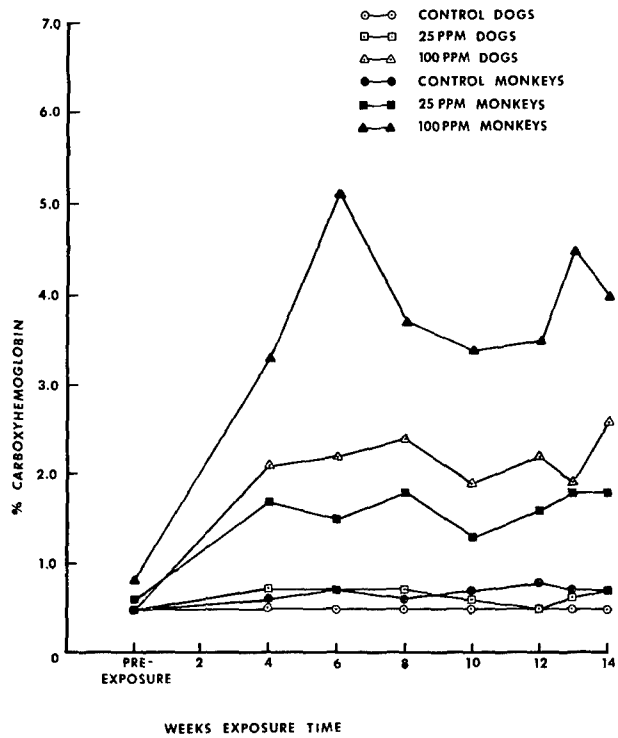

Figure 2. CARBOXYHEMOGLOBIN LEVELS IN DOGS AND MONKEYS EXPOSED TO 25 AND 100 PPM CH_2Cl_2.

TABLE IV
Dichloromethane in Blood of Dogs and Monkeys
Continuously Exposed at Low Levels
(μg/ml)

Dogs	6 Weeks	13 Weeks
Control	0.0	0.0
25 ppm Exposed	1.1	1.8
100 ppm Exposed	5.1	4.0
Monkeys		
Control	0.0	0.0
25 ppm Exposed	0.6	1.0
100 ppm Exposed	3.1	2.7

Some of the same information is expressed differently in table V which compares the calculated molar concentrations of CO and CH_2Cl_2 in the blood of dogs and monkeys, at 6 weeks, during continuous exposure. It can be seen that the molar concentrations of CO for monkeys at both exposure levels are almost 10 times higher than the equilibrium blood concentrations of CH_2Cl_2. In dogs, the molar concentrations are equal after exposure to 25 ppm CH_2Cl_2, and CO is 3 times higher after exposure to 100 ppm. The conclusion drawn is that (especially in the case of the monkeys) after blood and exposure atmosphere equilibrium has been established, a significant detoxification route is the conversion of CH_2Cl_2 to CO.

TABLE V
Molarities of CO and CH_2Cl_2 in Blood of Animals
Exposed to CH_2Cl_2 for 6 Weeks

Chamber Conc.	Dogs		Monkeys	
	CO	CH_2Cl_2	CO	CH_2Cl_2
25 ppm	2.0×10^{-5}	1.7×10^{-5}	6.1×10^{-5}	0.7×10^{-5}
100 ppm	17.0×10^{-5}	6.3×10^{-5}	34.0×10^{-5}	3.7×10^{-5}

The continuous 25 ppm and 100 ppm CH_2Cl_2 exposures described here were apparently low enough to avoid any major toxic alteration in the normal function of the animals exposed. However, some minor changes did occur at these levels. Mouse liver cytochrome enzymes were significantly different from controls at 30, 60 and 90 days exposure to 100 ppm CH_2Cl_2, while mice exposed to 25 ppm CH_2Cl_2 for the same periods showed no differences from controls. Carboxyhemoglobin levels were significantly higher in test animals compared to controls. Other minor changes were seen in several of the blood parameters from time to time during the course of the exposures, but these changes formed no pattern, and were not suggestive of any intrinsic damage. The level at which major toxic alterations will occur during continuous exposure lies somewhere between 100 and 1000 ppm CH_2Cl_2. At 100 ppm CH_2Cl_2, the animals were able to adapt and apparently reverse any changes that did occur. The space cabin TLV of 20 ppm for dichloromethane appears to be a satisfactory level for 100-day missions with a safety factor of at least 5.

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AMRL-TR-72-130

PAPER NO. 13

HEPATOTOXICITY OF DICHLOROMETHANE (METHYLENE CHLORIDE) WITH CONTINUOUS INHALATION EXPOSURE AT A LOW DOSE LEVEL*

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INTRODUCTION

Dichloromethane (CH_2Cl_2) is a common solvent and is generally regarded to be of low toxicity with single or intermittent inhalation exposures (Kutob and Plaa, 1962; Browning, 1965). Heppel et al. (1944) found that inhalation exposure to CH_2Cl_2 at 10, 000 ppm for four hours a day, five days a week, for periods up to eight weeks produced mild to moderate fatty change, but no necrosis in livers of several species and with no other organs involved. They also showed that intermittent exposure to CH_2Cl_2 at a dose level of 5, 000 ppm for seven hours a day, five days a week, for up to six months failed to produce toxic reactions as judged by light microscopy of many organs (Heppel et al., 1944; Heppel and Neal, 1944). Further evidence of the low toxicity of CH_2Cl_2 was obtained by Weinstein et al. (1971) who examined the ultrastructure of livers of mice exposed to 5, 000 ppm CH_2Cl_2, eight hours a day for four days, and found no pathologic alterations. Prolonged inhalation of CH_2Cl_2 at 5, 000 ppm and 1, 000 ppm dose levels is significantly more hepatotoxic than intermittent exposure to the compound (MacEwen et al., 1972; Weinstein et al., 1972) at the same dose levels. In order to further explore the hepatotoxicity of CH_2Cl_2 with prolonged inhalation, we have examined the effects of continuous inhalation of CH_2Cl_2 at a very low dose level, namely 100 ppm, for exposure periods up to 10 weeks.

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MATERIALS AND METHODS

Exposure Chambers

Two Thomas domes (McNerney and MacEwen, 1965; Thomas, 1965) were used for all exposures, one serving as a control chamber (called "control dome"). Both domes were operated at 725 mm Hg pressure to avoid leakage of gas, and air flow of 40 CFM was used in all instances. Dome temperatures were 24 ± 2 C, relative humidities were 50 ± 10%, and the CO_2 level never exceeded 0.2%. Dichloromethane in the experimental dome was maintained at a constant level of 100 ppm as described elsewhere (Weinstein et al., 1972). The level was continuously monitored with a Beckman Model 109A Hydrocarbon Analyzer and recorded six times per hour throughout exposures.

Chemicals

Chemical grade CH_2Cl_2 (Matheson, Coleman, and Bell, Norwood, Ohio) was used in all experiments.

Animals

ICR strain female mice (A. R. Schmidt Company, Madison, Wisconsin) weighing 17 to 25 grams were used for all experiments. Mice were fed ad libitum on Purina Laboratory Chow (Ralston Purina Company, St. Louis, Missouri) and were offered an unlimited supply of water. Mice were drawn from a randomized population and were numbered sequentially as they were placed in cages. Twelve groups of 16 mice each were maintained in the exposure domes for 3 days, or 1, 2, 3, 4, or 10 weeks. Animals were sacrificed by cervical dislocation within 5 minutes of their removal from the dome.

Tissue Triglyceride Determinations

At the termination of exposure periods the first 4 mice in each group were weighed and sacrificed; their livers were removed, weighed, and hemisected. One half of each liver was used for duplicate triglyceride determinations according to the method of Butler et al. (1961), and the other was used for light and electron microscopy.

Light and Electron Microscopy

Sections of neutral formalin-fixed paraffin-embedded liver were routinely stained with hematoxylin and eosin (H & E) and examined by light microscopy.

Cryostat sections of liver were stained for fat with Oil-red-O. Selected sections were stained with periodic-acid Schiff (PAS), and sections incubated with diastase prior to PAS staining served as controls. One micron sections of toluidine blue stained (Trump et al., 1961), Epon-embedded liver were examined by light microscopy.

For electron microscopy, one mm^3 blocks of liver were fixed by immersion in phosphate-buffered 1% osmic acid at pH 7.2 at 4 C. The fixative osmolality was adjusted to 320-330 milliosmoles with distilled water. Some tissue blocks were fixed at 2.5% glutaraldehyde buffer pH 2.4 with phosphate buffer and postfixed with 1% osmic acid in phosphate buffer. All samples for electron microscopy were dehydrated by serial passage through graded ethanol solution and embedded in Epon 812. Thin sections were cut on diamond knives, stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965), and photographed on a RCA EMU-4B electron microscope.

RESULTS

Liver and Body Weights

Table I compares body weights for groups of mice in the control and CH_2Cl_2 domes at 3 days and 1, 2, 3, 4, and 10 weeks of exposure. Body weights were not significantly different in the 2 domes except at 2 weeks, when the CH_2Cl_2 exposed mice were significantly lighter ($p < 0.05$, Student t test). Liver weights also were significantly lighter ($p < 0.05$) for the CH_2Cl_2 exposed mice at 2 weeks. However, liver/body weight ratios were not significantly different for control and CH_2Cl_2 exposed mice at any time interval.

Liver Triglycerides

Table II shows that liver triglycerides were normal for the first week of exposure to 100 ppm CH_2Cl_2, began rising at 2 weeks, were further elevated at 3 weeks of exposure ($p < 0.01$), and gradually declined, although remaining significantly elevated, after 10 weeks of continuous exposure to CH_2Cl_2 ($p < 0.01$).

Light Microscopy

One micron sections of Epon-embedded liver stained with toluidine blue and cryostat sections of formalin-fixed liver stained with Oil-red-O showed a few minute droplets of fat in scattered hepatocytes and Kupffer cells in control animals at all time intervals. Three days of exposure to 100 ppm CH_2Cl_2 produced no apparent change in mouse liver. At 7 days, however, definite changes

TABLE I

LIVER AND BODY WEIGHTS IN MICE WITH CONTINUOUS INHALATION OF 100 ppm DICHLOROMETHANE[a]

Weeks of Exposure	Body weight[b] (gm)		Liver weight[c] (gm)		Liver weight/ 100 gm body weight	
	Control	CH_2Cl_2	Control	CH_2Cl_2	Control	CH_2Cl_2
0.43	21.7 ± 0.31	21.4 ± 0.79	1.6 ± 0.08	1.4 ± 0.06	7.46	6.39
1	22.8 ± 0.48	22.6 ± 1.66	1.5 ± 0.12	1.3 ± 0.14	6.40	5.92
2	26.9 ± 0.65	23.4[d] ± 0.75	2.0 ± 0.11	1.3[d] ± 0.09	7.42	5.77
3	30.9 ± 0.79	28.4 ± 0.93	1.8 ± 0.07	1.6 ± 0.04	5.70	6.00
4	35.3 ± 0.59	31.1 ± 1.32	2.0 ± 0.15	1.9 ± 0.11	5.69	6.04
10	38.4 ± 1.27	36.1 ± 0.97	2.8 ± 0.09	2.7 ± 0.11	7.18	7.42

a Four female mice per exposure period in each chamber

b Mean body weight ± SE

c Mean liver weight ± SE

d Value is significantly different from the control value when tested by the Student t test at the 5% level of significance.

TABLE II

LIVER TRIGLYCERIDE LEVELS WITH CONTINUOUS INHALATION OF 100 ppm DICHLOROMETHANE

Weeks of Exposure	Control[a] (mg/gm wet weight)	100 ppm CH_2Cl_2[a] (mg/gm wet weight)
0.43	6.11 ± 0.90	6.43 ± 1.09[b]
1	6.10 ± 0.62	6.73 ± 0.92[b]
2	6.88 ± 0.72	16.70 ± 2.27
3	5.43 ± 0.43	22.88 ± 2.17
4	7.40 ± 0.50	14.0 ± 0.93
10	8.23 ± 0.59	11.80 ± 0.54

a Mean triglyceride level ± SE

b Value is not significantly different from the control value when tested by the Student t test at a 1% level of significance.

were apparent at the light microscopic level of resolution. All livers showed many small fat droplets in centrilobular hepatocytes and a decrease in hepatocyte glycogen. At 2 weeks, fat droplets increased in size and some droplets were larger than hepatocyte nuclei. Centrilobular glycogen depletion and fatty change persisted throughout the longer exposure periods (3, 4, and 10 weeks). Many hepatocyte nuclei appeared enlarged at 3, 4, and 10 weeks. Hydropic degeneration (so-called "ballooning") and hepatocyte necrosis were not observed.

Electron Microscopy

Thin sections of control livers at all exposure intervals showed essentially normal ultrastructure (figure 1). Electron microscopy confirmed the increase in lipid droplets and decrease in tissue glycogen in centrilobular hepatocytes with exposure to CH_2Cl_2. At 3, 4, and 10 weeks, small autophagic vacuoles were prominent (figure 2). Many of these vacuoles contained droplets or clumped membranous debris. Large autophagic vacuoles were present at 10 weeks of exposure in a few centrilobular hepatocytes. Elements of the smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER) appeared similar to the SER and RER in the control material. Other organelles were unremarkable.

DISCUSSION

Dichloromethane is generally regarded to be of low toxicity, although recent studies have indicated that it is more toxic than previously assumed (Stewart et al., 1972a; Stewart et al., 1972b; Weinstein et al., 1972). The toxicology of CH_2Cl_2 for prolonged exposures is particularly important in the spacecraft industry as CH_2Cl_2 is present in trace amounts in construction materials and may gas off into a closed space cabin atmosphere, eventually leading to a relatively high concentration. Inhabitants of spacecraft could be continuously exposed to gas off products for long time intervals. The 1972 Threshold Limit Value (TLV) for dichloromethane is 500 ppm, with a notice of intended change to 250 ppm, and the provisional spacecraft limit for 90- and 1000-day flights is 25 ppm and 5 ppm respectively. These levels are based on the lack of toxicity of CH_2Cl_2 at a 5000 ppm dose level with intermittent exposures.

Stewart et al. (1972a; 1972b) recently suggested that the TLV for CH_2Cl_2 be reassessed. They showed that 1 to 2 hours of human exposure to dichloromethane vapor is followed by a significant elevation in carboxyhemoglobin and suggested that carbon monoxide may be a metabolite of CH_2Cl_2. This observation is particularly worth noting in that concentrations of CH_2Cl_2 below the

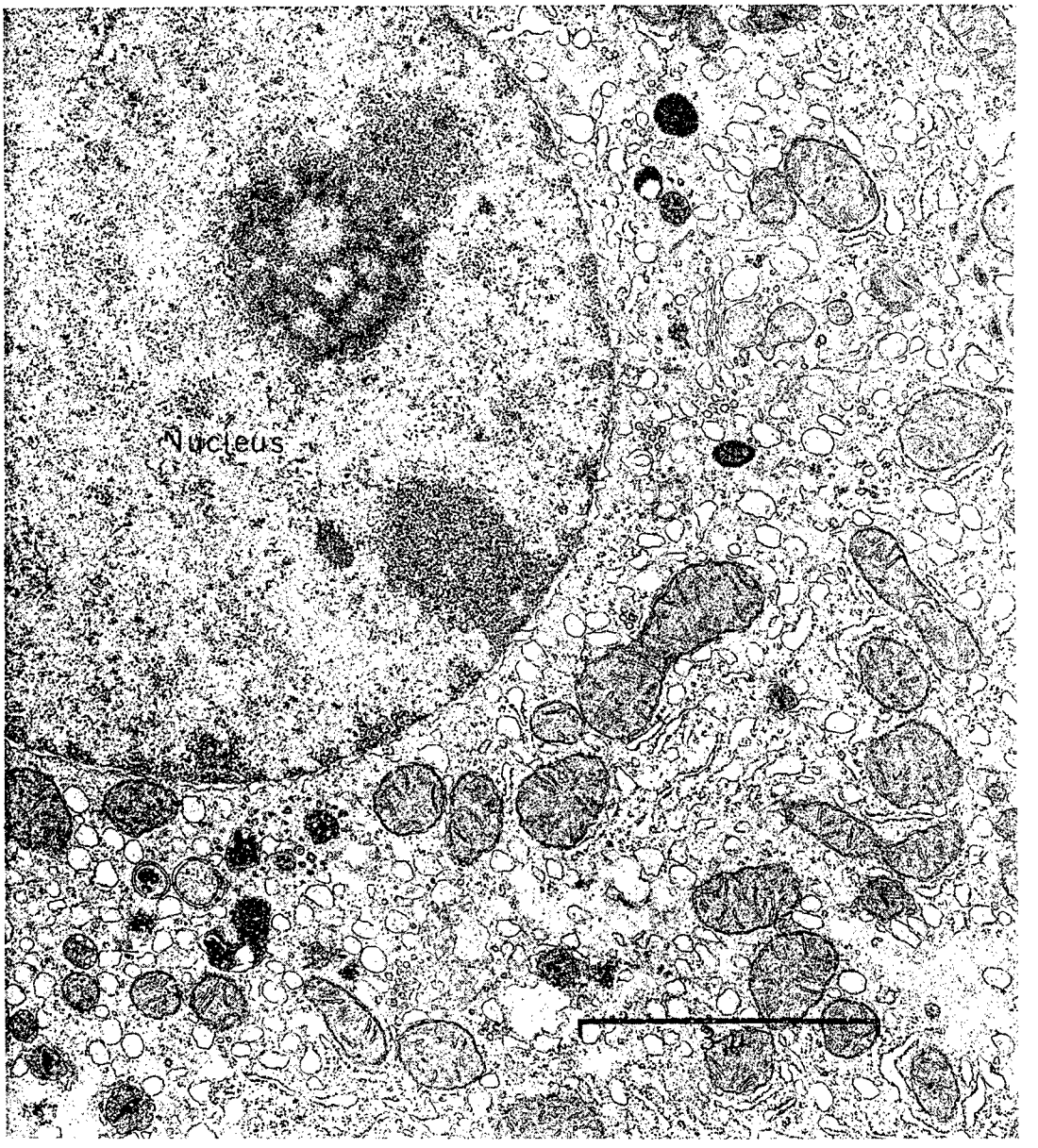

Figure 1. ELECTRON MICROGRAPH OF A CENTRILOBULAR HEPATOCYTE OF THE LIVER OF A MOUSE EXPOSED TO THE CONTROL DOME ENVIRONMENT FOR 21 DAYS. All of the organelles appear normal. x 13, 400

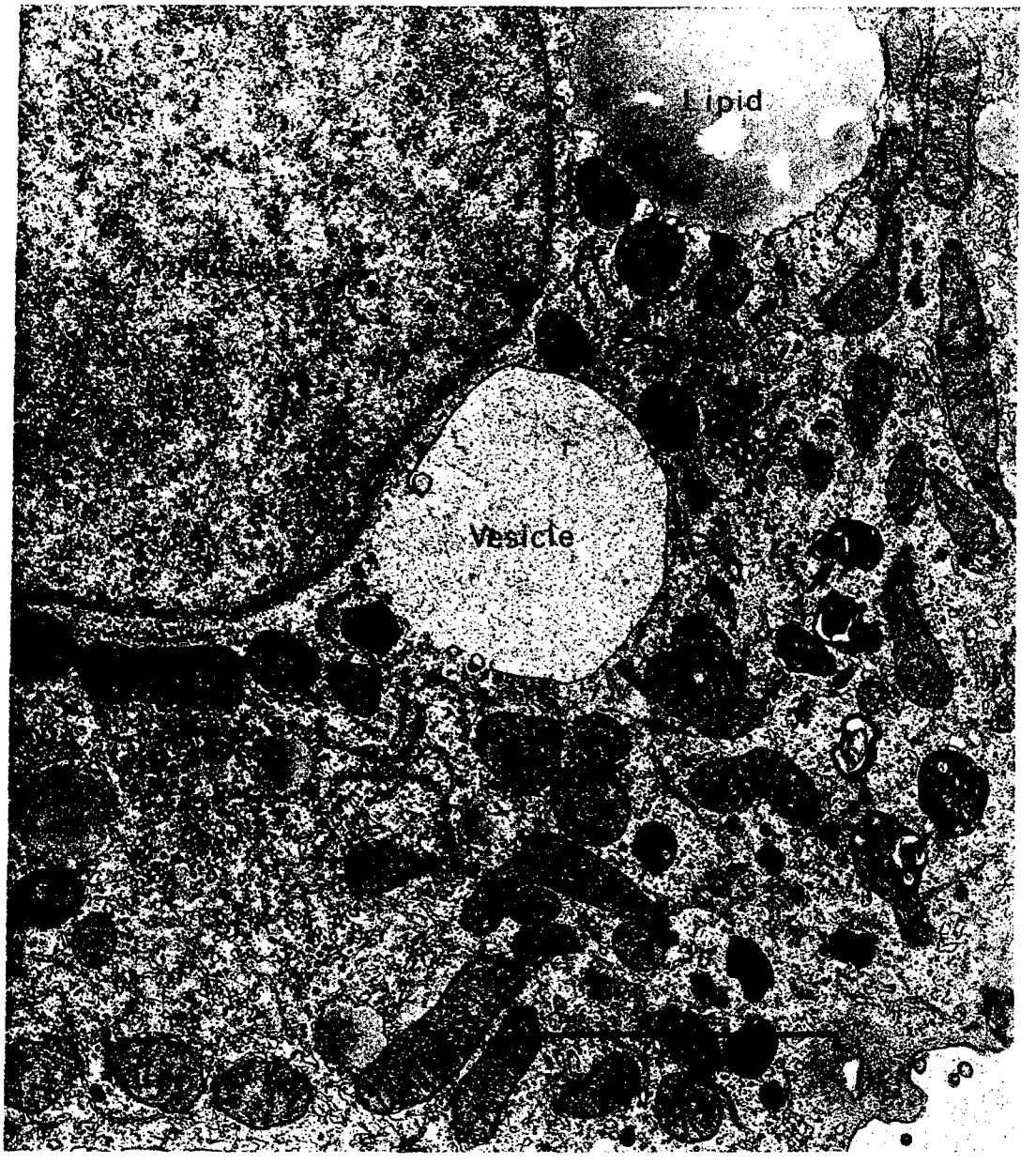

Figure 2. CENTRILOBULAR HEPATOCYTE OF A MOUSE EXPOSED TO 100 ppm CH_2Cl_2 FOR 21 DAYS. A large, empty-appearing vesicle is partially surrounded by a membrane. Many small autophagic vacuoles are filled with membranous debris (arrows). Lipid droplets vary in size. The nucleus, smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and mitochondria (Mit) appear unremarkable. x 13, 400

TLV for the compound result in a carboxyhemoglobin saturation level in excess of the allowable level for carbon monoxide itself (Stewart et al., 1972a; Stewart et al., 1972b).

Prolonged inhalation of dichloromethane is substantially more toxic than intermittent exposure to the vapor. Weinstein et al. (1971; 1972) found ultrastructural evidence of hepatic injury in mice after 12 hours of exposure to 5000 ppm CH_2Cl_2. This increased in severity with prolonged exposure, whereas repeated 8-hour exposures to 5000 ppm CH_2Cl_2 were shown to be nontoxic to the liver (Heppel et al., 1944). Haun et al. (1972) observed many fatalities in dogs, monkeys, and mice at 1 month of exposure at a 5000 ppm level and in dogs at a 1000 ppm dose level.

To further explore the hepatic effects of chronic inhalation of dichloromethane, mice were chronically exposed to dichloromethane at a dose level of 100 ppm. Animal growth proceeded at a normal rate at all time intervals examined, except at 2 weeks when there was a slight, but statistically significant, decrease in both body and liver weights with significant morphologic alterations observed in livers of animals. After 1 week of continuous exposure, hepatic triglyceride levels became elevated, then peaked at 3 weeks and declined, although they were still significantly elevated ($p < 0.01$) after 10 weeks of continuous exposure. Light and electron microscopy confirmed the presence of alterations that are associated with mild injury in the hepatocytes.

Although prolonged inhalation of CH_2Cl_2 at dose levels of 5000 and 100 ppm does produce partially reversible fatty change in mouse liver, the time course of change for each level is different. A level of 5000 ppm CH_2Cl_2 produces an immediate rise in liver triglycerides whereas onset of fatty change at the 100 ppm level is first detected by light and electron microscopy at 1 week and by tissue triglyceride level determinations at 2 weeks (figure 3). The delay in onset of fatty change provides us with a means of separating those changes induced by adaptation to the dome environment from those which are the direct result of CH_2Cl_2 toxicity, since adaptation apparently precedes the fatty change at the lower dose level. In earlier studies on the effects of prolonged inhalation of CH_2Cl_2 at a high dose level, it was suggested that some of the ultrastructural changes observed in mice could be the result of experimental manipulation of animals. Mice exposed to 5000 ppm CH_2Cl_2 rapidly became dehydrated and malnourished, which may account for at least part of their liver pathology (Weinstein et al., 1972). In this study CH_2Cl_2-exposed mice were clinically healthy. In the latter setting, the only ultrastructural alteration in liver was a slight decrease in tissue glycogen and increased fat.

Perturbation of protein synthesis in hepatocytes has been implicated in the development of fatty change in dichloromethane hepatotoxicity (Weinstein et al., 1972). Tritiated leucine incorporation into proteins is decreased, polyribosomes

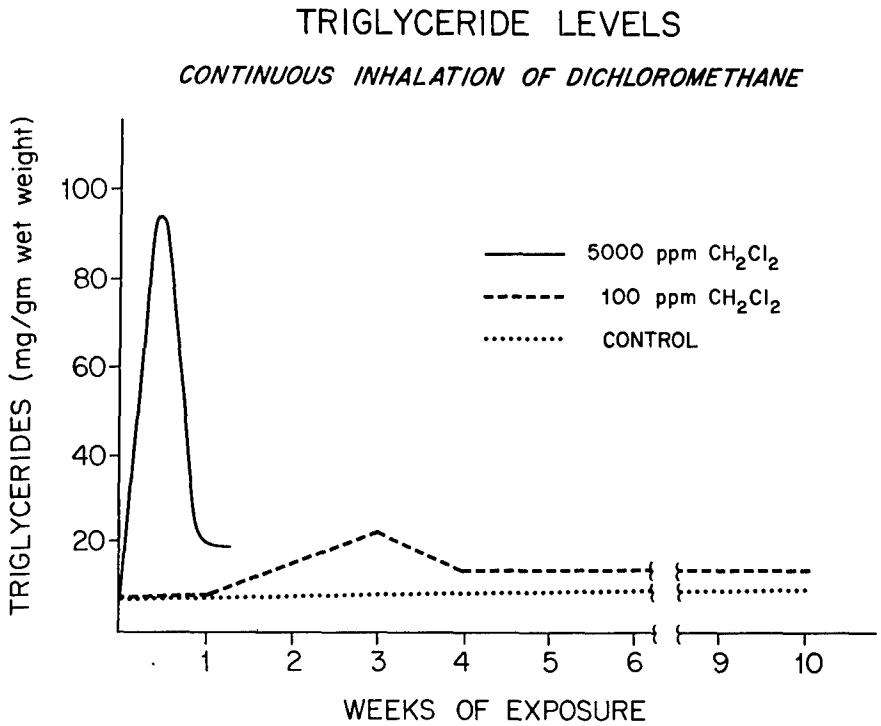

Figure 3. LIVER TRIGLYCERIDE LEVELS FOR CONTROL MICE AND MICE EXPOSED TO 100 ppm CH_2Cl_2 (DATA FROM THIS STUDY) OR 5000 ppm CH_2Cl_2 (DATA FROM WEINSTEIN et al., 1972).

dissociate into monoribosomes, and the RER undergoes hydropic degeneration with prolonged inhalation at the 5000 ppm dose level (Weinstein et al., 1972). Although leucine incorporation into hepatic proteins was not measured in the current study, elements of the RER appeared unaffected at the ultrastructural level, suggesting that a mechanism other than protein synthesis inhibition may be the cause of the hepatic fatty change observed in these animals.

The spontaneous partial reversibility of the hepatic fatty change supports the hypothesis that the injury produced by prolonged exposure to 100 ppm CH_2Cl_2 is mild, since severe injury of hepatocytes might be expected to compromise the cells' adaptive capabilities. On the basis of this study, we would speculate that the changes reported here may be totally reversible upon removal of the toxic material from the animal's environment and that permanent liver damage in mice is an unlikely sequela of prolonged exposure to 100 ppm CH_2Cl_2. However, continuous exposure at 100 ppm level of CH_2Cl_2 may not be totally innocuous.

For example, central nervous symptoms may appear during the time interval in which liver triglycerides become elevated, although the two events are not necessarily related as to cause and effect. Behavioral changes could account for the temporary retardation of growth rate of mice at 2 weeks of exposure, as observed in the study.

A delayed appearance of toxic reactions in conjunction with prolonged exposure to CH_2Cl_2 complicates the study of the toxicology of the compound. Since some toxic reactions are transient and the time of their appearance is dose-dependent, extensive sampling with respect to time is required. Predicting when a toxic reaction might occur in a complex chamber such as a space cabin would be difficult since dose levels may vary with time. Therefore, while toxic injury from continuous CH_2Cl_2 exposure may be nonpermanent, the possibility exists of such reactions impairing optimal function at a critical time in a mission.

ACKNOWLEDGMENTS

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DISCUSSION

DR. ROWE (The Dow Chemical Company): You mentioned the intermittent exposure to methylene chlorine producing no effects at 5000 ppm. This was reported, of course as you mentioned, by Heppel and others way back when. Have you conducted similar intermittent exposures and evaluated them by your techniques?

DR. WEINSTEIN (Tufts University School of Medicine): Yes, we have. We briefly mentioned that last year, we had done one experiment where we took similar groups of mice, exposed them to 5000 ppm dichloromethane for 8 hours a day for 4 consecutive days and sacrificed the animals at the end of the fourth exposure period. At the electron microscopic level we could detect no abnormalities. So we essentially confirmed their result although we didn't extend it over a large number of weeks.

DR. FASSETT (Eastman Kodak Company): Could I ask a brief question about the diet of these animals with reference to its fat content and whether it is present during the exposure. The nature of the question, of course, has to do with how much and what significance, if any, would the absorption of the highly lipid soluble methylene chloride into the 10% or so of the fat have to do with the possibility of oral ingestion?

DR. WEINSTEIN: I think Mr. Haun mentioned that quite obviously the food is exposed to the dichloromethane since the animals are never removed from the exposure chamber, and they are continuous experiments. The food is changed daily. I can't extend it any further than that. Perhaps Mr. Haun would like to comment on it. I think it's Purina Chow that they eat.

MR. HAUN (SysteMed Corporation): There isn't much I can add to what Dr. Weinstein has already said and what I've said in my paper. The food is changed every 24 hours during routine entries into the dome chambers. It's the Purina formulation for the rodents.

DR. ROWE: By chance, did you do any analysis on the food that had been in the chamber for a given period of time?

MR. HAUN: I seem to recall from last year's conference that Dr. MacEwen had some remarks on that. Am I right on that, Dr. MacEwen? Now, we did nothing on this this year, but it seems that last year when we reported the 5000 and 1000 ppm exposure, I think we had done some work on that. I simply don't remember the results.

DR. WEINSTEIN: We were very concerned with this problem last year and we regard it as a very important question. I think the point that could be made last year was that the animals effectively stopped eating at the end of 24 hours. They showed a rather dramatic loss in weight simply because they were very sick and couldn't eat. So I think that the amount of dichloromethane that they would be getting that way would be trivial simply because they weren't touching their food. I think with these experiments, it may well be true that they do get some dichloromethane via the oral route. It's a difficult thing to get around experimentally, because of the way the experiment had to be carried out to achieve truly continuous exposure. We did, at one point, debate the possibility of taking the animals out for an hour a day but in view of the dramatic difference in results between intermittent and continuous exposure, that would just clearly be a very, very different experiment so we didn't feel we could do it.

DR. HODGE (University of California): I'd like to ask whether the level of lipid in the liver in the 100 ppm exposure, 3 weeks and thereafter was approximately identical with the higher level established in the report of last year.

DR. WEINSTEIN: It was a bit higher last year. Last year, it was roughly 3 to 4 times elevated. This year it's roughly 1-1/2 to 2 times elevated. The answer to the question would have to be that last year we found a higher level; however, remember that last year, we stopped sampling at 7 days and this year we stopped sampling at 70 days. So it could very well be that within a couple of weeks we would achieve the level that we saw. In other words, the homeostatic level may be the same in both types of exposures after adaptation has taken place which is what I think you are driving at.

AMRL-TR-72-130

PAPER NO. 14

EFFECTS OF LOW LEVEL DICHLOROMETHANE EXPOSURE ON THE SPONTANEOUS ACTIVITY OF MICE

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INTRODUCTION

As a follow on study to the high level methylene chloride exposures reported in last year's conference proceedings (Thomas et al., 1971), a new continuous exposure study was designed to find a level of no toxicity. The pertinent exposure and recording parameters were essentially similar to last year's study, but concentrations of methylene chloride for the 100 day (14 week) exposures were reduced to 100 and 25 ppm, respectively. These experiments were conducted between 31 January and 19 May 1972.

METHODS

The closed circuit TV recording method for spontaneous activity of 10 mice each for the two exposure groups and for a single control group was exactly the same as described previously (Thomas et al., 1971).

Recording sessions were conducted on each regular workday for all 3 groups. The 25 ppm exposure group was always recorded from 0830 to 1130; the 100 ppm group from 1130 to 1330; and the control group from 1330 to 1530 hours. As before, the two exposure groups received a 2 week preexposure baseline measurement of spontaneous activity.

In each 2 hour daily recording session 7200 observations were made of spontaneous activity at 1 second intervals. Thus, each week 36,000 data points were collected for use in calculating the average daily activity for a week, and the total number of samples for a 14 week exposure was 504,000.

The 2 week preexposure baseline daily average activity for the 100 and 25 ppm groups was calculated from 72,000 observations. No such 2 week baseline was calculated for the control group; in calculating daily average activity the total 12 week observation period was used, consisting of 432,000 data points.

All mice used in the experiment were females, obtained by random selection from the same shipment. They were of the same age, and the average weight was 24 grams.

All mice were necropsied at the end of the experiment.

RESULTS

No death occurred in any of the 3 groups of mice. On routine visual observation, no clinical signs of toxicity could be detected, and activity was judged to be of the same intensity throughout the 2 week preexposure period. However, from the 9th week of exposure on, the 25 ppm group became visibly more active, with mice appearing to be climbing all over the cage most of the time. The 100 ppm and the non-exposed groups did not exhibit any trends toward increased or decreased spontaneous activity that could be noted by visual observation.

A week by week average of daily activity for the 3 groups of mice are summarized in table I.

Table I

Weekly Averages of Daily Spontaneous Activity in 3 Groups of Mice in Methylene Chloride Study

		100 ppm	Control	25 ppm
Preexposure Weeks	#1	217	N. D.*	122
	#2	333	N. D.	384
Exposure Weeks	#1	62	N. D.	381
	#2	178	N. D.	880
	#3	378	126	681
	#4	204	258	707
	#5	202	357	723
	#6	174	258	460
	#7	148	172	492
	#8	149	188	707
	#9	196	76	405
	#10	298	476	1383
	#11	437	778	2059
	#12	480	331	1095
	#13	286	320	1596
	#14	164	573	2202
Exposure Average		240	---	983
Control Average			326	

*N. D. - not done.

Figure 1 graphically illustrates the trends in individual exposure groups and the controls. It readily shows that there are no significant differences between the control and the 100 ppm exposure groups, but a significant increase of spontaneous activity takes place in the group of mice exposed to 25 ppm methylene chloride.

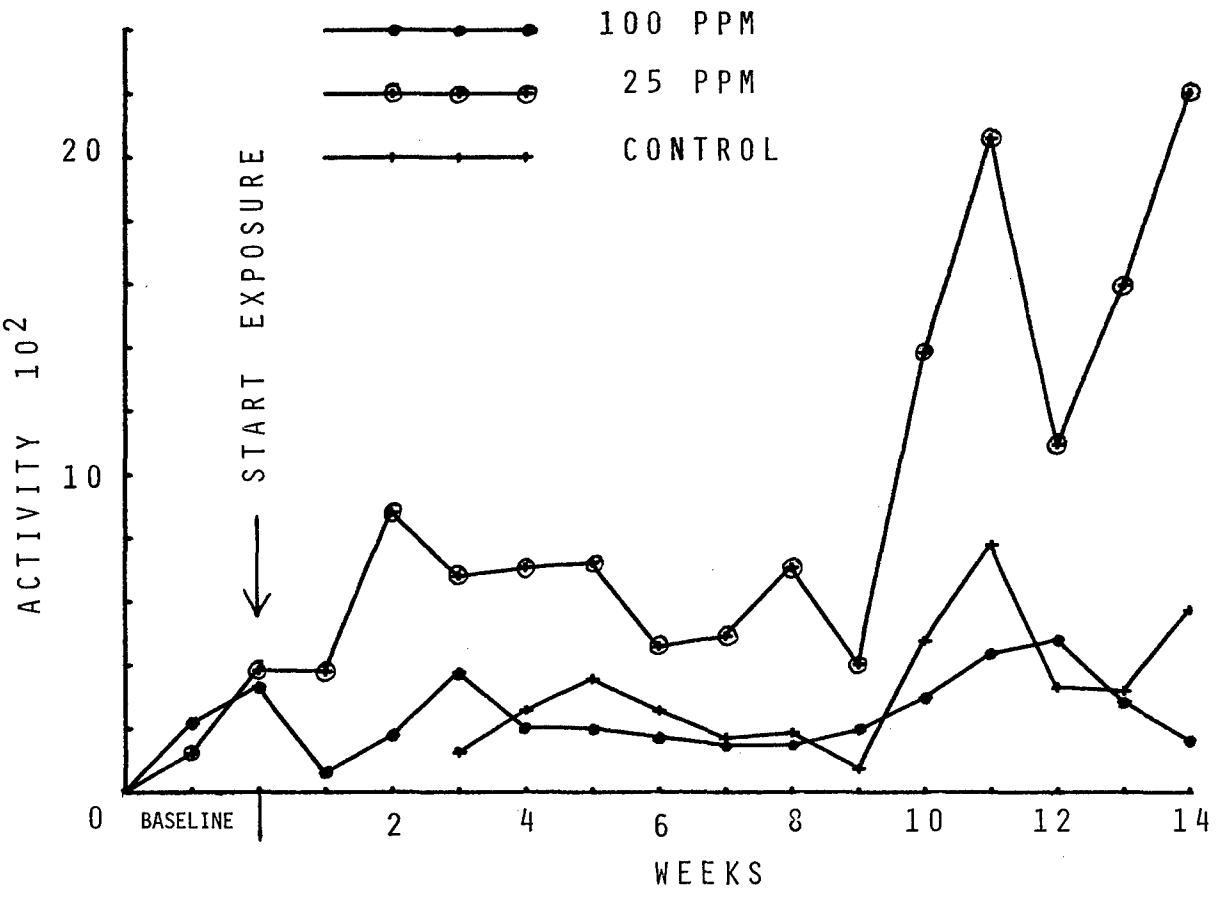

Table II summarizes the average spontaneous activity values for the baseline and total exposure period in the 100 and 25 ppm exposed groups, and compares these with the nonexposed control group during the last 12 weeks of the experiment. The parenthetical figures represent the lower and upper limits of weekly average for daily spontaneous activity. These data reinforce the absence of any effect in the group exposed to 100 ppm methylene chloride, when compared to their own baselines or to the control group.

Table II

Average of Daily Spontaneous Activity in Mice During Entire Methylene Chloride Experiment (114 Days)

	100 ppm	Control	25 ppm
Baseline Period	275(217-333)		253(122-384)
Exposure Period	240(62-480)	326(76-778)	993(381-2202)

Note: 1. Control group was recorded only during last 12 weeks.

2. Figures in parenthesis are for weeks of lowest and highest activity.

3. Baseline values were recorded for 2 weeks preexposure.

Gross and histopathology findings were negative.

DISCUSSION

This was the first experimental study employing an independent nonexposed control group housed in the control dome. There seems to be a trend toward slight increase in spontaneous activity with age in mice. These mice were 6 weeks old when delivered, spent 6 weeks in quarantine and 12 weeks in experiment, which would put the start of increased spontaneous activity at the 20th week of age.

A similar trend seems to appear in both exposed groups, although the marked increase in spontaneous activity in the 25 ppm exposure group cannot be explained on that basis alone.

The reason for the behavior of the 25 ppm groups is unknown at this time. These mice were at least three times more active than the control group at the 11th and 14th weeks of the exposure, even when one considers a possible aging effect. The assumption of a stimulant mechanism without further experimentation would be highly speculative at best.

SUMMARY

In contrast to the previous 100 day continuous exposure experiment, where depression of spontaneous activity was demonstrated at 1000 ppm methylene chloride concentration, this 100 ppm exposure level was without effect.

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DISCUSSION

DR. HODGE (University of California): I remember that Charlie Haun pointed out that the low level group weighed more at the end of the experiment and the lines diverged toward the latter part of the 14 week test period. Were those animals also observed to be hyperactive and do you suppose there was an increased appetite that went along with this somewhat greater growth?

DR. A. THOMAS (Aerospace Medical Research Laboratory): The problem was that those animals haven't been observed. Now, you have to keep in mind that in a 14 week period, we have over 600,000 observations, one second apart. That is a lot closer look than an animal handler can do by just looking at the cages once an hour or so. So, I couldn't tell that. But I went and looked at the other animals and they weren't as excited or running around as this particular group was.

DR. ROWE (The Dow Chemical Company): As I remember those growth curves, Mr. Haun, it looked to me as though the controls may have decreased a little bit because that curve wasn't smooth whereas the other two groups had smooth curves. Is that right?

MR. HAUN (SysteMed Corporation): That's correct and the curves were for rats, not mice.

DR. BACK (Aerospace Medical Research Laboratory): There is another possible explanation. The animals that are gang caged do not see a light intensity change anytime. Now, with Dr. Thomas' experiment, the thing is run at the same time of day each time, but a light comes on just before the camera starts. Now with low level CNS depressants, many times you see a startle effect and maybe you're inducing this startle effect with the low level CNS depressant. It may also happen with alcohol. I don't know but this is a possible explanation. The other animals might not pop like popcorn because they are always under the same light intensity.

DR. A. THOMAS: We standardized all the procedures. The light came on exactly one hour before the recording began. We controlled the dome entries so that the animals hadn't been handled. Remember that the recording cage had to be cleaned and so on. So what happened in the way of handling or lighting was standardized. Obviously, they weren't recorded at the same time and the 100 ppm group was recorded between 0830 and 1130, the 25 ppm group was recorded from 1130 and 1330 and the control group was recorded from 1330 to 1530. Looking at the baselines, they are all around 300 so it looks like something, but I hate to hang my hat on one run.

DR. HODGE: I'd like to make a comment. Going back just one moment changing the subject to yesterday's topic, here is apparently an effect on the rats at the low level, 25 ppm level, they seem to grow to slightly heavier rats. If this happens to be the most delicate response to the exposures to this toxic substance, what comment would those people charged with setting threshold limt values make on this change?

DR. A. THOMAS: They are healthier at 25 ppm than at 0 ppm.

DR. HENDERSON (Olin Corporation): As I pointed out yesterday, one of our major problems is obesity. All we're doing is triggering more of it with this exposure.

DR. STOKINGER (U. S. Public Health Service): In answer to Dr. Hodge's question, we'll do the same with it as we do with fluoride. We have a beneficial effect that occurs below the deleterious levels so we set a level for dental fluorosis, on the basis of the adverse effect with no problem.

OPEN FORUM

DR. ROWE (The Dow Chemical Company): Inasmuch as we are talking about methylene chloride in the papers this morning, Dr. Fassett had asked for a few minutes to bring us up to date on some work that they have been doing in his laboratory which I think is extremely pertinent with respect to the formation of carbon monoxide in the metabolic pathway of methylene chloride. So it might be apropos at this time so we can consider that in the discussions, because I know it is some work that we've all been waiting to hear about.

DR. FASSETT (Eastman Kodak Company): Just briefly, we've been quite interested in the metabolism of methylene chloride. This is one compound for which it would be practical to use biomonitoring as an index of control. When I heard earlier this year about Dick Stewart's fascinating work, at first I was a little skeptical because from what we knew about its metabolism, it seemed as though you would have to convert quite a high percentage (about 20%) of methylene chloride breathed in an 8-hour day at the TLV in order to reach the threshold limit of carbon monoxide. In other words, you need about 203 milligrams of CO. Only 14% of the methylene chloride molecule is carbon so you have to have quite a conversion to do that. It seemed strange, but knowing Dick Stewart's and Jack Peterson's reputation I thought we ought to confirm their findings. So, very briefly, there are a couple of preliminary studies which have been in progress the last few months and I wouldn't guarantee that the data won't change before we get through. In the first experiment, labeled methylene chloride was given to rats (i. p.) dissolved in corn oil, 1 ml per kg, and then these animals were sacrificed serially at 30-minute intervals. The blood was collected by heart puncture and carboxyhemoglobin determined using the Van Slyke. The animals indeed do, under these circumstances, generate carbon monoxide which reaches a plateau apparently in roughly 60 minutes. By the fact that the methylene chloride in the Van Slyke procedure is off gassed before you get to releasing the CO from the hemoglobin so that all we had to do was really capture the CO as it was released in Van Slyke procedure and measure the radioactivity. This study showed that the carbon monoxide in the rat apparently comes from methylene chloride itself. Another study was done in which we were interested to see whether methylene chloride interfered with phenobarbital metabolism and, very briefly, the results of this experiment showed that there was an increase in liver weights in the animals getting the phenobarbital and not in those inhaling methylene chloride itself. This fits in with the studies reported this morning. We measured plasma levels of phenobarbital in the animals concurrently getting methylene chloride and found that plasma levels aren't elevated. I'm not sure of the biochemical mechanism of this, maybe Bernie Astill will comment on that at this time. Briefly, we have started to look at some of our workers and this is still very preliminary. Because our exposures were actually pretty low - perhaps 50 to 100 ppm - we didn't expect much carboxyhemoglobin elevation. There is, however, a slight increase in nonsmokers over the working day. The statistics are still in the state of being accumulated.

DR. ROWE: Could you tell us what kind of levels you found in the nonsmokers?

DR. FASSETT: As nearly as I can gather from a very preliminary look without any statistics, it's maybe something in the order of twice the generally accepted control level of half a percent. Measurements are done with Van Slyke so at the TLV, I don't know what percent one would get. Dick Stewart said it's quite a bit but whether this would be the case or not I don't know. It seems to me rather doubtful whether this would be of major toxicological significance since the smoker's percentages certainly go up very high, as you well know.

DR. ROWE: Thanks, Dave, for your contribution. Now I should like to open all of the papers that were presented this morning to discussion.

DR. VERA THOMAS (University of Miami): I wonder if you saw any changes in the carboxyhemoglobin in animals pretreated with phenobarbital in comparison with the exposed group without phenobarbital.

DR. FASSETT: Your question was whether there was carboxyhemoglobin increase in the animals pretreated with phenobarbital? We didn't do that. We were measuring phenobarbital levels; we weren't measuring CO in those particular animals, but there's no doubt that in the other experiment that I described with the rats that you do get CO generated, but we weren't doing these on the same animals so we don't know that.

DR. VERA THOMAS: I asked because if microsomal enzymes are involved then their induction by phenobarbital should stimulate metabolism of methylene chloride.

DR. ASTILL (Eastman Kodak Company): Could I elaborate a little bit on those experiments? What we sought to do was to see whether in fact there was a cross relation between methylene chloride exposure and whether in actual fact we could have any effect on phenobarbital metabolism as being a typical substance which was hydroxylated by microsomal oxidases, and in consequence we found that at 50 ppm and 500 ppm methylene chloride together with phenobarbital we've got elevated phenobarbital levels in the plasma. Now this didn't happen of course where we didn't have any methylene chloride so we concluded that the methylene chloride in some way produces some material which interferes with the hydroxylation of phenobarbital, and I guess our conclusion is that this doesn't represent microsomal induction caused by methylene chloride. I think we're rather inclined to feel that it might be either methylene chloride binding to the enzymatic site or to the membrane in some way, phospholipid in the microsomal membranes. On the other hand, it might be due to carbon monoxide itself binding to cytochrome P-450 thereby reducing the amount of hydroxylation which is able to be carried out.

DR. DREW (National Institute for Environmental Health Services): We have done the exact studies that you asked the questions to. We have measured carboxyhemoglobin levels in rabbits exposed to dichloromethane and then, using the same

animals a few days later pretreated with either phenobarbital or SKF525A, which would be an inhibitor, neither of these compounds apparently affect the level of carboxyhemoglobin.

DR. SCHEEL (National Institute for Occupational Safety and Health): It'e entirely possible in the P-450 system where you are talking about phenobarbital metabolism to have another compound inhibit this system in such a way that it does not react to the inducer. This has been done with other materials, one of which is a drug used for treatment of malaria by the Army Chemical Center, and these inhibit the P-450 system so that it won't react to stimulants. I think here we have an experiment where the methylene chloride probably does inhibit the P-450 system and thereby makes it inactive and interferes in this way with the phenobarbital metabolism. I'd like to ask Dr. Hays from his experience whether cardiac arrhythmia is an all or nothing physiologic response or a dose-related physiologic response?

DR. HAYS (U. S. Department of Agriculture): My own feeling is that it is probably a dose-related response. We've thought for many years that there wasn't, for example, any decrement in the automaticity of the cells and the depolarization, but now I think it is certainly a dose-related phenomenon. It would appear to me, because of the wide variety of compounds, that many of these simply have a capacity to change the permeability of the cell and this could be done probably at very low doses, but then when you have on top of this increased contractility, this only causes a greater change in the ionic exchange of sodium and potassium and thus brings about the arrhythmia. So I have a feeling that it is dose-related.

MR. VERNOT (SysteMed Corporation): On that same score, I was a little uncertain about how to reconcile Dr. Van Stee's talk with Dr. Hays'. If I understood Dr. Hays' possible explanation for arrhythmia caused by anesthetics, the active substance had a positive inotropic effect. Dr. Van Stee's seems to indicate that bromotrifluoromethane has a negative inotropic effect, but still tends to cause arrhythmias under the right condition.

DR. HAYS: I quite agree and we recognized this many years ago that some of the agents actually depress the myocardial and depress the contractility. Chenoweth showed this years ago in the case of chloroform and yet in the presence of epinephrine it does produce the ventricular extrasystoles. I think we have to be very careful of what we choose as our criteria because there are so many types of arrhythmias and not all may have the same origin. I'm not convinced that they do, but I'm not convinced that they don't either. I wanted to specifically state that I've set as our criterion the level of ventricular extrasystoles, but this may not agree with those who studied ventricular fibrillation. There is an inconsistency and we recognize it but we don't know how to explain it.

DR. STOKINGER (U. S. Public Health Service): It is kind of hard for me to visualize a mechanism by which carbon monoxide is derived from methylene chloride. I wonder if any thoughts have been given to the effect of methylene chloride or its other metabolites on inhibiting the elimination of the endogenous carbon monoxide, thereby building it up in the system?

DR. ROWE: That's one of the first things that we thought about when we talked about this, but that's why the C-14 experiment was so important, because that shows pretty well that it comes right directly from it. Dr. Fassett, do you care to make any comment on that?

DR. FASSETT: I'm not sure we should get out on a limb now since we have a number of other experiments which I think will probably answer this question.

DR. ASTILL: We did take a look at this problem and I think the endogenous carbon monoxide comes from the exposed methyl group in porphyrins and it arises from glycine and our guess was that the turnover time is just not rapid enough for methylene chloride to affect endogenous carbon monoxide synthesis so that the carbon monoxide pool could be inhibited or built up in any way to produce this effect. And I guess the other point is that we feel fairly confident from our labeled work that the carbon monoxide which results from methylene chloride inhalation does, in actual fact, come from the methylene carbon and doesn't seem to come from the body pool. It appears too rapidly to be otherwise.

DR. HAYS: I'd like to ask Dr. Weinstein first if he has any studies to indicate whether this is a reversible process in the liver, and if so, how long does it take before recovery of the liver after stopping the continuous inhalation? Now, the second thing that I think is so important is the difference found in the continuous versus the intermittent exposures. As a bureaucrat and one who is responsible for regulatory matters, I was and still am constantly faced with this matter of what do you use in making your decisions about the particular compounds that are in the environment. I get very discouraged at times when I see that so much weight has been placed on the continuous exposure when we know very well that to many compounds one is not exposed continuously. Yet there are so few studies to make this distinction and here is a very clear-cut one of the difference between continuous exposure versus intermittent exposure, and I don't think we ought to ignore it. I have pleaded and pleaded with the authorities that be that it is not reasonable nor fair to simply take the maximum effect that one sees on a continuous study and say this is the basis on which we're going to make our judgment. I have a third question. Have you done any studies of the compounds that do produce hepatomas and what effect do you anticipate from the continuous versus the intermittent?

DR. WEINSTEIN (Tufts University School of Medicine): We really have not addressed ourselves to the problem of reversiblity. I can mention an experiment that was done almost incidentally at the beginning of the work and, since it wasn't done very systematically and was not repeated, shall we say that it is just relating an experience rather than a true result. When we first observed the marked effect of 5000 ppm dichloromethane with continuous inhalation we were rather amazed at the effect and, in fact, we initially wondered if something else was going on in the animals, we wondered whether they had some disease or something that was not toxic induced. At the end of the first week exposure, we decided to take some of the animals out of the dome, that is after four days. The following Monday we came in and decided to

sacrifice the animals and did do necropsies on the animals. Based on our light microscopic observation, it appeared that the lesions had almost totally reversed, but recall that even with continuous exposure at 5000 ppm the vast part of the lesion reverses during that same time interval. I think the experiment one would want to do would be to take animals out at 24 hours and 48 hours during the time when the lesions are normally getting worse and see what happens. So it wasn't done systematically, but it did seem that the lesions reversed in large measure. However, at that time you could still tell that there were some changes in that a few of the hepatocytes were engorged with lipid. So clearly the reversibility was not complete. We also measured thymidine indices in the livers at 5000 ppm. We took animals at one-day exposure, two-day exposures, four-day exposure, and seven-day exposures as well as the appropriate controls. Forty-five minutes before sacrifice within the dome, we injected thymidine. Of course, thymidine incorporates into the nucleic acid in the liver and gives one some measure of the mitotic index. In effect it amplifies it because at a given time far more cells would be taking up thymidine than will actually visibly be in mitosis since the end phase in a mitotic cycle is relatively short. We found at the end of one day that the experimental and control animals were taking up thymidine at comparable rates; however, at 2 days and 4 days there was a striking increase in mitotic activity in exposed animals, and we do tend to relate stimulation of mitotic activity to a potential to generate a tumor. Interestingly, at 7 days most of the animals had returned to their normal level, in other words we had induced a large burst of mitotic activity and then this disappeared so at the time when the livers looked better they also were not mitosing very actively. I might say that a similar experiment was started at 100 ppm and the animals were also injected with thymidine, but I went back into civilian service at about that time so I'm afraid the material hasn't been looked at. I don't know how far I would go out on a limb to say that one can rigorously correlate mitotic activity with tumorigenic potential. As a matter of fact, I don't know that it is terribly well correlated at all.

DR. ANTHONY THOMAS (Aerospace Medical Research Laboratory): I think that one portion of the question was not quite answered, and it is not scientific, rather purely managerial. Dr. Hays, you asked why we always tried to compare continuous exposure with interrupted exposure. First of all, this work was done for NASA and they need continuous exposure studies. Also, with other compounds, for example in the hydrazine family, we find that the CT relationship holds beautifully and you can directly compare continuous exposure with interrupted exposure. I have dedicated my life to trying to explore the hypothesis that with certain compounds you would be able to do chronic toxicity studies in a compressed time frame if the CT relationship holds. In other words, a six-hour per day, five-day per week chronic study at a particular concentration should cause the same amount of damage as in the continuous exposure at one-fifth that concentration. If we can do nothing else for industrial toxicology (and this is in my mind from the space program and the submarine program and other things), if we know the target organs and the mechanisms, we can get answers a lot faster. With the expanding chemical technology and with the dearth of government and industry inhalation exposure facilities, that would be one part of the solution to our problems.

DR. HAYS: Anton, I hope you didn't misinterpret my question. I think you have opened up an avenue here that we can address ourselves to this question of insisting that if you are going to make a decision based on some damage of some particular organ that we've got to compare just what you've done. You've got to compare continuous with the intermittent.

DR. FASSETT: Just a brief comment on Dr. Thomas' thought which is something that's interested us for many years too. The hitch is that some compounds are not metabolized the same way at high dosages and low dosages and unless you know these facts you can't make this judgment.

DR. ANTHONY THOMAS: Agreed. You've got to know enough about the compound.

DR. ROWE: Are there other questions?

DR. HAYS: I'd like to make just one more comment and that is not on this subject but on yesterday's discussion of the biologic standards. Col. Kittilstad raised the question about his being concerned. Dr. Culver, who I don't believe is here this afternoon, and myself are serving on a national task force that was appointed by the Council on Environmental Quality, and we have been dealing with this very problem of biologic standards for workers entering fields where pesticides have been applied and trying to establish what we considered to be a safe reentry period. Now what do you do? You can't use threshold limit values out in the open field so we are forced into a position of trying to come to grips with some biologic standards, and this is going to be a very difficult problem I'm sure. I think Col. Kittilstad had some reason to be concerned. We are meeting in Washington tomorrow, a rather high level meeting, Secretary's level, to discuss this whole question of occupational medicine of the worker in the fields. I thought you might be interested in that progress.

DR. ROWE: Thank you, I don't envy your problem. I'd like to ask Dr. Weinstein one question. Maybe I missed it in his presentation this morning, but did you describe any pathology examinations on the 25 ppm exposed animals?

DR. WEINSTEIN: No, I didn't describe them, but I might briefly comment on them. In all of our experiments large numbers of animals are randomized and broken into groups and a number of analyses are done on the groups at the time of sacrifice: liver weights, body weights, etc. Unfortunately the animals at 25 ppm turned out to be slightly but significantly larger than the animals that went in the dome at 100 ppm and the control animals. We were working with fairly small numbers. It didn't worry us tremendously except that we decided that since they were not statistically identical to the 100 ppm animals and the control animals, we decided not to deal with the data. However, I think it was actually worthwhile data, it just simply did not fit what we regarded in our initial protocol as acceptable data. So I might briefly mention it although I would not put it in the formal report simply because of that statistical problem. Triglyceride levels were determined at the same time intervals on animals exposed to 25 ppm dichloromethane. At no time were the triglyceride levels elevated. In other words, throughout the exposure period they were essentially the same as the control animals. We did examine the material light microscopically and, to a limited

extent, electron microscopically. We found that we could detect a lesion at 25 ppm. This was not reflected in triglyceride levels although you might recall from my talk this morning after 7 days at 100 ppm we could see triglyceride droplets in the tissue microscopically that were not reflected in triglyceride levels when measuring the tissue. In other words, we were just not sensitive enough with our chemical method to pick it up. So at 7 days and 14 days we could see enlarged lipid droplets in the tissue with 25 ppm. This was true in every animal that we looked at and we could separate our slides into groups and pick out the ones that had been exposed to 25 ppm so it was indeed a definite lesion and I really can't carry it further than that.

DR. ROWE: All right. I thought that maybe I had missed something this morning.

DR. HODGE (University of California): I'd like to ask Dr. Weinstein what the character of the regenerated liver cells was. Was it different in the animals that had been exposed to 5000 ppm than in the animals exposed to 100 ppm?

DR. WEINSTEIN: Again we're looking at the animals at different time intervals because the 5000 ppm experiment was compressed into a single week and the lower dose levels were spread out over 70 days so the sequencing is very different. At 5000 ppm we observed a real difference in the appearance of the smooth endoplasmic reticulum. We reported that the membranes looked different, the elements of the smooth endoplasmic reticulum appeared dilated with hypertrophy - clearly they just didn't look the same as our control smooth endoplasmic reticulum membranes. We didn't really see that alteration at 25 or 100 ppm. There may have been other changes. We have been very concerned with quantitation of microscopic data in our laboratory. One of the great problems is that there have to be enormous quantitative changes for one to really appreciate differences in electron microscopy. For example, we estimate that the microsomal membranes have to increase perhaps 25 or 30 percent in surface area before we can really tell the difference just by eye-balling micrographs. I mean it simply isn't that sensitive. We have a feeling that there are real changes in these tissues but until we really apply quantitative microscopic techniques we're not going to be able to document them very well. We do plan to make a very large effort to get into quantitative microscopy and I think we might better answer your type of question once we're doing that, but I think we've probably reached the limit of what we can do by eye-balling micrographs.

DR. ANTHONY THOMAS: I remember that three years ago one of our speakers really took Dr. Weibel to task about morphometry and about how representative a sample can be, especially in the lung. This point is what bothers us all. Most of the electron microscopy findings which you get on your desk say gee whiz look at this, look at that. But what does it mean? How much more is it? How much less is it? Unless we start doing that we are missing probably one of the finest and most sensitive tools in pathology. I think that in the occupational medicine area if we want to draw a fine line between reversible and irreversible injury we will have to start quantitating things in pathology and not just one plus, two plus, three plus, or four plus. That won't do anymore.

DR. SCHEEL: I'd simply like to emphasize that what Dr. Weinstein showed in terms of liver changes is really not that much different than what you can see in starvation or in high fat feeding or other things which constitute what I would call normal physiologic parameters as far as the liver is concerned. But when he goes on to show cell degradation, and a large amount of toxic response, such as an increase in mitosis which is sustained, then we are in a different ball park and I think we have to be very careful about drawing our lines on reversible and irreversible lesions and I'm still convinced that pathology is probably the best tool.

DR. WEINSTEIN: I'm delighted to hear you say that. Perhaps it represents some job security for many pathologists.

DR. ROWE: I still have one uneasy feeling and it was brought up this morning. I would certainly like to see an analysis on that food after it's been in those chambers say for 6, 12, 18 and 24 hours, just to relieve my worries.

MR. HAUN (SysteMed Corporation): I think we can probably arrange that. I back checked to the publication of last year's proceedings and we didn't do it last year.

DR. ROWE: I know we talked about it last year.

DR. FASSETT: I'd like to go back to the cardiac arrhythmias for a moment to Dr. Hays and Maj. Van Stee. Years ago when I was a young pharmacologist, I had the pleasure of working on this problem myself in developing methoxamine. My question is which comes first, the chicken or the egg. There is no doubt but that potassium/sodium ratios are terribly important and it seems like the contractile forces are also very important. But which is the chicken, and which is the egg in this case? The other factor that I didn't hear any discussion of is coronary flow. The coronary flow, as you all know, is during diastole so the type of contraction that one gets has to do with the amount of flow and of course the diastolic pressure has a lot to do with this. So I wonder if the solvent itself is affecting the flow. I don't know whether anybody has investigated that at all.

DR. HAYS: Well, I can't answer that either, Dave, but let's take class one and class three. We have in the first group compounds that act directly on the myocardium and they produce a very forceful contraction. These are epinephrine, norepinephrine, and isoprel, even though it is a hypotensive agent. We measured the contractile force with isoprel and found an increase. On the other hand, methoxamine acts by peripheral vasoconstriction. It has no direct affect on the myocardium and one would expect then that it would not produce arrhythmias, and indeed that turned out to be the case. But all those compounds that have increased pressure by virtue of peripheral resistance do not produce cardiac arrhythmias. Now with regard to the coronary, there are beta receptors in the coronary and if you give any one of the beta adrenergic blocking agents you get a very excellent protection from all of the compounds that produce arrhythmias if you pretreat first with the blocking agent. You don't get this if you use the alpha adrenergic blocking agents such as dibenamine. You get some

protection in some animals but mostly you get very little protection so I don't know at this point what part the coronary plays in the production of arrhythmias. It may well have some effect.

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PAPER NO. 15

CARDIAC FUNCTION IN MICE FOLLOWING EXPOSURE TO HALOALKANE PROPELLANTS ALONE AND IN COMBINATION WITH BRONCHODILATORS

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INTRODUCTION

In recent years, there has been considerable concern over the incidence of sudden death following the inhalation of volatile hydrocarbons (Bass, 1970). Along with the sniffing of glue, paint thinner and volatile solvents, the inhalation of aerosol products in an attempt to get "high" has become widespread. There has also been, during the past decade, an increase in the incidence of sudden death in patients suffering from asthma, particularly in Great Britain. The rise in the number of such cases has been associated with the introduction and wide use of aerosol bronchodilator preparations. It has been proposed that the deaths resulted from cardio-toxic effects of these preparations (Taylor and Harris, 1970). It has also been suggested that the propellants used in aerosol products are toxic to the heart and that their inhalation might lead to cardiac arrhythmias and arrest which could account for the sudden deaths reported in both asthmatics and aerosol "sniffers." Taylor and Harris (1970) have reported that inhalation of haloalkane propellants sensitizes the hearts of mice to asphyxia induced sinus bradycardia and A-V block. The purpose of the present investigation was to duplicate the technique of Taylor and Harris in an attempt to provide additional information to be used in answering the question of possible cardiotoxicity of the haloalkane propellants.

METHODS

Seventy-two male mice of the ICR strain weighing from 35-45 g (39. 4 ± 0. 4, mean ± S. E.) were anesthetized with sodium pentobarbital, 50 mg/kg i. p. Needle electrodes were subcutaneously inserted into the four extremities for ECG recording (usually lead II) with a Grass polygraph. After a 55 second control period, the mice were exposed for five seconds to a haloalkane propellant, with or without a bronchodilator agent, or of nitrogen. The propellant was administered by placing the mouthpiece of the nebulizer (pocket-type dispenser) over the snout of the animal, discharging it once, and removing it after the 5 second exposure. Nitrogen gas was delivered for 5 seconds at a slight positive pressure into a small mask that covered the snout of the animal.

Immediately after withdrawing the nebulizer or nitrogen mask, the animal was asphyxiated by covering the snout with a form-fitting plastic bag (Taylor and Harris, 1970). Control animals were exposed to the nebulizer in an identical manner prior to asphyxiation, but the nebulizer was not discharged.

It was found that under these conditions the control animals failed to survive for two minutes, whereas Taylor and Harris (1970) reported that control animals treated in what appeared to be a similar manner survived for four minutes, without signs of marked sinus bradycardia or A-V block. We, therefore, treated another group of animals (control, propellant alone, or nitrogen) by exposing as before but the plastic bag covering the animal's snout was fastened somewhat less securely and permitted the passage of a limited amount of air. This condition we called partial asphyxia.

In some of the experiments 30 seconds elapsed between the exposure to propellant (alone) or nitrogen and the initiation of asphyxia.

The results were treated by analysis of covariance. Duncan's Multiple Range Test was then used to compare the groups. Comparisons to control or to nitrogen are the only ones of any realistic value and thus only significant differences in these comparisons are reported.

The aerosol propellants were graciously supplied in pocket-type plastic nebulizers by the Schering Corporation, Bloomfield, New Jersey. Most of the dispensers delivered 70-77 mg of propellant per activation containing 28% trichloromonofluoromethane (Freon 11) and 72% dichlorodifluoromethane (Freon 12). In addition to the placebo dispensers (propellant alone), some of the dispensers delivered 100 μg/activation of isoproterenol or 100 μg/activation of salbutamol (albuterol) [2-t-butylamino-1(4-hydroxy-3-hydroxymethyl)-phenylethanol], a non-catechol adrenergic β-stimulant bronchodilator (Brittain et al., 1968). In some of the experiments, a commercially available isoproterenol nebulizer was used (Vapo-N-Iso Metermatic®, USV Pharmaceutic Corporation, 70 μg isoproterenol/activation) (denoted in the text, figures and tables as isoproterenol #2).

RESULTS

Figures 1 and 2 show the heart rates (adjusted by analysis of covariance, the covariate being initial heart rate) of mice recorded at 0.1 minute intervals during the period of asphyxia. In figure 1, heart rates of control mice are compared to animals exposed to nitrogen, haloalkane propellant or commercial isoproterenol preparation #2. A decline in heart rate was evident almost immediately in all mice except those receiving isoproterenol in which heart rate remained at control levels for one-half minute. Beyond this point the heart rate declined in all groups to a range of 130-230 at the end of the first minute, or less than 50% of control values for all groups. Heart rate continued to decline more slowly during the second minute of asphyxia. The data summarized in figure 2 permit a comparison of control mice and those exposed to nitrogen to animals inhaling haloalkane propellants with isoproterenol or salbutamol. Changes in heart rate followed the same general pattern, although the degree of bradycardia was slightly less in the mice exposed to salbutamol. Treatment of the data summarized in figures 1 and 2 by analysis of covariance at each 0.1 minute interval revealed no significant differences in heart rate.

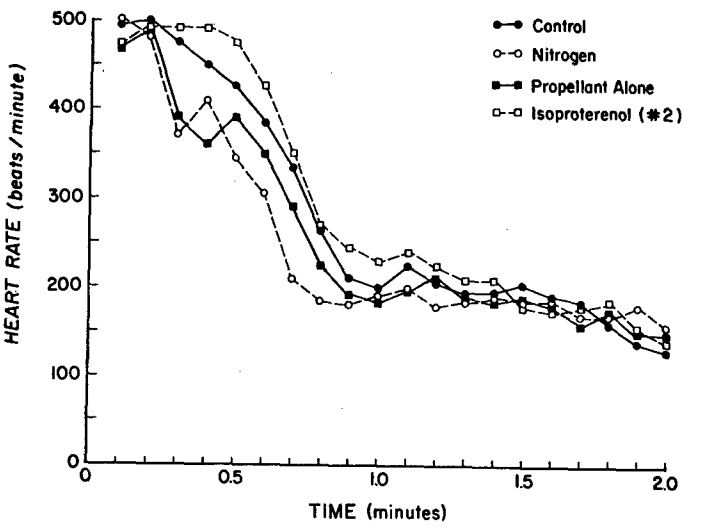

Figure 1. HEART RATES DURING ASPHYXIA OF CONTROL MICE AND ANIMALS EXPOSED TO NITROGEN, HALOALKANE PROPELLANT ALONE OR PROPELLANT WITH ISOPROTERENOL #2.

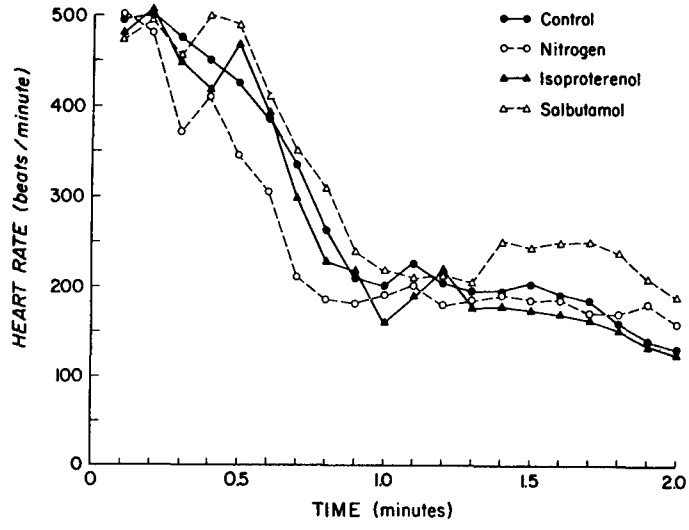

Figure 2. HEART RATES DURING ASPHYXIA OF CONTROL MICE AND ANIMALS EXPOSED TO NITROGEN, HALOALKANE PROPELLANT WITH ISOPROTERENOL OR PROPELLANT WITH SALBUTAMOL.

The mean number of instances of 2:1 A-V block occurring during the last 1.5 minutes of asphyxia in each group is summarized in table I. The means ranged from 1.16 for salbutamol to 5.55 for nitrogen. In comparing all other means to control, the nitrogen group was significantly higher and the salbutamol group significantly lower ($P < 0.01$). The other groups were not significantly different from control (asphyxia group). A comparison was also made of all other means to the mean for the nitrogen group and all were found to be significantly lower ($P < 0.01$).

TABLE I
2:1 A-V Events Occurring During the Last 1.5 Minutes of Asphyxiation

	X[1]	S.E.
Asphyxia Alone Control	2.86	±0.20
Nitrogen	5.55	±0.29[2]
Propellant Alone	3.45	±0.52
Isoproterenol	3.42	±0.42
Isoproterenol #2	2.69	±0.53
Salbutamol	1.16	±0.47[3]

[1]Mean number of ventricular omissions per minute.
[2]Significantly greater than all other means ($P < .01$).
[3]Significantly less than all other means ($P < .01$).

The results are also expressed in table II according to the format used by Taylor and Harris (1970). As in our table, they (Taylor and Harris) recorded the time to onset of "either 2:1 A-V block or life-threatening sinoatrial (SA) bradycardia." We found this definition somewhat unsatisfactory since occasionally single QRS omissions were noted quite early, even during the control period (before asphyxia). Thus, the onset of 2:1 A-V block was arbitrarily taken as the occurrence of at least five ventricular omissions in a 0.1 minute period. Furthermore, rather than depend on a subjective evaluation of "life-threatening," a fall in heart rate to 50% of the control value was taken as the onset of "marked" bradycardia. Thus, the figures shown in table II represent the time to either 2:1 A-V block or marked bradycardia (as previously defined) whichever occurred first. Also tabulated is the number of mice exhibiting bradycardia or A-V block first, although all mice eventually developed irreversible bradycardia and some degree of A-V block. Also tabulated for comparison with the data of Taylor and Harris (1970) are the heart rates 24 seconds after the onset of asphyxia expressed as a percent of the control (pre-asphyxia) values.

Neither the time to onset values nor the percent heart rate values showed any significant differences among the various treatments. Also included in table II are the results of the experiments wherein asphyxiation was delayed for thirty seconds after the administration of propellant or nitrogen. There were no significant differences between either of these groups and control.

TABLE II
Cardiac Responses (Mean ± S. E.) of Mice to
Asphyxia, Propellant, Nitrogen and Aerosol Mixtures.
Complete Asphyxiation.

Condition	No. of Mice	% Control Heart Rate at 24 Seconds After Asphyxiation[1]	Event[4] A-V Block[2]	Bradycardia[3]	Time to Onset of Event[1, 5] (Minutes)
Propellant (Alone)	8	71 ± 7	1	7	0. 66±0. 09
Propellant and Isoproterenol	6	89 ± 10	3	3	0. 67±0. 06
Propellant and Isoproterenol #2	6	101 ± 3	3	3	0. 73±0. 06
Propellant and Salbutamol	6	103 ± 10	0	6	0. 93±0. 08
Asphyxia without Propellant	11	94 ± 7	2	9	0. 77±0. 08
30 Second Delay					
Propellant (Alone)	4	88 ± 12	1	3	0. 77±0. 06
Nitrogen	4	70 ± 7	0	4	0. 78±0. 08

[1] These data were subjected to an analysis of variance and statistically significant differences were not found.

[2] A-V block = 2:1 block and at least 5 per 0. 1 min.

[3] 50% decrease from control.

[4] Number of mice exhibiting event.

[5] Event: bradycardia (50% fall from control heart rate) or 2:1 A-V block (at least 5 per 0. 1 min.) whichever occurred first.

In table III, the data from the experiments previously described as "partial asphyxiation" are summarized in the same fashion as in table II. Although the time to onset of bradyarrhythmias was somewhat longer, there were no significant differences among these groups. There were also no significant differences among the groups with respect to heart rate 24 seconds after the onset of asphyxia. As was the case with complete asphyxia, all animals eventually developed irreversible bradycardia and some degree of A-V block.

TABLE III
Cardiac Responses (Mean ± S. E.) of Mice to
Asphyxia, Propellant and Nitrogen.
Partial Asphyxiation.

Condition	No. of Mice	% Control Heart Rate at 24 Seconds After Asphyxiation[1]	Event[4] A-V Block[2]	Bradycardia[3]	Time to Onset of Event[1, 5] (Minutes)
Propellant (Alone)	10	110 ± 6	4	6	1.80±0.14
Nitrogen	5	113 ± 15	1	4	2.50±0.29
Asphyxia (Control)	13	104 ± 5	11	2	2.51±0.20

[1] These data were subjected to an analysis of variance and statistically significant differences were not found.

[2] A-V block = 2:1 block and at least 5 per 0.1 min.

[3] 50% decrease from control.

[4] Number of mice exhibiting event.

[5] Event: bradycardia (50% fall from control heart rate) or 2:1 A-V block (at least 5 per 0.1 min.) whichever occurred first.

DISCUSSION

The results of the present study clearly do not support the contention that inhalation of haloalkane propellants under the conditions employed by Taylor and Harris (1970) makes cardiac activity in mice more sensitive to the effects of asphyxia. Others have questioned the conclusions of the Taylor and Harris study. Silverglade (1971)* has suggested that the mice died of asphyxia without the aid of a propellant, with the tightness of the head mask being a critical factor in the development of hypoxia and decreased arterial oxygen tension. Azar et al. (1971)* point out that a mouse whose head is enclosed in a tightly fitting plastic bag will be asphyxiated whether exposed to propellant or air and furthermore that animals preexposed to the inhalation of any vapor of low oxygen content will be hypoxic to some degree before having a plastic bag placed over head. Therefore, such animals may indeed reach a critical level of hypoxia before animals preexposed to air alone. These authors further state that if Taylor and Harris had preexposed animals to an inert gas such as nitrogen they would have observed the same effects they describe in mice preexposed to propellant and which they concluded to be cardiotoxic effects of the propellant. Azar et al. (1971)* performed experiments of this type in anesthetized mice, including preexposure to nitrogen, and these results did not indicate any specific cardiotoxicity of propellant compounds in the concentrations employed.

*Letters to the editor, J. Amer. Med. Asso., 215:1501-1503, 1971.

In the present study, a marked bradycardia developed in all groups of mice within the first minute of asphyxia and remained until the end of the asphyxiation period. There were no significant differences between any of the groups in the development and pattern of the bradycardia. The heart rate did remain near control levels slightly longer in the mice inhaling isoproterenol. However, differences in heart rate with isoproterenol were not significant.

The summary of the mean number of instances of 2:1 A-V blockade do not give any indication that prior exposure to haloalkane propellants enhances the induction of such events when asphyxia is produced. However, prior preexposure to the inert gas nitrogen resulted in a significantly higher number of A-V events during the period of asphyxiation. Apparently exposure to nitrogen hastened the development of A-V block by depriving the mice of small amounts of oxygen that would otherwise have been available as asphyxia began. It is interesting to note that the development of A-V blockade in the mice receiving salbutamol along with a propellant was significantly less than in the control group. Salbutamol has been shown to have bronchodilator activity comparable to isoproterenol with a longer duration of action (Brittain et al., 1968). It has also been reported that it does not increase cardiac output or heart rate, as isoproterenol does, nor does it reduce PaO_2 levels when airway obstruction is relieved (Palmer and Diament, 1969; Kelman et al., 1969). The explanation of any apparent protective effect of salbutamol under the conditions of the present study obviously requires further investigation.

The question of possible cardiotoxic effects of aerosol products leading to sudden death and resulting from haloalkane propellants appears to be an unresolved controversy at this time. Inhalation of such compounds can sensitize the heart to catecholamines leading to serious cardiac arrhythmias (Reinhardt, 1971). However, relatively high concentrations may be required. In addition, the pharmacological actions of bronchodilators and asphyxia are two additional factors that must be considered in explaining sudden deaths in asthmatics or those "sniffing" aerosol products.

It is felt that the results of the present study provide additional evidence that the conclusions of the Taylor and Harris investigation, based on the techniques employed, constitute an unwarranted indictment of haloalkane propellants.

ACKNOWLEDGEMENTS

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PAPER NO. 16

SENSITIZATION OF THE HEART TO CATECHOLAMINE - INDUCED ARRHYTHMIA

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This work was undertaken as a part of a cooperative effort between the Institute of Experimental Pathology and Toxicology of Albany Medical College and the Manned Space Flight Center of NASA, to find a fire-suppressant, or at least a fire-retardant, that can be present within the atmosphere of a space vehicle without significant hazard to the occupants. A group of fluorinated halocarbon compounds was selected for further study on the basis of the existing information on their antipyral and toxic activities.

The specific task of my group was to determine the effects of candidate chemicals on the cardiovascular system. In view of previous work showing that such halogenated compounds as chloroform (Levy and Lewis, 1911; Brow et al., 1930; Morris et al., 1953; Raventos, 1956), halothane (Raventos, 1956; Hall and Norris, 1958; Katz et al., 1962; Flacke and Alper, 1962), methoxyflurane (Bamforth et al., 1961), and other fluorinated halocarbons (Taylor and Harris, 1970; Reinhardt et al., 1971) are capable of sensitizing the heart to catecholamines, study of the effects of the candidate compounds on the response of the heart to infused catecholamines seemed particularly pertinent.

Methods

Two sorts of studies with guinea pigs, cats, and dogs anesthetised with Na pentobarbital were performed. In both, the animals have been made to breathe a mixture of a halocarbon compound and air, made up in a calibrated spirometer, through an intake-output valve system attached to a cannula inserted into the trachea just below the larynx. In one type of experiment, represented schematically in figure 1, epinephrine was infused into the femoral vein of one hind leg at a rate of about 3.5 μg/kg/min during periods of two minutes before and after exposure to a halocarbon compound during 15 minutes, inhalation of the halocarbon continuing during the second infusion. The second type of experiment was similar except that the infusions of epinephrine were replaced by intravenous injections into the femoral vein of one hind limb of 5, 10, or 15 μg/kg of epinephrine before and at various times after inception of exposure to a halocarbon.

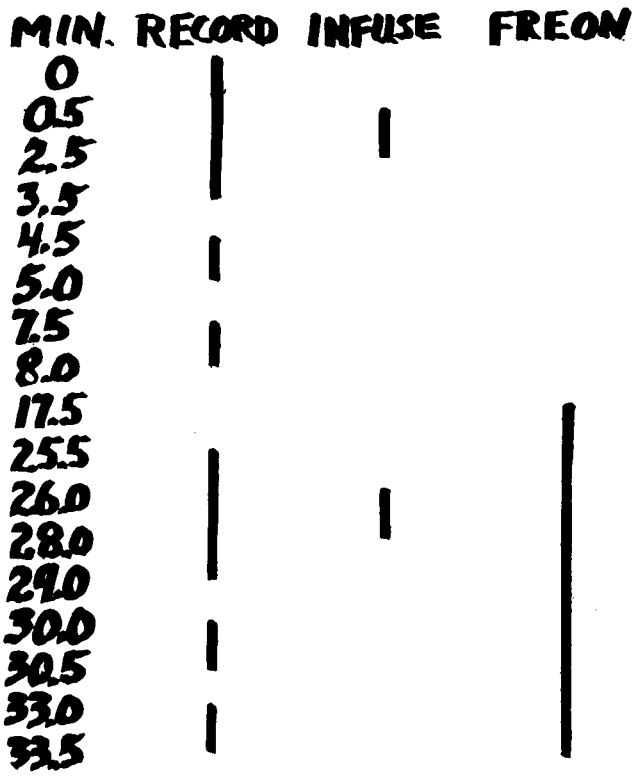

Figure 1. SCHEMATIC REPRESENTATION OF THE SCHEDULE FOR A TYPICAL EXPERIMENT WITH TWO INFUSIONS OF EPINEPHRINE, INDICATING THE TIMES AT WHICH RECORDINGS OF THE ECG (IN SOME CASES OF BLOOD PRESSURE ALSO), INFUSIONS OF EPINEPHRINE, AND INHALATION OF VAPORS OF A HALOCARBON COMPOUND DILUTED IN AIR WERE PERFORMED.

The halocarbon compounds studied were: I = Freon® 11 = trichloro fluoromethane, II = Halon® 1301 = trifluorobromoethane, III = Halon® 2402 = 1, 2-dibromotetrafluoroethane, IV = Freon® 116 = 1, 2-hexafluoroethane, and V = Freon® C-318 = cyclic octafluorobutane. Some studies have been made also with Freon® 12 = difluorodichloromethane. Halocarbon-air mixtures have been analyzed by gas-liquid chromatography.

Calcium and potassium have been estimated by flame photometry. Norepinephrine has been estimated by the method of Lund (1950), using extraction with perchloric acid, adsorption on alumina, and oxidation with potassium ferricyanide instead of manganese dioxide.

Magnesium chloride, calcium chloride, and MK-486 [L-2-hydrazino-2-methyl-3-(3, 4-dihydroxyphenyl) propionic acid] have been injected or infused intravenously. The MK-486 was provided kindly by Alexander Scriabine, M. D., of Merck Sharp and Dohme.

Results

Figure 2 shows the responses of a dog to duplicate infusions of epinephrine before and after a 15-minute period of inhalation of a 0.87% (v/v) concentration of Freon® 11 in air, the times indicated beneath the various sections of record being the times elapsed since the start of the infusion. The upper record represents limb lead I in each pane and the lower record limb lead II. The only effect of the infusion in the unexposed animal (left) was some slowing of the heart's rate, whereas in the same animal after exposure to the Freon® compound the duplicate infusion of epinephrine produced initially a more marked slowing of the rate and then the appearance of ectopically generated ventricular contractions and fairly marked arrhythmia. The exposure to the halocarbon compound of itself had little effect on the ECG recordings.

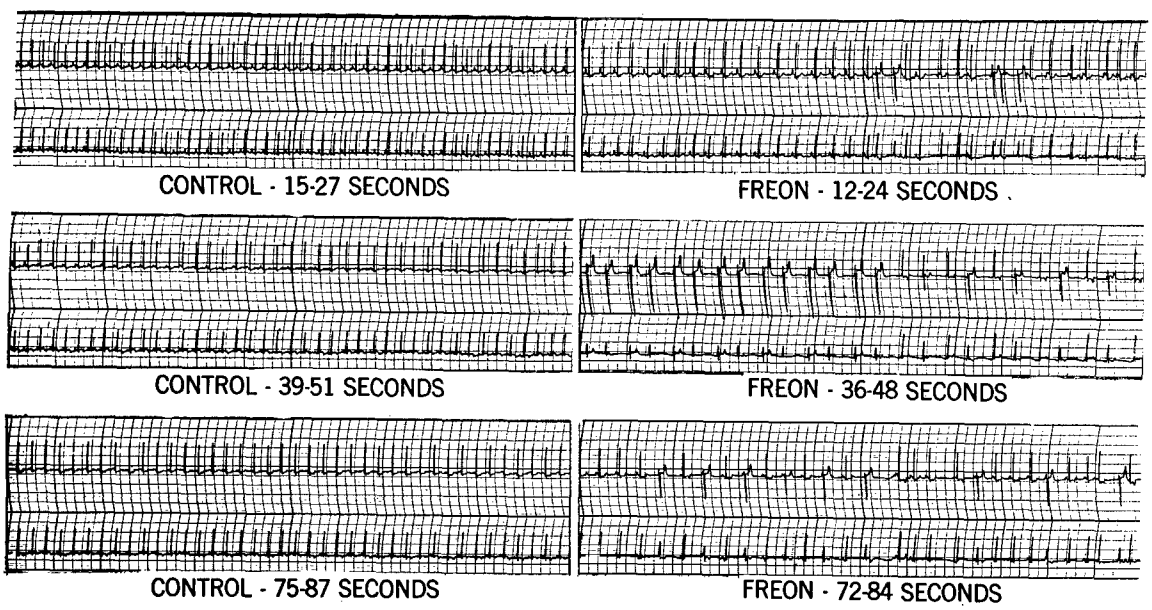

Figure 2. ECG RECORDS MADE AT VARIOUS TIMES AFTER THE BEGINNINGS OF INFUSIONS OF EPINEPHRINE BEFORE (LEFT) AND AFTER (RIGHT) EXPOSURE OF THE ANIMAL TO A VAPORIZED HALOCARBON COMPOUND BY INHALATION.

When the various halocarbon compounds were compared in the type of experiment illustrated above, the results set forth in table I were obtained. The experiments with II and IV in the guinea pig show that although the sensitizing effect of exposure to a halocarbon compound is dose related, the relationship is far from a proportional one; quadrupling the concentration of halocarbon produced only a little more than an eleven percent increase in the number of animals showing sensitization to the infusion of epinephrine, on the average between compounds II and IV. In the case of compound IV, this change in the concentration of the halocarbon compound produced less than a 35 percent increase in the number of animals showing sensitization. This table shows also that the dog and the guinea pig are not entirely identical in their responses to the various halocarbon compounds. Freon® 11 (I) is clearly the most potent of the five chemicals within this table. It is probably followed in order of decreasing effectiveness as a sensitizer to catecholamines by V, II and IV, and III, although the choice among the last four compounds on this basis is not clear.

Table I

Effect of Inhalation of Halocarbon Compounds on Arrhythmia Induced by Infusion of Epinephrine

Compound	Concentration in Air (v/v)	Arrhythmia Increased Guinea Pig	Dog
I	0.87%	6/6	
II	2.2	4/10	3/4
	8.7	2/6	
III	1.8	3/10	1/4
IV	2.2	5/10	2/4
	8.7	2/3	
	33.8	2/2	
V	2.2	7/11	1/4

In one cat, exposure to Freon® 11 in a concentration of 1.7% produced arrhythmia without infusion of epinephrine. In a similar experiment in which 8.8% Freon® 12 was administered, spontaneous arrhythmia appeared also. Two cats responded to this concentration of Freon® 12 in this way. These three experiments are the only ones in which arrhythmia has appeared without infusion of epinephrine, although much higher concentrations of some halocarbon compounds have been used [for example, 44% (v/v) of Freon® 116 (IV)]. A comparison of Freons® 12 and 116 (IV) and Halon® 1301 (II) in concentrations of 2.6% (v/v) in the inspired air disclosed that Freon® 12 sensitized more than II to the hypertensive and pulse-pressure elevating effects of infused epinephrine, but less to its chronotropic action. Freon® 12 sensitized less than IV to the hypertensive, pulse-pressure elevating, and chronotropic actions of the infusion of epinephrine. Freon® 12 under these conditions induced arrhythmia during infusion of epinephrine in 1/6 cats whereas the other two halocarbon compounds produced arrhythmia in 0/6 animals.

In experiments in which discrete doses of epinephrine were injected intravenously at various times after beginning exposure to 0.87% (v/v) I, the maximum arrhythmia was found to occur at around 10 minutes of exposure to this most potent of the halocarbon compounds studied; thereafter, despite continued exposure to I, the response decreased. When the exposure to I was terminated, the enhanced responsiveness to epinephrine disappeared rapidly (table II, +5 min. = 5 minutes after termination of the exposure at 15 minutes after its start).

Table II

RESPONSES TO I.V. DOSES OF EPINEPHRINE AT VARIOUS TIMES (MINUTES) AFTER BEGINNING EXPOSURE TO 0.87% (V/V) I

DOSE OF EPINEPHRINE	RESPONSE*				
(μg/kg)	0 MIN.	5 MIN.	10 MIN.	14 MIN.	+5 MIN.
5	4	29	31	21	4
10	30	42	88	28	21
15	73	111	153	123	33

* NUMBER OF ARRHYTHMIC HEART BEATS AFTER EPINEPHRINE.

Because of the well-known interaction of anoxia and epinephrine in the genesis of arrhythmia (see, for example McNamara et al., 1952), a series of animals was given a standard concentration of I (0.87%) made up in air enriched by either nitrogen or oxygen. Sensitization to injected epinephrine was enhanced by low oxygen and decreased, but not abolished, by high oxygen. Section of the vagi, vagal activity being known to enhance the effect of anoxia (McNamara et al., 1952), also reduced, but did not remove entirely, the sensitizing action of I on the chronotropic effect of epinephrine (table III).

Table III

EFFECT OF VAGOTOMY ON THE NUMBER OF ARRHYTHMIC HEART BEATS INDUCED BY I.V. EPINEPHRINE AT VARIOUS TIMES DURING EXPOSURE TO 0.87% (V/V) I

DOSE OF EPINEPHRINE	CONTROL				VAGOTOMISED			
	0 MIN.	5 MIN.	10 MIN.	14 MIN.	0 MIN.	5 MIN.	10 MIN.	14 MIN.
5 µg/kg	2	11	8	0	8	11	7	2
10	60	28	72	0	60	17	6	5
15	116	116	144	104	116	21	9	7

In attempting to identify the change in the heart elicited by exposure to a halocarbon compound, we have used Freon® 11 (I) as the most potent representative of its general chemical type. Whether it is a typical representative remains to be seen, although we have some indication that it is not too different from the others in its qualitative actions.

One obvious factor in sensitization of the heart to extraneous catecholamines is the intrinsic concentration of norepinephrine. To study the effect of exposure to halocarbon compounds on this possible variable, we exposed anesthetised guinea pigs to I and IV by inhalation through a tracheal cannula during a period of 15 minutes. At the end of that time, the animals were killed quickly by intravenous injection of an overdose of Na pentobarbital; the hearts were excised rapidly and put immediately into previously cooled beakers held in an ice bucket. The hearts were homogenized in an all-glass homogenizer and analyzed for norepinephrine. Control animals were treated similarly except that they inhaled normal room air. The results are given in table IV, where each mean represents a group of 6 guinea pigs. Although the concentrations of the two halocarbon compounds used were both known from previous work to be capable of sensitizing the heart of the guinea pig to infused epinephrine, the hearts from the animals exposed to the halocarbon materials did not differ significantly in catecholamine concentration from those from the control animals.

Table IV

Effect of Inhalation of Freon in Air on the Concentration (μg/gm) of Norepinephrine within the Heart of the Guinea Pig

Freon	Concentration	Control	Experimental
116	5.0%	2.29 ± 1.04	2.19 ± 0.81
11	0.4%	1.60 ± 0.36	1.89 ± 0.60

To study the possibility that dopamine contributes to the sensitization of the myocardium by halocarbon compounds, cats were given i.v. doses of 25 mg/kg of MK-486 five minutes before the beginning of exposure to I. This treatment did not modify the response of the heart to infusion of epinephrine at the end of the 15-minute exposure. This result is in sharp contradistinction to those obtained with MK-486 in arrhythmia induced by the administration of DOPA (Mars and Krall, 1971).

Another possible factor in the sensitizing action of the halocarbon compounds is alteration of the relation of the heart to potassium. We have determined that our standard 15-minute exposure to I (in a v/v concentration in air of 0.4%) does not alter significantly the concentration of potassium within blood plasma (table V). We are in the process of determining the effect of this treatment on the concentration of potassium within the heart muscle, but do not have final figures yet.

Table V

Effect of Inhalation of Freon® 11 in Air on the Concentration (meq/liter) of Potassium in Plasma of the Cat

	Control	After Freon
	4.1	4.1
	6.4	4.8
	6.2	4.6
	4.2	4.3
	3.7	3.8
	6.6	5.2
	4.6	4.0
Mean	5.1 ± 1.2	4.4 ± 0.5

We have examined also the effect of i.v. injection of $MgSO_4$ (6 ml/kg of 3.3%) on sensitization of the heart to epinephrine by I, with the result that this dose of $MgSO_4$ did not alter the sensitization. This finding suggests that the halocarbons, unlike digitalis glycosides (Langer and Serena, 1970; Seller, 1971), do not facilitate arrhythmogenic activity through shifting intracellular potassium into the plasma. If the analyses of heart muscle for potassium corroborate this tentative conclusion, we think that we will have good evidence that alteration of the permeability of the myocardial membrane to potassium is not a factor in sensitization of the heart to catecholamines by halocarbon compounds.

We have just begun to study the possibility that the relationship of the myocardial membrane to calcium may be modified by exposure to a halocarbon compound. About all that I am in a position to say with fair certainty at present is that by infusing $CaCl_2$ into cats at a rate of 5 mg/kg/min we have been able to obtain about as great sensitization of the heart to infused epinephrine as we can obtain by the standard exposure to I. A different sort of experiment will be undertaken soon to attempt to determine more directly whether calcium is involved in sensitization by halocarbon compounds, possibly by enhancing release of catecholamines from secretory granules (Greenberg and Kolen, 1966).

In another connection, we have been interested in a compound that has both alpha- and beta-adrenergic blocking abilities, the beta-blocking activity being somewhat the greater, as well as an anticholesterolemic action. This compound has been found to prevent the induction of arrythmia by infusion of epinephrine after exposure to halocarbon compounds. Both phenoxybenzamine and propranolol also have this ability. It is apparent, therefore, that both alpha- and beta-receptors may be involved in the sensitization of the heart by halocarbon compounds to the arrhythmogenic action of extraneous epinephrine.

Moe et al. (1948) showed clearly the involvement of the peripheral alpha-receptors of the cardiovascular system in the induction of idioventricular rhythms by epinephrine administered during cyclopropane anesthesia. Kunkel et al. (1951) found that veratramine, which did not alter the pressor actions of epinephrine but did reduce its cardioaccelerator effect, was capable of preventing ventricular fibrillation induced by epinephrine in cats that had been exposed to vapors of benzene. It is possible, therefore, that effects on both inotropic and chronotropic properties of the myocradium may be involved in such phenomena as the sensitization by halocarbon compounds that we are studying. Depression of myocardial contractility without significant beta-adrenergic blocking action actually is capable of mitigating the arrhythmogenic action of daunomycin (Burka et al., 1970).

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PAPER NO. 17

EFFECT OF FREONS® ON ACETYLCHOLINESTERASE ACTIVITY AND SOME COUNTER MEASURES

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and

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The classical studies of halocarbons by Levy (1911; 1913-14) and Levy and Lewis (1911-12) have recognized that many hydrocarbons, either unsubstituted or halogenated, can sensitize the heart to sympathetic nervous stimulation or to exogenous epinephrine at ordinarily nonhazardous dose levels.

Freon 12, a well known aerosol propellant, and Freon 13, a well known fire extinguishing agent, have aroused the general public alertness because of their hazardous nature. From both chronic and acute point of view, Freon 12 and Freon 1301 should be handled with caution, as there is considerable evidence that these two gases interfere with the enzyme activity at the postsynaptic junctions. The Freon molecule, per se, may interfere with the ionic transportation and thereby impairs the neuroregulatory mechanism of the cardiac performance. Van Stee and Back (1969) have demonstrated some cardiac irregularities and Carter et al. (1970) have shown decrement in performance in Freon - treated animals. It is well known that chloroform is likely to develop irregularities ranging from extrasystole to ventricular fibrillation which can be prevented or abolished by section of the cardiac sympathetic nerves and provoked by stimulation of these nerves or by injection of epinephrine (Brown et al., 1930). Most of the Freons have similar properties to chloroform because of their similarities in structure.

Bass (1970) recently reported on 110 cases of sudden death in youths inhaling various vapors, and it has been suggested by Taylor and Harris (1970) that cardiac arrhythmias might be responsible, particularly if asphyxia, due to respiratory arrest and the resultant hypoxemia and hypercapnia, released endogenous catecholamine.

NOTE: Freon® is a DuPont trade name.

Freons have been widely used as aerosol propellants and fire-retarding agents and there are frequent reports of accidents concerning Freons. It seems important to investigate the effect of some of the Freons commonly used in the laboratory; it may shed light on the basic mechanism of how Freon behaves in the biological system. In view of the fact that Freons will be around for some time, it appears appropriate to develop some counter measures whenever possible. The present study is, therefore, directed specifically to the effect of Freons on cardiac performance and its modification of the neuroconducting system in the heart, and to seek counter agents to relieve the sensitization effect.

MATERIALS AND METHODS

Rana pipiens were used for this study. The vagal heart preparation and the perfusion techniques were essentially those previously described (Young, 1969). The nerve was stimulated with a pair of platinum electrodes via a Grass S-8B stimulator. The heart was prepared by triple cannulation to allow for introduction of the substances under investigation, e.g., ACh, physostigmine, etc. The pulsating heart exerts a force on the Statham transducer which, in turn, transmits a signal to a Massa recorder. Quantitative measurements of force of cardiac contraction were possible by transmission of the transducer signal to an integrating digital voltmeter. The product of the peak digital voltmeter reading and the actual displacement reading on the recorder gives a value representing the energy, or work done, of the cardiac contraction (figure 1).

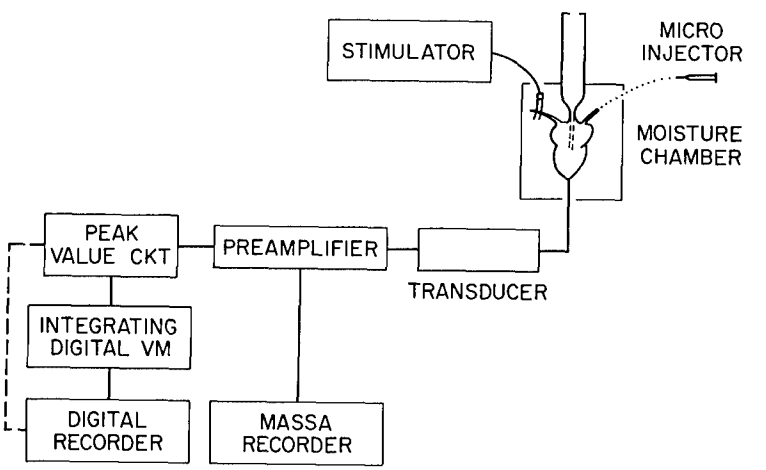

Figure 1. A BLOCK DIAGRAM OF THE VAGAL HEART EXPERIMENT WITH THE NECESSARY RECORDING EQUIPMENT.

Freons were obtained from Matheson Company. The Ringer solution was saturated with the specific Freon in question. The concentration of Freon in the solution was determined by its solubility. An aliquot of the Freon -saturated Ringer was introduced to the preparation. The effect was quantitatively estimated by area increment and decrement for monoamine oxidase and acetylcholinesterase activity respectively.

RESULTS

Effects of Freons to Sympathetic Stimulation and Exogenous Epinephrine Challenge

1. Cardiac sensitization of Freon 12 to sympathetic stimulation.

When the sympathetic nerve is stimulated, there is a typical response in the sympathetic heart system as shown in figure 2. The effect of Freon 12 on sympathetic stimulation is also shown in figure 2. There is a definite disturbance in the response to sympathetic stimulation after Freon 12 , as shown by the arrow. The possible reason for such a response will be explained in the mechanism section.

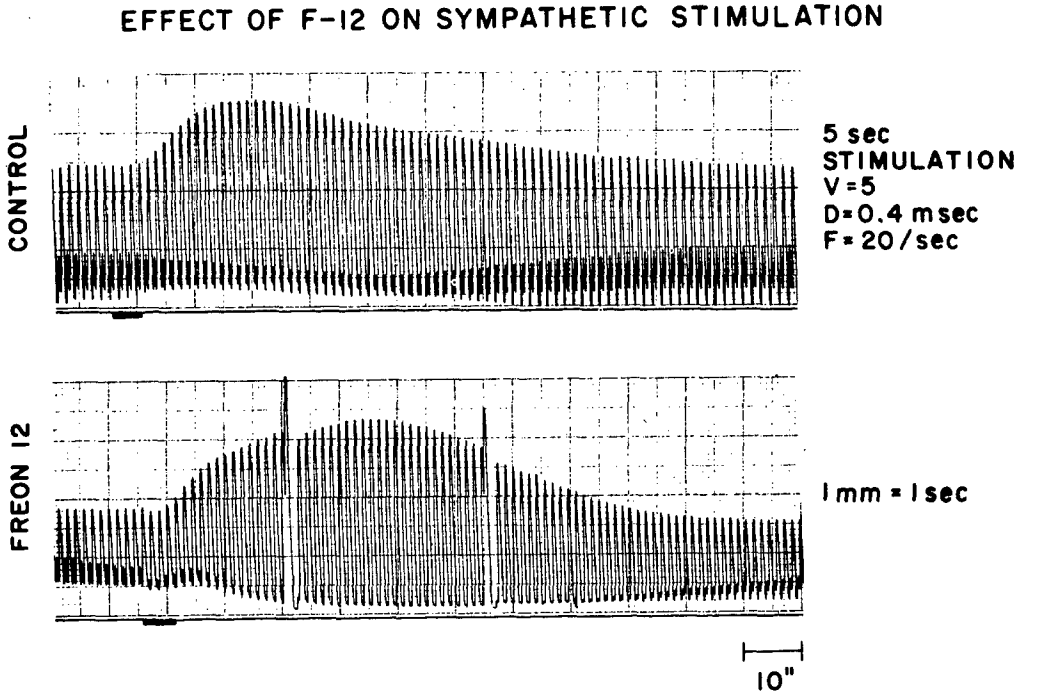

Figure 2. EFFECT OF FREON 12 ON CARDIAC SENSITIZATION TO SYMPATHETIC STIMULATION.

2. Cardiac sensitization of Freon 12 to exogenous epinephrine challenge.

Figure 3 shows the effect of Freon 12 on cardiac sensitization to epinephrine injection. Figure 3(a) shows the typical response of a perfused heart to addition of 1 x 10^{-7} gram of epinephrine. After introduction of Freon 12 [figure 3(b)], the contractility of the heart decreases. The rate of the contraction is also decreased. Upon the challenge of epinephrine there is an initial positive inotropic effect, followed by partial A-V block and then complete A-V block, and finally, cardiac arrest [figure 3(c)].

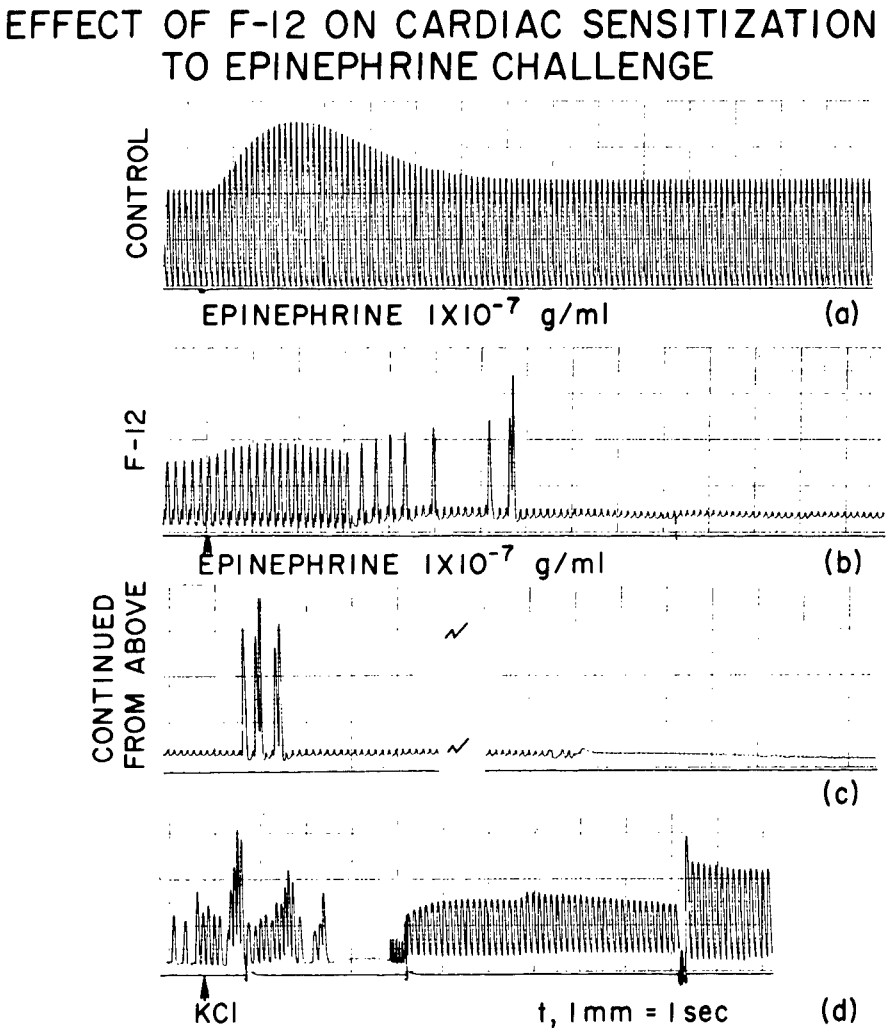

Figure 3. EFFECT OF FREON 12 ON CARDIAC SENSITIZATION TO EXOGENOUS EPINEPHRINE CHALLENGE.
a. Control; b. Post Freon 12; c. Continued from b.; d. Potassium Chloride restores the Cardiac Rhythmic Contraction.

The Effect of Freon 12 on the Velocity of the in situ AChE and Cardiac Contractility

Figure 4 shows the effect of Freon 12 on the AChE activity and contractility. Before addition of Freon 12 the AChE activity and contractility remain remarkably constant. As soon as Freon 12 is introduced into the perfusate, there is immediate rise of enzyme activity by about 6 fold and the contractility decreases by about one-third. The AChE activity remains quite high as long as Freon 12 is in the system. After Freon 12 is removed from the system, the contracting force of the heart recovers almost immediately, whereas the AChE activity is higher than normal even two hours after removal of Freon 12.

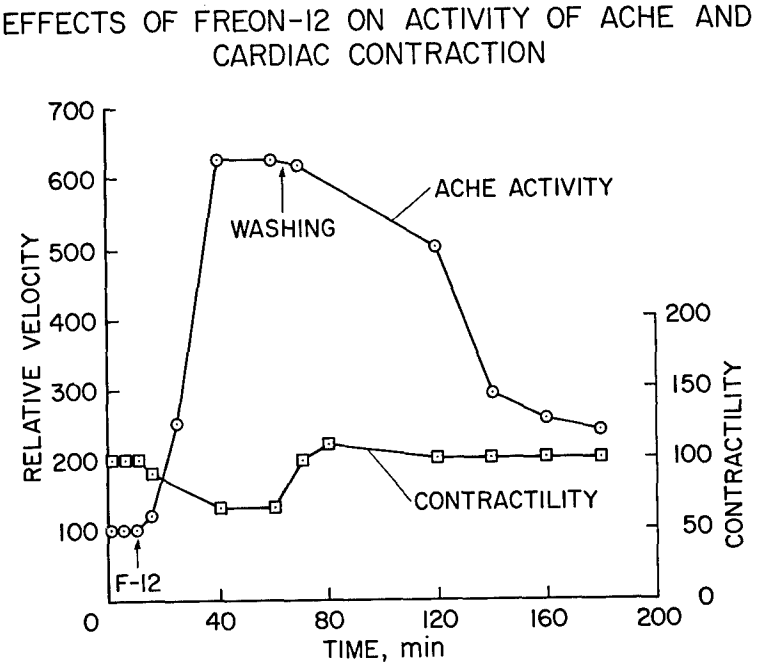

Figure 4. EFFECT OF FREON 12 ON THE ACTIVITY OF AChE AND CARDIAC CONTRACTION.

ED_{50} of Various Freons on the AChE Activity

Table I shows a comparison of various Freons studied in the vagal heart system. It also compares the published values of the corresponding Freons. The values of ED_{50} represent the relative toxicity of each individual Freon. The lower the value the higher the toxicity. The ED_{50} values are converted to ppm for comparison of the published values in the 6th column from different sources as indicated in the table. It is interesting to note that our values across the board are lower than the values recommended by the American Conference of

Governmental Industrial Hygienists and those of Haskell Laboratory. Freon 113 was originally recommended as 1, 000 ppm. It was only recently the change was made from 1, 000 ppm to 200 ppm due to some careful reexamination and scrutinizing. This value is quite close to our value 160 ppm.

TABLE I

COMPARATIVE TOXICITY OF FREONS ON AChE IN THE VAGAL HEART SYSTEM AND OTHER PUBLISHED VALUES

FREONS	FORMULA	AChE ACTIVITY	ED_{50}	PPM	TLV* (PPM)	CLASS**
Freon 11	$CFCl_3$	Increase	6×10^{-4}	600	1000	5a
Freon 12	CF_2Cl_2	Increase	3×10^{-5}	30	1000	6
Freon 13	CF_3Cl	Decrease	5.6×10^{-5}	56	1000***	6
Freon 1301	CF_3Br	Decrease	2.4×10^{-4}	240		6
Freon 14	CF_4	Decrease	5×10^{-5}	50	1000***	6
Freon 22	HCF_2Cl	Decrease	1×10^{-3}	1000	1000	4-5
Freon 113	CF_3001_3	Increase	1.6×10^{-4}	160	200	4-5
Freon 114	CF_4001_2	Increase	6.9×10^{-5}	69		6

* TLV, Threshold Limit Value assigned by the American Conference of Governmental Industrial Hygienists, 1968 values.

** Classified according to Underwriters' classification. The higher the value, the lower the toxicity.

***Based on data from Haskell Laboratory

Table II compares the relative potency of Freons as activator and inhibitor of AChE. Freon 11 , Freon 12 , Freon 113 , and Freon 114 are nonspecific activators whereas Freon 13 , Freon 14 , Freon 1301, and Freon 22 are nonspecific inhibitors of AChE in the vagal heart system.

TABLE II

COMPARISON OF RELATIVE TOXICITY OF FREONS ON AChE ACTIVITY

Toxicity of Freons Decreasing in Order

Freon 12	> Freon 114	> Freon 113	> Freon 11	>	Excitatory (increase AChE activity)
Freon 14	> Freon 13	> Freon 1301	> Freon 22	>	Inhibitory (decrease AChE activity

Kinetics Studies of Freons

Kinetically speaking, all the Freons tested so far are nonspecific. Figure 5 shows the double reciprocal plot of the effect of Freon 12. The intercepts at the 1/v axis have different values, V_{max} changes after Freon 12. Both the control and experimental curves meet at one point at the 1/(s) axis indicates strongly that Freon 12 is nonspecific activator. Figure 5 also shows the effect of Freon 13. The V_{max} value after Freon 13 treatment is higher than that of the control. Again the experimental line meets at the same point as that of the control. It also suggests that Freon 13 is a noncompetitive inhibitor.

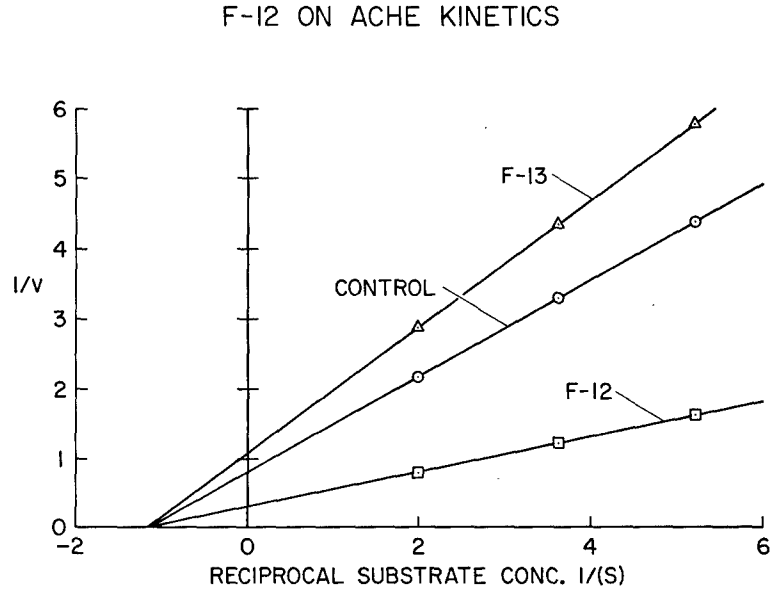

Figure 5. DOUBLE RECIPROCAL PLOT OF VELOCITY AND SUBSTRATE CONC.
Ordinate: Reciprocal of relative velocity.
Abscissa: Reciprocal of substrate concentration.

Counter Measures of Freon Sensitization to Epinephrine Challenge

Arrythmia induced by epinephrine in Freon 12 sensitized heart can be stopped by addition of potassium chloride [figure 3(d)]. This is probably due to the fact that KCl displaces the Freon molecule which is held by the zwitterion of the phospholipid of the synaptic membrane (figure 6). Since the Freon still blocks the glycolysis cycle, the energy source is inadequate, hence KCl can only restore the rhythm but not the normal strength of contraction. Preliminary experiments indicate that Freon 12 molecules are preferentially dissolved in or interact primarily with phosphatidylethanolamine but less with lecithin or sphingomyelin. Figure 7 compares the relative association of Freon 12 with the three major phospholipids. Phosphatidylethanolamine has a stronger zwitterion form than lecithin or sphingomyelin. The latter two phospholipids have choline radicals in the phospho-tail. The physical hindrance of choline radical to the Freon molecule may account for the less affinity of Freon to lecithin and sphingomyelin.

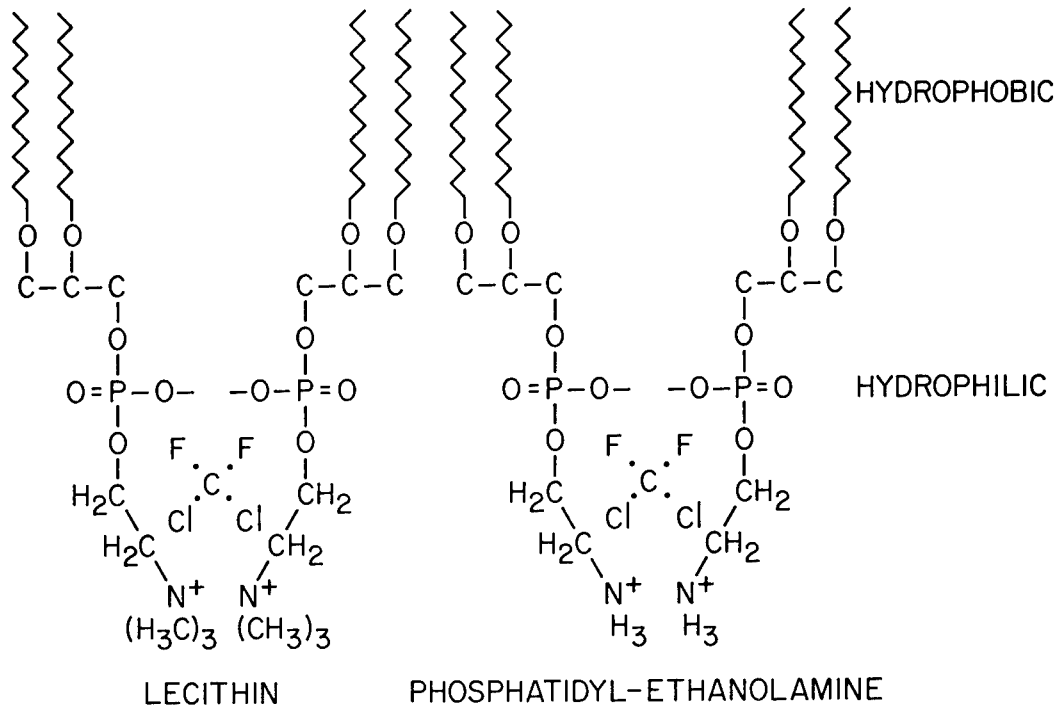

Figure 6. INTERACTION OF FREON MOLECULE WITH PHOSPHOLIPID.

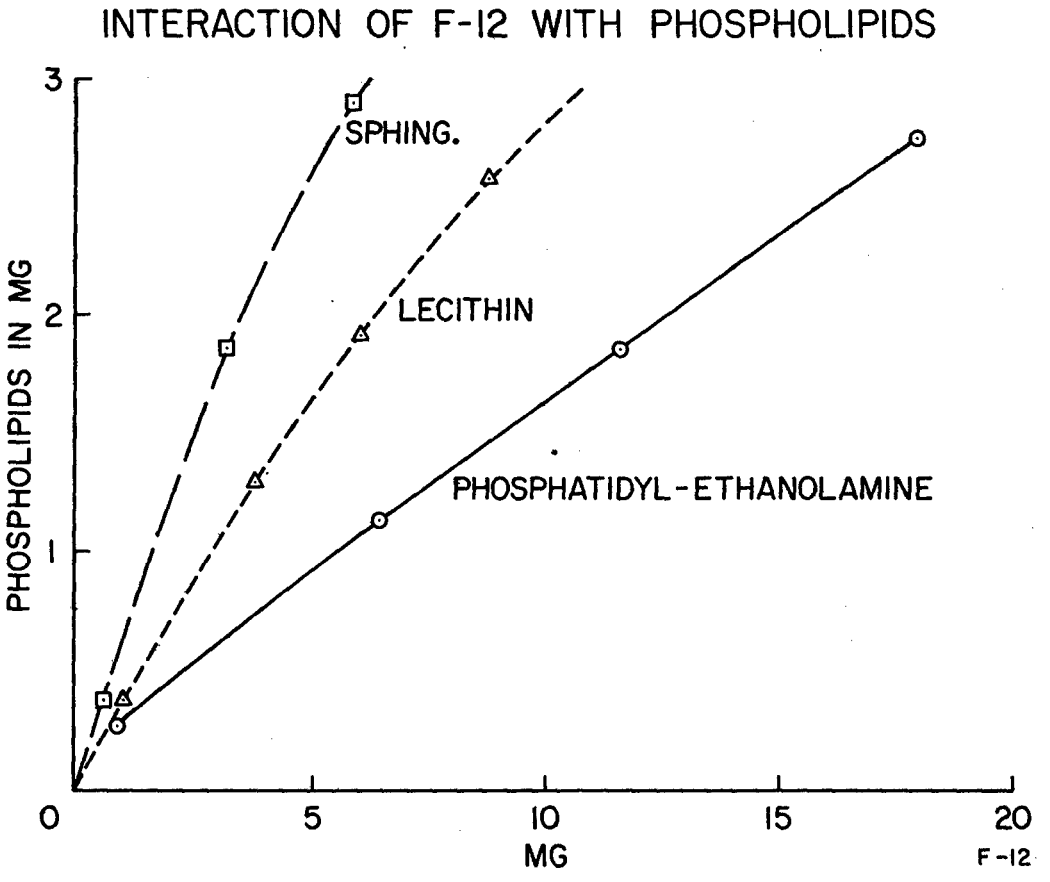

Figure 7. SOLUBILITY OF FREON 12 IN PHOSPHOLIPIDS: PHOSPHATIDYLETHANOLAMINE, LECITHIN, AND SPHINGOMYELIN.

Addition of glucose is intended to supply energy which is usually derived from glycolysis and now seems to be blocked by Freon. Since each mole of glucose can supply 38 moles of ATP, which is equivalent to the energy of about 19 electron volts when ATP is split to ADP and inorganic phosphate.

DISCUSSION

Aforementioned data clearly indicate that Freons have strong effects on enzyme activity and cardiac contractility. The block of Freon on glycolysis is very interesting. The accumulation of fructose diphosphate (Rhoden and Garbriel, 1972) suggests that there is a block just below FDP step of the glycolysis sequence.

However, injection of glucose completely counters the adverse effect of Freon and indicates that the blocking of glycolysis perhaps at the stage of phosphorylase or phosphomutase stage. The possibility that Mg^{++}, which is essential for these two enzymes, may interact with Freon molecules, seems to be slim. Addition of Mg^{++} does not improve arrhythmia induced by epinephrine in sensitized heart. The activity of phosphorylase should be checked under Freon sensitized condition. There is also considerable accumulation of lactate in the Freon treated heart. It suggests that there may be a blockade of aerobic oxidation of pyruvate. Addition of acetyl-CoA does not relieve the arrhythmic contraction after Freon 12, indicates that aerobic pathway is not impaired. Perhaps the accumulation of lactate is due to slightly hypoxia state under Freon exposure.

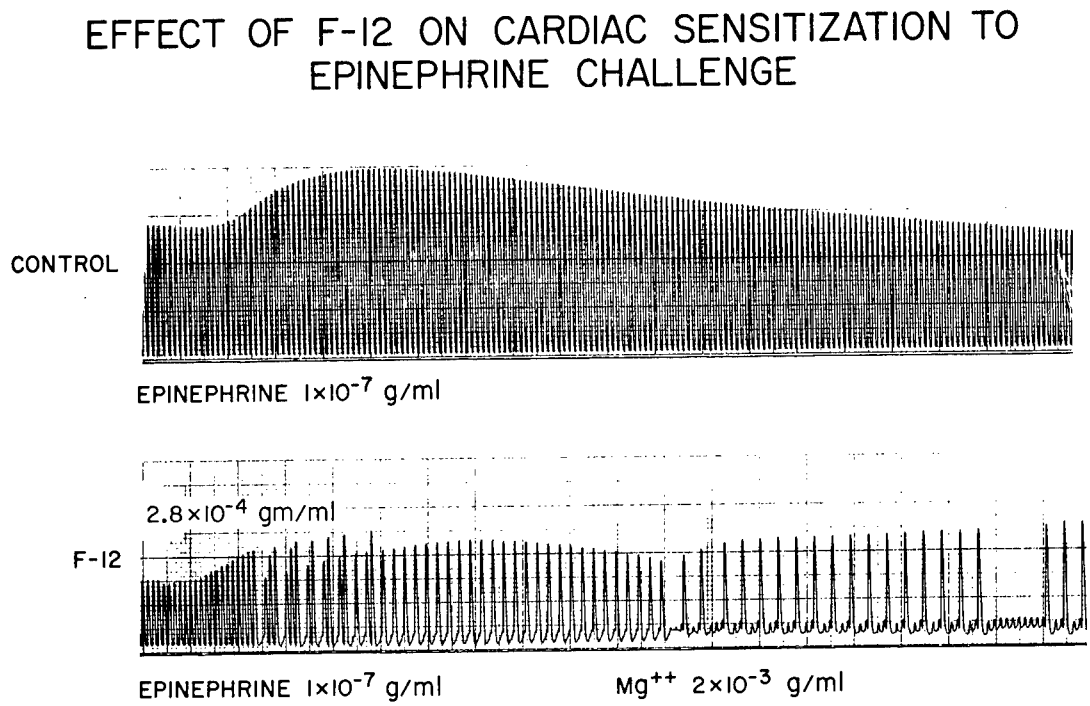

Figure 8. EFFECT OF Mg^{++} ON EPINEPHRINE CHALLENGE IN A FREON 12 SENSITIZED HEART.

The ED_{50} of Freon 1301, Freon 13 and Freon 14 are 240, 56 and 50 ppm respectively suggest that the affinity of these Freons to the receptor is largely an electronic force. Freon 1301 is the least effective among the three indicates that Freon molecules are rather loosely bound to the zwitterion of the membrane phospholipids. The ED_{50} values of three Freons run parallel with the bonding energies of C-Br, C-Cl and C-F. The well known antifibrillation agent, lidocaine, cannot be used as a counter measure for Freon 12 cardiac sensitization to epinephrine challenge, because lidocaine does not relieve the arrhythmia already induced

by Freon 12 epinephrine challenge. If lidocaine is administered previously, however it does prevent the onset of arrhythmia. The fact that lidocaine reduces the contractility and cardiac rate so drastically prohibits using lidocaine as an antidote for Freons.

The most effective counter measure for Freon sensitization to epinephrine challenge seems to be potassium ions and glucose. To a large extent glucose supplies the energy whereas potassium displaces the Freon molecules from the active sites tentatively assigned as phosphatidylethanolamine. Phosgene and carbonyl fluoride also produce arrhythmia without epinephrine challenge. Glucose and potassium seem also able to restore phosgene and carbonyl fluoride arrhythmia just as well. The basic concept of Freon and phosgene poisoning seems essentially that they deprive the cardiac muscle of energy supply and cripple the conducting elements at higher concentrations. At low concentration Freons as well as phosgene act strictly at the enzyme level.

SUMMARY

An isolated vagal sympathetic heart system has been successfully used for the study of the effect of Freons on cardiac performance and in situ enzyme activity. Freon 12 sensitizes this preparation to sympathetic stimulation and to exogenous epinephrine challenge. Partial and complete A-V block and even cardiac arrest have been induced by epinephrine challenge in the Freon-sensitized heart. Potassium chloride alone restores the rhythmicity but not the normal contractility in such a heart. Addition of glucose will, however completely restore the normal function of the heart, which is sensitized by Freon 12.

The ED_{50} value of AChE activity which is used as a measure of the relative effectiveness of Freons is compared with the Maximum Permissible Concentration. Kinetics studies indicate that all the Freons tested so far are noncompetitive.

TABLE III

RELATIONSHIP BETWEEN PHYSICAL PROPERTIES OF FREONS AND THEIR ED_{50} OF AChE ACTIVITY

Freons	Formula	Bond Length	Energies	Electronegativity	ED_{50}
F-1301	CF_3Br	C-Br 1.93	69	1.76	240
F-13	CF_3Cl	C-Cl 1.76	85	1.36	56
F-14	CF_3F	C-F 1.36	122	1.06	50

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OPEN FORUM

DR. AZAR (Haskell Laboratory, E. I. duPont): I was a little disturbed by this comparison of the threshold limit value with the concentration of fluorocarbon 12 in a bath. Unless my calculations are in error here, I would like to know how Dr. Young converted from the concentration in the bath to the TLV value in the air because on the last slide there he showed a concentration of Freon 12® of 2.8×10^{-4} g/ml and I get that down as 280 micrograms/ml and we've got blood levels on exposing dogs to 100,000 ppm of Freon 12® for 10 minutes and the blood levels are less than 50 micrograms/ml. I don't have the exact data with me but it's an order of magnitude that's way, way different.

DR. YOUNG (Ames Research Center): We dissolved the Freon® in the solution. That's the number I got.

DR. AZAR: This is a concentration in a solution. How do you take that then to the concentration in the air which is the TLV?

DR. YOUNG: No, I just quoted that for comparison, that's all.

DR. AZAR: Well, that's very misleading, I'm sorry.

DR. HODGE (University of California): I need a little information. I think, Dr. Young, you said that Freon® increased the acetylcholinesterase activity in the heart. Now, I know we have parasympathetic endings in the auricle. Are there parasympathetic endings in the ventricle?

DR. YOUNG: Well, this was a whole heart.

DR. HODGE: The heart is mostly ventricle in its weight.

DR. YOUNG: Well, actually I stimulated the vagus nerve so that the vagus nerve acted as an innervator of the sinus. So, we're talking about the sinus, the pacemaker.

DR. HODGE: Does the ventricular muscle contain acetylcholinesterase?

DR. YOUNG: I don't think so.

DR. HODGE: I hadn't heard of it.

DR. YOUNG: No, I don't think so.

DR. HODGE: So all of the change that you found must have been an auricular muscle, atrial muscle.

DR. YOUNG: I'm talking about the post junctional membrane. You stimulate the nerve, then the nerve releases acetylcholine and outside of the nerve ending you have a post junctional membrane, and acetylcholinesterase acted essentially in the post junctional membrane, so that's where the action takes place.

DR. HODGE: I don't quite understand how you get an increase in acetylcholinesterase activity.

DR. YOUNG: I measure the time of inhibition, the velocity of acetylcholinesterase.

DR. MAC NAMARA (Department of the Army): What about the possibility that the effect of the acetylcholine is not showing up on the heart muscle even though it's not being destroyed?

DR. YOUNG: As long as acetylcholine remains in the heart, the heart would have a different function. You would lower the contractility and also slow down contraction.

LT. COL. CARTER (NASA Manned Spacecraft Center): I think one thing we ought to realize is that in your parasympathetic preparation you're stimulating the preganglionic nerve and your assay for acetylcholinesterase as I understand it from your presentation is the time that it takes the response to return to normal, the heart response. Is that correct?

DR. YOUNG: Yes.

LT. COL. CARTER: Well, there's a ganglion in the middle of this situation also which you have to consider. And most of these compounds do have ganglionic blocking properties. So I don't really feel that's a good way of doing cholinesterase activity, because you have to take into consideration that this ganglion is sitting here in the middle of your preganglionic fiber where you're doing your cholinesterase assay.

DR. YOUNG: In answer to your question, the concentration we're dealing with is much lower than that which would have an effect on the ganglia. I can show some slides showing physostigmine inhibition. This is a typical enzyme activity plot. We're dealing with 10^{-9} molar of physostigmine. The increase would indicate the intercept of the 1/(v) axis. So the physostigmine is a specific inhibitor of this particular preparation so I don't think you have much to argue against acetylcholinesterase. The same thing with DFT. You get exactly the same intercept.

MAJ. VAN STEE (Aerospace Medical Research Laboratory): I'd like to ask Dr. Young about the sympathetic half of this experiment. Did I understand correctly that you were using the time of recovery from the effect of sympathetic stimulation as an index of MAO activity? Is this correct?

DR. YOUNG: We're using time area increment as an indicator for that.

MAJ. VAN STEE: What I'd like to point out is that the action of catecholamine, the neural transmitter, is terminated by 3 distinct mechanisms. About 60% of it is terminated by reuptake in the terminals, about 35% by MAO activity, and about 5% by catecholmethyltransferase. This has been shown in the classic experiments, I think of Copen using cat's spleen a number of years ago. I don't know if this has been confirmed specifically for the heart, but in any event the deamination only represents about 1/3 of the total termination of the action and you are, in effect, measuring the total termination here. So really I can't see how you can distinguish among the 3 different mechanisms of the termination, particularly the reuptake which accounts for more than half of the termination of the action of the neural transmitter.

DR. YOUNG: Well, in this particular preparation, 95% to 99% of the transmitter is epinephrine. This is different from a dog or other mammals.

DR. AZAR: This is the first time to my knowledge that anyone has ever come close to demonstrating the slightest change in anything below the TLV of Freon 12®. I want to know how that jump was made from a concentration in a tissue bath to a TLV in the air and why humans have been exposed to concentrations well above the TLV for fluorocarbon 12 and nothing has been seen and this is very disturbing. The frog does act differently and I think that's one of the points I'm trying to make. Making the jump from the tissue bath to inspired concentrations leads to results that do not agree with data that I've seen elsewhere.

DR. YOUNG: I think the air is pretty much equivalent with the blood especially at low concentrations.

DR. SMITH (Federal Aviation Administration): I'm somewhat disturbed about the low concentrations of the Freons® that appeared to cause problems. I'm frequently asked about the practicability of the use of fire suppressants. It's a very important subject with commercial airlines. There's even been talk from time to time of making a trade-off between oxygen systems which fortunately are seldom required and installation of a fire suppressant. So it is disturbing to find such low concentrations of the Freons® triggering arrhythmias. I have several questions having at one time worked a little bit with arrythmias and animals. In the constitution of an artificial mixture in which a Freon® is included, I wonder if account has been taken of the fact that an insidious hypercapnia can

occur in an oxygen-rich mixture in an anesthetized animal and I am confident that this can contribute to the occurrence of an arrhythmia and I believe that this should be carefully controlled in any kind of experiment in which an artificial mixture is used. Second, I wonder if anybody has observed what I believe I have seen many times under nembutal anesthesia, dogs are hypertensive with a tendency toward tachycardia which might be predisposing. Third, is the dog the right experimental subject? Are they or are they not more susceptible to arrhythmias? Fourth, I wonder how practical a catecholamine infusion is or how it relates to the practical situation of maximal endogenous release?

DR. WILLS (Albany Medical College): I'm not sure that I can remember all the questions that were asked but as far as our own work is concerned, I can't really say whether the dog is the proper animal to use. We have used cats, dogs and guinea pigs and we find all 3 respond in much the same way. I think we can say that we're getting fairly typical responses but whether they're typical of man or not, I can't say because we haven't used any men yet. It's possible that we will eventually, but we haven't done it yet. We have used a few monkeys, not very many, only 2 in fact, but in 2 monkeys we got very much the same effects that we got in the other animals. As far as the effects of anesthesia go, we have done a few unanesthetized animals and we do find that there are some quantitative differences but no qualitative differences in response. In an unanesthetized animal, we can induce the effect with somewhat less epinephrine than we can in an anesthetized animal but otherwise, the responses are quite similar. There were two other questions.

DR. SMITH: Specifically, sodium pentobarbital as an anesthetic and I guess my last one was the relationship between an infusion and the maximum endogenous release which might be considered possible under this kind of stress.

DR. WILLS: Well, I don't know what the maximum response is and there is some evidence that the normal rate of release of epinephrine or catecholamines from the adrenal glands and from the other related tissues in the body is somewhere on the order of 2 micrograms per kilo per minute. Now the dose that we've used is somewhat above that. I don't know whether our dose is really a realistic dose or not because I haven't been able to find any good statement about what the maximal release rate is. I would say that Dr. Van Stee's experiment in which he has run dogs about as hard as they can stand running would indicate that at least by that procedure he doesn't get as much release of epinephrine as we have used in our infusion experiments. This is an inference; it's not a fact.

DR. SMITH: I think my real point is that under this kind of stress the two could be superimposed.

DR. WILLS: That's possible. I wouldn't expect an animal under anesthesia which would be producing any unusual amount of epinephrine but I haven't actually measured it.

DR. YOUNG: In our preparation, we found some anesthetics would decrease the acetylcholinesterase activity. That would counter some of the effect of the Freons® which are activators.

MAJ. MC NUTT (Aerospace Medical Research Laboratory): I was interested by what Dr. Wills said about being able to run up some compounds to 80% concentration with the other 20% being oxygen and not getting spontaneous arrhythmias. I wonder what chemical compound this was. It sounds like it would be a fairly good control compound to use for some of these complicated metabolic studies where you're really interested in whether the metabolic effect you're seeing is pertinent to arrythmias that you see in the whole animal or not. I wonder if he could elucidate what some of these compounds are and whether you see the same effect on spontaneous arrhythmia formation that you do on sensitization of the heart to the catecholamine infusion.

DR. WILLS: The compound that I had in mind when I made that statement was Freon 116® . We haven't done very many experiments at these high concentrations of that compound because the concentration that we're really interested in, or that NASA is interested in, is somewhere around 20% but we have run up in just a couple of experiments to much higher levels just to see what happens.

DR. AZAR: A lot of this information I've said to people in this group before, so I apologize if it's old to them. I'd just like to take a minute. If you go back to look at, let's say, Freon 12® , I should point out also that's a duPont trademark. You can buy it under Genetron, Arcton, or what have you. But back when this compound first came out, rats were exposed to 80% fluorocarbon 12 and 20% oxygen for 4 to 6 hours. They were anesthetized but they didn't die. So everyone thought this sounded like a good compound. And then in World War II, Professor Kehoe at Cincinnati carried out some controlled human exposures where he had humans that were exposed to 4 to 11% of fluorocarbon 12 or Freon 12® , whatever you want to call it, for about 80 minutes. Now, it is interesting there that at about 10 to 11% concentration, in about 10 to 11 minutes, I think he was the subject, he became unconscious. If you go back and read his report and look at his EKG tracing, you can see he developed a bigeminal rhythm. Now that's at 10%. Then we heard about the sniffing deaths and Bass suggested a cardiac sensitization and we had presented prior to the Taylor and Harris article, data showing that, indeed, the fluorocarbons did cause cardiac sensitization and if I might on a board show you the concentrations of fluorocarbon 12 that we saw. We took dogs and they were breathing this, they got a slug of epinephrine of 8 micrograms per kilogram in 9 seconds which is about 10 times what Starling has calculated as maximum secretion. So there were zero out of 12 dogs that showed any cardiac sensitization. Then we went to 5% and 5 out of 12 dogs showed it. So then we were asked the question, "Is the dog an appropriate animal?" We investigated the work of Professor Meek; he had studied various species and looked at various anesthetic compounds, and found that of the various species, the dog data closely approximated that which they were seeing in anesthesia, the human cases.

So he felt that the dog was the best animal. Then we went to the question about "How does this relate to maximum secretion?" So we took these dogs and we ran them on a treadmill. We ran them for about 15 minutes at 5%, 2.5% and none of these dogs fibrillated. We couldn't see anything. We went up to 10% running on a treadmill and we saw 1 of 6 dogs develop a burst of ventricular tachycardia. Now, it's amazing that this 10% with the endogenous running was quite similar to what was seen in the only human that I know of that developed a recorded arrhythmia. So I would say that this data fits in with the pattern quite well. Now, somebody says five minutes isn't long enough. Well, we then did another study where we went back and we exposed these dogs to these same concentration. They had a cannula in their carotid artery and one in their jugular vein. Every minute we took a blood sample and we analyzed it for fluorocarbon 12 and if you do that, this is what you see. It initially starts to go up. At about 3 to 5 minutes, it starts to reach its peak. And about 7 to 10 minutes which would go along with Dr. Wills demonstration that that's when he's seeing the maximum sensitization. This is where it leveled off. At 10 minutes we stopped it and you see the arterial and venous levels come down. This data has been presented. It has been submitted to the Journal of the American Industrial Hygiene Association for publication. This, I think, fits in very well with what Dr. Wills has seen. So I think the dog is an appropriate animal. I think that the unanesthetized state is certainly something to consider. Phenobarbital has been shown to affect cardiovascular response just by itself. I'm sorry if I took too long but I thought it was important to get this thing into some perspective.

DR. SMITH: I don't agree with the statement that 5 minutes isn't long enough. We're talking about a different order of magnitude in time here. If we can find a fire suppressant that humans can tolerate, 90 seconds is the time required for evacuation of an aircraft after a crash. In theory, at least, all uninjured persons are out, but it's the fire that frequently complicates an aircraft crash that really worries us. So when you run experiments for 5, 10, 15, or 30 minutes, it's not apropos.

DR. WILLS: I think it is apropos from the standpoint of NASA, although not from the Federal Aviation Agency.

LT. COL. CARTER: I'd like to ask Dr. Wills one technical question. You said you induced spontaneous cardiac arrythmias with Freon 11® . Correct?

DR. WILLS: In one case.

LT. COL. CARTER: How long did it take from the start of exposure? Do you remember?

DR. WILLS: I couldn't be absolutely certain but I would say that it was not more than 5 minutes.

DR. BACK (Aerospace Medical Research Laboratory): I'd like to ask about the effects of calcium on Dr. Young's preparation. Does the increase of calcium in your fluid have an effect?

DR. YOUNG: I haven't done anything with calcium in my Freon® studies. But I did some studies with calcium alone. The calcium increases the inhibition of acetylcholinesterase. Five millimole increases it up to 100%. If we reduce that to 0.1 millimole there's very little inhibition.

DR. BACK: If you use potassium chloride which would be the obvious way to go, there are two ways of protecting against an arrhythmia once started I suppose, and one of them would be to increase potassium and the other would be to decrease calcium. You didn't do the obvious other one. How much calcium do you have in your perfusate or didn't you add any?

DR. YOUNG: My perfusate was 0.2 millimoles.

DR. BACK: So have you tried say, to use a compound like EDTA at that moment in time?

DR. YOUNG: No, I didn't.

DR. FASSETT (Eastman Kodak Company): Just a brief comment about Dr. Smith's question about the barbiturates. Back in the early days when we were working on this thing in cyclopropane and so forth, anesthetists at that time certainly had a feeling that pretreatment with barbiturates actually protected against arrhythmia.

MAJ. VAN STEE: I might add something in response to that question. It's been shown that pentobarbital, at least, has an atropine like effect in the dog. This is rather readily demonstrated crudely if you observe the sinus rhythm of a dog before pentobarbital and you'll notice that a normal dog has a sinus arrhythmia and upon giving pentobarbital, there's immediately a large increase in the heart rate which is a reflection of the decrease of vagal inhibition of the heart. In view of the fact that atropine and vagotomy have been shown to have a sparing effect on arrhythmia formation, the mechanism then of this supposed protective effect of pentobarbital might be by way of this atropine like action of the pentobarbital.

SESSION III

PYROLYSIS AND ROCKET EXHAUST PRODUCTS

Chairman

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PAPER NO. 18

INTERRELATIONSHIP OF METHEMOGLOBIN, REDUCED GLUTATHIONE AND HEINZ BODIES IN MONOMETHYLHYDRAZINE-INDUCED ANEMIA. IN VITRO STUDIES ON HUMAN RED CELLS.

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INTRODUCTION

The use of monomethylhydrazine (MMH) in rocket propulsion systems has stimulated an intensive investigation into the toxic effects of this compound. A substantial amount of data has been reported on the effects of both acute and chronic exposures to MMH by various routes of administration in several animal species including man, as reviewed by Clark et al. (1968).

Exposure to acute doses of MMH can cause a variety of symptoms and pathological changes depending on the concentration of MMH and the animal species. Symptoms include diarrhea, emesis, salivation, irritation of mucous membranes, dyspnea, and convulsions. At lethal doses, cause of death is respiratory depression. Findings at necropsy on dogs and monkeys exposed to lethal and near lethal levels of MMH are variable but may include pulmonary congestion with hemorrhage, hepatic congestion, and kidney damage ranging in severity from mild swelling of the renal tubular epithelium to vacuolization and coagulative necrosis of these cells (MacEwen et al., 1969). The range between a lethal or near lethal concentration of MMH, producing major pathological changes and overt symptoms, and a concentration causing minimal effect is very narrow; the dose response curve is extremely steep.

The only effects found consistently after acute or chronic exposure to various levels of MMH given by ingestion, injection, or inhalation involve the hematologic system. Haun et al. (1968) exposed dogs and rhesus monkeys to various concentrations of MMH in inhalation chambers and determined hemoglobin, hematocrit,

and red blood cell and reticulocyte counts before and after exposure. Dogs surviving a 60-minute exposure to 92 ppm MMH had moderate to severe anemia with concurrent reticulocytosis, reaching a maximum 7 days postexposure, 24 days after which the values returned to near normal levels. Monkeys exhibited the same hemolytic response but to a milder degree. In another experiment, Haun (1970) exposed beagle dogs to 5 ppm and 2 ppm MMH 6 hours a day, 5 days a week, for 26 weeks, and observed a significant decrease in hematocrit, hemoglobin, red cell count, and an increase in reticulocytes and osmotic fragility, as well as in methemoglobin and Heinz body production. These changes peaked at 2 weeks exposure to 5 ppm and 4 weeks exposure to 2 ppm and returned to normal postexposure. Monkeys under the same experimental conditions exhibited similar changes but to a much milder degree, indicating a distinct difference in susceptibility for dogs and monkeys. MacEwen et al. (1970), in a study designed to validate Emergency Exposure Limits, exposed human volunteers to 90 ppm for 10 minutes. There was no change in hematocrit, hemoglobin, red cell count, reticulocyte count, or methemoglobin level immediately postexposure or at periods up to 60 days. However, all subjects showed Heinz bodies in 3 to 5 percent of their red cells by the 7th day; these disappeared by the 14th postexposure day.

Clark and De La Garza (1967) reported on the difference in red cell susceptibility of various species, as well as the formation of methemoglobin in red cells exposed to MMH in vitro and in vivo. The dog red cell produces the highest level of methemoglobin, followed by man, rat, and monkey. Leahy (1970) confirmed this species difference and demonstrated the necessity for an adequate supply of oxygen for methemoglobin production during MMH exposure.

Many anemias that are associated with methemoglobin formation and Heinz body production are also associated with alterations in glutathione metabolism. The temporal connection between the appearance of methemoglobin and Heinz bodies and the net loss of reduced glutathione (GSH) in these anemias is variable and a direct mechanistic relationship between these events is uncertain. The mechanism by which red cells become increasingly susceptible to hemolysis in these anemias is also unclear, although it has been widely speculated that alterations in the red cell membrane may play an important role in the premature removal of injured cells from the circulation (Rifkind, 1965).

In this study we have used an in vitro system to examine the effects of MMH on the human red cell; GSH levels, methemoglobin, and Heinz body production were measured. Biological reduction in the red cell is maintained by GSH and various reductases which require either NADH from the Embden-Meyerhof pathway or NADPH from the hexose monophosphate shunt (HMP) pathway. The activities of glutathione reductase (GSSG-R) and two enzymes in the HMP pathway, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconic dehydrogenase (6PGD), were determined since a decrease in HMP enzyme activity could partially explain

the striking susceptibility of mammalian red blood cells to MMH exposure and a decrease in GSSG-R could explain the decrease in GSH. Osmotic fragilities of red cells were examined as a measure of red cell membrane integrity. The development of Heinz bodies was followed by light microscopy and freeze-cleave electron microscopy, and an attempt was made to correlate morphologic alterations in red cells exposed to MMH with biochemical and functional changes.

MATERIALS AND METHODS

Whole blood was drawn by venipuncture from normal human subjects using heparin as an anticoagulant. The blood was centrifuged at 1000 g and the buffy coat removed. The red cells were washed three times at 6 C with isotonic phosphate-buffered saline, pH 7.4, containing 0.011 M glucose (Jacob et al., 1968) and resuspended in buffered saline to a hematocrit of 40-50%. The white cell count was less than 100 per cu mm. Monomethylhydrazine (MMH) (Eastman Organic Chemical Company, Rochester, New York) was diluted with the same buffered saline. MMH solution was added to the red cell suspension yielding a final concentration of 0.01 M MMH in the suspension. In some experiments, additional samples were exposed to a concentration of 0.001 M MMH. Controls were run concurrently with buffered saline substituted for the MMH solution. Both the control and test red cell suspensions were incubated aerobically at 37 C. The cell suspensions were then washed two times with buffered saline and resuspended to a hematocrit of 20% for enzyme determinations and to 40-50% for GSH, methemoglobin, and electron microscopic examination. An aliquot was taken for a red count, hematocrit, and hemoglobin determination using standard hematologic techniques.

Light Microscopy

Wet smears of control and MMH-exposed red cells were prepared after 30-minute and 1-, 2-, 4-, 6-, and 24-hour incubation periods. Heinz bodies were demonstrated by supravital staining of cells with 0.5% brilliant green, 0.5% neutral red, or 1% crystal violet in either 0.9% NaCl or 50% methanol.

Electron Microscopy

Buffer-incubated control cells and MMH-incubated cells were packed by light centrifugation at 1000 g, suspended in 20% glycerol containing 0.90% NaCl for 15 minutes at 4 C, pelleted at 2000 g for 5 minutes. Droplets of packed cells were frozen in Freon®22 at -150 C. The cells were freeze-cleaved and replicas of the fresh fracture faces were prepared either in a Balzers 360M Freeze Etch machine, according to the technique of Moor (Moor and Muhlethaler, 1963), or in a Type II Bullivant device (Bullivant et al., 1968). In heat-etch runs the

specimen was maintained at -100 C for approximately 1 minute. In nonetch experiments, the specimen was maintained at or below -150 C during cleavage and replication procedures. The replicas were retrieved from the tissues with Clorox®, washed with distilled water, and retrieved on uncoated 200- or 400-mesh copper grids. Replicas were photographed in an RCA-EMU-4B electron microscope. Micrographs of replicas were printed as positives so that accumulations of platinum appear as dark and shadowed regions, and areas devoid of platinum appear white.

Enzyme Activity Determinations

Cell suspensions were lysed by adding 1.0 ml water, 0.3 ml saturated digitonin solution, and 0.7 ml 0.05 M triethanolamine buffer, pH 7.5, per 1.0 ml red cell suspension which was placed in the refrigerator at 4 C for 20 minutes. The lysate was centrifuged 15 minutes at 1000 g to remove cell stroma. G6PD activity was measured on the lysate by the method of Lohr and Waller (1963), following the formation of NADPH at 340 $m\mu$ at 25 C spectrophotometrically. Cells and lysate were prepared in a similar manner for determination of 6PGD activity, except that a glycylglycine buffer, 0.25 M, pH 7.5, was used. The activity was measured according to the method of Marks (1961). Glutathione reductase activity was determined using the method outlined by Salkie and Simpson (1970). The results were calculated and reported as milli-International enzyme units per 10^9 red blood cells.

GSH and Methemoglobin

The GSH levels of the cell suspensions were measured by the Alloxan 305 method (Patterson and Lazarow, 1955) and results reported as μg GSH/10^9 red cells. To determine the glucose requirement and to assure that adequate substrate was available for NADPH production by the EMP pathway, a limited number of experiments were run at glucose concentrations of 0, 25, 100, 200, and 400 mg% in the buffered saline. Methemoglobin was determined by a modification of the Evelyn and Malloy method as described by Hainline (1965).

Osmotic Fragility

Osmotic fragilities of control cells and cells incubated for 30 minutes, 2 hours, and 4 hours with 0.01 M MMH were determined using the spectrophotometric method outlined by Wintrobe (1951).

Gravimetric Analysis of Heinz Bodies

Red cell ghosts were prepared according to the method of Dodge et al. (1963) from MMH-exposed red cells and from control red cells. The ghosts were dried in vacuo over P_2O_5 and weighed. The dry ghost pellet was dissolved in 0.1 N KOH. Total protein was measured by the method of Lowry et al. (1951) and the hemoglobin remaining in the ghosts was measured as pyridine hemochromogen.

RESULTS

Light Microscopy

The number of Heinz bodies per cell, sizes of Heinz bodies, and the percentage of cells containing Heinz bodies were related to MMH concentration in the incubation medium. Incubation of cells with 1×10^{-4} M MMH produces no apparent Heinz bodies at 2, 4, or 6 hours and only small Heinz bodies in less than 20% of those cells at 24 hours. Incubation of cells with 0.001 M MMH produces small Heinz bodies in less than 20% of the cells at 2 hours but 1 to 4 large Heinz bodies in over 90% of the cells at 24 hours. Incubation with 0.01 M MMH produces 1 to 9 small Heinz bodies per cell in 95 to 100% of the cells in 1 hour. These appear to increase in size at 2 hours and many of the Heinz bodies were as large as 2 microns at 24 hours. Heinz bodies could not be demonstrated by light microscopy with certainty after 30 minutes incubation in any of the concentrations of MMH used here. (They were demonstrable by electron microscopy at 30 minutes.)

Control red cells retain a normal biconcave configuration throughout the 24-hour incubation period. Alterations in the configuration of red cells are apparent in MMH-exposed cells. They consist of distortion of cells and loss of their central concavities (figures 1 and 4). These changes are related to concentration of MMH since they were observed earliest with 0.01 M MMH.

Electron Microscopy

The appearance of the cytoplasm and membranes of normal human red cells in freeze-cleave preparations has been previously reported (Weinstein and McNutt, 1970). Briefly, cleavage planes through pellets of frozen red cells can pass directly through the cytoplasm of individual red cells or be deviated for some distance by the cell membrane. The cleavage along a "frozen" membrane is within the matrix of the membrane. Red cell membranes are hemisected by freeze-cleaving and two fracture faces are generated by the process. By convention, the fracture face of the juxtacytoplasmic leaflet of the membrane is called the "A-Face" and the fracture face of the outer leaflet of the membrane is called

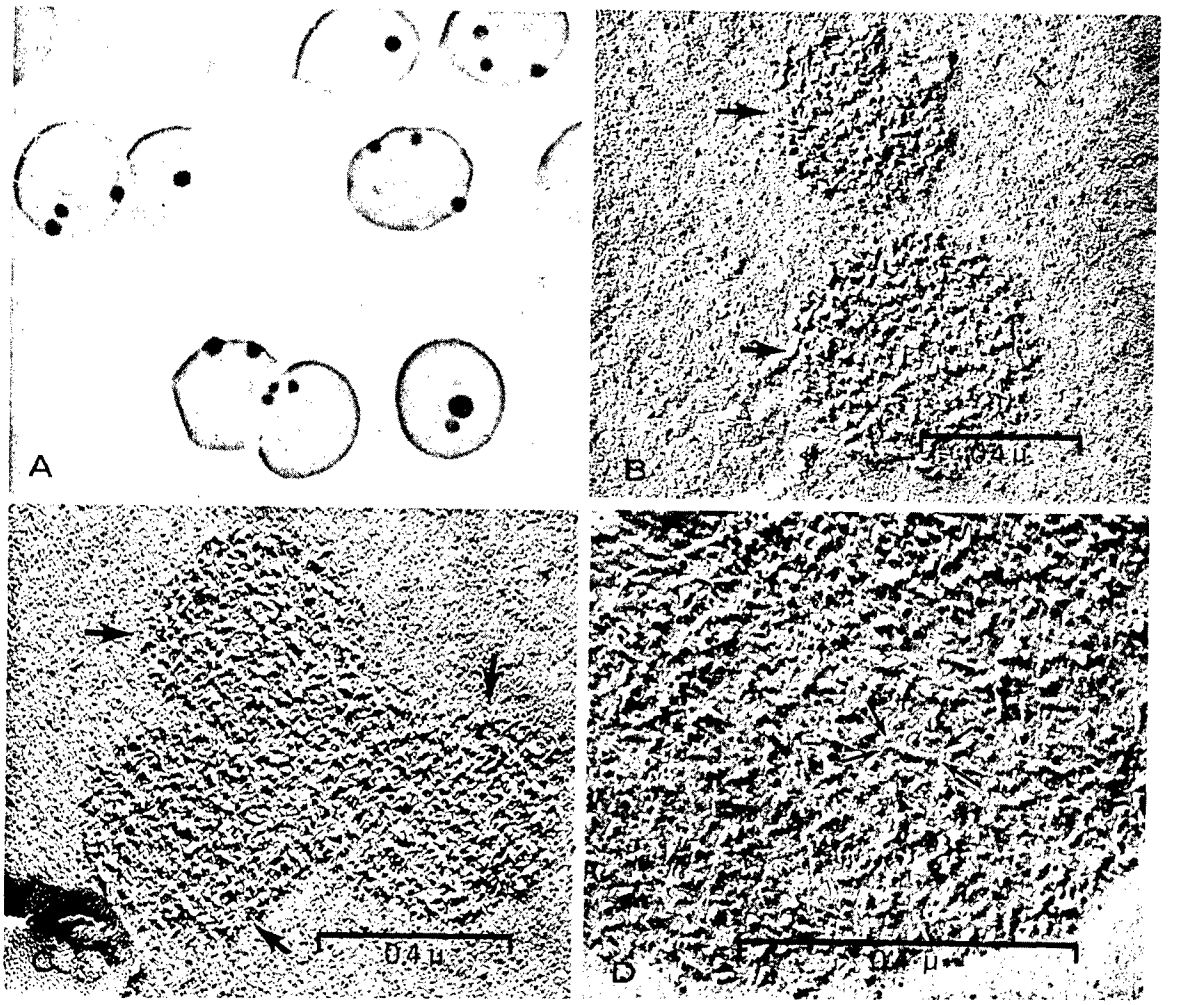

Figure 1. A: LIGHT MICROGRAPH OF HUMAN RED BLOOD CELLS INCUBATED WITH 0.01 M MONOMETHYLHYDRAZINE (MMH) FOR 2 HOURS. The cells are deformed, have lost their biconcave configuration and contain Heinz bodies. The proportion of Heinz bodies at cell membranes is in excess of what is seen in most microscopic fields. Brilliant green supravital stain. x 1700. B: ELECTRON MICROGRAPH OF A CARBON-PLATINUM REPLICA OF FREEZE-CLEAVED HEINZ BODIES IN THE CYTOPLASM OF A RED CELL EXPOSED TO 0.01 M MMH FOR 30 MINUTES. Two round granulo-fibrillar Heinz bodies are illustrated (arrows). x 51, 000. C: RED CELLS EXPOSED TO 0.01 M MMH FOR 30 MINUTES. Three Heinz bodies (arrows) appear to be fusing. x 62, 000. D: HIGH MAGNIFICATION ELECTRON MICROGRAPH OF A HEINZ BODY IN A HUMAN RED CELL EXPOSED TO 0.01 M MMH FOR 2 HOURS. 70-100 A in diameter fibrils are prominent (arrows). x 110, 000.

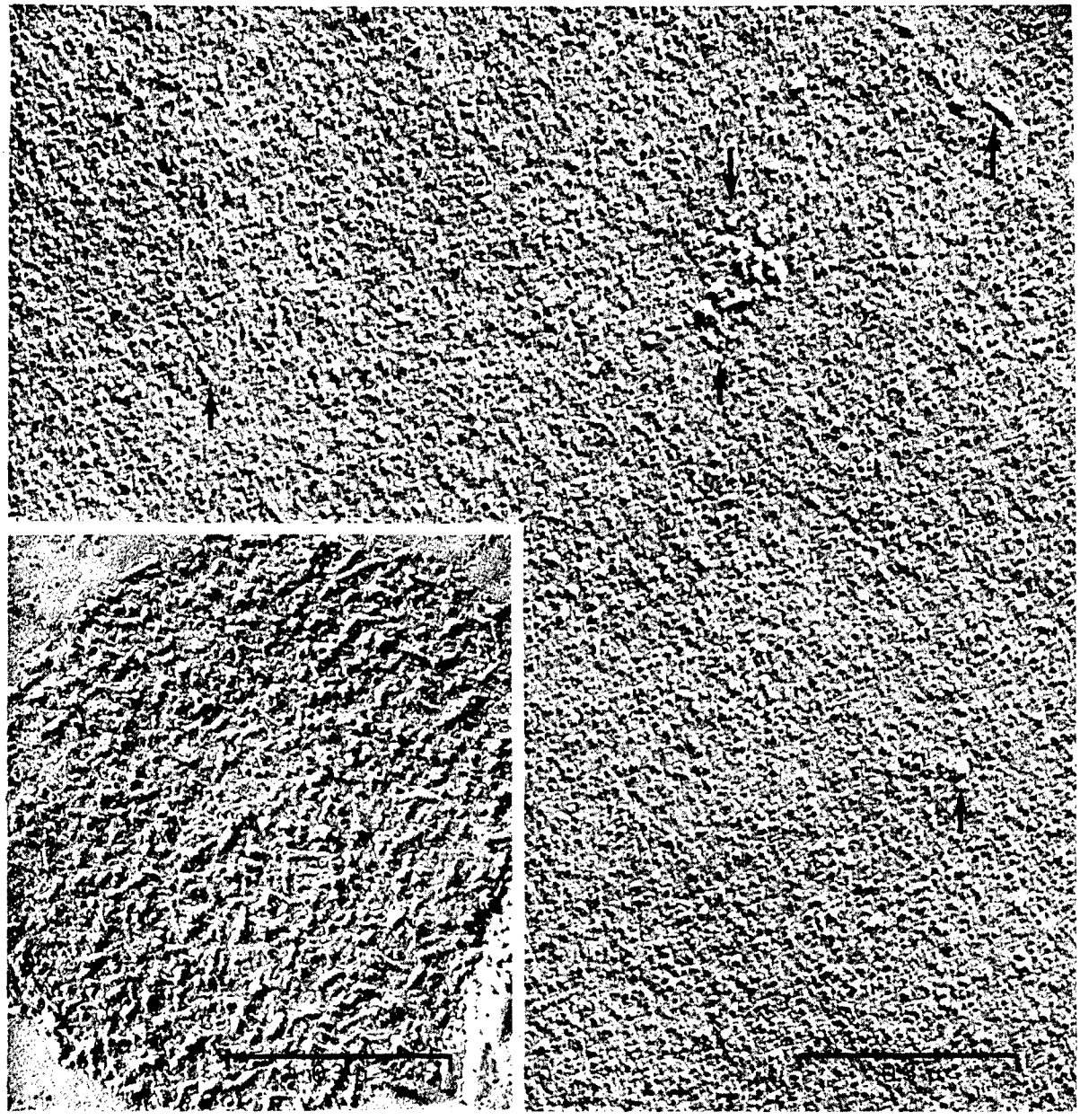

Figure 2. UPPER RIGHT: CYTOPLASM OF A FREEZE-CLEAVED RED CELL EXPOSED TO 0.01 M MMH FOR 1 HOUR. Single fibrils (single arrows), provisionally interpreted as polymers of hemoglobin, and aggregates of fibrils (apposing arrows) may represent the earliest stage of Heinz body formation. x 110,000. INSERT: LARGE HEINZ BODY SHOWING MANY RANDOMLY-ORIENTED FIBRILS. The Heinz body is not limited by a membrane. x 110,000.

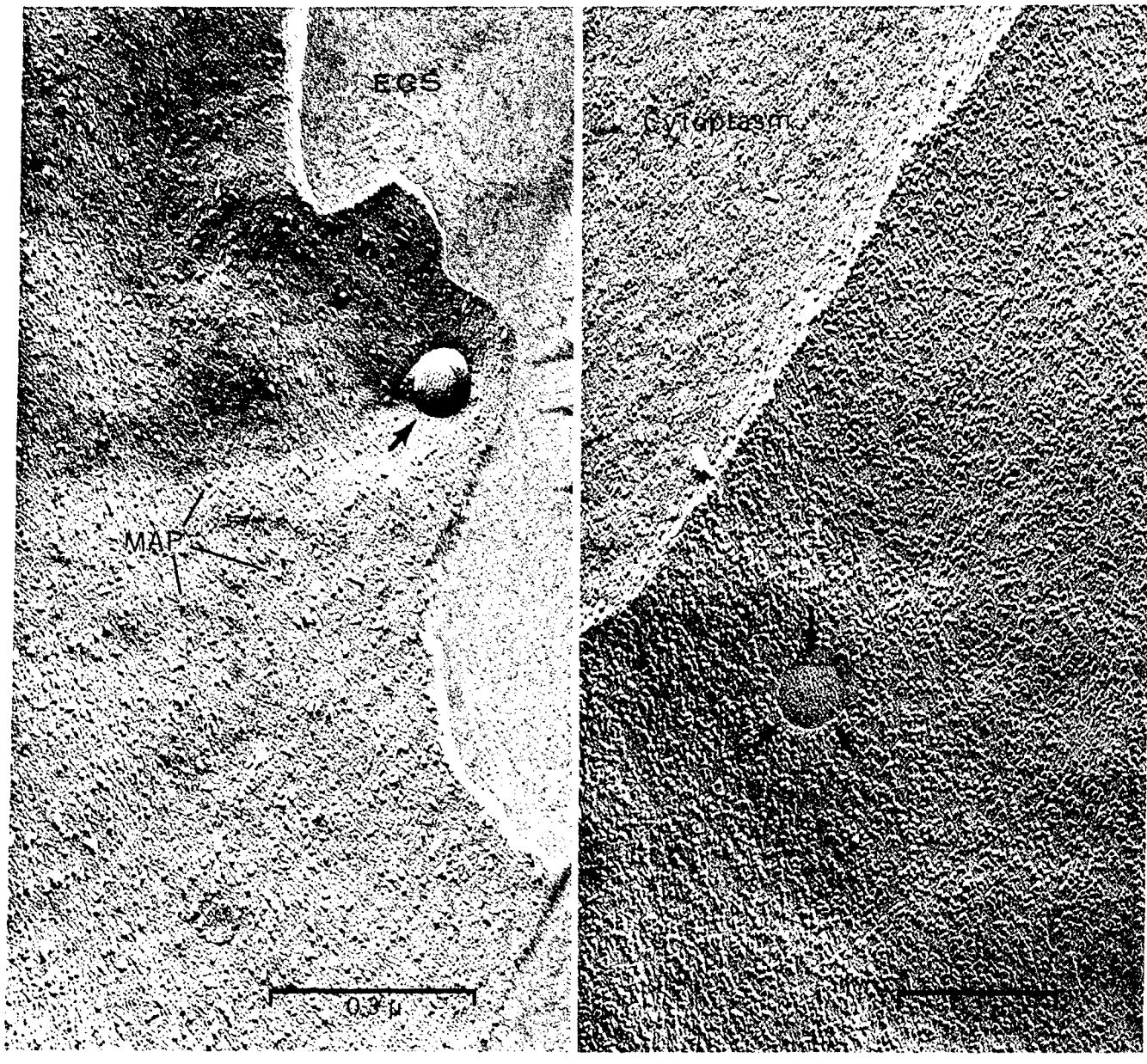

Figure 3. LEFT: B-FACE OF A FREEZE-CLEAVED RED CELL AFTER 6 HOURS INCUBATION IN 0.01 M MMH. A smooth endocytotic vesicle is budding toward the cell's interior (arrow). The extracellular compartment (ECS) appears smooth. Small membrane-associated particles (MAP) are present at fracture faces of control and MMH-exposed red cell membranes. x 100, 000. RIGHT: A-FACE OF A FREEZE-CLEAVED RED CELL SHOWING A SMOOTH-BASED CRATER (ARROWS) APPEARS AS TIGHTLY-PACKED PARTICLES AND IS SHOWN IN THE UPPER LEFT OF THE MICROGRAPH. x 80, 000.

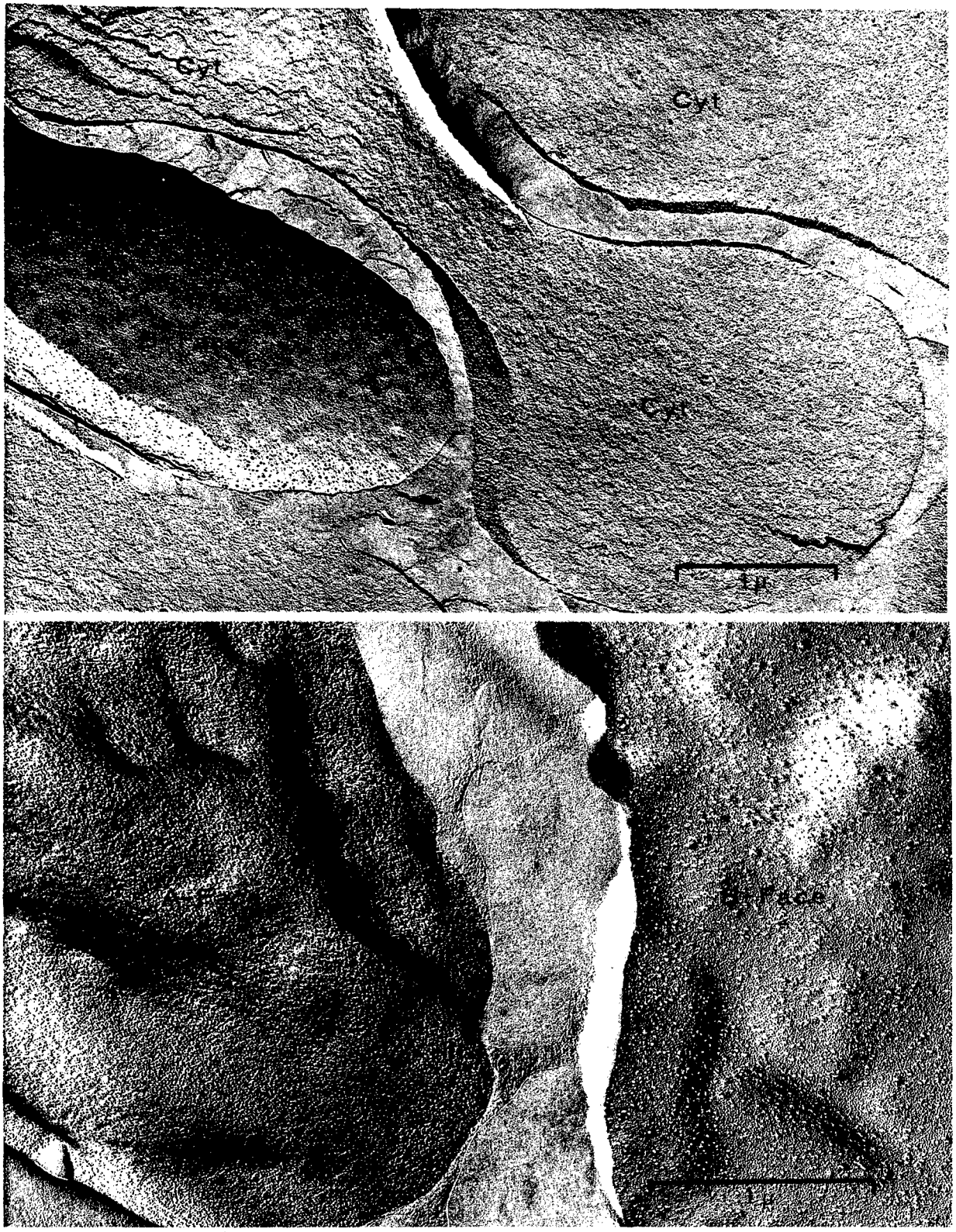

Figure 4. TOP: CONTROL, FREEZE-CLEAVED, RED CELLS. The red cells are in a biconcave configuration. Cleavage planes are flat within the cell cytoplasm (Cyt). x 27, 000.
BOTTOM: CELL MEMBRANES OF ERYTHROCYTES INCUBATED IN 0. 01 M MMH FOR 4 HOURS. The cell membranes are grossly distorted and appear as wrinkled sheets. x 38, 000.

the "B-Face". Both of the fracture faces generated by cleaving membranes bear a prominent small particulate component. These membrane-associated particles of red cells average 85 Å in replica diameter and are believed to represent intramembranous proteins. There are more particles on the A-Face than are on the B-Face. Red cell cytoplasm as seen in freeze-cleave replicas contains closely-packed spherical particles that measure 70 to 100 Å in replica diameter. It is generally accepted that these particles represent replicas of molecular hemoglobin (Haggis, 1961; Weinstein and Bullivant, 1967; Weinstein and McNutt, 1970).

Discrete areas of cytoplasm are modified in red cells incubated with MMH, as viewed in freeze-cleave preparations. The areas correspond in size and distribution to the densely stained areas that are demonstrated by supravital staining preparations with the light microscope and, therefore, are believed to represent Heinz bodies. The areas appear coarsely granulofibrillar and are not limited by a membrane (figure 1). Within these areas are loosely-aggregated small fibrils that are 70 to 100 Å in replica diameter and measure up to 700 Å in length. There are also 70 to 100 Å spherical structures that cast long shadows and may represent cross-fractured fibrils. The fibrils rarely branch and are unordered.

The formation of Heinz bodies was followed by freeze-cleaving (figure 2). Small, round Heinz bodies, approximately 0.5 microns in diameter, are present and well-formed in cells after exposure to 0.01 M MMH for 30 minutes, the earliest exposure interval examined in this study. The demonstration of Heinz bodies at 30 minutes is noteworthy since they were not convincingly shown by light microscopy at this time. The vast majority of the Heinz bodies first appear away from the cell membrane. Heinz bodies may occur singly, although more frequently they appear in replicas to be fusing with other Heinz bodies (figure 1). Attachment of Heinz bodies to the cell membrane is rarely observed at 30 minutes or 1 hour, but even at 24 hours the majority of Heinz bodies observed are still not attached to the membrane.

Effects of MMH incubation on red cell membrane were surveyed by freeze-cleaving. Incubation with MMH results in a loss of the biconcave configuration associated with normal red cells. A few small endocytotic and exocytotic vesicles devoid of membrane-associated particles are observed "budding" into the intracellular and extracellular compartments (figure 3). These are occasionally seen in control cells but are more prevalent in MMH preparations. Another feature occasionally encountered at the A-Face (the fracture face of the membrane inner leaflet) of cleaved red cell membranes is small, shallow flat-faced craters. The craters measure up to 1500 Å in diameter (figure 3).

Enzyme Activity

The results of the measurement of activity of G6PD, 6PGD, and GSSG-R as shown in table I indicate that exposure to 0.01 M MMH for 2 hours at 37 C has no significant effect on the activity of these enzymes.

TABLE I

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD), 6-PHOSPHOGLUCONIC DEHYDROGENASE (6PGD), AND GLUTATHIONE REDUCTASE (GSSG-R) ACTIVITIES FOR CONTROL AND MMH-EXPOSED HUMAN RED BLOOD CELLS.

Effects of MMH on Red Cell Enzyme Activity

m I.U./10^9 red cells[a]

	G6PD	6PGD	GSSG-R
Control[b]	140 ± 29	84.9 ± 8	84.0 ± 6.7
Exposed[b]	137 ± 20	79.5 ± 10	91.4 ± 8.8

a. Values are mean ± S. D. n = 10.

b. Control cells incubated with buffered saline 2 hours 37°C.
Exposed cells incubated with MMH, 10mM, 2 hours 37°C.

Reduced Glutathione Levels

The cells exposed to MMH in an incubation medium containing 100 mg% glucose exhibit a decrease in GSH of approximately 40% at 2 hours with a subsequent return to baseline levels at 4 hours incubation (table II and figure 5). The cells exposed to MMH in an incubation medium with no added glucose show a progressive decrease in GSH over 4 hours with almost total depletion of GSH at 4 hours. Control levels of GSH in samples with and without glucose added to the incubation medium are quite stable over the 4-hour period. However, those with added glucose show a slight rise at 4 hours which is probably due to the reduction of existing GSSG to GSH by the GSSG-R system. Both control cells and exposed cells exhibit extremely low levels of GSH at 24 hours when no glucose is added to the medium (not illustrated).

Methemoglobin Levels

The formation of methemoglobin in cells exposed to 0.01 M MMH and 0.001 M MMH is shown in figure 6. In the cells exposed to 0.01 M MMH, maximum levels of methemoglobin are reached after 30 to 60 minutes of incubation followed

TABLE II

GSH LEVELS IN HUMAN RED BLOOD CELLS INCUBATED WITH MMH

Effects of MMH on GSH after Various Exposure Times

μg GSH/10^9 red cells[a]

	Baseline	1 hr.	2 hr.	3 hr.	4 hr.
Control[b]	63.74	61.85	60.50	59.42	71.35
Exposed[b]	61.72	48.81	38.27	57.27	60.89
Control[c]	68.28	66.15	61.21	70.94	69.46
Exposed[c]	68.28	36.61	25.39	11.20	7.49

a. Mean values n = 5.

b. Control cells incubated with buffered saline containing 200 mg% glucose.
Exposed cells incubated with MMH, 10mM, containing 200 mg% glucose.

c. Control cells incubated with buffered saline with no glucose added.
Exposed cells incubated with MMH, 10mM, with no glucose added.

by a gradual decrease over a 3-hour period. Cells exposed to 0.001 M MMH reach a peak level between 90 and 120 minutes. Levels are still above baseline at 6 hours for both concentrations. This is probably due to the presence of chromogens commonly classified as sulfhemoglobins since they are resistant to conversion to cyanmethemoglobin.

Osmotic Fragility

Osmotic fragilities of red cells are unaltered by exposure to 0.01 M MMH for 30 minutes, or 2 or 4 hours.

Gravimetric Analysis

Ghosts of cells exposed to MMH plus "free" Heinz bodies (not attached to ghost membranes) have only approximately 1.5 times as much protein and 3.4 times as much hemoglobin as ghosts of control cells, indicating the relatively small percentage of hemoglobin precipitated as Heinz bodies.

DISCUSSION

MMH is a strong reducing agent that produces biologic effects on red cells that have been considered to be characteristic of oxidative damage. These changes include methemoglobin production, hemoglobin denaturation in the form of Heinz bodies, and oxidation of GSH to GSSG. Other similar compounds, such as phenylhydrazine and acetylphenylhydrazine are also highly reactive redox compounds which produce some of these same changes in red cells. All of these compounds oxidize in the presence of oxygen into intermediates that may be responsible for damaging red blood cells.

Recent studies by Kosower et al. (1969) suggest that a free radical mechanism may explain the kinds of changes that we have observed in MMH-exposed red cells. They produced intracellular oxidation of GSH to GSSG with an "azoester", methylphenyldiazinecarboxylate ($C_6H_5N{=}NCOOCH_3$). Azoester in excess of that required to oxidize GSH causes oxidation of ferrohemoglobin to ferrihemoglobin and Heinz body formation. According to Kosower et al. (1971) these biological effects are explained on the basis of free radical formation: hydrolysis of the azoester produces phenyldiazine which reacts with oxygen to generate up to 4 reactive radicals (i.e., $C_6H_5N{=}N\cdot$, $C_6H_5\cdot$, $C_6H_5OO\cdot$, and $HOO\cdot$). GSH may be oxidized to GSSG by radical coupling. For example, equations 1 and 2:

$$C_6H_5\cdot + GSH \longrightarrow C_6H_6 + GS\cdot \qquad (1)$$

$$GS\cdot + GS\cdot \longrightarrow GSSG \qquad (2)$$

Methemoglobin formation could be mediated by free radicals through one of two reactions: a double, one-electron transfer reaction, equation 3:

$$C_6H_5\cdot + Fe^{++}O_2 \longrightarrow C_6H_5OO^- + Fe^{+++} \qquad (3)$$

(Oxyhemoglobin) (Methemoglobin)

or by a direct one-electron transfer, equation 4:

$$C_6H_5OO\cdot + Fe^{++}O_2 \longrightarrow C_6H_5OO^- + Fe^{+++} \qquad (4)$$

Free radicals could also provide a mechanism for the denaturation of hemoglobin and the formation of Heinz bodies through the formation of bonds within protein molecules which could lead to the unfolding of peptide chains and the formation of bonds between hemoglobin molecules. Bonds could form as the result of dimerization of protein thiol· radicals, equation 5:

$$\text{Protein S}\cdot + \text{Protein S}\cdot \longrightarrow \text{Protein S-S protein} \qquad (5)$$

Homolytic reactions may generate free radicals from MMH in our in vitro system. The degradation of MMH to CH_4, N_2, NH_3 and methanol is very rapid in the presence of oxygen at room temperature (Vernot et al., 1967). It has been suggested that the initial reaction of MMH and oxygen involves the formation of methyldiazine. Methyldiazine, $CH_3N{=}NH$, may react with oxygen to produce several free radicals, equations 6 through 8:

$$CH_3NH{=}NH + O_2 \longrightarrow CH_3N{=}N\cdot + HOO\cdot \quad (6)$$

$$CH_3N{=}N\cdot \longrightarrow CH_3\cdot + N_2 \quad (7)$$

$$CH_3\cdot + O_2 \longrightarrow CH_3OO\cdot \quad (8)$$

The methyldiazine may also undergo a bimolecular coupling reaction to form symmetrical dimethylhydrazine which, in the presence of excess oxygen, is oxidized to azomethane (dimethyldiazine, $CH_3N{=}NCH_3$), a potent free radical source. Hydrogen peroxide generated by the system could in turn form free radicals, although it is rapidly destroyed catalytically by catalase, an enzyme found in great abundance in red cells.

It is well established that adequate levels of GSH have an essential role in maintaining the integrity of the red cells. GSH serves (1) as an agent for the maintenance of enzyme SH groups in the reduced form, (2) as a catalyst for structural rearrangements in proteins through disulfide interchange reactions, and (3) as a hydrogen donor to potentially harmful free radicals. Decreased GSH levels are observed in several different clinical settings. Persistent low GSH levels are associated with a spectrum of nonspherocytic hemolytic anemias that are caused by deficiencies of hexose monophosphate shunt enzymes such as G6PD, 6PGD, etc. Inadequate HMP activity results in a deficiency of NADPH, an essential cofactor for GSSG-R and methemoglobin reductase. GSH levels are also lowered in congenital Heinz body hemolytic anemias. In these anemias unstable mutant hemoglobins spontaneously precipitate as Heinz bodies.

It has been postulated that the cysteines in the beta chains of the abnormal hemoglobins excessively bind GSH in stable mixed disulfide linkages resulting in diminished intracellular GSH which in turn apparently stimulates HMP metabolism (Jacob et al., 1968). It has been reported that GSH levels must be markedly lowered before red cells are adversely affected. For example, Jacob and Jandl (1966) lowered red cell glutathione content to 10% of normal with n-ethylmaleimide without affecting red cell metabolism in vitro or red cell survival in vivo. Also Kosower et al. (1971) found that oxidation of 70-80% of intracellular GSH with azoester produces little observable effect. MMH lowers the intracellular GSH approximately 40% at 2 hours. In the presence of adequate glucose, GSH levels are restored to normal at 4 hours of incubation. The transient fall in GSH may result from binding of GSH to denatured protein during the formation of Heinz

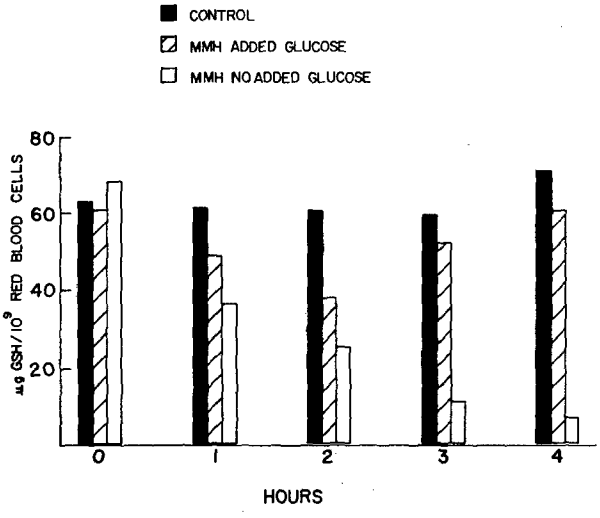

Figure 5.

GSH LEVELS IN HUMAN RED BLOOD CELLS INCUBATED WITH MMH. Bar Graph of the data in table II. GSH levels are transiently lowered in the presence of glucose and progressively decline in the absence of glucose.

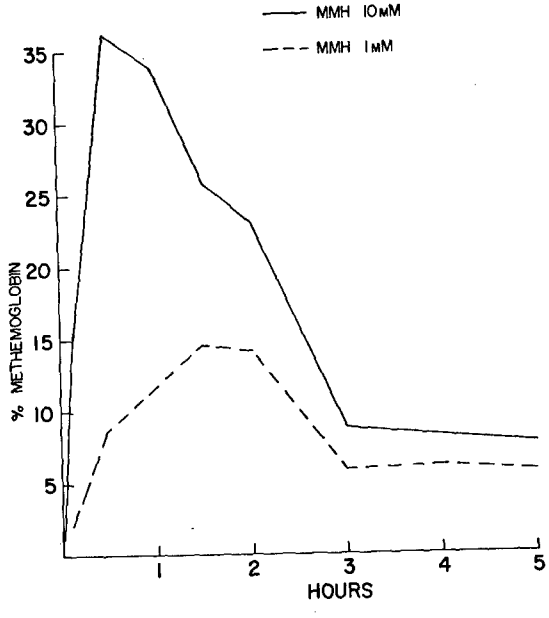

Figure 6.

RED BLOOD CELL METHEMOGLOBIN LEVELS DURING INCUBATION OF CELLS WITH MMH.

bodies and/or by oxidation to GSSG. Recovery of GSH levels in the presence of glucose may be related to the stimulation of the HMP although shunt activity was not measured in these studies. However, the competence of the HMP shunt is supported by results of this study that show normal levels of HMP shunt enzymes after incubation of red cells with high levels of MMH.

The relationship between the oxidation of ferrohemoglobin to ferrihemoglobin, methemoglobin, and the precipitation and denaturation of the hemoglobin in the form of Heinz bodies is uncertain. Jandl et al. (1960) suggested that methemoglobin invariably occurs in the early stages of Heinz body anemias and that those instances in which methemoglobin is not observed can be explained by the transient nature of the reaction. Allen and Jandl (1961) proposed that oxidant drugs and chemicals produce the following sequence of events: oxidation of GSH to GSSG; oxidation of ferrohemoglobin to methemoglobin; oxidation of sulfhydryl groups on the hemoglobin molecule and binding of GSSG to hemoglobin sulfhydryl groups resulting in an alteration of the configuration of the hemoglobin molecule; oxidation of other hemoglobin sulfhydryl groups; and, finally, precipitation of the altered hemoglobin as Heinz bodies. In this scheme, oxidation of both ferrohemoglobin and GSH play an essential role in the mechanism of Heinz body formation. Rentsch (1968) has questioned the validity of the Jandl scheme. He believes that methemoglobin formation may occur concurrently with or before Heinz body production but that it is not a prerequisite in the sequence of events leading to hemoglobin precipitation. He based his argument on the observation that certain chemicals, i.e., sodium nitrite, produce methemoglobin without GSH oxidation or Heinz body formation and that the time of appearance of Heinz bodies and the time of methemoglobin production and disappearance varies with exposure to different compounds.

The current study on MMH-induced Heinz body anemias provides further examples of the lack of strict correlation between methemoglobin production, Heinz body formation, and decreased GSH levels. In our in vitro system, ferrohemoglobin is oxidized to methemoglobin and GSH levels are slightly diminished during Heinz body growth. Near normal levels of GSH are present at the time of initiation of growth (i.e., at 30 minutes of incubation) in 0.01 M MMH and Heinz body growth continues to be prominent after 2 to 4 hours of incubation with high levels of MMH although methemoglobin and GSH levels are returning to normal. In this regard, it is also noteworthy that there are large species differences in the response to MMH. Monkey and rat red cells produce little methemoglobin but do show decreases in GSH levels and the formation of Heinz bodies. Inhalation of MMH by humans produces no measurable increase in methemoglobin although Heinz bodies are present in red cells up to 2 weeks after exposure (MacEwen et al., 1970).

The results of our morphologic studies on Heinz bodies in MMH-exposed red cells are significantly different from the changes reported by Kosower et al.

(1969;1971) for azoester-treated red cells and the usual findings in nonspherocytic hereditary anemias in which Heinz body production is associated with oxidative stress in the face of low GSH levels. Kosower et al. (1971) found few Heinz bodies produced in their in vitro system when GSH oxidation by azoester was incomplete. With MMH exposures, Heinz bodies become prominent when intracellular GSH levels are in excess of 50% of normal, a level usually considered as adequate. Therefore, the production of Heinz bodies by MMH seems to be independent of the cellular GSH concentration. This finding would seem to argue against a free radical mechanism as an explanation for Heinz body formation since intracellular GSH levels in red cells in our system should be adequate to scavenge harmful free radicals. However, we suggest that a free radical mechanism may be involved in producing damage to red cells even in the face of adequate levels of GSH. Free radical reactions can be extremely rapid and GSH may only act as a scavenger when it is in close proximity to free radicals. Our data are consistent with the hypothesis that GSH is not uniformly distributed in the cytoplasm of red cells. This would explain the focal vulnerability of hemoglobin to denaturation and precipitation as Heinz bodies. Only small areas of cytoplasm need be depleted of GSH since Heinz bodies represent a minor component in MMH-exposed cells, as is indicated by our gravimetric analyses which show that a small percentage of cell protein is incorporated into Heinz bodies.

The direct effect of MMH on the cytoplasm of red cells is manifested by Heinz body formation. Heinz bodies attaching to the cell membrane may provide a mechanism for membrane injury (see review by Jacob, 1970). It is also conceivable that MMH directly injures the red cell membrane since free radicals could interact with membrane lipid and SH-containing membrane proteins. Alterations in membranes including gross deformation and increased endocytosis and exocytosis were observed in this study. These changes appear unrelated to intracellular Heinz bodies since the Heinz bodies produced in our experimental system are typically some distance from the cell membrane. The suggestion that membrane alterations are the result of direct membrane injury via a free radical mechanism could be tested by examining cell membranes for the development of abnormal conjugated dienes which would be indicative of lipid peroxidation.

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PAPER NO. 19

TOXICOLOGICAL STUDIES ON A BERYLLIUM CONTAINING EXHAUST PRODUCT

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INTRODUCTION

The major toxicological portion of the beryllium propellant development program of the U. S. Air Force Rocket Propulsion Laboratories was performed by The Dow Chemical Company during the period from 1963 to 1968 (Spencer et al., 1968). Results of long-term studies on rats, injected intratracheally with well-characterized key samples of beryllium oxide prepared by calcining beryllium hydroxide for 10 hours at 500, 1100, and 1600 C, respectively, showed clearly that there is a definite gradation in biological response depending upon the oxide administered. Thus, the oxide calcined at 500 C was highly active as judged by histopathological examination of the lungs, incidence of tumors, and translocation of beryllium from the lungs to other tissues. In contrast, the oxide calcined at 1600 C showed much less severe effects. Dose-response studies were carried out, using carefully prepared subsamples of "respirable particle size" of the three key oxides; results showed a definite gradation in response which diminished with decreasing dosages of the administered oxide. Investigations on motor exhaust products have shown that most samples have chemical, physical, and toxicological properties similar to the beryllium oxide calcined at 1600 C. On the other hand, other samples were heterogeneous, contained considerable quantities of water-soluble beryllium, and varied in toxicity.

The present research study* was designed to define the physical and chemical characteristics and toxicological properties of an exhaust product collected from a beryllium-fueled NASA-JPL High Energy Upper Stage (HEUS) motor. Comparisons have been made with selected exhaust products previously studied as well as with key beryllium oxide samples of varying physical properties and biological activity. The sample studied will hereafter be identified as "BeO exhaust product".

*Contract No. F33615-70-C-1811

SAMPLE STUDIES

The physical and chemical properties determined on the "BeO exhaust product" are given below, along with the analytical methods employed:

Specific surface area - nitrogen adsorption.
Average crystallite size - X-ray diffraction.
Crystallinity - polarized light microscopy.
Refractive index - dispersion staining.
Density - sink-float method.
Major components of sample - X-ray powder diffraction.
Trace elements in sample - emission spectroscopy and X-ray fluorescence.
Sample solubility - gravimetric method.
Determination of major and trace components in soluble and insoluble portions - X-ray powder diffraction and emission spectroscopy.
Determination of amount of beryllium soluble in water, 0.1 N HCl and 6 N HCl - emission spectroscopic analysis of filtrate.
Elemental analysis -
beryllium by gravimetric method
carbon by microcombustion
hydrogen by microcombustion
oxygen by neutron activation
chloride by microvolumetric method
Particle characterization - light microscopy, transmission electron microscopy, and scanning electron microscopy.
Particle size - photomicrographs and Coulter Counter technique.

The physical properties of the "BeO exhaust product" are presented in table I. Data on the key samples of BeO calcined at 500, 1100, and 1600 C are included for comparative purposes. Surface area of the "BeO exhaust product" is slightly greater than that of the key BeO calcined at 1100 C, and density is midway between that of the key BeO calcined at 500 C and the density of the 1100 C material. The "BeO exhaust product" most closely resembles the key BeO calcined at 1600 C in average crystallite size and refractive index. It is slightly less crystalline than the key BeO samples calcined at 1100 and 1600 C.

The physical properties of the "BeO exhaust product" are compared with those of a group of selected exhaust products previously studied in table II. The "BeO exhaust product" is higher in surface area, smaller in average crystallite size, and slightly less crystalline than the exhaust products previously studied. Other exhaust products previously studied, with properties similar to those of ARC Nos. 1, 22, and 24, were not measured for surface area due to limited

TABLE I

PHYSICAL PROPERTIES OF "BeO EXHAUST PRODUCT"
COMPARED WITH KEY SAMPLES OF BeO

PROPERTY	BeO CALCINED FOR 10 HOURS AT:			"BeO EXHAUST PRODUCT
	500 C	1100 C	1600 C	
Specific Surface Area M^2/G	50.8	2.2	1.3	2.51
Average Crystallite Size Å	150	1500	1600	2000 ± 300
Crystallinity %	<10	100	100	95
Refractive Index	1.683 ± 0.003	1.704 ± 0.002	1.711 ± 0.007	1.712 ± 0.002
Density G/ML	2.87 ± 0.07	2.98 ± 0.02	3.00 ± 0.03	2.92 ± 0.03

TABLE II

PHYSICAL PROPERTIES OF "BeO EXHAUST PRODUCT"
COMPARED WITH EXHAUST PRODUCTS PREVIOUSLY STUDIED

PROPERTY	ARC NO. 1	ARC NO. 22	ARC NO. 24	"BeO EXHAUST PRODUCT"
Surface Area M^2/G	<2.5	0.8	0.7	2.51
Average Crystallite Size Å	>5000	>5000	>5000	2000 ± 300
Crystallinity %	100	100	100	95
Refractive Index	1.704 ± 0.004	1.709 ± 0.003	1.709 ± 0.003	1.712 ± 0.003
Density G/ML	2.80 ± 0.05	2.99 ± 0.01	2.93 ± 0.07	2.92 ± 0.03

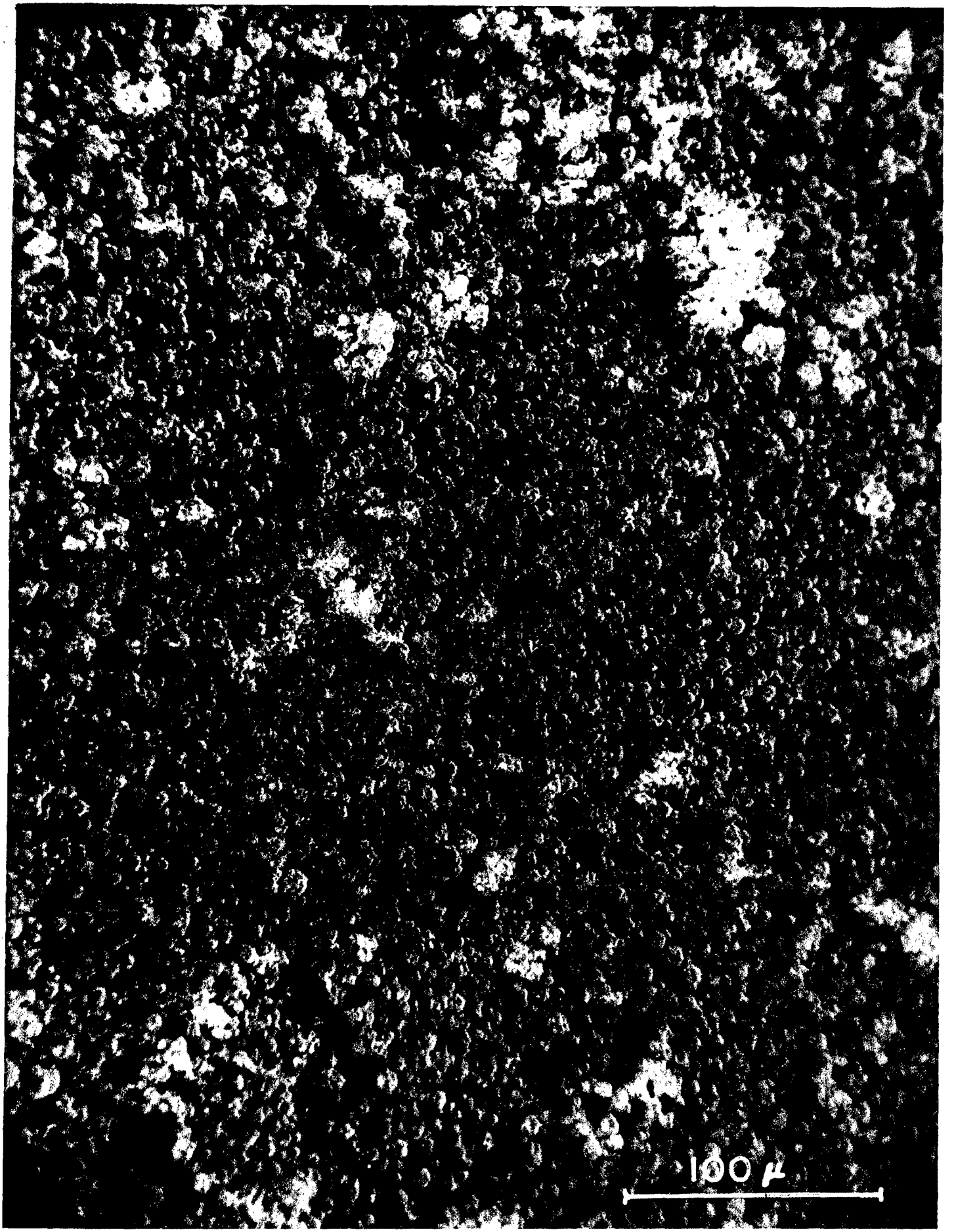

Figure 1. SCANNING ELECTRON PHOTOMICROGRAPH OF "BeO EXHAUST PRODUCT". x 500

Figure 2. SCANNING ELECTRON PHOTOMICROGRAPH OF "BeO EXHAUST PRODUCT". x 5000

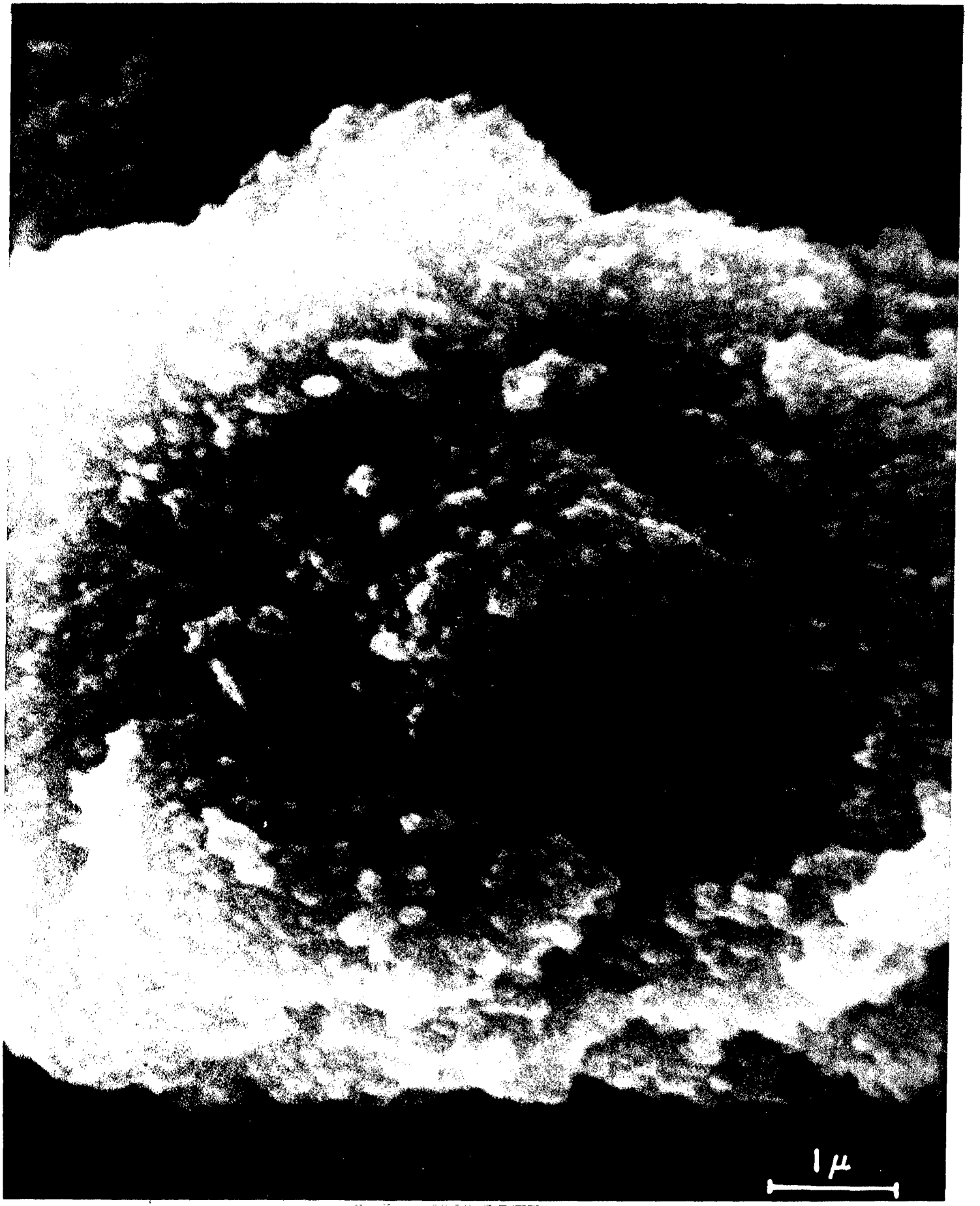

Figure 3. SCANNING ELECTRON PHOTOMICROGRAPH OF "BeO EXHAUST PRODUCT". x 25, 000

TABLE III

EXPERIMENTAL DESIGN FOR FEMALE RATS RECEIVING "BeO EXHAUST PRODUCT" BY INTRATRACHEAL ADMINISTRATION

SAMPLE	DOSE (Mg/Kg)	NUMBER OF RATS TREATED	NUMBER OF RATS SCHEDULED FOR NECROPSY			
			25 Weeks	50 Weeks	75 Weeks	100 Weeks
"BeO Exhaust Product"	50	60	10	10	10	All Survivors
	10	60	10	10	10	All Survivors
	2	60	10	10	10	All Survivors
BeO Calcined at 500 C	50	60	10	10	10	All Survivors
Saline Control	0	60	10	10	10	All Survivors
TOTAL		300	50	50	50	

TABLE IV

BERYLLIUM CONCENTRATION IN TISSUES OF RATS KILLED 25 WEEKS FOLLOWING INTRATRACHEAL ADMINISTRATION OF "BeO EXHAUST PRODUCT"

SAMPLE	DOSE Mg/Kg	BERYLLIUM CONCENTRATION (μ g/g TISSUE)			
		LIVER	KIDNEY	SPLEEN	BONE
"BeO	50	1.7	0.01	1.7	0.52
		0.07	0.01	0.09	0.45
		0.16	0.01	0.26	0.47
		0.07	0.01	0.10	0.31
	10	<0.01	<0.01	0.03	<0.1
		0.02	<0.01	0.04	<0.1
		1.3	<0.01	0.84	<0.1
		0.32	<0.01	0.40	<0.1
	2	<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1
Saline Control	0	<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1

sample quantities and are, therefore, not included in the comparative tabulation. These samples were ARC No. 3, AC No. 2 (Aerospace Corporation), "Aerospace Corporation BeO Sample from Large Motor Firings" received from Wright-Patterson Air Force Base in October, 1966, and a sample of beryllium exhaust products from Aerospace Corporation - "BeO, June 29, 1967".

Determination of the other properties studied on the "BeO exhaust product" gave unremarkable findings. The sample is primarily BeO, with low levels of impurities. Water solubility of the sample is low, with 0.71% of the total sample soluble in water (chiefly $CaSO_4 \cdot \frac{1}{2} H_2O$) and only 4 ppm soluble beryllium. Determination of particle size distribution by Coulter Counter showed 87% of the sample mass to be less than 11 μ in diameter, while examination by microscopy revealed that most of the particles were less than 5 μ in diameter.

Scanning electron photomicrographs of the "BeO exhaust product" are presented in figures 1, 2, and 3. Photomicrographs of key samples of BeO and exhaust products previously studied may be found in the report of the earlier work (Spencer et al., 1968). Figure 1 (x500) can be compared with figures 21, 24, 91, 93, and 96 of the report by Spencer et al. (1968); figure 2 (x5000) can be compared with figures 22, 25, 92, 94, and 97; and figure 3 (x25,000) with figures 23, 26, 27, 95, and 98 of the 1968 report.

ANIMAL STUDIES

The biological activity of the "BeO exhaust product" was evaluated by intratracheal administration of single doses of 50, 10, and 2 mg/kg of the sample to groups of female rats, followed by a 100-week test period. Another group of rats received 50 mg/kg of the key sample of BeO calcined at 500 C as a positive control, and a final group received saline as a negative control. The experimental design is summarized in table III. Pathological examinations were conducted on groups of rats necropsied 25, 50, 75, and 100 weeks following treatment. Studies on the translocation of beryllium to extrapulmonary tissues were carried out at 25 and 100 weeks.

Results of translocation studies on the "BeO exhaust product" 25 weeks following treatment are presented in table IV. Beryllium was detectable in all 4 tissues analyzed at the 50 mg/kg dose, while only liver and spleen showed measurable amounts at the 10 mg/kg dose. Tissues from rats treated with 2 mg/kg showed no detectable quantities of beryllium. Translocation studies 100 weeks following treatment with "BeO exhaust product" showed a similar decrease in beryllium concentration with decreasing dose (table V). The analyses conducted after 100 weeks also showed slight increases in beryllium concentration over the levels at 25 weeks.

TABLE V

BERYLLIUM CONCENTRATION IN TISSUES OF RATS KILLED 100 WEEKS FOLLOWING INTRATRACHEAL ADMINISTRATION OF "BeO EXHAUST PRODUCT"

SAMPLE	DOSE Mg/Kg	BERYLLIUM CONCENTRATION (μg/g TISSUE) LIVER	KIDNEY	SPLEEN	BONE
"BeO Exhaust Product"	50	0.26	0.02	1.0	0.51
		11.0	0.04	34.0	1.3
		1.2	0.03	0.89	0.49
		0.22	0.02	0.44	0.51
	10	1.5	<0.01	3.0	<0.1
		0.07	<0.01	0.12	<0.1
		0.02	<0.01	0.05	<0.1
		0.15	<0.01	0.25	<0.1
	2	0.01	<0.01	0.02	<0.1
		0.01	<0.01	0.03	<0.1
		1.4	<0.01	1.6	<0.1
		0.02	<0.01	0.04	<0.1
Saline Control	0	<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1
		<0.01	<0.01	<0.01	<0.1

Translocation of beryllium from the "BeO exhaust product" 25 weeks following treatment is compared with data obtained after similar time periods on key BeO samples and exhaust products previously studied in table VI. Levels of beryllium in tissues from rats treated with the key BeO calcined at 500 C are greater than those found in tissues from rats treated with "BeO exhaust product", while the latter sample in turn shows slightly greater translocation than found from the key BeO calcined at 1600 C or ARC Nos. 22 and 24. (Note that the doses of the key BeO calcined at 1600 C, ARC No. 22 and ARC No. 24, were approximately twice that of the "BeO exhaust product".) A comparison of the 100-week translocation data on the "BeO exhaust product" with long-term data on key BeO samples and exhaust products previously studied is presented in table VII. Although the data are more variable, the same general comparative relationship that was observed in the 25-week data is evident. (Note again the differences in dose.)

TABLE VI

TRANSLOCATION TO EXTRAPULMONARY TISSUES OF BERYLLIUM FROM "BeO EXHAUST PRODUCT" AT 25 WEEKS COMPARED WITH KEY BeO SAMPLES AND EXHAUST PRODUCTS PREVIOUSLY STUDIED

SAMPLE	DOSE Mg/Kg	WEEKS POST TREATMENT	BERYLLIUM CONCENTRATION (μg/g TISSUE) LIVER	KIDNEY	SPLEEN	BONE
Key BeO Calcined at 500 C	50	25	3.2	0.11	3.6	6.0
			1.3	0.12	1.1	5.0
			0.15	0.11	0.18	5.0
			0.21	0.11	0.36	6.5
"BeO Exhaust Product"	50	25	1.7	0.01	1.7	0.52
			0.07	0.01	0.09	0.45
			0.16	0.01	0.26	0.47
			0.07	0.01	0.10	0.31
Key BeO Calcined at 1600 C	~100	24	0.022	< 0.003	0.10	0.10
			0.053	< 0.003	0.11	0.06
			0.070	0.003	0.26	0.11
			0.028	0.004	0.16	0.11
			0.010	0.004	0.040	0.14
			0.007	0.004	0.038	0.09
ARC No. 22	~100	21	0.010	< 0.003	0.019	0.28
			0.005	0.004	0.037	0.60
			0.004	< 0.003	0.015	0.23
			0.010	< 0.003	0.063	0.48
ARC No. 24	~100	21	0.096	0.004	0.014	0.43
			0.004	0.004	0.029	0.16

TABLE VII

TRANSLOCATION TO EXTRAPULMONARY TISSUES OF BERYLLIUM FROM "BeO EXHAUST PRODUCT" AT 100 WEEKS COMPARED WITH KEY BeO SAMPLES AND EXHAUST PRODUCTS PREVIOUSLY STUDIED

SAMPLE	DOSE Mg/Kg	WEEKS POST TREATMENT	BERYLLIUM CONCENTRATION (μg/g TISSUE) LIVER	KIDNEY	SPLEEN	BONE
Key BeO Calcined at 500 C	50	100	0.11	0.15	0.39	3.2
		100	0.46	0.27	1.3	5.7
		100	0.24	0.20	0.97	5.2
		100	1.4	0.30	7.9	8.5
"BeO Exhaust Product"	50	100	0.26	0.02	1.0	0.51
	50	100	11.0	0.04	34.0	1.3
	50	100	1.2	0.03	0.89	0.49
	50	100	0.22	0.02	0.44	0.51
Key BeO Calcined at 1600 C	50	80	1.1	0.009	0.92	0.4
	50	80	0.029	$<$0.003	0.12	0.5
	$\backsim$100	98	0.60	0.008	1.0	0.75
	$\backsim$100	98	0.60	0.009	0.90	0.30
	$\backsim$100	98	0.34	0.006	0.64	0.20
	$\backsim$100	98	0.43	0.007	0.70	0.70
ARC 22	$\backsim$100	77	0.012	0.004	0.022	0.2
	$\backsim$100	77	0.006	0.003	0.037	0.3
	$\backsim$100	77	0.005	0.004	0.042	0.4
ARC 24	50	55	0.008	$<$0.003	0.009	0.4
	50	55	0.007	$<$0.003	0.035	0.5
	50	65	0.013	$<$0.003	0.036	0.4

Histopathological examination revealed a typically severe pulmonary response in rats treated with 50 mg/kg key BeO calcined at 500 C. This reaction can be described as a progressive proliferative response characterized in the earlier phases by fibrotic and epithelial metaplastic changes, advancing into neoplastic changes as indicated by the development of pulmonary carcinomas. The associated lymph nodes from these rats showed evidence of fibrotic proliferation. The pulmonary lesions observed in the lungs of rats treated with 50 mg/kg "BeO exhaust product" were less severe than those induced by the key BeO calcined at 500 C, but were similar in nature. The severity of the pulmonary response diminished with a decrease in dose of the "BeO exhaust product", with only focal, minimal changes observed at the 2 mg/kg dose. No evidence of a proliferative response was observed in the associated lymph nodes from rats treated with even the highest dose of "BeO exhaust product".

The occurrence of primary pulmonary tumors in rats treated with 50 mg/kg key BeO calcined at 500 C or with 50 mg/kg "BeO exhaust product" is presented in table VIII. The vast majority of all these tumors were carcinomas, with occasional metastases to the associated lymph nodes. A few tumors were more appropriately classified as adenomas. Since the earliest primary pulmonary tumor was noted 58 weeks following treatment in the group of rats that had received 50 mg/kg of the key BeO calcined at 500 C, the tabulation was started at that point. For comparative purposes, 58 weeks was used as the starting time in tabulation of tumors in the group treated with 50 mg/kg "BeO exhaust product", although the first tumor was not noted in this group until the 75-week necropsy. The tabulation shows a higher occurrence of tumors in the group of rats treated with 50 mg/kg key BeO calcined at 500 C than was found at the same dose of the "BeO exhaust product". Only one pulmonary tumor was observed in the group of rats treated with 10 mg/kg "BeO exhaust product". None of the rats treated with 2 mg/kg "BeO exhaust product" had pulmonary tumors.

TABLE VIII

PRIMARY PULMONARY TUMORS IN RATS FOLLOWING SINGLE INTRATRACHEAL ADMINISTRATION OF "BeO EXHAUST PRODUCT" AND KEY BeO CALCINED AT 500 C

	CUMMULATIVE TOTALS					
	KEY BeO 500 C 50 Mg/Kg			"BeO EXHAUST PRODUCT" 50 Mg/Kg		
WEEKS POST TREATMENT	NUMBER RATS EXAMINED	NUMBER RATS W/TUMORS	% RATS WITH TUMORS	NUMBER RATS EXAMINED	NUMBER RATS W/TUMORS	% RATS WITH TUMORS
58	1	1	100	1	0	0
67	5	2	40	1	0	0
73	7	3	43	3	0	0
75	17	9	53	13	5	38
80	21	11	52	16	6	38
86	23	11	48	19	7	37
87	27	15	56	23	9	39
89	28	16	57	28	10	36
93	30	18	60	29	10	35
95	31	19	61	29	10	35
98	32	20	62	29	10	35
100	37	25	68	39	19	49

In comparison with the three key BeO samples (calcined at 500, 1100, and 1600 C), the "BeO exhaust product" is less active, as far as tumorigenicity is concerned, than the key BeO calcined at 500 C, but is more active than the key BeO samples calcined at 1100 and 1600 C. Data regarding the tumorigenicity of the three key samples of BeO are presented in the report of the earlier work (Spencer et al., 1968).

SUMMARY

Physical and chemical characterization studies on an exhaust product collected from a beryllium-fueled NASA-JPL High Energy Upper Stage motor ("BeO exhaust product") showed the material to be similar to the key samples of BeO calcined at 1100 and 1600 C, and to a group of exhaust products previously studied (ARC Nos. 1, 22, and 24). A slightly higher surface area, smaller crystallite size, and lower crystallinity of the "BeO exhaust product" were the notable differences from the exhaust products previously studied.

The biological activity was evaluated during a 100-week period following intratracheal administration of the "BeO exhaust product" to rats in single doses of 50, 10, and 2 mg/kg. Criteria used for judgment were analysis of beryllium in extrapulmonary tissues and histopathological examination of the lung.

Studies of the translocation of beryllium to extrapulmonary tissues (liver, kidney, spleen, and bone), conducted 25 and 100 weeks following treatment, showed the translocation of beryllium from the "BeO exhaust product" to be considerably lower than that of the key BeO calcined at 500 C, but slightly greater than that of the key BeO calcined at 1600 C and ARC Nos. 22 and 24.

Histopathological examination 25, 50, 75, and 100 weeks following treatment revealed a pulmonary response less severe than that induced by the key BeO calcined at 500 C, but with lesions similar in nature. Evaluation of tumorigenicity of the "BeO exhaust product" showed fewer tumors than were found in rats treated with the key BeO calcined at 500 C. The tumors found in rats treated with the "BeO exhaust product" also occurred later than those induced by the key BeO calcined at 500 C.

ACKNOWLEDGMENTS

The authors wish to acknowledge the valuable contributions of Dr. G. L. Sparschu and Dr. Paul Gross in the pathological evaluations, and to thank the personnel in various analytical laboratories of The Dow Chemical Company for their work on the physical and chemical characterization of the sample.

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DISCUSSION

DR. THOMAS (Aerospace Medical Research Laboratory): Was that a perfect static firing, or was that the one which blew up half way?

DR. BACK (Aerospace Medical Research Laboratory): No, this was a perfect firing. For those of you who don't know, the ARC (Atlantic Research) material was fired in a tank at altitude and that's why it's so clean. It was fired in a tank at altitude through a water scrubber. It was I suppose almost a hard vacuum. If they fire in a closed system, they have to fire into a vacuum. That's why it's so clean. Now, this other thing was fired outside and maybe the reason it looks so amorphous is because of the way it finally cooled. Cooling one way through a water scrubber at altitude as opposed to cooling out in the tropics, outside in the ambient air, probably gave us the differences. That's what we're looking for, obviously. We just made three new samples with the good help of Dow Chemical. But we don't know whether somebody is going to let us work on them or not since beryllium is a bad name. But if we ever get any money to do it and if somebody will let us, we are certainly going to put two years effort into this, and I hope we can continue this work. Since we are this far along, it would be a shame not to get the full answer.

AMRL-TR-72-130

PAPER NO. 20

DETERMINATION OF TRACE LEVELS OF BERYLLIUM OXIDE IN BIOLOGICAL MEDIA

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INTRODUCTION

Beryllium oxide is a potentially toxic compound which appears as the major component of the exhaust of solid fuel rocket motors which contain beryllium metal as a propellant additive. Spencer (1968) at Dow Chemical has shown that the carcinogenic activity of beryllium oxide is a function of the temperature to which the oxide has been exposed. Low-fired BeO (calcined at 500 C) is highly carcinogenic while high-fired BeO (calcined at 1600 C) is essentially inert. In order to further investigate the toxicity of beryllium oxide and to assess the amount present in the environment as a result of rocket motor firings, it is desirable to have a simple analytical method capable of determining trace levels of both the high-fired and low-fired forms of the oxide.

One of the simplest and most sensitive methods for measurement of beryllium was pioneered in the Aerospace Research Laboratories at Wright-Patterson AFB by Dr. Robert Sievers (Mosier and Sievers, 1965). This is the gas chromatography of trifluoroacetylacetone chelates of beryllium with detection by electron capture. Taylor and Arnold (1971), working here at the Toxic Hazards Division, developed a simple version of this method for the determination of soluble forms of beryllium in aqueous, blood, and tissue media. They carried out all reactions in sealed ampoules, thus keeping the manipulations to a minimum and allowing for maximum sensitivity by permitting the use of small solution volumes. Following the success of this method for soluble beryllium, we desired to see if it could be employed for the analysis of the various forms of the insoluble oxide.

This extension of the method to beryllium oxide was done as a joint effort of the Chemical Hazards Branch of the Toxic Hazards Division together with an outside contractor, the Monsanto Research Corporation. Funds for the contract were obtained from the Air Force Rocket Propulsion Laboratory. The Principal Investigator for Monsanto was Dr. William G. Scribner, who was responsible for the preparation of radioactive beryllium oxide and all radiochemical measurements in this work. I would also like to acknowledge the extensive work done by technicians Tom Ctvrtnicek of the Monsanto Laboratory and Sgt. Rod Ford in my own lab.

EXPERIMENTAL APPROACH

In previous experiments at the Monsanto Laboratory, we observed that radiolabeled high-fired beryllium oxide could be dissolved by concentrated trifluoroacetylacetone, and we suspected that it should be possible to use the chelation-gas chromatography procedure for direct analysis of the oxide.

To prepare samples of known small amounts of beryllium oxide for the development of an analytical method proved to be rather difficult. Unlike solutions of soluble Be^{+2} ion, suspensions of beryllium oxide cannot be diluted, as the small solid oxide particles tend to be adsorbed on the walls of the containers and pipettes and are nonuniformly distributed in the various dilution steps. Attempts to make samples of low enough concentration to be detected by the sensitive electron capture gas chromatographic method failed repeatedly. This problem could be solved by synthesizing ^{7}Be-labeled beryllium oxide and measuring the amount of oxide present in a dilute suspension radiochemically.

The following goals for a joint USAF-Contractor research program were set:

A. Synthesis of ^{7}Be-labeled low-fired and high-fired beryllium oxide.

B. Investigation of the parameters of chelate concentration, and the length and temperature of heating to optimize the ampoule method of Taylor and Arnold for the analysis of beryllium oxide in blood and tissue.

C. Application of the developed method to organs of animals containing beryllium translocated from a dose of the oxide injected into the lungs.

D. An attempt to determine whether translocated beryllium is in a soluble or insoluble form.

RESULTS AND DISCUSSION

Low-fired ^{7}Be-labeled BeO was prepared by precipitating beryllium hydroxide from a beryllium sulfate solution containing $^{7}BeCl_2$ and calcining at 500 C for 10 hours. The product was ground and then sized to a 1-5 micron size range by Stokes Law sedimentation. Under light microscopic examination it appeared flaky, translucent, with a rough, mottled surface.

High fired ^{7}Be-labeled BeO was prepared by taking 500 C-calcined BeO prepared in the fashion just described and calcining it an additional 10 hours at 1600 C in a Lucifer high temperature furnace. The product was ground and sized to a 1-10 micron size range and appeared distinctly different from the low-fired preparation, being composed of transparent, sharply fractured, crystalline material.

We first attempted to employ the ampoule reaction technique of Taylor and Arnold on samples of aqueous beryllium oxide suspensions. After several months of work we had to abandon this approach, as interferences which would mask the beryllium peak at the trace levels we desired to measure could not be eliminated. I will briefly describe why this promising approach did not work out so you can see why we abandoned it for a more complicated procedure.

Table I describes the ampoule procedure for direct chelation.

TABLE I

DIRECT CHELATION (AMPOULE) PROCEDURE

1. Add 0.2 ml of BeO-containing sample and 0.2 ml of neat H(tfa) to an ampoule made by sealing one end of a DISPO-brand pasteur pipette in a flame.

2. Flame seal open end of ampoule, shake, and place in an oven at 175 C overnight.

3. Break open ampoule, add 0.5 ml of benzene and wash through a small column containing Sephadex G-10 saturated with 1.0 M NaOH.

4. Inject 1.0 microliter of benzene effluent into gas chromatograph. The beryllium peak appears in about 2 minutes, and the amount present is determined by comparison with peak heights of beryllium trifluoroacetylacetonate standards.

Table II describes the parameters of gas chromatographic operation.

TABLE II

PARAMETERS OF GAS CHROMATOGRAPHIC OPERATION

1. Column: 6-ft., 2-mm i.d. with glass U-tubes, silanized with dimethyldichlorosilane, packed with 60-80 mesh 5% SE-52 on Gas Chrom Z.

2. Carrier Gas: J. T. Baker prepurified nitrogen at 100 ml/min.

3. Instrument: Varian 2100 biological GC with a DC-H^3-ionized electron capture detector.

4. Chromatographic conditions: Column temp. 110 C, Injector temp. 140 C, Detector temp. 180 C. Direct on column injection. Unknowns measured by injecting 1 microliter samples with a Hamilton Syringe and comparing peak heights with similar injections of appropriate standards prepared by dissolving twice resublimed beryllium trifluoroacetylacetonate in benzene at the appropriate concentration.

Our studies of the variation of parameters of (a) trifluoroacetylacetone concentration, and (b) length and temperature of heating showed that high-fired beryllium oxide could be completely chelated using neat trifluoroacetylacetone at 175 C for 16 hours. Low-fired beryllium oxide required somewhat less stringent conditions.

Table III shows the amounts of beryllium found by gas chromatography in an experiment conducted under such conditions.

TABLE III

PER CENT RECOVERY

Sephadex Column	1:5 NH_5OH Extraction
82	99
82	92
98	100
81	99. 5
57	98. 5
81	96. 5

The right-hand column shows the percent recovery of beryllium oxide as the chelate when ammonia is used to remove excess trifluoroacetylacetone from a greatly diluted sample. It indicates that chelation is complete. For a sample containing 1 nanogram BeO/gram, the Sephadex column procedure must be used as the sample cannot be so diluted, and so much ammonia would be required that the ammonium salt of trifluoroacetylacetone would precipitate. Note that the column cleanup gives lower and less reproducible recoveries.

Figure 1 shows an actual chromatogram from a dried aqueous suspension chelated by the ampoule technique. The peak on the left-hand side is a beryllium trifluoroacetylacetonate peak from the injection of a standard. Note that in the samples there is another, larger peak which comes out just before the beryllium peak and interferes with it. This compound is an unidentified breakdown product of the chelating reagent. Its presence cannot be avoided under any conditions of chelation which are sufficient to dissolve beryllium oxide.

It is possible to separate the two peaks by reducing the column temperature for the sample shown in the figure. These conditions correspond to a beryllium oxide concentration of 1. 0 microgram/gram, and at the level of 1 nanogram/gram the interference peak would be so much greater than the beryllium peak that even a lower column temperature would not be useful.

Because of this problem it seemed that some means of dissolving beryllium oxide would be necessary so that the less severe chelating conditions required for soluble beryllium could be employed. Three approaches were possible:

1. Acid Solution - This is slow and unlikely to work for the refractory oxide.

2. Base Solution - The effectiveness of this approach was unknown.

3. Molten Salt Fusion - This is a very slow and messy procedure.

At this point we became aware of a recent publication by Eisentraut, Griest, and Sievers (1971), which described a procedure for the gas chromatographic analysis of beryllium in samples of lunar dust using a 75% sodium hydroxide solution procedure at high temperature. They reported that this procedure would dissolve beryllium oxide from rocket exhaust, and since that material is similar in its properties to high-fired BeO, we decided to develop a base solution procedure for analysis in blood and tissue.

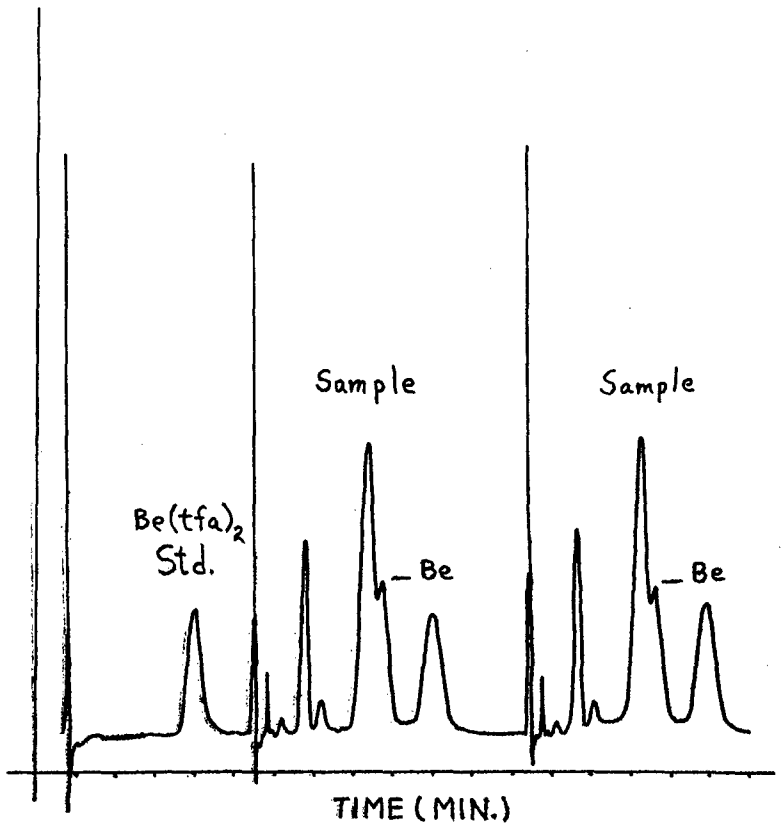

Figure 1. CHROMATOGRAPH FROM AMPOULE REACTION.

Table IV shows the details of the procedure we eventually developed for rendering low-fired and high-fired beryllium oxide in a soluble form. Once the beryllium oxide is converted to soluble Be^{+2} ion, a room temperature chelation procedure can be used, and this is so mild that there is no danger whatever of forming the interference peak which caused problems in the direct reaction approach. This chelation recipe is described in table V. Note that we are able to use the more satisfactory ammonia treatment for removal of the lower concentration of excess trifluoroacetylacetone.

TABLE IV

BASE DISSOLUTION PROCEDURE

1. Pipette 0.2 ml of blood or tissue homogenate into a 10 ml Pyrex Erlenmeyer flask.

2. Add 6 pellets of NaOH, cover top of flask with microscope slide cover glass, place on a hot plate, and melt NaOH.

3. After material in flask is completely fluid, allow it to boil with swirling for exactly 2 minutes.

4. Place flask on wood block to cool, and before the solution freezes carefully add about 1 ml of distilled water with a dropper.

5. Transfer solution to a 1 oz polyethylene bottle with rinsings of HNO_3 and neutralize to a phenolpthalein endpoint with concentrated HNO_3 (attempt to keep total volume to within 5 ml).

TABLE V

ROOM TEMPERATURE CHELATION

1. Add 2 ml of 1.0 M sodium acetate buffer and 2 ml of 0.05 M sodium EDTA solution to about 5.0 ml of sample solution of Be^{+2} in a 1 oz polyethylene bottle.

2. Add exactly 5.0 ml of a solution of 1% trifluoroacetylacetone in benzene and shake for 15 minutes at room temperature.

3. Pipette 1.0 ml of the benzene layer into a glass stoppered test tube, add 1.0 ml 2.8% NH_4OH solution and shake vigorously for 10 seconds.

4. Centrifuge and inject 1 microliter of upper (benzene) layer into gas chromatograph. The beryllium peak appears in about 2 minutes, and the amount present is determined by comparison with peak heights of beryllium trifluoroacetylacetonate standards.

Figure 2 shows samples of beryllium oxide in liver homogenate which have been prepared by the base solution technique. Note the cleanliness of the chromatogram, which is indistinguishable from a standard. These peaks correspond to samples containing about 1 microgram BeO/gram of sample, which was the smallest level which could be measured by the radiochemical method. The chromatogram appears sufficiently clean that samples at the 1 nanogram of BeO/gram level could be detected and measured. Once the details of the method had been worked out, an extensive series of radiochemical-gas chromatographic comparative analyses were made on samples of dog blood and rat liver homogenate spiked with suspensions of low-fired and high-fired beryllium oxide. In these experiments, the amounts of beryllium recovered and lost during the base solution and chelation steps were followed radiochemically, and each step was found to proceed quantitatively. The amount of beryllium chelate present in the final benzene extract was measured radiochemically at Monsanto. The same counted sample was then provided to our laboratory for gas chromatographic analysis and the results were compared on a double blind basis. The percent recoveries (i. e., GC versus radiochemistry) for this series of experiments is summarized in table VI.

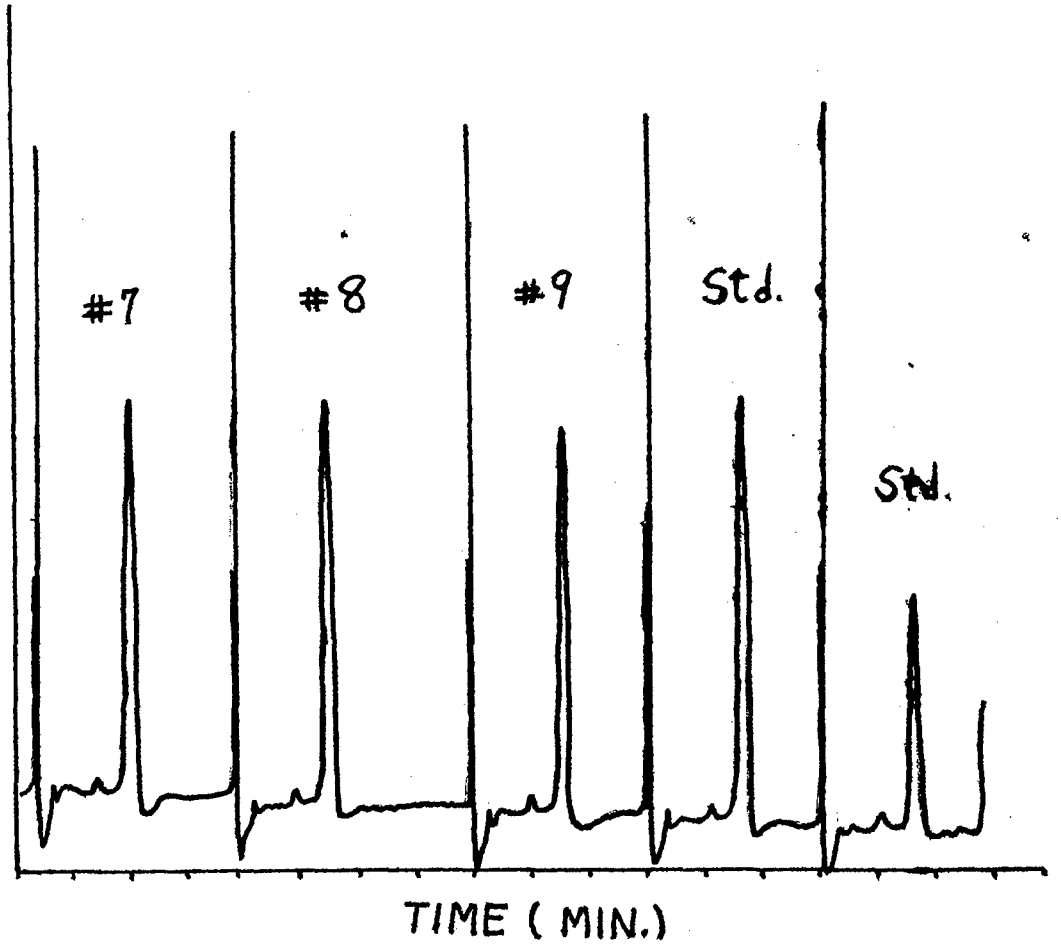

Figure 2. CHROMATOGRAPH FROM BASE SOLUTION REACTION.

TABLE VI

PERCENT RECOVERY (GC VERSUS RADIOCHEMISTRY)

OF BeO IN SPIKED TISSUE AND BLOOD

	High-Fired (1600 C) BeO		Low-Fired (500 C) BeO	
Sample	Dog Blood	Liver Homogenate	Dog Blood	Liver Homogenate
1	107 112 97	101 100 106	111 118 106	109 103 104
2	99 98 89	110 112 118	106 101 105	106 105 104
3	106 107 90	112 108 104	103 110 112	101 99 99
4	98 89 101	106 107 113	101 107 103	106 104 104
5	99 100 99	110 106 109	106 104 94	104 103 96
MEAN	99.4%	108.1%	105.8%	103.1%
SD	±4.1%	±3.9%	±4.2%	±2.6%

The samples ranged from 1 to 10 microgram BeO/gram of blood or tissue, and three replicates of the entire procedure were run on each sample. The levels of beryllium oxide were made this high to obtain good counting statistics, but the large dilutions of the same sample which were made in our lab to prepare the samples for gas chromatograhic analysis imply that beryllium oxide could have been measured at levels three orders of magnitude lower. The recoveries tended to be

slightly high, and the standard deviation of the results was about 4%. An analysis of variance was performed on these data, and it showed that there was no significant dependence of the results on the firing mode of the beryllium oxide, the two biological media tested, or the concentration of BeO over the range taken.

In order to assess the base solution technique on tissues from actual animals exposed to beryllium oxide, two series of 16 rats each were intratracheally administered 50 milligram/kilogram of body weight doses of high-fired or low-fired ^{7}Be-labeled beryllium oxide in normal saline suspension. The animals were individually housed in metabolic cages for a period of 21 days before being killed for the extraction of blood, lungs, liver, kidney and spleen, and radiochemical measurement of the beryllium content of these organs. Daily accumulations of urine and feces were separated automatically by the cage assembly and their beryllium content was measured 1, 2, 7 and 21 days postexposure. The excretion pattern was uniform in all rats tested with the great majority of the excreted beryllium appearing in the feces during the first several days. Urinary excretion was less than 0.1% of this initial fecal excretion rate and remained constant throughout the holding period, while the fecal rate eventually declined to about the urinary rate of 1-2 microgram BeO/day after 21 days. The massive elimination of beryllium through the digestive tract indicated by the initial high fecal excretion rates probably reflected that portion of the intratracheal injection which was not firmly implanted in the lungs. Especially high excretions could be correlated with poor injections as indicated by low lung counts in the extracted organs of certain rats.

The levels of translocated beryllium found in the organs of rats dosed with low-fired BeO showed that each rat that received a substantial amount of BeO as indicated by the lung level also showed noticeable translocation of a small portion of this beryllium, especially to the liver. In the case of rats injected with high-fired BeO, no such substantial translocation was observed.

The experiment was repeated with another series of 16 rats, with similar results except that in the case of three rats in the high-fired BeO subseries, substantial levels of beryllium were found in the liver. Because of the sharp differences in apparent translocation of beryllium in the rats of the second high-fired subseries, it seems possible that these animals may have been injected with a high-fired BeO or soluble beryllium. At present we have no other explanation to offer for this anomaly.

To test the effectiveness of the base solution analytical procedure on actual samples of translocated beryllium, we homogenized half of the livers of Rat #9 (Series I) and Rat #13 (Series II). These contained the highest levels of translocated beryllium found, but it was still necessary to count the homogenate for a period of 1 hour to get an accurate radiochemical measurement of its beryllium concentration. Gas chromatographic recoveries of beryllium from aliquots of this homogenate were about 115% of the amount measured radiochemically. The peak heights of the extensively diluted samples injected into the chromatograph correspond to levels of beryllium oxide in a sample containing 1-10 nanogram/gram. This together with the cleanliness of the chromatograms indicates that the base solution technique could successfully analyze translocated beryllium in liver at such levels.

Application of the room temperature chelation procedure to samples of these homogenates without a previous base dissolution step resulted in recoveries of up to 50% of the beryllium determined to be present by radiochemistry. This implied that at least this much if not all the beryllium translocated from the low-fired BeO in the lungs was in a very soluble form. Several chelations were performed on these homogenates after they had been subjected to concentrated nitric acid digestion to oxidize and break up the tissue to make the beryllium more available for chelation. This somewhat more stringent presolution step should not dissolve the oxide. While it does not provide a perfectly clean chromatogram, since the electron capturing compounds from the liver homogenate are not completely destroyed by the digestion, the beryllium peak can usually be measured. The recoveries of beryllium following chelation of these acid-digested tissues are shown in table VII. These figures show more clearly that all of the translocated beryllium is probably not in the form of the original BeO injected into the lungs but some more soluble form.

TABLE VII

PERCENT RECOVERIES OF TRANSLOCATED Be

IN LIVER HOMOGENATE DIGESTED IN HNO_3

Sample #	Rat #9 (0.909 μg BeO/g)	Rat #13 (1.904 μg BeO/g)	
1	105%	102%	
2	99%	92%	95%
3	101%	105%	106%
4	97%	107%	112%

We are presently extending this comparison of beryllium recoveries in both acid- and base-dissolved tissues from other rats exposed to both high-fired and low-fired beryllium oxide.

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AMRL-TR-72-130

PAPER NO. 21

AUTOMATED MASS SPECTROMETRIC THERMAL ANALYSIS

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INTRODUCTION

This paper describes a mass spectrometric thermal analysis instrument combination incorporating a computerized data handling system for the analysis of potentially toxic thermal degradation products of nonmetallic materials. Basically, this instrumentation package can be divided into three distinct components: a thermogravimetric analyzer, a double focussing magnetic mass spectrometer, and a dedicated data acquisition and processing system with library search capability. A detailed description of the functional interplay of each component with emphasis on the accomplished interfacing is given in the first part of this paper. During the course of an analysis, the weight loss of the sample under investigation is recorded, with respect to increasing sample temperature, by the thermogravimetric analyzer. Simultaneously, the evolved gaseous thermal decomposition products are introduced into the mass spectrometer which produces mass spectra repetitively at scan speeds of the operator's choice. The mass spectrometer is coupled to a computer for continuous on-line data acquisition, thus producing a complete mass spectrometric record of the released breakdown products. All original data are stored on magnetic tape. When an analysis is completed, there may be as many as 600 scans recorded. An executive program, stored in the core of the computer, permits the operator to call for subroutines that calibrate the system, compute mass, display the computed data in digital as well as graphical form, perform a library search for spectrum identification, and manipulate the data in several different ways such as subtract background or any specified scan from another, ignore up to four peaks, print a significant peak table, etc. A typical example of a complete mass spectrometric thermal analysis is discussed in the second part of this presentation.

Due to rapid progress in computer hardware technology, "mini"-computers suitable for dedicated applications to a broad variety of analytical instruments have recently become commercially available. With a dedicated mode of operation in mind, the classification "mini" applies only to physical dimension and initial purchase price of these specialized third-generation computing devices. Consequently, more and more analytical instruments can be automated economically because of a tremendous increase in efficiency and gain in additional capabilities. To answer

the question: "Why are you using an instrument combination of a thermal analyzer and a mass spectrometer?" I can point to a simple reason. Any physical or chemical process can be followed directly by continuous monitoring of the thermal functions of state. This makes thermal analysis a powerful research tool for a wide spectrum of scientific disciplines.

We are applying thermogravimetry to toxicology for the identification of potentially toxic thermal decomposition products of all kinds of nonmetallic materials that are being utilized in enclosed habitable environments. The most basic question in thermal analysis as we are performing it is: "What happens to a particular sample on heating?" If anything happens, the next questions become more and more complex and at the same time are much harder to answer. Decomposition of a material is easily recognized, but what are the degradation products that are formed, at what temperature, and at what rate are they released? How many compounds are formed and are these primary decomposition products or do they derive from secondary reactions?

To answer all these questions, thermal analytical methods alone are not sufficient and one has to consult additional analytical techniques. Our choice for the identification of evolved gases from the sample is mass spectrometry. Both analytical operations are being performed simultaneously on the same sample so that the results can be correlated unequivocally.

INSTRUMENTATION

Basically, our instrument package can be divided into three distinct components: a thermogravimetric analyzer (TGA), a double focussing magnetic mass spectrometer (MS), and a dedicated data acquisition and processing system with library search capability, each coupled together by an appropriate interface.

Figure 1 shows a simplified block diagram of our instrument arrangement. Let me briefly describe the individual components and explain their functional interplay.

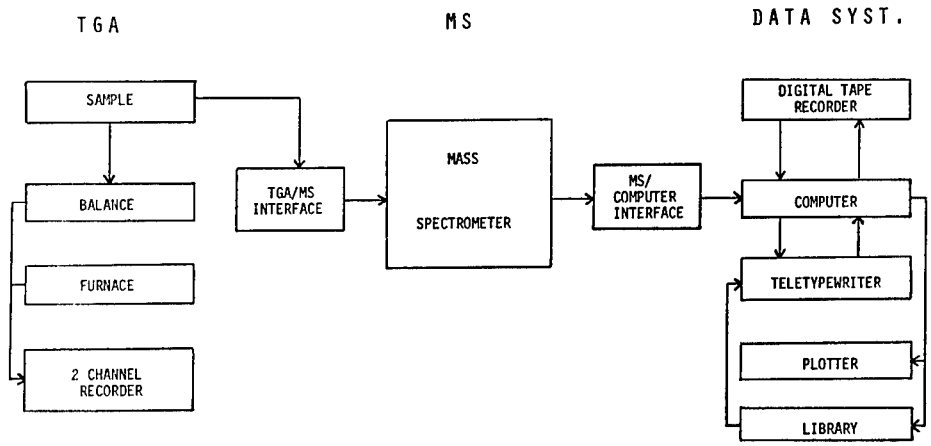

Figure 1. Block Diagram of Thermal Analyzer Mass Spectrometer System.

A sample of about 10 mg is loaded into the automatic recording electrobalance Model Cahn RH and positioned inside a clam shell furnace. Then a temperature program typically of an increase of 10°/min. is initiated and a two-channel recorder is used to trace the temperature increase and the concurrent weight change on the same strip chart. At the same time the duPont 21-491 mass spectrometer starts to acquire mass spectral scans typically at a rate of 10 sec/decade between 600 and 18 amu. The sample is being introduced into the mass spectrometer through the TGA/MS interface. Signals generated at the detector/multiplier end of the mass spectrometer are channeled directly into a Hewlett-Packard 2100A computer through the MS/computer interface. The acquired original or raw data are stored on a magnetic tape of a Hewlett-Packard 7970B Digital Magnetic Tape Recorder. The teletype with a punch-tape terminal serves as the input-output device for the computer. By using conversational language, the operator calls into use the various programs available for data acquisition and processing. The plotter is used for the presentation of data in the form of charts and bar graphs. The library consists of a second tape recorder and a magnetic tape. This system compares mass spectra taken with the 21-491 MS with 6000 mass spectra stored on tape and reports a quantitative measure of similarity.

A very vital role in this instrument combination is played by the two interfacing devices. First, the TGA/MS interface. The sample in the TGA is being analyzed under ambient pressure in room air to be as close as possible to a "real world" environment. The mass spectrometer, however, has to be maintained under a reduced pressure of about 10^{-7} torr. In order to provide a constant sample flow into the ion source of the mass spectrometer without compromising the reduced pressure requirement, a stainless steel capillary line with a restricting orifice at the tip close to the sample location is employed to mate the thermal analyzer and mass spectrometer. The dimensions of inner diameter, length, and orifice opening of this line are such that viscous flow exists to avoid mass discrimination (Geiger and Kleineberg, 1972).

The second interface that deserves discussion is the MS/computer hookup. The data arriving at the detector/multiplier of the mass spectrometer are in digital form, the ions are discrete entities and therefore the signal may be detected by counting equipment. The instrumentation employed consists of a pulse amplifier discriminator (PDA) and appropriate binary counters. Data counts are accumulated for a period which is controlled by the operator at rates varying from 2 to 10 KHz. As each resolved ion beam strikes the electron multiplier, the PDA produces a pulse for each ion impact. Every burst of energy is counted as one ion for a preset "window" (time). The utilization of counting equipment in this fashion overcomes the shortcoming of digital to analog and analog to digital conversion.

Before the computer can be put to use for its intended purpose, it has to be calibrated initially. This is accomplished in the following manner. Acetone is introduced into the mass spectrometer and the raw data acquired are printed out in a special build table format which consists of line numbers, time windows, and ion current intensities of all peaks registered. The molecular - and parent-peak

of this compound at m/e = 58 and 43 are readily recognized by their respective intensities. The next step is to introduce perfluorotributylamine into the mass spectrometer and obtain an additional build table. This compound gives rise to peaks ranging from 614 to 69. Using the information obtained from the position of the acetone fragments in a simple mathematical equation the exact centroids for additional peaks can be calculated and the acquired information fed into the computer through the punchtape terminal. Between 30 to 40 calibration points are required for a mass range of up to 600. Right now we have to perform this procedure by hand, but in the near future a computer program will become available to accomplish this tedious task automatically. Once a successful initial calibration is completed, an automatic recalibration subroutine can be utilized to compensate for minor shifts in the mass spectrometer scan circuit electronics as soon as errors in the calibration show up.

During the second part of my presentation, I want to present as an example the results that we obtained from a sample of polyvinylchloride (PVC) that was analyzed with the instrument system described.

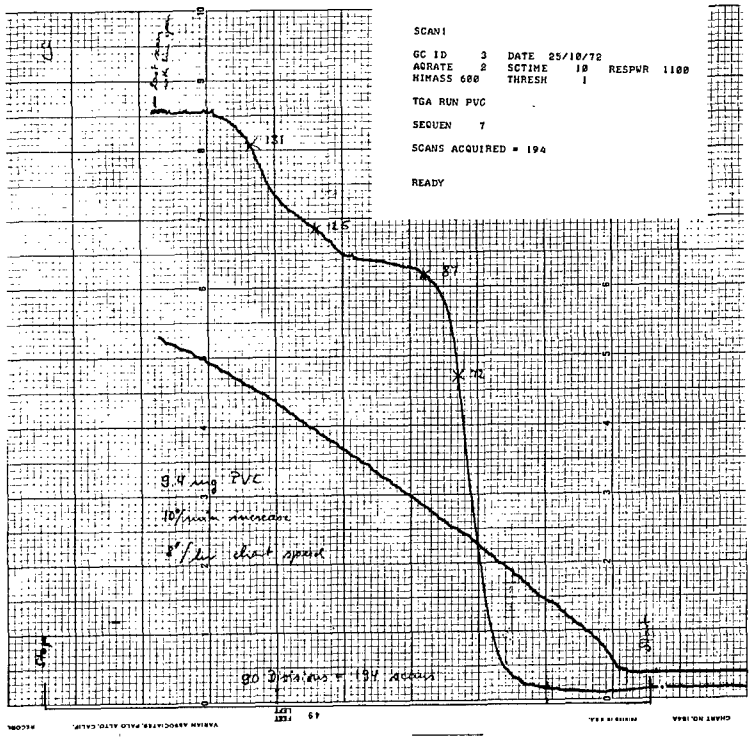

Figure 2. Thermogram of Polyvinylchloride Sample.

Figure 2 represents a strip chart recording obtained from the TGA for 9.4 mg of PVC heated from room temperature to 630°C at a rate of 10° increase per minute.

During the 68 minutes of analysis time a total of 194 mass spectral scans, one for about every 21 seconds, were acquired and stored on magnetic tape. About 500 feet of tape were filled with data at this point, which means that with a capacity of 800 bits per inch, close to 5,000,000 data points were recorded. Looking at the weight loss trace, at least 3 different steps of thermal degradation are apparent. I have marked 4 points with the notation of the respective scan number recorded at that time. Please keep these in mind for the subsequent discussion.

After completion of the data acquisition, the computer is available for conversation through which we can find out what happened during the analysis. The first thing one will do is to kindly ask the computer to normalize and assign mass numbers to all the data that it has stored on tape. Only an "old-fashioned" mass spectroscopist can appreciate what a laborious task that is. The data system accomplishes this command in about 15 minutes including read and write steps with only milliseconds of actual central processing unit time. This task accomplished, the analyst is already ahead weeks of time.

The next sensible question to ask the computer is to present a list of significant peaks. This program prints a list of significant peaks vs. scan number. It tabulates mass, maximum normalized intensity, first scan in which the ions of that mass reached the maximum intensity indicated, and total number of ions of that mass in all scans.

The list obtained for the subject PVC sample is shown in table I. Note that the option of ignoring certain peaks was exercised to eliminate the mass spectrometer response to room air. Also a "millout" of 200 was called for resulting in an abbreviated table containing only those masses that contributed an ion current of at least 20% of that of the most abundant ion.

The ion species for m/e = 94, 78, 128, 44, and 30 were selected for further investigation. The fragment ion of m/e = 94 for $[C_6H_5O + H]^+$ was chosen because, in this case, it is indicative of a phenylphosphate derivative that was used as a plasticizer in the PVC sample. Calling for the program to present the mass chromatogram of this ion species, the plot as shown in figure 3 was obtained. From this it can be seen that the plasticizer is gassing off at its maximum rate when scan #72 was recorded. With this evidence on hand, one certainly wants to examine more closely what specific plasticizer is being released from the sample at this point. To accomplish this end, one calls for the plotted mass spectrum recorded at that time.

Table I

Significant Peak Table

```
SIGNFPK
GC ID EB      3    DATE  25/10/72
AQRATE        2    SCTIME     10    RESPWR  1100
HIMASS      600    THRESH      1

TGA RUN PVC

IGNORE     32,  40,  44,    0
MILOUT 200  SEQU   172
```

MASS	MAX INTN	FIRST OCCUR	SUM IONS *2** 3
33	1000	1	25244
34	1000	2	26541
41	1000	183	4640
43	1000	69	8463
55	1000	62	5197
57	1000	63	4538
31	948	87	2522
36	879	191	4871
94	797	72	1651
69	739	186	3327
67	667	188	1164
97	652	186	1696
95	588	193	1450
70	515	61	1873
81	500	186	1383
78	484	76	1184
71	478	186	1742
83	478	186	2233
56	455	191	1913
85	364	191	1035
42	349	62	5159
109	346	187	913
96	333	191	624
45	318	139	2033
39	316	70	1570
77	303	191	508
79	303	191	522
111	295	184	1020
82	294	193	697
123	268	190	570
149	265	193	322
125	256	188	468
80	242	191	164
141	242	191	122
133	235	193	227
38	231	187	3484
105	220	190	611
37	215	74	3666
66	213	72	416
119	212	187	408
113	206	193	198
53	205	188	510

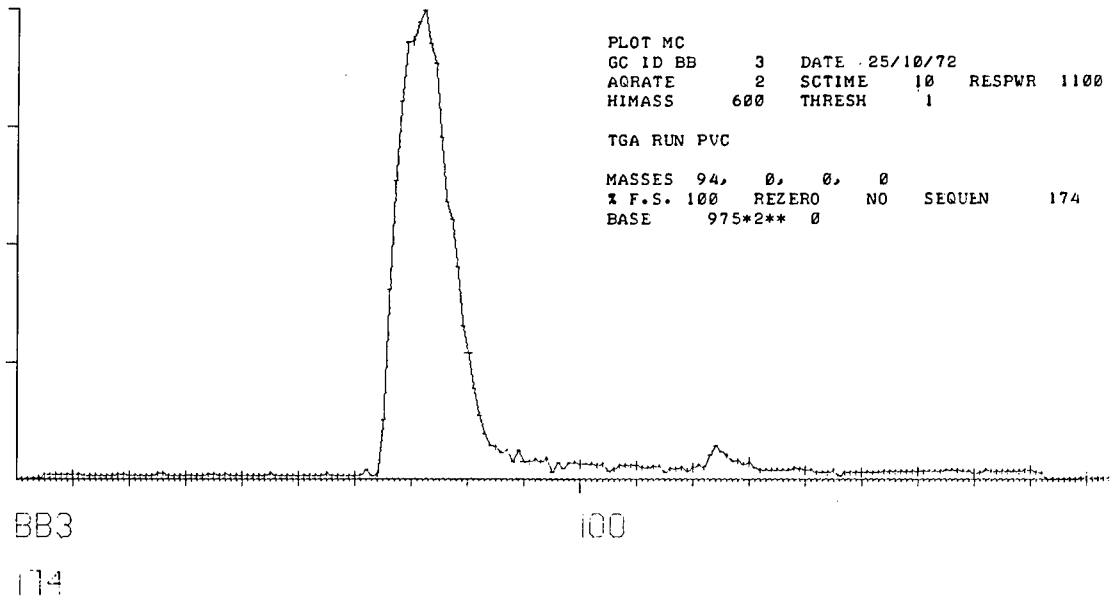

Figure 3. Mass Chromatogram for Fragment Ion with m/e = 94.

Figure 4 represents the spectrum that was obtained as scan #72.

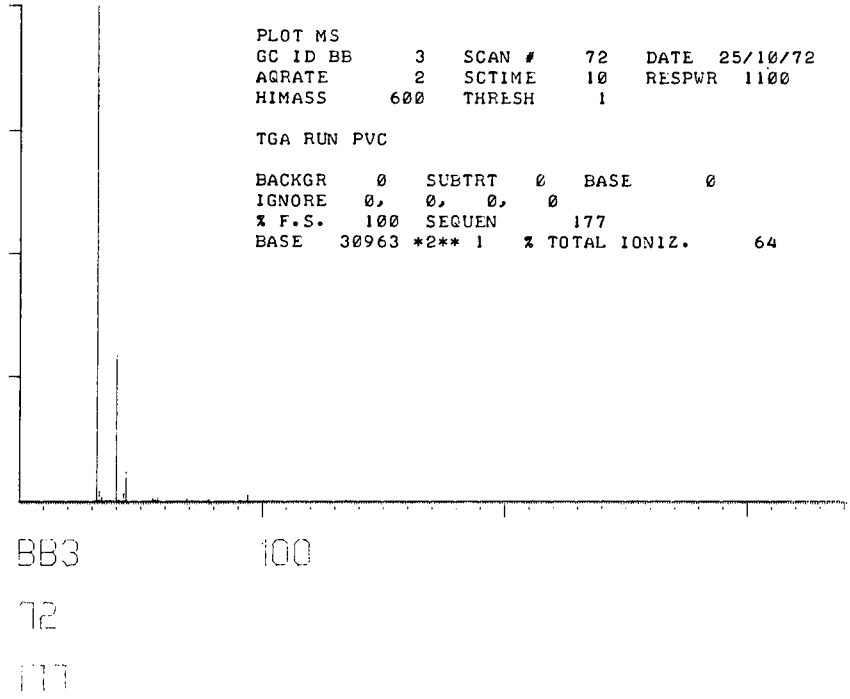

Figure 4. Mass Spectrum Recorded as Scan #72.

This information is somewhat disappointing because the best that one can make out of this is that the mass spectrometer saw normal air at this time. But that is what it actually sees all the time at a concentration of some 99.9%. The degradation products are only present at trace levels. To obtain a mass spectral presentation of this trace, one can tell the computer to subtract the background that it sees all the time. In doing just that, the mass spectrum obtained is depicted in figure 5.

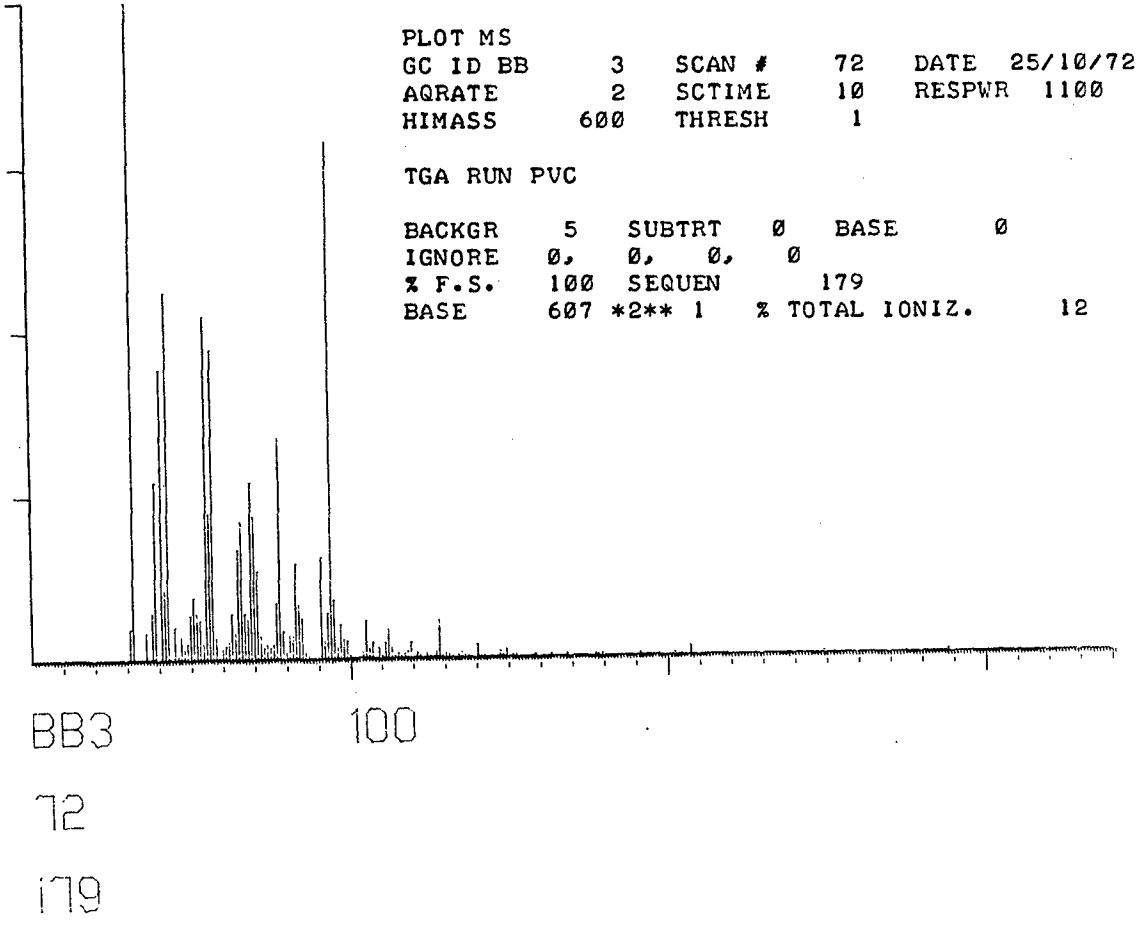

Figure 5. Mass Spectrum Recorded as Scan #72 with Scan #5 Substracted as Background.

Closer examination reveals that aliphatic hydrocarbon moieties are present as indicated by the peak cluster of 14 atomic mass unit intervals at 41-42-43, 55-56-57, 69-70-71, and 83-84-85. This makes it highly probable that a mixed aliphatic aromatic phosphate ester was used as a plasticizer.

Figures 6 and 7 show mass chromatograms for the molecular ion species of m/e = 78 and m/e = 128 representing benzene $[C_6H_6]^+$ and naphthalene $[C_{10}H_8]^+$, respectively. These compounds are being formed as secondary reaction products during the thermal degradation of the studied polymer. Primarily, complete dehydrochlorization takes place leaving a polyene chain. This chain breaks up and the fragments stabilize themselves through cyclization yielding the detected stable aromatic hydrocarbons.

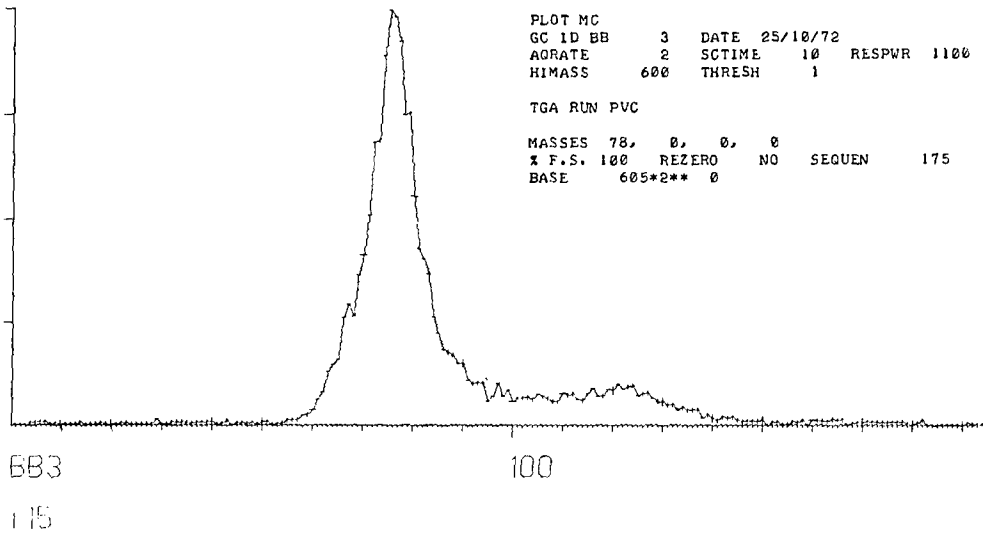

Figure 6. Mass Chromatogram for Molecular Ion with m/e = 78.

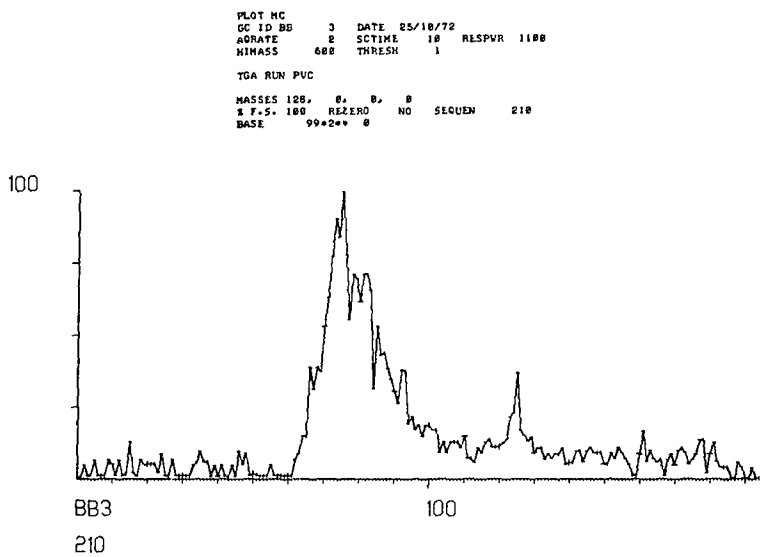

Figure 7. Mass Chromatogram for Molecular Ion with m/e = 128.

Figure 8 shows a curiosity. The computer was asked to look for the ion species of mass 30, and this is what came out, a sharp peak with its maximum appearing in scan #87. The species presented here is probably NO (nitric oxide). A total ion count of only 16 was registered for this compound. This could mean, if later on we are able to reproduce this result, that the system is capable of real trace analysis.

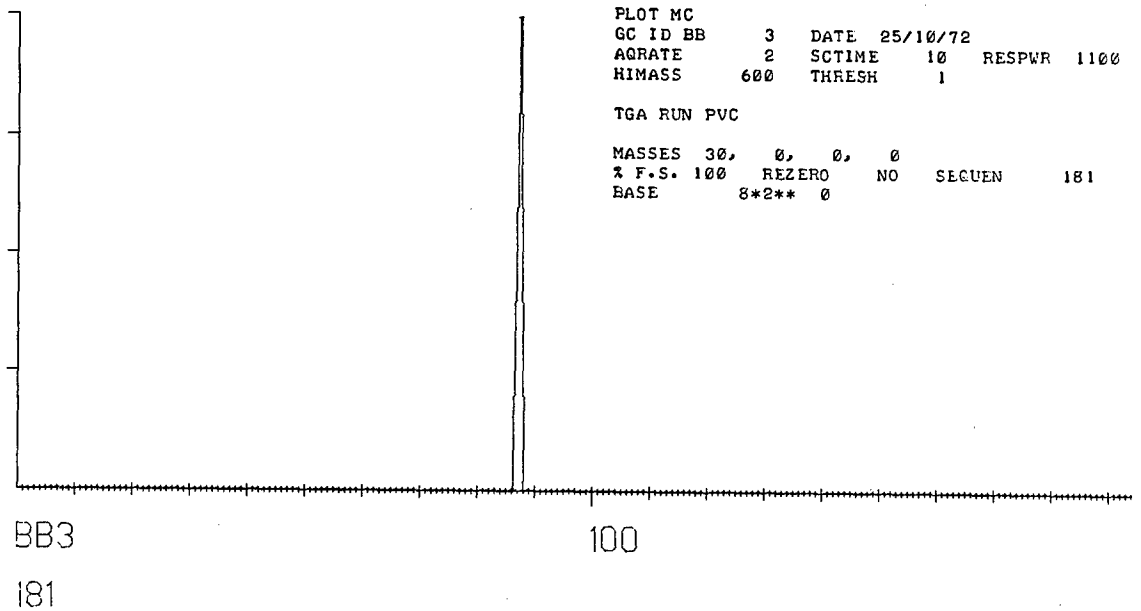

Figure 8. Mass Chromatogram for Fragment Ion with m/e = 30.

Additional valuable information concerning the fate of our sample inside that furnace is contained in figure 9, showing the mass chromatogram for the ion species of m/e = 44. This curve shows a plateau with insignificant fluctuations in the first part. Between scan #120 and 150 it has two pronounced peaks with two additional discernible maxima between them. The latter portion of the trace plateaus at about the same level as the first part. One has to assume that the identity of the ion species plotted here for the mass of 44 amu is carbon dioxide. The plateau represents the carbon dioxide content in room air which is coming into the mass spectrometer during the entire analysis as mentioned before. The peaks above this background are due to the formation of carbon dioxide as it is being formed by oxidation of thermal degradation products from the PVC sample.

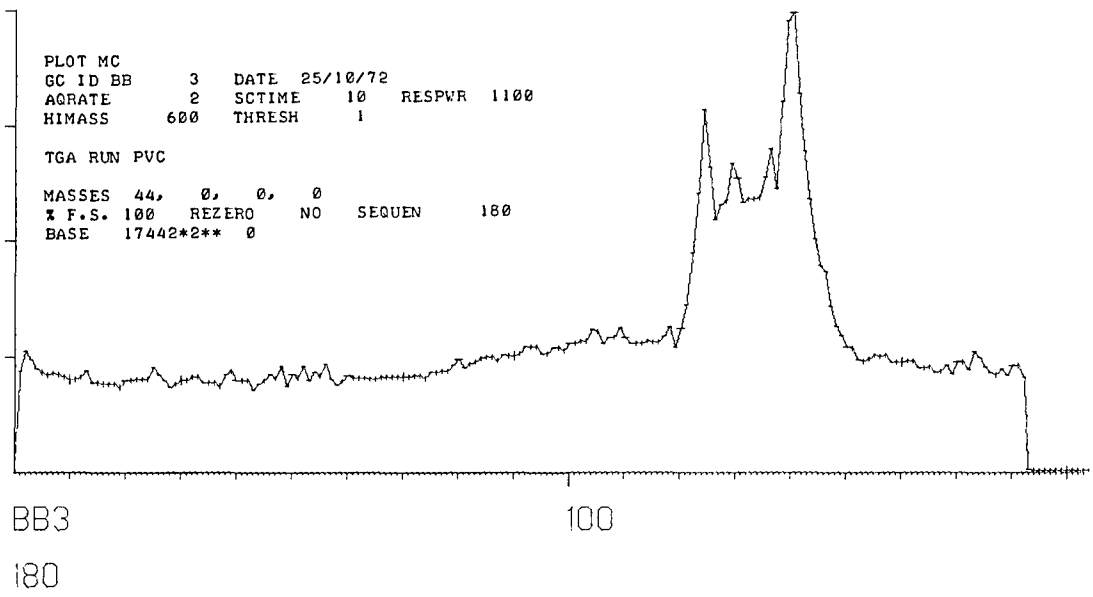

Figure 9. Mass Chromatogram for Fragment Ion with m/e = 44.

The assurance for this and the fact that CO_2 is the only compound formed from the time on scan #120 was recorded comes from the information obtained next from the computer. Figure 10 shows the mass spectrum recorded as scan #131 with scan #5 subtracted as background.

The mass spectrometer was tuned to stop scanning at mass 31 which explains the absence of peaks at amu 12 and 16 which are present in a complete CO_2 mass spectrum.

At this point a library search program was called up and the computer was instructed to compare the mass spectrum of figure 10 with information stored on the library tape. The information obtained is presented in table II.

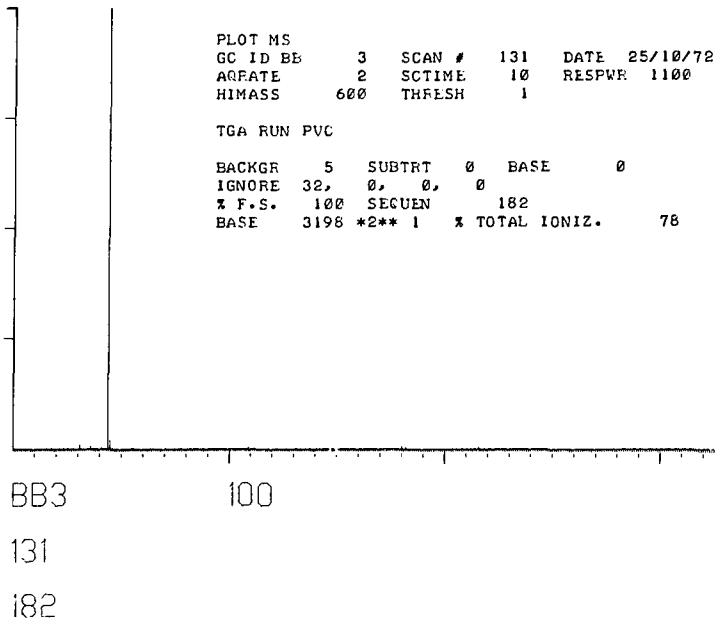

Figure 10. Mass Spectrum Recorded as Scan #131 with Scan #5 Subtracted as Background.

All five possible fits indicate that the mass spectrum recorded at the specified time during the TGA analysis was indeed closest to that of carbon dioxide. The poor "goodness" numbers (1000 being a perfect fit) are explained by the absence of peaks below amu 31 in the recorded spectrum that was compared to literature data.

Table II

Library Search Print-Out for Scan #131

```
LIBRARY SEARCH

GC ID BB      3    DATE  25/10/72
AQRATE        2    SCTIME    10    RESPWR  1100
HIMASS      600    THRESH     1

TGA RUN PVC

IGNORE  32,   0,   0,   0
SEQUEN      183
INDEX    SCAN  BACKGR   SUBTR   BASE

   0      131       5       0       0

LAST INDEX PROCESSED  0
SCAN #      131     BASE       44
GOODNESS  ID. NO.  PAGE
   343  API-1582 0011 CARBON DIOXIDE (GAS)
   293  DOW-0017 0011 CARBON DIOXIDE
   288  BIE-0589 0444
   278  API-0157 0011
   275  DOW-1974 0294
```

One fact that I did not mention up to this point is that we did not see a peak at amu 36 for the large amount of HCl that is released during thermal decomposition of PVC. This is explained by the fact that the HCl is reacting with metal surfaces beyond the port of introduction into the line directly behind the jet separator that we are bypassing. The same stainless steel capillary was successfully employed for the detection of HCl in our previous instrumentation using a Bendix Time-of-Flight mass spectrometer (Kleineberg and Geiger, 1971). We will investigate this phenomenon further and correct the situation.

We had to go through much frustration and anguish to develop the installation to the point where we are today. But I feel it was well worth every bit of it considering the increased capabilities that we have on hand right now. I don't know how many of you have noticed that all data that I presented here were acquired and processed in one day, two days prior to this presentation, that is.

REFERENCES

1. Geiger, D. L., and G. A. Kleineberg; "Threshold Response of a Thermogravimetric Analyzer-Mass Spectrometric System to Selected Degradation Products of Polymeric Materials;" Proceedings of the Twentieth Annual Conference on Mass Spectrometry and Allied Topics, Dallas Texas, June 1972, Paper H7 (Also AMRL-TR-72-58).

2. Kleineberg, G. A., and D. L. Geiger; "Tandem Thermogravimetric Analyzer-Time-of-Flight Mass Spectrometer System Designed for Toxicological Evaluation of Nonmetallic Materials;" Proceedings of the Third International Conference on Thermal Analysis, Davos, Switzerland, 1971, in Thermal Analysis, Vol. 1, Advances in Instrumentation, Hans G. Wiedemann (Ed.), Birkhauser Verlag, Basel, Switzerland, 1972, pp 325-336. (Also AMRL-TR-71-71).

AMRL-TR-72-130

PAPER NO. 22

SHORT-TERM ANIMAL EXPOSURE TO CARBON MONOXIDE (CO) AND HYDROGEN CYANIDE (HCN) SINGLY AND IN COMBINATION

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INTRODUCTION

A number of aircraft crashes and fires have resulted in passenger deaths which could not be attributed to traumatic injuries. Pathologic examinations and blood tests for cyanide and carboxyhemoglobin levels have also failed to establish a clear-cut cause of death other than smoke inhalation or severe burns. The cause of death in such cases may very likely be the result of inhalation of dangerous pyro-decomposition products present in the atmosphere of the burning aircraft cabin. Such exposures would be brief in nature since passengers would either escape or suffer fatal burns within a few minutes of the outbreak of the fire. Since it is impractical to monitor atmospheric concentrations of the pyro-decomposition products during actual aircraft fires, only the blood levels of the compounds in the victims can be examined and an attempt made to correlate these with the conditions necessary to produce them.

Individually measured blood cyanide and carboxyhemoglobin values, although below those levels considered lethal, may be indicative of an additive or synergistic toxic response. There were, however, no published experimental data available to confirm this possibility. Acute toxicity tests on inhaled hydrogen cyanide for 5-minute exposures conducted in combination with carbon monoxide did not show any increased toxic response at carboxyhemoglobin levels of 25% saturation.

To determine whether there was a toxic interaction of some of the principal combustion products of aircraft fires, studies were conducted to (1) define blood levels of HCN and CO necessary to cause death in laboratory animals under controlled conditions, (2) determine the rate of uptake of each into the blood and (3) to determine whether exposure to both compounds simultaneously represented a greater hazard than each separately. From this data, it was hoped to define blood levels of each compound, and combinations of blood levels at which death or survival would be possible, above which survival would be impossible and below which survival would be assured.

MATERIALS AND METHODS

The experimental animals used in these studies were 200-300 gram male Sprague-Dawley rats. Each group of animals was subjected to quality control examinations prior to use and appeared to be in good health.

All exposures were made in a modified Rochester Chamber (Leach et al., 1959; Haun et al., 1969) under dynamic airflow conditions. The experimental set-up is shown in figure 1. For combined exposures an appropriate amount of carbon monoxide was passed through a flowmeter and mixed with metered amounts of hydrogen cyanide just prior to introduction into the chamber airflow. The hydrogen cyanide was supplied in liquid form in a cylinder. This cylinder was pressurized to about 40 lbs. with nitrogen to permit uniform flow of small volumes of liquid HCN through a flowmeter. The HCN was then vaporized in a heated glass evaporator using a flow of 5 cfm predried air. The total chamber airflow was maintained at 50 cfm, 45 cfm from the preconditioning system, and 5 cfm from the contaminant generation system. A slight negative pressure was maintained in the chamber at all times to prevent leakage of the contaminants into the laboratory air.

Chamber concentrations of both HCN and CO were monitored continuously during all exposures. The HCN was monitored with a cyanide specific electrode after collection in 0.1 N NaOH absorber solution. Carbon monoxide was monitored with a nondispersive infrared analyzer, and concentration recorded on a strip chart recorder. It was determined experimentally that neither contaminant interfered with the analysis of the other for the exposure concentration range tested.

The desired concentrations of each contaminant were established in the chamber and then the rats were inserted into the chamber using the sliding cage drawers shown in figure 2 (DiPasquale and Davis, 1971). All exposures were timed with a stopwatch. Each exposure had 10 rats, 5 per sliding cage. Individual rats were observed for toxic signs and mortality during exposure, and time to death was recorded.

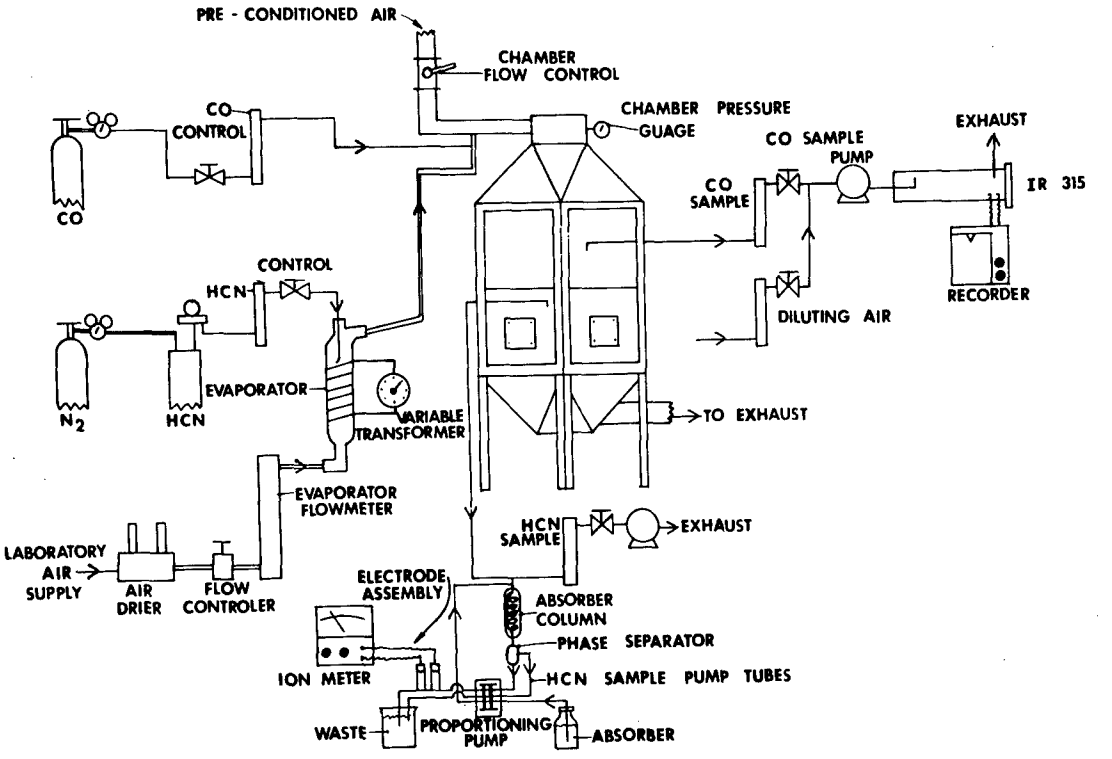

Figure 1. ROCHESTER CHAMBER SYSTEM FOR HCN AND CO COMBINATION EXPOSURES.

Immediately upon completion of exposure, the rats were removed from the cages and bled with heparinized syringes by cardiac puncture. Bleeding the 10 animals required no longer than 2 to 2. 5 minutes. It was determined that no significant change in carboxyhemoglobin occurred for this length of time post-exposure.

Blood cyanide concentrations were determined by placing 1 ml of heparinized blood into the outer ring of a Conway diffusion dish. One ml of 0. 1 N NaOH was then placed in the central ring and 0. 5 ml of 10% sulfuric acid was added to the blood. The top was sealed immediately after addition to the acid, to keep the liberated cyanide in the diffusion dish. These dishes were then mixed and allowed to sit for 3. 5 hours. After this waiting period, which was found to be optimal for maximum recovery of HCN, the lids were removed and the NaOH in the central ring was transferred with a Pasteur pipette into the cyanide ion electrode cell for analysis.

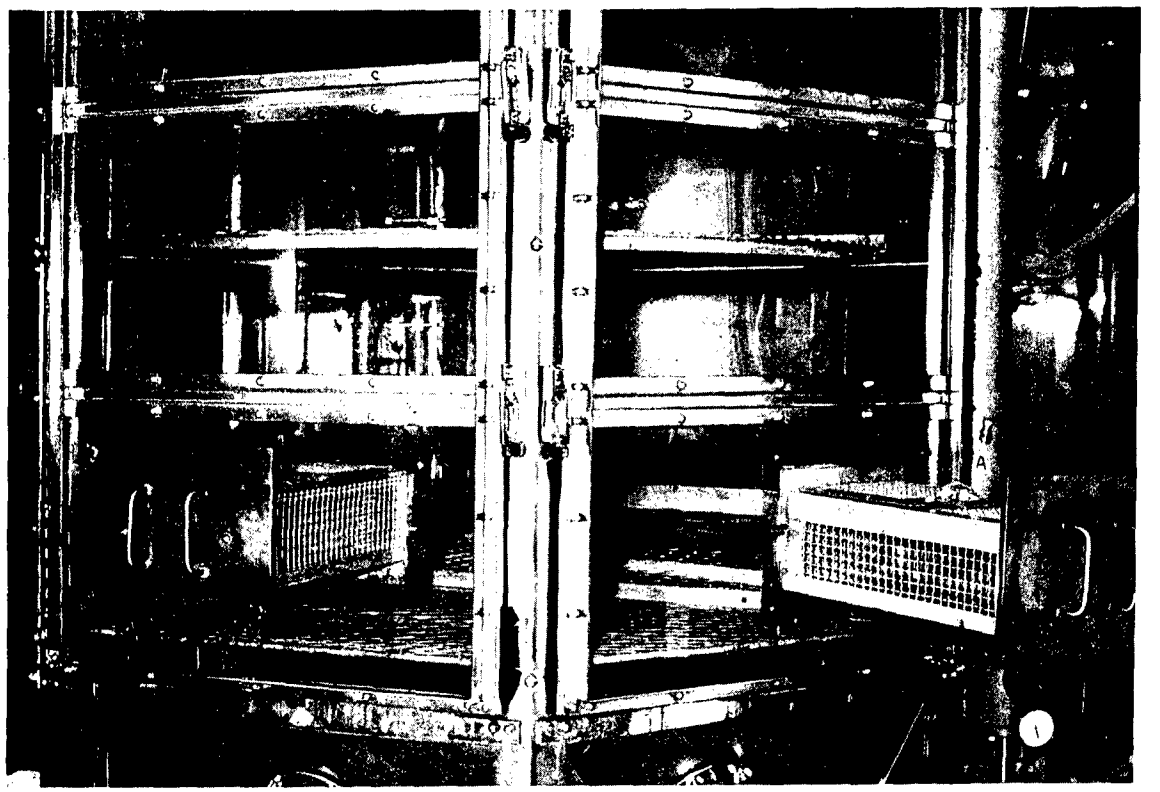

Figure 2. ROCHESTER CHAMBER MODIFIED TO ACCEPT SLIDING CAGE DRAWERS.

The amount of CO in blood was determined immediately following exposure by measuring carboxyhemoglobin content using a CO-Oximeter® standardized for rat blood.

The first step in carrying out this study was the determination of LC_{50} values for HCN and CO individually. The LC_{50} values were determined for 5-minute exposures since this was thought to be a maximum exposure period for a victim of an airplane fire. All subsequent concentrations used in this study were based on the results of the 5-minute mortality curve. Figure 3 shows the mortality data for rats exposed to HCN. The important concentrations used in the balance of the study are marked on the curve. Figure 4 shows the mortality data for rats exposed to CO. Again, the important concentrations are marked on the curve.

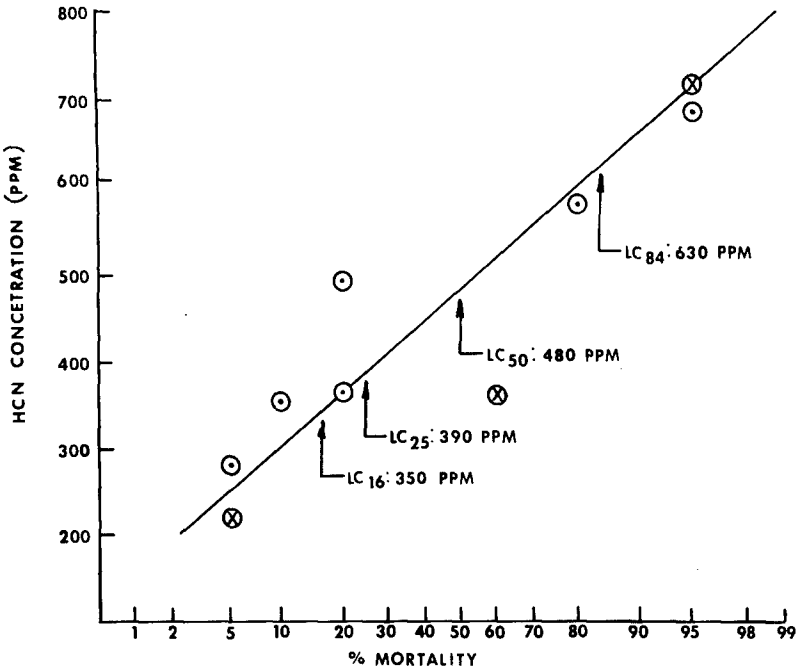

Figure 3. RAT MORTALITY FROM 5 MIN. EXPOSURE TO HYDROGEN CYANIDE.

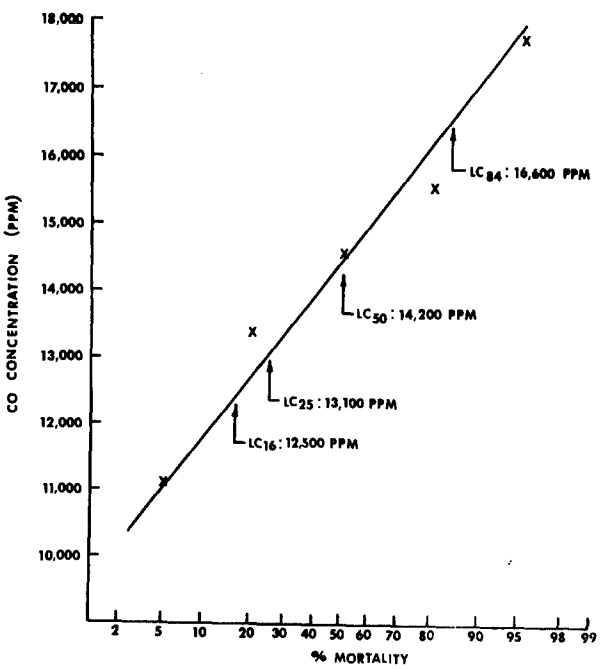

Figure 4. RAT MORTALITY FROM 5 MIN. EXPOSURES TO CARBON MONOXIDE.

To determine the rates of uptake of HCN and CO into rat blood, various concentrations of each were selected, and groups of 10 rats were exposed to these concentrations for varying lengths of time. The conditions used for examination of cyanide uptake into blood are shown in table I. Rats were exposed to the 5-minute LC_{50} concentration for HCN for 4, 3.5 and 2 minutes. They were exposed to the LC_{25} concentration for 6, 5.5 and 4 minutes, to the LC_{10} for 7, 6, 5 and 4 minutes, the LC_2 for 4 and 3 minutes and the LC_0 for 4 minutes. For the determination of CO uptake rate, the rats were exposed to the conditions shown in table II. This consisted of exposure to the 5-minute LC_{50} concentration for 5, 4, 3 and 2.5 minutes, the LC_{25} for 7.5, 6.5, 5 and 2.5 minutes, and the LC_2 for 8, 7, 6 and 5 minutes.

TABLE I
Conditions Used to Examine HCN Uptake into Rat Blood
(N = 10)

HCN Concentration	Exposure Duration (min)
5 min. LC_{50} (480 ppm)	4.0, 3.5, 2.0
5 min. LC_{25} (390 ppm)	6.0, 5.5, 4.0
5 min. LC_{10} (300 ppm)	7.0, 6.0, 5.0, 4.0
5 min. LC_2 (200 ppm)	4.0, 3.0
5 min. LC_0 (100 ppm)	4.0

TABLE II
Conditions Used to Determine CO Uptake into Rat Blood
(N = 10)

CO Concentration	Exposure Duration (min)
5 min. LC_{50} (14, 200 ppm)	5.0, 4.0, 3.0, 2.5
5 min. LC_{25} (13, 100 ppm)	7.5, 6.5, 5.0, 2.5
5 min. LC_2 (10, 000 ppm)	8.0, 7.0, 6.0, 5.0

For the combination exposures, 5 groups of 10 rats each were exposed to each selected combination of HCN and CO, making 50 data points for each combination. Table III shows the first group of combinations. The 5-minute LC_{50} of CO was combined with the 5-minute LC_{16}, LC_{50} and LC_{84} of HCN, and the 5-minute LC_{50} of HCN was also combined with the 5-minute LC_{16}, LC_{50} and LC_{84} of CO. Each of these combinations was used for exposure of 50 rats for 2.5 minutes. Table IV shows the second group of combinations. This consisted of exposing 50 rats to the 5-minute LC_{16} of HCN with the LC_{25} of CO for 1.5, 2.5 and 3 minutes, the two LC_{25} concentrations for 2.5, 4 and 5 minutes, and the HCN LC_{25} with the CO LC_{16} for 2.5, 3.5 and 5 minutes.

TABLE III
Concentrations of HCN and CO Used in Simultaneous
Combination Exposures of Rats
(N = 50)

Exposure Duration (min)	HCN Concentration (ppm)	CO Concentration (ppm)
2.5	350 (LC_{16} 5 min)	14,200 (LC_{50} 5 min)
2.5	480 (LC_{50} 5 min)	14,200 (LC_{50} 5 min)
2.5	630 (LC_{84} 5 min)	14,200 (LC_{50} 5 min)
2.5	480 (LC_{50} 5 min)	12,500 (LC_{16} 5 min)
2.5	480 (LC_{50} 5 min)	16,600 (LC_{84} 5 min)

TABLE IV
Concentrations of HCN and CO Used in Simultaneous
Combination Exposures of Rats
(N = 50)

Exposure Duration (min)	HCN Concentration (ppm)	CO Concentration (ppm)
1.5, 2.5, 3.0	350 (LC_{16} 5 min)	13,100 (LC_{25} 5 min)
2.5, 4.0, 5.0	390 (LC_{25} 5 min)	13,100 (LC_{25} 5 min)
2.5, 3.5, 5.0	390 (LC_{25} 5 min)	12,500 (LC_{16} 5 min)

RESULTS AND DISCUSSION

The first part of this study not only yielded information about rates of uptake of the two compounds into blood, but also was valuable for information regarding exposure to each compound individually. Since one of the objectives of this study was to determine blood values of each compound separately that would cause death, the uptake exposures provided that information. Table V shows the lowest blood values of each compound at which death occurred. Also, it shows the highest blood values at which survival occurred. The value of 12.2 μg/ml for blood cyanide represented the highest blood cyanide level observed for the entire study. The highest COHb level found during the study was 77% and every rat that reached that level died during exposure. The data also show an interesting fact about the two compounds; the lethal range for CO alone was very narrow, while that for HCN was extremely wide.

The rate of uptake of HCN into rat blood is shown in figure 5. The dose is represented as concentration times minutes of exposure (CT) in terms of ppm-minutes and the response in terms of μgCN/ml in blood. A rapid, almost linear, rise up to about 6 μg/ml was observed which then leveled off to a more exponential rise. A CT dose of about 1000 ppm-minutes was required to reach the minimum lethal blood cyanide level.

TABLE V
Range of Lethal Blood Levels in Rats
Exposed to HCN or CO Alone

	HCN	CO
Lowest Lethal Blood Value	4. 95 μg/ml	72% COHb
Highest Non-Lethal Blood Value	12. 20 μg/ml	76% COHb

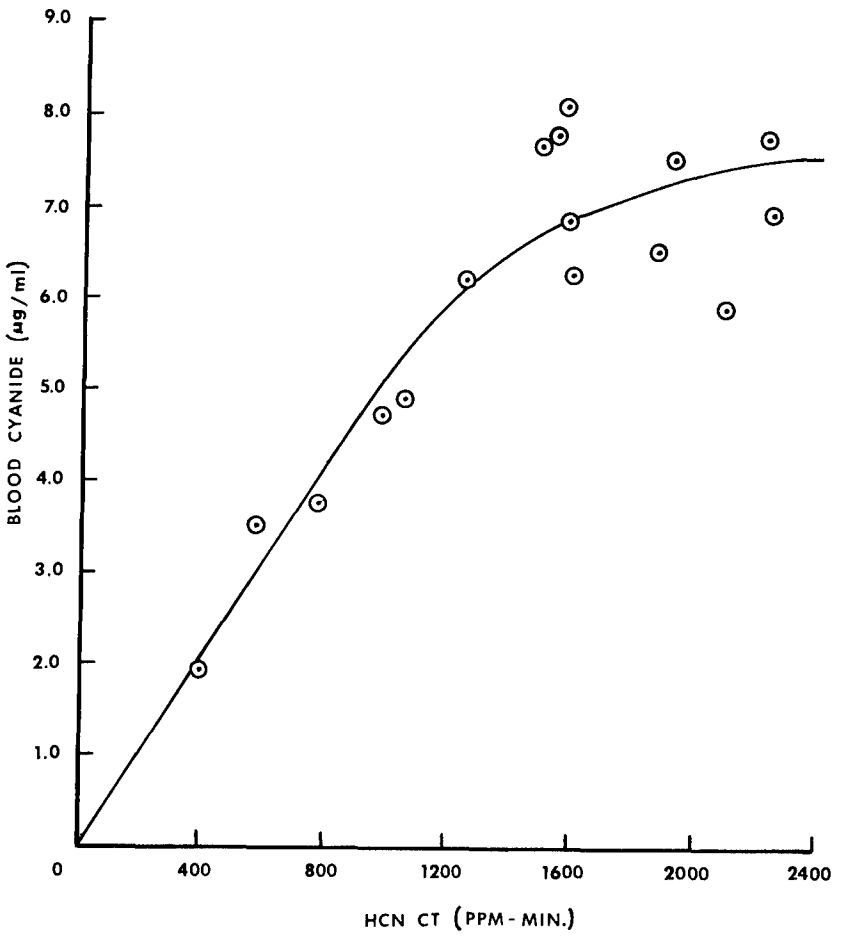

Figure 5. HCN UPTAKE INTO RAT BLOOD, MEAN VALUES FROM SURVIVORS ONLY (N = 10).

The rate of uptake of CO into rat blood is shown in figure 6. Again, the dose is represented in terms of ppm-minutes and the blood CO level is in % COHb. The initial rate of CO uptake into rat blood was extremely fast as would be expected. Exposure to the 5-minute LC_a concentration (10, 000 ppm) for 5 minutes resulted in a COHb level of 57%. Deaths from CO occurred in a very narrow range of 72-77% COHb.

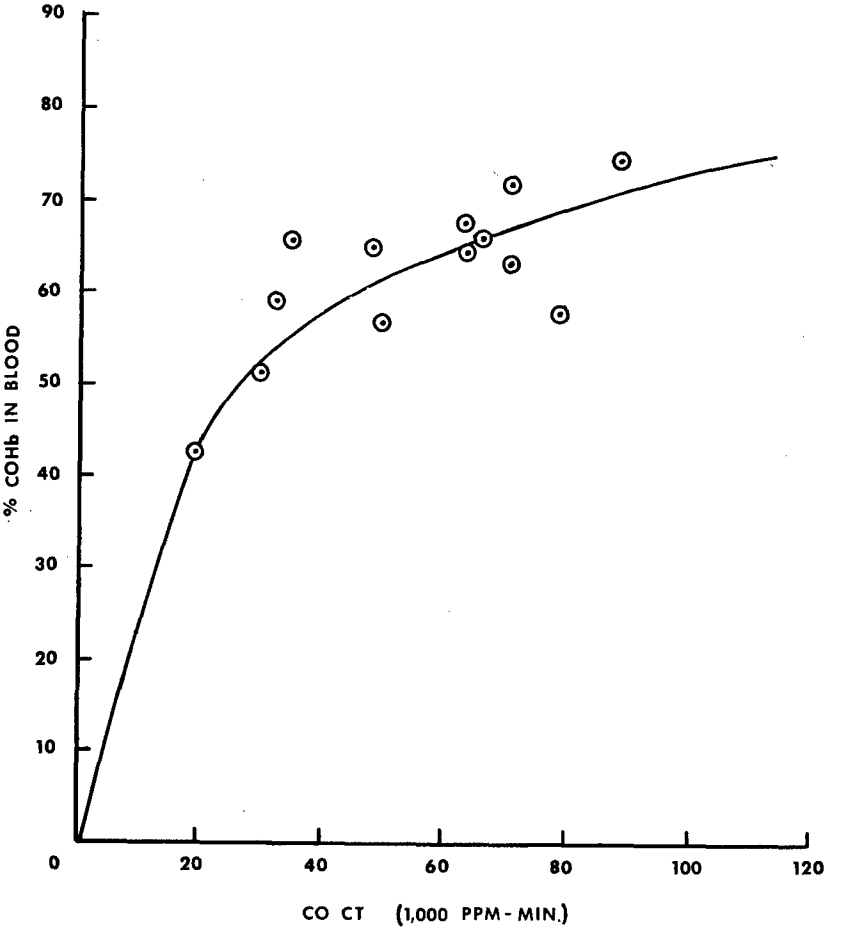

Figure 6. CO UPTAKE INTO RAT BLOOD, MEAN VALUES FROM SURVIVORS ONLY (N = 10).

The results of combination HCN and CO exposures are still being evaluated. During the course of conducting exposures, it became apparent that the variability of CN^- recovery from the blood was greater than expected from data developed in singly exposed animals. It was necessary to group results of analyses from animals in each test run to minimize the effect of this variable. The result of this

grouping has led to the tentative conclusion that the toxic responses to HCN and CO are independent in action although occasional groups (4 of 59 test runs) appear to suggest some interaction.

Further experiments will be conducted on the method of cyanide analysis and additonal animal exposures will be performed to clarify these tentative conclusions.

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DISCUSSION

DR. SMITH (Federal Aviation Administration): I believe the scatter is in part due to the difficulties of the cyanide measurement and it is not too surprising in view of some of the experiences we have had in our own lab attempting to use the selective ion electrode. And it is not out of line with figures in the literature for blood cyanide levels in deaths from fires in which both gases were present. I think it is a pretty elegant piece of work considering the difficulties, and the precision of the exposures is most impressive.

DR. HENDERSON (Olin Corporation): There is one other factor, I believe, that you might want to consider and that is the fact that you almost immediately lose all of the oxygen in a very fast, hot fire so that you not only have high carbon monoxide and cyanide levels but you also don't have any oxygen because your fire has just consumed all of the oxygen in the enclosed space.

MR. DARMER (SysteMed Corporation): I think that is an important point.

OPEN FORUM

DR. BACK (Aerospace Medical Research Laboratory): Before we start Dr. Erickson would like to show a couple of slides on some of the work he is doing down at Brooks on carbon monoxide.

MAJ. ERICKSON (USAF School of Aerospace Medicine, Brooks AFB): I would like to take a couple of minutes describing some of the methods that we have been using to look at the effects of carbon monoxide and altitude hypoxia on the heart and the coronary circulation and discuss the results we have obtained. We place a Doppler ultrasonic flow transducer around the circumflex branch of the left coronary artery to measure flow. We implant a high fidelity solid state pressure transducer in the apex of the left ventricle in order to measure left ventricular pressure and compute left ventricular dp/dt, an index of myocardial contractility similar to what Maj. Van Stee described yesterday. Then we place catheters in the left atrium and in the coronary sinus of the heart in order to measure arterial blood supplying the heart and venous blood draining from the heart. This permits us to look at the AV difference across the heart. We then bring the transducer leads out through the fourth intercostal space, pass them subcutaneously up into the neck region, and permit the dog to recover for a period of at least two weeks so that we can study these stresses in a conscious unanesthetized preparation. This isn't always feasible in many experimental studies, but I think it's something we should try to attain, if possible. It eliminates the effects of anesthetics and things of this sort which we discussed to some extent yesterday.

The heart is different from other organs in that it utilizes 80% of the oxygen supplied to it as opposed to the other organs which utilize approximately 25% so the heart must respond primarily with an increase in coronary blood flow. There's really very little oxygen reserve which it can call upon. I think we also need to remember that this is a healthy young individual that has this 4- or 5-fold increase in coronary blood flow. As we age, this is actually reduced probably 50%. May we see the next slide please?

This shows the effects of carbon monoxide on that reserve coronary oxygen delivery. You can see that with 10% carboxyhemoglobin the arterial saturation is down to this point, with 20% carboxyhemoglobin arterial saturation is down to this point. The coronary venous saturation, however, increases to this point with 10% COHb and to this point with 20% COHb. With altitude hypoxia it goes in the other direction and this reserve can be used, so the heart must compensate by increasing coronary flow and now we have only this reserve oxygen delivery as opposed to the large area here. Let's see the next one.

Earlier studies were done with 1500 ppm carbon monoxide. We repeated some of these studies breathing 100 ppm carbon monoxide and this is coronary blood flow as represented percent increase. We have at 3.2% COHb a 10% increase in flow, and at 8.3% COHb a 20% increase in flow. May we see the last slide?

There was also some discussion yesterday about the interaction of stresses and what effects these might have on our biological limits. This shows the combination of altitude hypoxia for a dog breathing 10% oxygen, equivalent to approximately 18,000 feet, and breathing carbon monoxide alone. This is the response from zero or one up to 20% COHb and the top line is the combination of carbon monoxide plus 10% oxygen so we can see that with this combination we really have an added stress on the heart. Thank you.

DR. BACK: Thank you very much. Now we'll entertain any questions from the floor on any subject either today or yesterday.

MR. VERNOT (SysteMed Corporation): This is to Dr. Weinstein. It appeared that all the work you did in trying to find the site of monomethylhydrazine injury to the red cell, investigation into the various enzymes systems and such, was unsuccessful, you really didn't find it. Have you been speculating since then, do you have any other areas where you think you could go where the site of the injury might lie?

DR. WEINSTEIN (Tufts University School of Medicine): I think that we were surveying the enzyme systems as any good clinician does, and we had definitely established that the enzymes were not involved in the process, which we regard as almost a positive result. I think that at the initiation of the study we were very concerned with why monomethylhydrazine seemed to be particularly effective in producing lesions in red cells but obviously I think other hydrazines such as acetyl phenyl hydrazine on a mole to mole basis are probably pretty much as effective as monomethylhydrazine. I don't think the effect is all that different. I think that we still feel that the most important lesion occurs at the cell membrane and I think the last slides that I showed indicated that there is some evidence that the cell membrane is deranged in the face of monomethylhydrazine. I think that Heinz body binding to the membrane is important and I think that we realized very late in the course of the work that our system really wasn't very adequate for demonstrating this. We realized that in our system Heinz bodies more typically were in the center of the cell or near the membrane but not really attached to the membrane and at the time we completed this work, of course, I returned to Boston and Miss George remained here, and I think we independently began thinking about the work and came to an identical conclusion.

We really failed to consider in setting up our protocol that a test tube really isn't very much like what happens in the human body and one doesn't really simulate the situation just by putting cells in a test tube. As a red blood cell

circulates through the body it goes through the spleen and goes through pores that exist between the splenic cords and splenic sinusoids and these pores typically have a very small orifice something in the range of 3. 2 microns. Then we also reflected that in our preparations the Heinz bodies were frequently not at the membrane and yet when one looks at the peripheral blood of the patient who has a Heinz body anemia, the Heinz bodies are almost invariably at the membrane. So I think that the weakness in our work, or the oversight, is that we should have put a step in whereby we generate Heinz bodies and then pass the red cells through a small orifice, forcing the Heinz bodies over to the cell membrane. I think by doing this we may more closely simulate what happens in the body and then I think having added that step to the protocol, that looking at the cell membrane for damage may be far more fruitful than what we have attempted to do so far and I think that is the next step in our protocol.

MR. WANDS (National Academy of Sciences): I wanted to ask Dr. Kleineberg what he's going to do about this copper tubing he's got inside of his mass spectrophotometer. Will it corrode out the whole brand new gadget in the first week's analyses? Is it possible to modify the equipment to eliminate that scrubbing action towards HCl and similar materials?

DR. KLEINEBERG (Aerospace Medical Research Laboratory): Well, we have to learn a lot to use it properly, but I think that modifications to that extent can be made and we will attempt to do that.

MR. GEIGER (Aerospace Medical Research Laboratory): With respect to your question, Mr. Wands, the copper tube that we're speaking of is on the exit side of the separator or the entrance line coming in from the thermogravimetric balance and it passes on into the source area of the mass spectrometer. Now duPont in building this thing felt that they did not want any condensation to take place after the separator portion in the actual source area of the mass spectrometer, and they felt that copper, due to its conductivity properties, would be the best material to use; they could heat this and the heat would transfer right down the copper line into the source area of the mass spectrometer. We did not know this when we purchased the instrument because everything else is made out of stainless steel and we expected this to be made out of stainless steel also. Had we known this in advance then we would have specified stainless steel or some other material or at least copper coated inside with platinum or some other nonreactive material. Personally, as a mass spectroscopist and an instrumentalist, I feel that this was a very poor choice of materials and I was surprised that the duPont engineers who built this equipment would actually use a copper line. This is one of the things that we are definitely going to change. We'll have to get together with duPont or make some special arrangements, because for our purposes this is totally unsatisfactory.

MR. DI PASQUALE (SysteMed Corporation): Dr. Weinstein, I know we discussed this over the coffee break, but I'm still a little bit fuzzy on a few points. If we assume that a morphometric change in the cell membrane of the erythrocyte

triggers the spleen to pull it out of circulation, do we attribute a qualitative or quantitative function to the Heinz body in the production of this deformation?

DR. WEINSTEIN: I don't know. It could be qualitative or quantitative or both. I think that as a membranologist I would suggest that a single lesion in the membrane, a single abnormality in the membrane, may be quite adequate to allow the spleen to recognize the cell as abnormal. In a strict sense, this may be neither quantitative nor qualitative, it might be topological. For example, if there are determinants on the surface of the cell and they change their arrangement, come a little closer together, that may be adequate to label the cell as being an abnormal cell. In the recent literature on cell membranes there are a large number of papers that point out that the cell membrane is probably a very fluid structure and that proteins that are intercalated within the membrane probably swim quite freely through a lipid sea, if you will, and the distribution of surface charge and different moieties on the surface of the membrane may be extremely important in determining how the cell will function. And a local redistribution of determinants may represent a kind of information that the spleen can recognize and act upon and if this is true, and I think there's enough evidence in the literature to at least suggest it as a viable hypothesis, then perhaps a single Heinz body at a membrane may be quite adequate to account for the spleen's ability to recognize the cell. I would, however, emphasize that at this point we cannot be at all certain that Heinz bodies are required for the process. Remember, if free radicals are being generated in the system, it's quite conceivable that the damage is directly to the cell membrane and has nothing to do with Heinz bodies at all. Free radicals certainly can produce autooxidation of lipids within membranes and those types of changes also may alter the mechanical and chemical characteristics of the membrane and again present the spleen with a picture that it can recognize as aberrant.

DR. BACK: I would like to ask Dr. Spencer a question concerning his paper. You've gotten pretty good translocation of the compound from lungs to other body tissues. Were there any evidences of changes in other organs? Did you see other than lung tumors? Or any other manifestation either in bones or in spleen where you got high levels or in kidney or liver?

DR. SPENCER (The Dow Chemical Company): As far as the active material was concerned, there were metastases to at least the regional lymph nodes. We have not seen them in other organs. Also, as you know, there are translocations to the lymph nodes and we have not quantitated that here simply because we are concerned about the other organs, but there is no doubt about the metastasis. Quite probably also from the active material there was the proliferative response in the lymph nodes in the 500^{o} material but not in the exhaust product.

DR. SLONIM (Aerospace Medical Research Laboratory): I just want to ask Capt. Frame about the time of analysis. Also, do you foresee any limitations or disadvantages in application to biological fluids or other fluids? In other words, do you feel now that there is still room for improvement?

CAPT. FRAME (Aerospace Medical Research Laboratory): The time, including the base dissolution procedure, which I assume is the one you're talking about, is about an hour and a half, two hours when we work with the acid digestions, and I can make the point that all our evidence so far seems to indicate that when we are dealing with beryllium translocated from beryllium oxide which is injected into the lungs, the translocated material is in its soluble form and capable of being put in solution by simple acid digestion. We can then speed up our analysis because the acid digestions can be carried out en masse, in other words, we can put up maybe 20, 25, 30 samples in flasks, digest them all at once over a period of two hours, and then proceed to analyze them perhaps one every 10 minutes as fast as we can get them in the chromatograph. The base dissolution procedure is really an art, has to be watched closely, it only takes 5 or 10 minutes of actual time on the hot plate, but each one of these has to be done individually, and I would say the complete time for taking a single sample, from beginning to end, is an hour or so. I don't see any significant limitations in biological tissues. The work we reported was in two representative biological tissues, blood and liver homogenate. This base dissolution procedure dissolves even high fired beryllium oxide and it certainly dissolves all the tissue and as you saw from the slides the sample is extremely clean. We are, of course, working with the acid solution techniques, trying to make them as gentle as possible, so that we can distinguish between soluble and insoluble forms of beryllium and, under those conditions, we are not completely digesting the tissue and we're having a few problems going down as low as we'd like.

DR. SPENCER: Capt. Frame, I'd like to ask if you anticipate any difficulty in bone?

CAPT. FRAME: Well, I don't know. I think we'd have to do the experiment. There would probably be a lot of phosphate in bone that may precipitate or sequester the beryllium. There's also going to be a lot of calcium and there are calcium chelates which don't chromatograph too well. I think it can be done, but I think we might very well discover that we'd have to alter our chelation procedure.

MR. WANDS: I'd like to ask Capt. Frame two questions please. First of all, in your alkali digestion in pyrex glass, are you getting any extracted beryllium out of the glass?

CAPT. FRAME: No. We've run blanks and we don't find beryllium.

MR. WANDS: How large a tissue sample do you use?

CAPT. FRAME: Two tenths of a gram of tissue homogenate, which contains perhaps one tenth of a gram of actual tissue.

DR. CAMPBELL (U. S. Public Health Service): I wouldn't want to leave Mr. Darmer out so I have one for him. Were you able to observe the temporal pattern

of deaths in the animals that died in your experiment? If so, did you notice any pattern or any interactions concerning the time to death? Was it different for two materials singly and in combination?

MR. DARMER (SysteMed Corporation): One of the limitations on observing the time to death in the experiment which we did was that we sacrificed the animals immediately after exposure in order to obtain the blood. If the animal died during the exposure we did keep track of the time as accurately as we could. I would say that definitely there is a pattern of increasing mortality during exposure when we used the two compounds together at the lower combinations, like the LC_{25} and the LC_{16}, and so on, for the short periods of time. You might have noticed that most of the times of those exposures were considerably less than 5 minutes. If this were a strictly additive type of toxicity, for an LC_{16} of HCN combined with an LC_{25} of CO, we could expect something like 45% mortality, but in actuality we observed total mortality at 5 minutes, so we had to expose for shorter periods of time. And even at those shorter periods of time, we did observe in many cases high mortality rates during the exposure. Does that answer your question?

DR. CAMPBELL: Yes.

DR. MAC FARLAND (Bio-Research Laboratories Ltd., Quebec): I wanted to ask Mr. Darmer the same question Dr. Campbell has asked. In doing your studies first of all with the carbon monoxide for which you showed us your equipment you establish a concentration of carbon monoxide in the chamber and shove the animals in and leave them there for 5 minutes and withdraw them. Let us suppose that it transpires that the concentrations are such that the 5-minute exposure turned out to be the LC_{50} when did the animals die? I assume, of course, that in these studies there was no manipulation of the animals after exposure of low blood drawn.

MR. DARMER: No. On the LC_{50} determinations, we did not draw any blood. We simply exposed the animals, removed them at the proper time after 5 minutes, and observed them. At the LC_{50} concentration the animals die near the end of the exposure or even shortly after the exposure. Animals that did not die by 5 to 10 minutes following the end of the exposure, recovered in experiments with both gases, singly and in combination.

DR. MAC FARLAND: Yes, with carbon monoxide it is very unusual to get deaths after you've withdrawn animals from the gas.

MR. DARMER: Yes, that's right.

DR. MAC FARLAND: They start blowing it off. You might be interested in the discussion on this same point that was made a year or so ago in the proceedings of last year's conference.

DR. HODGE (University of California): Dr. Weinstein, are the Heinz bodies distributed in the hemoglobin?

DR. WEINSTEIN: Yes.

DR. HODGE: And presumably it's intact, native hemoglobin, it isn't a decomposed kind of altered hemoglobin.

DR. WEINSTEIN: I'm sure it's altered. I'm sure it's denatured hemoglobin. The Heinz bodies are not a single entity, they are a morphologist's description. They are round dense bodies that pick up supravital stains that are frequently associated with certain kinds of injuries. For example, there is a class of Heinz body anemias that is related to congenital abnormalities in hemoglobins, and in these situations there are alterations in polypeptide chains, abnormalities in sequencing' and so forth, and the hemoglobin is unstable. It usually cannot retain the heme moiety, the heme is lost from the molecule and the globin then precipitates, so in these situations it comes down as a protein and typically it loses its red color so it has a different appearance, that would be one kind of Heinz body. Heinz bodies can be produced by drugs and in these cases some people had suggested that they are composed of denatured methemoglobin, other people just don't think one really has to go to methemoglobin before one gets denaturization and so forth. So there are a number of different ways that Heinz bodies can be formed and they probably have different compositions and are probably dependent on the mechanism of injury in a given case.

DR. HODGE: Mr. Darmer, the lethal dose of cyanide is usually considered to be a very precise value with quite small variabilities. You had very much larger variabilities than that and this presumably is a result of experimental conditions, differences in exposure, or differences in the responses of individuals.

MR. DARMER: I mentioned at the time that that merely represented the individual data from the individual rats for all the exposures, not taking into account time of exposure or concentration to which they were exposed and that does have some bearing on it. In other words, the ones where the CO concentration in the blood was quite high obviously were the ones exposed to the higher concentrations of CO, and similarly with the cyanide, although there just seems to be a great deal of variation with the cyanide and I think it simply has to do with the response of the individual animals to this compound. We tried to be as consistent as possible in our measuring of the blood cyanide levels, so I think it simply is an animal response and it is not directly related to the conditions of exposure.

DR. MAC EWEN (SysteMed Corporation): I would like to make one additional comment too. As Ken Darmer pointed out earlier this morning, the animals were bled immediately after removal from exposure. Now that's within 2 minutes. On the hydrogen cyanide portion of the study, deaths did occur later postexposure than was possible with CO alone. We really don't know whether some of those we

plotted as survivors might not have died if we had waited 10 minutes before killing them. We were trying to get the samples in a uniform time period for comparison before the detoxication process had gone too far. I think that spread is probably a little greater than it would normally be because of that.

DR. BACK: Of course this is the real life situation and this is what you were looking for. You wanted to find out if you would indeed die or not die if you found x amount of one or x amount of the other in the blood, and that's what you found out - that there was a wide variation.

DR. MAC EWEN: We had very few animals that died in less than 2 minutes. It takes a little while for tissues to die, so some of them might have received a lethal dose at the end of the exposure and still be counted as survivors, but they didn't have time to die, because we exsanguinated them by cardiac puncture.

DR. MAC FARLAND: I think another source of this variability you see, I'm assuming that everyone is aware of the fact that if you put 10 animals in your chamber for 5 minutes at a concentration of let us say 20, 000 ppm of carbon monoxide and that, in the course of the 5 minutes, 5 of the 10 animals die and then you withdraw the animals and have no further deaths, this is not the LC_{50} or the LCT_{50} that you have determined. This again is a point that I raised a year or two ago here and in characterizing something like that as being an LCT_{50} this is not only a rather loose way of speaking, it is in fact quite incorrect, and I exhorted you a year or so ago to consider using LT_{50} information rather than LC_{50} information when you are working with carbon monoxide so that those things that you've described as LCT_{50} , LCT_{25}, LCT_{84}, and so on, are not really that at all, if you had deaths during the 5 minutes exposure.

DR. ANTHONY THOMAS (Aerospace Medical Research Laboratory): We did not forget what you said last year, Dr. MacFarland, but unfortunately this was a study designed only to get forensic medicine information. In other words, what does a certain blood level of carboxyhemoglobin or cyanide mean after a crash? In designing this study and trying to keep to a cost of $10, 000 we didn't have much choice.

DR. FASSETT (Eastman Kodak Company): Just a brief comment on the variability with nitriles. We've had quite a lot of experience studying different types of nitriles and it is true there is a lot of variability. Now the problem is really that these nitriles that act like hydrogen cyanide have a very profound effect on the respiration itself. These animals develop a peculiar gasping respiration. So I think this is part of the variability, you don't have control over the input of the dose.

MR. DARMER: That's a good point and we've observed this in our exposures. The animals did exhibit this type of respiration pattern during the exposures.

DR. HODGE: I'd like to direct a question to Dr. Spencer, perhaps also to Dr. Back. I listened with very considerable interest to discussions of some new preparations of beryllium oxide - three new samples that Dr. Back referred to. As I listened, it seemed to me the interesting possibility was raised that perhaps there is a truly inert beryllium oxide. This just academically is a question that I'd like to see explored but suppose we look at it from a somewhat more practical point of view. Even if these samples, or one of them, was shown to be truly inert physiologically by a good thorough study, would the pattern or the possibilities of beryllium oxide use be altered and would this change our attitude on beryllium motors?

DR. SPENCER: I'll ask Dr. Back first on that, he's much closer to the motor situation.

DR. BACK: Probably not. We did make three new species. Dow made them just recently. What we tried to do was get the largest amount of mass in the smallest package, which obviously is a sphere, and I think they were quite successful in that chore. These compounds were made under extremely high temperatures (2300 C) and they were done in argon in an inert atmosphere and cooled in argon and they are extremely beautiful materials - very clean. It would be academically very good to pursue this. But even if we found that this was totally inert, I would seriously doubt that it would make any difference at all to the powers that be in allowing people to use it. If you could tailor a motor that would spew out that kind of compound then you'd be way ahead. Even if you could build a motor that would do that, I still doubt very seriously that the medical authorities would change their minds. But I still think it ought to be done.

DR. HODGE: From where the Chairman has been sitting the past three days, I'd like to comment that this has been a very interesting program with some new ideas, some new facts, and new reasons for thinking or worrying that have come to me. I've enjoyed the papers and I certainly want to thank all the people who have been presenting.

DR. MAC EWEN: It would be very difficult to summarize all of the things that we have heard in the last few days and I won't even try. I would like to thank you all very much for your excellent participation in this conference and to tell you how much we appreciated your coming. We wish you bon voyage and hope you come back next year. Thank you very much.

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