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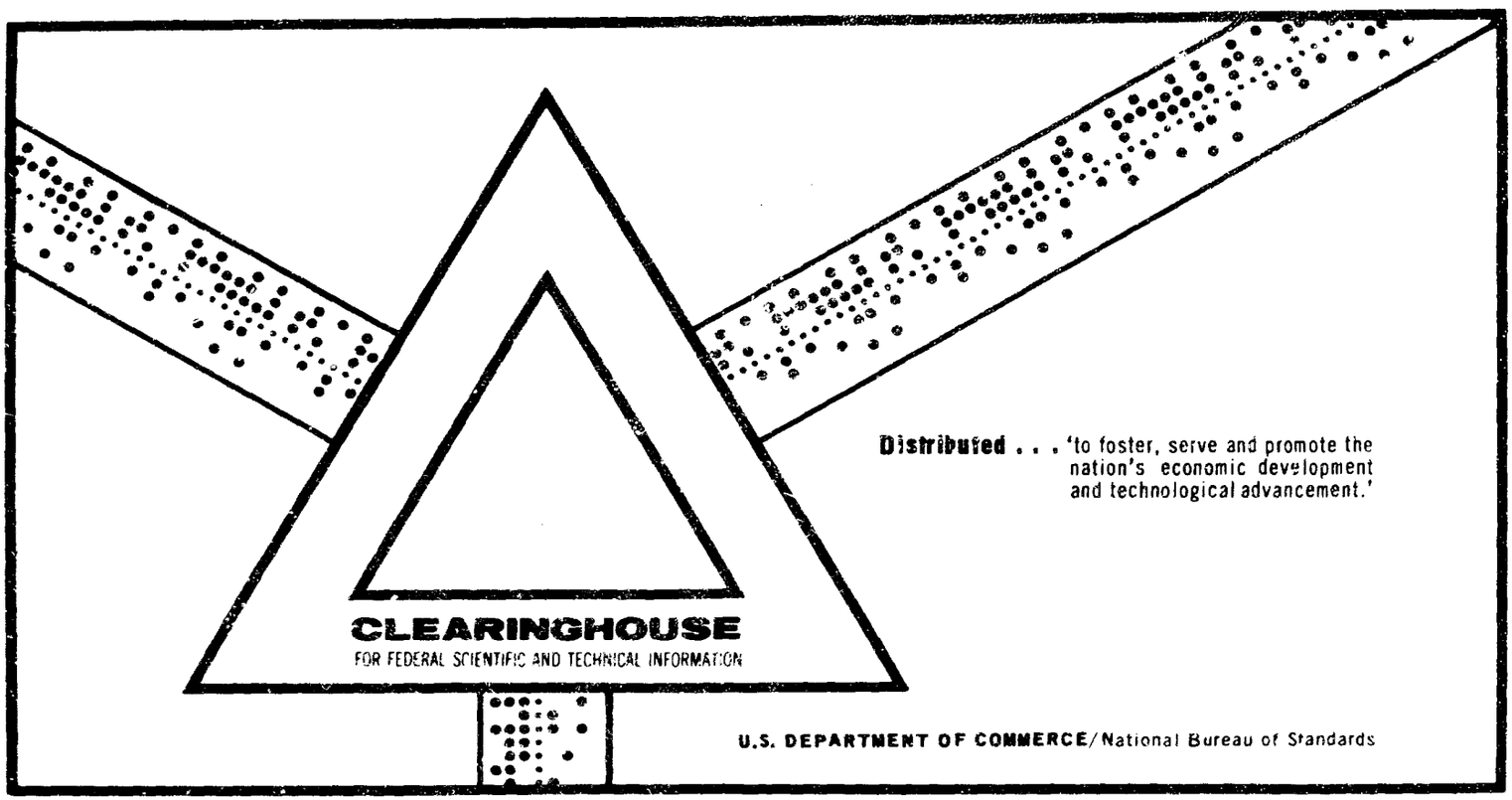
RESEARCH PROGRAM FOR ALERTING, DETECTION AND IDENTIFICATION OF PATHOGENS

J. R. Gould, et al

General Electric Company
Syracuse, New York

September 1969



ELECTRONICS LABORATORY • SYRACUSE, N.Y.

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Period Covered

1 July 1968 to 1 July 1969

Reporting Date

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Prepared by

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FOREWORD

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ABSTRACT

The objective of this program is to assess the feasibility of rapidly detecting and identifying pathogens when present in small populations. As in the past progress reports, the technique used is that of gas chromatography in conjunction with ultrasensitive detectors. Of particular concern in this reporting period are studies to:

(1) Investigate the feasibility of the detection and identification of viruses by a chromatographic analysis of the virus-induced changes in tissue cultures.
(2) Investigate the early detection of bacterial infections by a gas chromatographic analysis of blood sera.
(3) Determine the effects of varying the culture conditions and extraction procedures on the signature.
(4) Evaluate the use of sampling the enclosed space (head space) above the culture medium as a method of obtaining gas chromatographic signatures.

It was definitely established that present methods of ether extraction and silylation of the supernatants and lysates of tissue cultures are not conducive to obtaining reproducible results. Work reported discusses methods of preventing emulsions during ether extraction of serum and techniques of preparing samples for chromatographic analysis.

Studies using halogenated organic acids as media supplements in tissue cultures offers promise of being a useful technique. An evaluation of ten acids showed that at least one distinctive peak unique to the parent compound was observed in the supernatant of infected cells in eight of the ten tested. The prospect of using other than the halogenated acids as substrates is under consideration to further increase the number of reaction products defining the infection.

At no test period after infection of rabbits with Klebsiella-Aerobacter strain did the ether extracts of blood sera show peaks which were not present in the control sample taken prior to inoculation. It is believed that the precipitation of protein to avoid emulsions acts to getter the products of interest.

The conditions of incubation, extraction and GC analysis and the pH value of the medium were varied to obtain a more complex signature. With but one exception, a completely new set of peaks were obtained with eight strains of bacteria when growth was altered from aerobic to anaerobic. Four new peaks were observed with a reduction in pH under anaerobic conditions and three upon alkaline extraction.

Preliminary experiments with head space sampling show conformity to the conventional mathematical model and provides a technique which is adequate to distinguish two strains of bacteria. However, the state-of-the-art is subject to improvement in the analysis of metabolites in the presence of water vapor.

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0.0 INTRODUCTION

Work under this program for the Office of Scientific Research has resulted in the initiation of methods for the rapid detection and identification of small numbers of microorganisms. These methods show considerable promise in meeting the needs of the Department of Defense in the areas of clinical pathology; water, air and soil contamination; and BW defense. The research has been continued during this reporting period with emphasis on investigations of those techniques that lead to the capability of expressing the results at a high level of confidence and to those which provide insight to the basic phenomena.

The successful efforts this program has enjoyed have been based on approaches that are not only unique to the solution of problems, but also those that are amenable to attaining extremely high sensitivity levels. This is not to say that a particular scheme involving a particular detector or method of isolating organisms is the only approach to a desired result; however, it does emphasize that extremely high sensitivity is considered a necessary ingredient.

Consistent with the above philosophy, gas chromatography has been used to undertake investigations in the following areas.

PART I - VIRUSES

The organic mass of virus particles is generally too small to detect or measure by direct chemical or physical analysis. Consequently, methods for detection and identification of viruses usually rely on the biological response as an amplification system. A wide variety of indicators and reactions have been employed by virologists. These include first the products produced by the infected cells or tissues----antibody, interferon, new cell antigens, cross-reacting proteins, etc. Another type of indirect indicator of viruses is the macroscopic response of tissue culture cells, i.e., rounding up and other cytopathic effects, hemadsorption, syncytial formation, agglutination and inability to concentrate or retain dilute dyes such as neutral red. Other miscellaneous types of cell responses have been employed for identification of viruses (although their use may be limited to specific types of viruses), interference with the multiplication of an unrelated challenge virus, absorption of fluorescent antibody, necrotic pocks in monolayers of cells, DNA and RNA annealing experiments, etc.

Most of the methods cited above suffer from slowness, lack of specificity, and/or a low degree of sensitivity. Since we are faced with the urgent need to rapidly identify viruses, work was continued during this reporting period using gas chromatography in conjunction with ultrasensitive detectors to detect and identify viruses based on the premise that the host-virus interaction products will reflect the type of virus in the host. Experiments were conducted in which we attempted to reinforce virus-induced changes in

metabolic pools by addition of glucose, serum components, and halogenated organic acids. We attempted to find culture systems which exhibited unusually high levels of metabolic activity and might be expected to have elevated levels of extractable metabolites. And finally we evaluated various aspects of the gas chromatographic system in an effort to improve the extraction procedures and to achieve greater reproducibility.

PART II - BACTERIA

Investigations in the area of detection and identification of bacteria by a gas chromatographic analysis of the products of microbial metabolism were continued during this reporting period, with emphasis on: 1) early recognition of bacterial infection using blood sera, and 2) increased complexity of signatures by variation of the culture conditions and extraction procedure.

PART III - HEAD SPACE

Head space refers to the confined area above the culture media. Inasmuch as the analysis of the vapors above the media could result in a rapid, non-perturbing, and an easily implemented system, a mathematical model for head space sampling was developed and tested.

1.0 PART I - VIRUSES

1.1 EXPERIMENTAL

The following experiments describe efforts to measure the production of volatile, virus-specific metabolites of infected cells and to describe qualitative changes in the nutrient solutions overlaying such cultures. It is reasonably well established that the regulatory interactions between metabolic pathways also change the allosteric controls operating within specific pathways (1). Virus infection clearly changes both of these control mechanisms by altering substrate levels available to various enzymes. The extent to which unique low molecular weight components are synthesized in this process is the objective of these tests.

1.1.1 Experiment 1 - Glucose Enriched Media

Objective

An experiment was undertaken to determine the effect of glucose enriched media upon the infecting agent Sindbis virus. A comparison of ether and silylation methods of preparing samples from the supernatant and lystate phases for chromatographic analysis was also made. The samples were prepared at Syracuse University as outlined in the flow diagram (Chart 1).

Materials and Methods

Chick embryo filbroblast (CEF) cells were grown in MEM + 10% calf serum supplemented with 0, 0.5, and 1.0% glucose for 48 hours at 37 C. The medium was decanted and the cell monolayer washed with Hank's balanced salt solution. The cell sheet was 1) sham exposed, placed under 15 ml of MEM with and without 2% serum and a 0, 0.5, or 1.0% glucose supplement and incubated for 24 hours at 37 C; or 2) subjected to an absorption period (Sindbis CEF preparation, titer 512 Hemagglutinating units, 1 ml of virus/bottle) of 2 hours at 25C, washed twice with BSS, and maintained as (1).

Infection was >95% after 24 hours as judged by the number of cells exhibiting cytopathic effects (CPE).

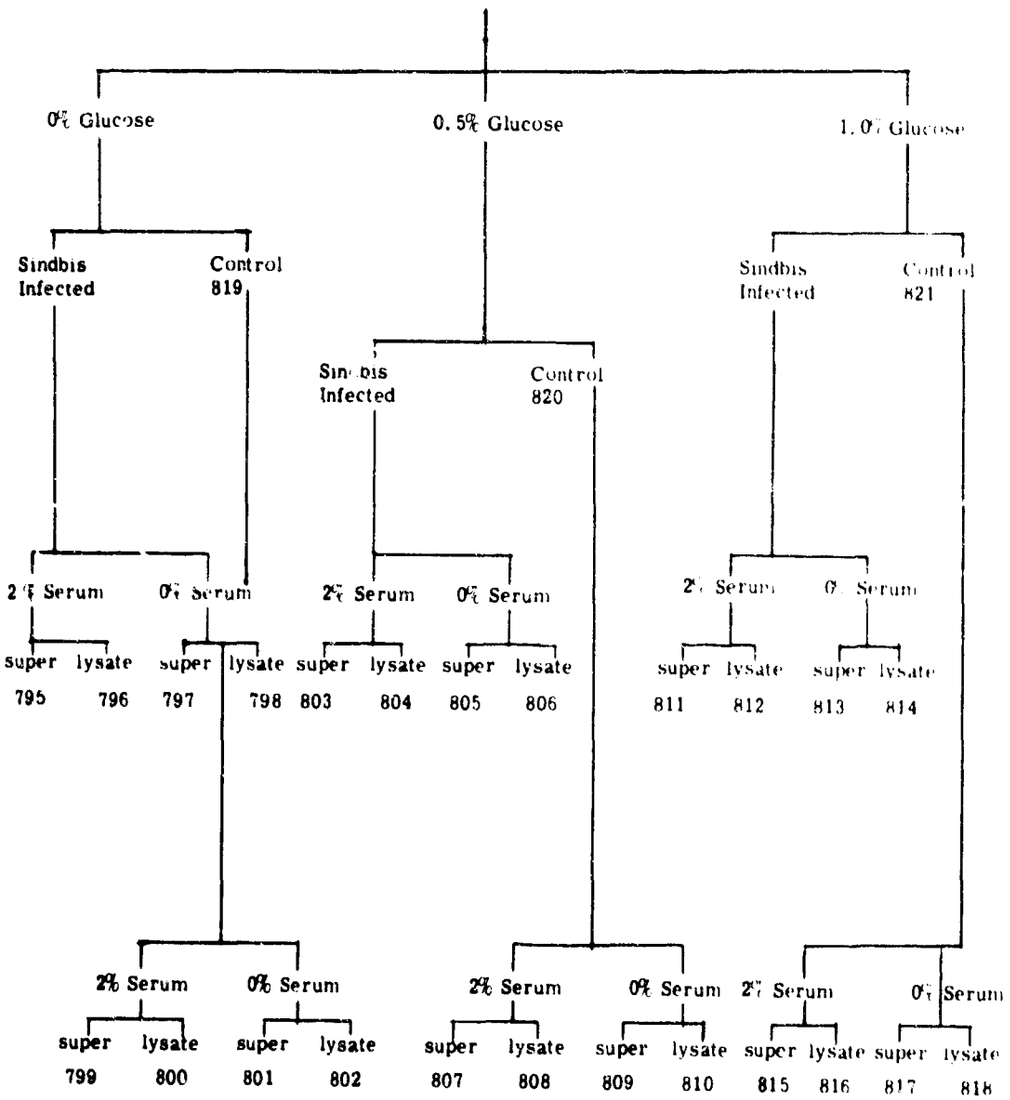
Upon completion of maintenance in the media shown (Chart 1), the tissue culture media were centrifuged at 1,000 × G for 10 minutes and 10 ml of the supernatant withdrawn for GC analysis. The supernatant was adjusted to pH 2 with 1N HCL and extracted with an equal volume of ether. The ether phase was concentrated to 0.5 ml by evaporation and injected into the GC in 5 μl aliquots. These methods are similar to those previously reported.

The aqueous phase was then used for silylation by evaporating to dryness in a rotary evaporator, drying an additional 14 hours in a dessicator, dissolving in 5 ml of NaOH dried pyridine for 5 minutes at 110C,

Chart 1

GLUCOSE ENRICHED MEDIA

MEM MAINTENANCE MEDIA



adding 0.5 ml of Regisil [Bis (trimethylsilyl) trifluoroacetamide] at room temperature and reacting at 110C for 5 minutes. A 3 μl aliquot of the silylated sample was used in the chromatography system.

The lysate samples for ether extraction and silylation were prepared by washing the cell layer with BSS, adding 10 ml of the appropriate maintenance media, freezing at -20C, and thawing rapidly to 37C. The samples were then extracted in a manner similar to that used for the supernatants.

The GC system used to analyze the ether phase contained a 6' × 1 4" stainless steel column of 10% Carbowax 20 M TPA on 120/140 Chromosorb W-AW. It was operated at 140 C with a N_2 flow of 52 ml/min.

The GC system used to analyze the silylated samples contained a 4' × 5 mm i.d. glass U-tube packed with 10% DC-200 on 80/100 Chromosorb W-AW. This column was operated at 180C with a N_2 flow rate of 52 ml/min.

Discussion

A summary of the signatures of infected cells for both ether extracts and silylated samples of supernatants and lysates is presented in Table I. In order to gain a conception of the extent to which the uninfected cells are metabolizing as measured by the above procedures, the data distinguishing the controls with and without cells at 0.0% serum are presented in Table II. Control samples at 2.0% serum were not examined. Inspection of the data indicates that metabolic products observed in ether extracts a) have the largest variety and greatest reproducibility in the supernatant samples, b) show a consistent trend to increase in number in glucose, and c) vary in kind at different levels of glucose. Silylated samples, on the other hand, show a) considerable variability in the data, b) distinctions more dependent on the depletion of background peaks than addition of new peaks, and c) two peaks (D & I) that were not observed in ether extracts.

Table III shows the effect on the signatures of Sindbis-infected cells of varying the glucose concentration at constant serum concentrations of 0.0 and 2.0%. When the ether data are compared with the observations from the uninfected cells (Table II), it appears that a) there are fewer peaks to distinguish the cells from their controls, and b) the maximum differences occur at 0.5% glucose, similar to ether extracts, but in the lysates rather than the supernatants. Once again, no trend in the silylated samples is observed.

In summary, it appears that a) the ether data are more informative than the silylation data for both infected and uninfected cells, b) usually signatures from unifected cells are more informative when taken from the supernatants rather than from the lysates, information being maximized at 0.5% glucose, and c) signatures from infected cells are best exemplified by samples from the lysates, information being maximized at 0.5% glucose and 2.0% serum.

TABLE I

GLUCOSE ENRICHED MEDIA
SIGNATURE SUMMARY

ETHER EXTRACTS

E. C. D.

Infected		Control		Final	% Glucose	% Serum	Phase
795	ABCEF	799	ABCEF	---	0	2	Super
796	ABC	800	BC	A	0	2	Lysate
797	ABCEF	801	ABCEF	---	0	0	Super
798	BC	802	BC	---	0	0	Lysate
803	ABCEF	807	ABCEFH	H	0.5	2	Super
804	ABCEF	808	BC	AEF	0.5	2	Lysate
805	ABCEFH	809	ABCEFGH	G	0.5	0	Super
806	BCG	810	BCG	---	0.5	0	Lysate
811	ABCEF	815	ABCEFH	H	1.0	2	Super
812	BC	816	C	B	1.0	2	Lysate
813	ABCEFH	817	ABCEFH	---	1.0	0	Super
814	C	818	BC	B	1.0	0	Lysate

Controls for 0% Serum Media
0% Glucose 819 BC
0.5% Glucose 820 B
1.0% Glucose 821 BC

SILYLATION

F. I. D.

Infected		Control		Final	% Glucose	% Serum	Phase
795	EFGHI	799	EFI	GH	0	2	Super
796	AEGHI	800	DGH	ADEI	0	2	Lysate
797	DEFI	801	EFI	D	0	0	Super
798	G	802	G	---	0	0	Lysate
803	DEFHI	807	BDEFHI	B	0.5	2	Super
804	Lost	808	Lost				
805	DEFHI	809	EFHI	D	0.5	0	Super
806	EFI	810	BDEFI	BD	0.5	0	Lysate
811	DEFI	815	BDEFI	B	1.0	2	Super
812	EFI	816	EFI	---	1.0	2	Lysate
813	BCEFI	817	BDEFI	CD	1.0	0	Super
814	EFI	818	EFI	---	1.0	0	Lysate

Controls for 0% Serum Media
0% Glucose 819 EFI
0.5% Glucose 820 BCEFI
1.0% Glucose 821 BCEFI

Note: A indicates peak A absent from sample, but present in control.
--- indicates no distinction.

TABLE II

GLUCOSE ENRICHED MEDIA CONTROLS

UNINFECTED CELL METABOLISM				
ETHER EXTRACTS				
			Signature	
% Glucose	% Serum	Sample-Control	Super	Lysate
0.0	0.0	801-819	AEF	
		802-819		-----
0.5	0.0	809-820	ACEFGH	
		810-820		CG
1.0	0.0	817-821	AEFH	
		818-821		-----
SILYLATION				
			Signature	
% Glucose	% Serum	Sample-Control	Super	Lysate
0.0	0.0	801-819	---	
		802-819		GEFI
0.5	0.0	809-820	BCH	
		810-820		DC
1.0	0.0	817-821	DC	
		818-821		BC

TABLE III

GLUCOSE ENRICHED MEDIA
SIGNATURES FROM INFECTED CELLS
GLUCOSE VARIABLE AT CONSTANT SERUM

ETHER EXTRACTS

% Serum	% Glucose	Super	Lysate
2.	0.0	---	A
	0.5	H	AEF
	1.0	H	B
0.	0.0	---	----
	0.5	G	----
	1.0	---	B

SILYLATION

% Serum	% Glucose	Super	Lysate
2.	0.0	GH	ADEI
	0.5	B	Lost
	1.0	B	----
0.	0.0	D	----
	0.5	D	BD
	1.0	CD	----

1.1.2 Experiment 2 - Blood Serum Variable

Objective

This experiment was designed to investigate the effect of various concentrations of serum on the signature of Sindbis virus. Included is a study of signature changes with incubation time. Chart 2 shows the various serum concentrations and the maintanance incubation periods.

Materials and Methods

The CEF cells were grown in 0.5% lactalbumin hydrolysate (LAH), 10% calf serum, 1.0% Earle's BSS, and 1% PSN antibiotics for a period of 48 hours at 37°C. The supernatant was decanted, the monolayers washed twice with Earle's solution, infected as in experiment (1), and maintained as shown in Chart 2.

The methods used in the preparation of ether extracts for gas chromatography were similar to those used in experiment (1). Silylation of these samples has not yet been completed.

A 6' × 1/4" ss column packed with 10% Carbowax 20M TPA on 120/140 Chromosorb W-AW extracts operating at 150C with a N_2 flow rate of 40 ml/min was used to analyze the ether extracts. Only the ECD data is reported, since the FID chromatograms showed only trace peaks and therefore were termed unsuitable as signatures.

Discussion

The results of an ECD analysis of the extracts showed no correlation among the controls (Table IV). Ideally, there should have been a trend of peak increases or decreases with a change in serum concentration within a given set. The media control set should produce the most consistent trend since all other controls would be subject to the variability introduced by the uninfected cells. However, there is no evidence of a trend in the data. In addition, a correlation among cell controls of each set of serum concentrations in relation to incubation time was expected. This trend was also not evident.

Emulsions encountered upon ether extraction may be responsible for the inconsistancy of the data. The existance of an emulsion may act to getter reaction products of interest and since this may not be a uniform effect from sample to sample, the emulsion may be the cause of the inability to show a trend in the data. Work has been initiated to devise both physical and chemical methods of breaking these emulsions. Under consideration are high acidities, salts, de-emulsifying agents such as Brij 35 and Triton X-100, centrifugation, and filtering.

Summary

Inasmuch as the data (Table IV) lacks expected trends, further treatment is not presented. The results of the GC analysis of silylation, which avoids the emulsion problem, of these and or similar samples will be presented at a later date.

Chart 2

CALF SERUM VARIABLE

Sindbis Infected				Control			
Media	Sample Time			Sample Time			Media
	T_0	T_{18}	T_{24}	T_0	T_{18}	T_{24}	
0	822	837	847	827	841	852	0
2	823	838	848	828	842	853	2
4	824	839	849	829	843	854	4
8	825	845	850	830	846	855	8
Control	826	840	851	831	844	856	Control

Media		Sample
0	undiluted serum - 14 ml serum 1 ml PSN bottle	832
2	50% serum, 50% MEM maintenance media	833
4	25% serum, 75% MEM maintenance media	834
8	12.5% serum, 87.5% MEM maintenance media	835
Control	0% serum, 100% MEM maintenance media	836

Time

T_0 - zero hours post infection

T_{18} - 18 hours post infection

T_{24} - 24 hours infection

TABLE IV

BLOOD SERUM VARIABLE

CONTROL COMPARISONS

Media Controls

Serum Concentrations	Sample No.	Signature
100% Serum	832	CDFJMOR
50%	833	DJO
25%	834	EHJLNOP
12.5%	835	EHJLNOP
0%	836	EH

Sample Control 100% Serum

Sampling Time (hrs.)	Sample No.	Signature
T_0	827	---
T_{18}	841	N
T_{24}	852	EIN

Sample Control 50% Serum

Sampling Time (hrs.)	Sample No.	Signature
T_0	828	----
T_{18}	842	LNP
T_{24}	853	FIN

Sample Control 25% Serum

Sample Time (hrs.)	Sample No.	Signature
T_0	829	EHJLOPS
T_{18}	843	D
T_{24}	854	EFIKMNP

Sample Controls 12.5% Serum

Sample Time (hrs.)	Sample No.	Signature
T_0	830	EHLPS
T_{18}	846	DEFGJKNP
T_{24}	855	BDIKN

TABLE IV - concluded

BLOOD SERUM VARIABLE

CONTROL COMPARISONS

Sample Controls 0% Serum		
Sampling Time (hrs.)	Sample No.	Signature
T_0	831	EHS
T_{18}	844	DEGKMO
T_{24}	856	DFILNP

1.1.3 Experiment 3 - Egg Inoculation

Objective

This experiment was designed to determine if it is feasible to eliminate many of the steps in sample preparation by using fluids from embryonated eggs serving as the host for different viruses. This was an attempt to reduce the number of possible variables inherent to tissue cultures and to improve reproducibility. Chart 3 shows the inoculation route used for each virus.

Materials and Methods

Ten-day old embryonated eggs were inoculated with 0.1 ml of a 1:100 dilution of stock virus in tryptose phosphate broth.

Stocks: WSN - Allantoic Fluid - 8×10^7 P.F.U./ml

Sindbis - Mouse brain - 3×10^9 P.F.U./ml

NDV - Allantoic fluid - 1280 H.A.U./ml

After inoculation, the eggs were sealed with wax, incubated for 48 hours at 37C, and refrigerated overnight. The respective fluids were then harvested.

The ether extracts of samples were prepared for GC in the same manner as prior experiments.

The column used was 6' × 1/8" ss packed with 5% FFAP on 90/100 Anakrom ABS run at 150C with a N_2 flow rate of 50 ml/min.

Discussion

The only peak shown on the chromatograms had a retention time of 1.8 cm as detected by the ECD. This is constant through all samples run with each virus. Since the peak was also in the controls,the conclusion

Chart 3

EGG INOCULATION

Inoculum	Inoculation Route	Designation of Harvested Fluid
1. Influenza A WSN	0.1 ml - allantoic cavity	allantoic - 1030 amniotic - 1031
2. NDV	0.1 ml - amniotic cavity	allantoic - 1023
3. Sindbis	0.1 ml - amniotic cavity	amniotic - 1029
4. Control Sindbis uninfected mouse brain	0.1 ml - amniotic cavity	amniotic - 1033
5. Control WSN uninfected allantoic fluid	0.1 ml - allantoic cavity	amniotic - 1032 allantoic - 1027
6. Control NDV uninfected allantoic fluid	0.1 ml - amniotic cavity	allantoic - 1027

must be drawn that these viruses, in the presence of either the allantoic or amniotic fluid, produced no products that could be detected either by FID or ECD using the FFAP.

These results are not consistent with those summarized in previous reports.[2] An experiment designed to identify the virus Sindbis and influenza WSN by ECD used allantoic fluid as the carrier media for the viruses. These results showed at least 2 ECD peaks from the allantoic fluid control, so on this basis a slight background from the controls of this experiment was expected. However, as stated, only one constant peak appeared in each sample.

It is possible that different column conditions are responsible for the inconsistency since the column used in the earlier experiment was 10% Carbowax 400-TPA on Chromosorb W-HMDS 80/100 with a column temperature of 100C and a N_2 flow rate of 60 cc/min. This possibility will be fully investigated before a final decision is made regarding the feasibility of using chick embryos within the egg as the host cells. The latter host would be employed for non-dermatropic viruses, which normally grow poorly in epithelial cells of the amniotic sac or the CAM.

1.1.4 Experiment 4 - Organic Acids as TC Supplements

Objective

This experiment was conducted to determine the effect of certain halogenated organic acids, which were used as a media supplement, upon tissue culture signatures using Sindbis virus as the infecting agent. The following acids were used at a final concentration of 0.1% to help increase the detection by GLC of the products formed (Chart 4):

dibromosuccinic	bromohexanoic
bromovaleric	dichloropropionic
chlorovaleric	alpha-chlorobutyric
dichlorosuccinic	chlorosuccinic
bromocaprylic	

Materials and Methods

The ether extracts for GC were prepared at pH_2 in the usual manner.

Growth and infection were conducted as in the glucose experiment with the organic acids added to the maintainance medium.

The column used was 10% Carbowax 20 M TPA . 120/140 Chromosorb W-AW packed in 6' × 1/4" ss operated at a temperature of 155C with a N_2 flow rate of 40 ml/min.

Discussion

Studies of the signature patterns (Table V) indicate that there is a distinction between infected and control samples in both the lysate and supernatant extracts.

Chart 4

ORGANIC ACIDS AS TC SUPPLEMENTS

MEM MAINTENANCE MEDIA

Sindbis Infected		754	Control	
di-cl-succinic	super 755 lysate 756	775 super 776 lysate		di-cl-succinic
di-cl-propionic	super 757 lysate 758	777 super 778 lysate		di-cl-propionic
control-no acid	super 759 lysate 760	779 super 780 lysate		control-no acid
di-br-succinic	super 761 lysate 762	781 super 782 lysate		di-br-succinic
α-cl-butyric	super 763 lysate 764	783 super 784 lysate		cl-butyric
cl-valeric	super 765 lysate 766	785 super 786 lysate		cl-valeric
br-caprylic	super 767 lysate 768	787 super *788 lysate		br-caprylic
br-valeric	super 769 lysate 770*	789 super *790 lysate		br-valeric
cl-succinic	super 771 lysate 772	791 super 792 lysate		cl-succinic
br-hexanoic	super 773 lysate 774*	793 super *794 lysate		br-hexanoic

*monolayer sloughed - no lysate

TABLE V

ORGANIC ACIDS AS TC SUPPLEMENTS

ECD SIGNATURE COMPARISONS

	Sample Designation			Qualitative Distinctions	
	Supernatant		Lysate	Supernatant	Lysate
Dichlorosuccinic	755	Infected	756	F CI	C
	775	Control	776		
Dichloropropionic	757	Infected	758	J FN	No Lysate
	777	Control	778		
Dibromosuccinin	761	Infected	762	H	ABDEGJ
	781	Control	782		
α-chlorobutyric	763	Infected	764	I CDGH	C
	783	Control	784		
Chlorovaleric	765	Infected	766	No Distinction	I BGK
	785	Control	786		
Bromocaprylic	767	Infected	768	N	No Lysate
	787	Control	788		
Bromovaleric	769	Infected	770	P	No Lysate
	791	Control	792		
Chlorosuccinic	771	Infected	772	FGK AM	No Lysate
	789	Control	790		
Bromohexanoic	773	Infected	774	ABDEGH	No Lysate
	793	Control	794		
No Acid	759	Infected	760	F	No Difference
	779	Control	780		

A Represents a peak depleted from the control

It was also found that the following four acids, at the concentration used, gave a reduced yield relative to the control:

1. Dichloropropionic yielded no virus and caused the monolayer to slough.
2. Bromohexanoic yielded no virus and caused the monolayer to slough.
3. Bromocaprylic yielded no virus.
4. Bromovaleric yielded no virus.

Data obtained regarding deletion of peaks from the control in comparing it with the infected samples possibly indicates a unique signature associated with a particular combination of Sindbis virus with a halogenated organic acid. The acid that appeared to add the greatest number of positive peaks (peaks present in the infected sample and not in the control) was chlorosuccinic acid. This increase is evident in only the supernatant extract inasmuch as the monolayer in the lysate sample sloughed.

Before any of the designated acids are used as a permanent intricate component of the maintenance media, additional replicate experiments will be conducted employing a wider range of concentrations, a greater variety of halogenated acids and a replacement schedule to remove the excess reagent if necessary.

1.1.5 Experiment 5 - Canine Viruses

Objective

The purpose of this experiment was to evaluate the changes in the signatures from tissue cultures with incubation time for the FV-7233, DK, and PK-14 strains of infectious canine hepatitis virus (ICHV). These strains were previously evaluated.[3]

Materials and Methods

Primary dog kidney cell cultures (DKC) were prepared from 4 week SPF Beagle pups. The growth medium was Earle's saline solution (ESS), 0.5% lactalbumen hydrolyzate, and 10% new-born lamb serum. The monolayer was washed with ESS and each culture inoculated with 0.1 ml of virus ($10^{6.3}$ $TCID_{50}$). After a 2 hour absorption period the cells were washed 3X with ESS (25%) and normal pup serum (75%), and then maintained in 1.5 ml of the wash solution. Cultures of each virus were harvested at 0, 20, and 43 hours after absorption. Each sample was centrifuged for 15 minutes at 1500 rpm and the supernatant removed for storage at -60C. The samples were prepared for GC by rapid thawing, adjusting to pH 2 (0.2 ml of 5N HCl and 1.0 ml of 0.2M HCl-CK1 buffer, pH 2), and extracting with an equal volume of ether.

No cytopathic effects (CPE) or bacterial contamination was observed in the controls, no CPE at T_0, CPE ± (occasional cell beginning to show adeno-CPE) at T_{20}, and CPE + 2 (approximately 50% of cells showing typical adeno-CPE) at T_{43}.

The above samples were supplied by Dr. L. E. Carmichael of the Cornell Veterinary Virus Research Institute.

The ether extracts were chromatographed using a Varian Aerograph 1800 with 10% Carbowax 20 M TPA 100 200 packing in a 6' × 1/8" stainless steel column at a temperature of 150C using nitrogen as a carrier gas at a rate of approximately 35 ml/min.

Discussion

The experimental protocol is outlined in Chart 5 and the resulting data from the chromatograms are portrayed in Tables VI-VIII. These data certainly indicate, in particular that exhibited by the ECD, that the three stains of ICHV may be distinguished. (Table VIII). However, it is evident (Table VII) that these distinctions are the result of the presence or absence of products excreted by the control cells rather than induced reaction products resulting from the infection. Inasmuch as this information is the result of one of four replicates, further evaluation of the ICHV strains is contemplated. Such investigations will not only include ether extracts but also silylated samples of the supernatant for GC analysis.

Chart 5

Canine Viruses

Strain	FV-7233			DK			PK-14		
Incubation (hrs.)	0	20	43	0	20	43	0	20	43
Infected	1035	1039	1043	1036	1040	1044	1037	1041	1045
Control	1034	1038	1042	1034	1038	1042	1034	1038	1042

TABLE VI

CHROMATOGRAM DATA

Sample No.	No. of Peaks	Flame Data A	B	C	D	E	F	G	H
1034	6	2.9		5.6	6.6	---	10.7	13.7	25.6
1035	4	2.9	---	5.7	6.8	---	----	13.8	----
1036	7	2.9	---	5.7	6.8	8.8	10.8	13.8	----
1037	3	---	---	5.8	6.8	---	----	13.7	----
1038	5	3.0	---	5.8	6.8	---	10.8	13.9	----
1039	5	3.1	---	5.8	6.9	---	10.8	13.9	----
1040	5	3.0	---	5.7	6.7	---	10.8	13.7	----
1041	5	3.0	5.1	5.7	6.8	---	----	13.7	----
1042	4	2.9	---	5.7	6.7	---	----	13.8	----
1043	5	3.0	---	5.7	6.7	---	10.6	13.6	----
1044	5	3.0	---	5.7	6.9	---	10.9	13.9	----
1045	4	3.1	---	5.9	6.9	---	----	14.0	----

Sample No.	No. of Peaks	Electron Capture Data A	B	C	D	E	G	H	I	J	K	L	M
1034	10	1.6	0.8	2.6	3.2	5.3	---	6.5	8.0	10.4	12.6	13.3	----
1035	9	1.4	1.7	2.6	3.2	5.4	---	6.4	---	10.5	12.7	13.3	----
1036	9	1.4	1.7	2.6	3.1	5.3	---	6.5	---	10.5	12.7	13.4	----
1037	9	1.3	1.6	2.7	3.2	5.4	---	6.6	8.0	10.4	12.8	13.4	----
1038	10	1.3	1.7	2.6	3.2	5.4	6.3	6.6	8.1	10.4	12.7	----	15.7
1039	7	1.2	---	2.7	3.2	5.4	6.3	---	---	10.6	12.4	----	----
1040	9	1.3	1.7	2.6	3.2	5.3	6.3	---	8.0	10.3	12.6	----	----
1041	8	1.3	1.7	2.6	3.2	---	6.3	---	7.9	10.3	12.4	----	----
1042	9	1.3	1.7	2.6	3.2	5.4	6.4	---	8.0	10.5	12.5	----	----
1043	11	1.3	1.7	2.6	3.1	5.3	6.3	6.6	8.0	10.4	12.6	----	13.6
1044	9	1.3	1.8	2.6	3.2	5.4	6.4	6.7	---	10.6	12.9	----	----
1045	5	1.4	1.7	2.7	3.3	---	6.5	---	---	10.3	12.9	----	----

Retention time for each peak expressed in cm.

TABLE VII

CANINE VIRUSES
SIGNATURE* SUMMARY

ECD

FV-7233		DK		PK-14	
T_o	I	T_o	I	T_o	---
T_{20}	B H I M	T_{20}	H M	T_o	E H M
T_{43}	H M	T_{43}	H I	T_{43}	E I

FID

FV-7233		DK		PK-14	
T_o	F H	T_o	E H	T_o	A F H
T_{20}	---	T_{20}	---	T_{20}	B F
T_{43}	F	T_{43}	F	T_{43}	---

* Sample peaks (Table VI) - controls

TABLE VIII

CANINE VIRUSES

COMPARISON OF VIRUSES

Electron Capture				Flame Ionization			
	T_0				T_0		
	FV-7233	DK	PK-14		FV-7233	DK	PK-14
FV-7233	---	---	(I)	FV-7233	---	E(F)	(A)
DK	---	---	(I)	DK	E(F)	---	(A)E(F)
PK-14	(I)	(I)	---	PK-14	(A)	(A)E(F)	---
	T_{20}				T_{20}		
	FV-7233	DK	PK-14		FV-7233	DK	PK-14
FV-7233	---	(B)(I)	(B)(E)(I)	FV-7233	---	---	B(F)
DK	(B) (I)	---	(E)H	DK	---	---	B(F)
PK-14	(B) (E)(I)	(E)	---	PK-14	B(F)	B(F)	---
	T_{43}				T_{43}		
	FV-7233	DK	PK-14		FV-7322	DK	PK-14
FV-7233	---	(I)M	(E)H(I)M	FV-7233	---	---	F
DK	(I)M	---	(E)H	DK	---	---	F
PK-14	(E)H(I)M	(E)H	---	PK-14	F	F	---

1.1.6 Experiment 6 - Comparison of Methods of Sample Evaporation

Objective

The purpose of this experiment was to compare various methods of evaporating serum samples and to compare samples with and without ether extraction prior to silylation (Chart 6).

Methods and Materials

Since an adequate lyophilization unit was not available, freeze-drying of the samples was postponed temporarily. Samples prepared for lyophilization were, instead, dried in a vacuum oven at 54 C for 2 hours and left overnight under vacuum at 24C. The samples prepared for vacuum oven drying were dried overnight at 50C. Attempts to dry samples on a rotary evaporator were not successful.

Each sample contained 5.0 ml of calf serum, 0.5 ml of 5 N HC1, and 2.5 ml of 0.2 M HC1-KC1 buffer (pH 2). Duplicate samples for each method of drying contained high or low concentrations of asparagine, glycine, and methionine, and acetic acid. The asparagine was dissolved in water (0.022 g/ml), and to the appropriate samples was added 0.5 ml of the asparagine solution (to give a final concentration of 2×10^{-6} g/μ1 of solution to be injected into the column). A 1:2 dilution of the above asparagine solution was made for samples receiving a low concentration of asparagine, giving a final concentration of 1×10^{-6} g/μ1. Similarly, methionine (0.022 g/ml) and glycine (0.022 g/ml) were dissolved in acetic acid (1×10^{-8} g HAc/μ1 of final solution to be injected into the GC), and 0.5 ml of this solution was added to the appropriate samples. Dilution (1:2) of this solution in HAc was used for the low concentration solution of methionine and glycine.

The appropriate samples were extracted with 10 ml of ether, and the aqueous layers were dried as described above. Silylation was accomplished (see Experiment 1) as follows:

1. Pyridine (5.0 ml) was added to each sample.
2. Test tubes were imbedded in sand (110C) for 5 minutes.
3. Silylating agent (Regisil), 0.5 ml, was added to each sample, and test tubes were again imbedded in sand (110C) for 5 minutes.

Silylated samples were stored at room temperature for at least 24 hours before chromatographic analysis. Ether extracts were kept at 0C.

The column used was a 10% Carbowax 20 M-TPA on 120 140 Chromosorb W-AM (see Experiment 1) operated at 80C and a N_2 flow rate of 21 ml/min.

Chart 6

METHODS OF SAMPLE EVAPORATION

Sample No.	Method of Drying	Contents
8	Vacuum oven; (50C overnight)	low conc. of amino acids HAc
9		low conc. of amino acids HAc
10		high conc. of amino acids HAc
11		high conc. of amino acids HAc
12		low conc. of amino acids HAc
13		low conc. of amino acids HAc
14		control (No amino acids or HAc)
15	Vacuum oven; (54C for 2 hours)	low conc. of amino acids HAc
16		low conc. of amino acids HAc
17		high conc. of amino acids HAc
18		high conc. of amino acids HAc
19		low conc. of amino acids HAc
20		low conc. of amino acids HAc
21		control conc. of amino acids HAc
22		distilled H_2O (used for Asn sgl. + dilution of HAc)

Samples numbered 12, 13, 19 and 20 were not extracted with ether prior to silylation.

Discussion

The samples dried via vacuum oven at 54C for 2 hours then left overnight at 24C produced a greater number of peaks than those dried overnight in a vacuum oven at 50C (Tables IX-XII). It was also evident using either method that reproducible signatures were not obtained for similarly prepared samples and that extraction with ether prior to silylation did not tend to stabilize the results.

The data demonstrate that these methods of vacuum drying are not suitable for preservation of the sample characteristics.

TABLE IX

METHODS OF SAMPLE EVAPORATION

RETENTION TIMES IN MM

Peak No.	R_t	Peak No.	R_t	Peak No.	R_t
1	1-2.5	16	44-47	31	191-200
2	3-4	17	47-49	32	201-215
3	5-6	18	50-53	33	250-350
4	7-8	19	54-58	34	351-400
5	9-10	20	66-78	35	401-500
6	11-12	21	79-82	36	501-600
7	13-14	22	83-93	37	601-700
8	15-19	23	120-125	38	651-799
9	17-19	24	126-136	39	800-825
10	20-23	25	138-147	40	826-850
11	24-26	26	148-150	41	851-900
12	27-28	27	151-159	42	901-925
13	29-31	28	161-170	43	926-950
14	33-36	29	171-180	44	951-1000
15	39-42	30	181-190	45	1600-1750

TABLE X

METHODS OF SAMPLE EVAPORATION
ECD PEAK SUMMARY

	Sample No.	
Dried Via Vacuum Oven at 50C Overnight	8L	1, 7, 17, 43, 44, 45
	9L	1, 7, 15, 39, 40, 41
	10H	1, 6, 15, 24, 28, 38, 39, 40
	11H	1, 6, 15, 39, 40, 41
	*12L	1, [illegible], 8, 17, 23, 25, 28, 33, [illegible]
	*13L	1 [illegible], 19, 28, 32, 45
	14 Control	1, [illegible] 9, 17, 37 [illegible]
Dried Via Vacuum Oven at 54C For Two Hours	15L	1, 18, 30, 32
	16L	1, 5, 8, 11, 16, 17, 44, 45
	17H	1, 8, 17, 19, 22, 44
	18H	1, 5, 8, 12, 18, 19, 41, 42 43, 44
	*19L	1, 3, 5, 8, 9, 13, 14, 18, 19, 34, 35, 44
	*20L	1, 3, 4, 9, 12, 18, 19, 22, 27, 29, 31, 36, 41, 44
	21 Control	1, 3, 5, 9, 13, 18, 19, 22, 29, 31, 33, 37, 39, 44

L Low concentration of amino acids
H High concentration of amino acids
* Samples were not extracted with ether prior to silylation

TABLE XI

METHODS OF SAMPLE EVAPORATION
ECD SIGNATURES

	Sample	
Dried Via Vacuum Oven at 50C Overnight	8L	7, 8, 9, 37, 39, 43, 44, 45
	9L	7, 8, 9, 15, 17, 37, 40, 41
	10H	6, 8, 9, 15, 17, 24, 28, 37, 38, 40
	11H	6, 8, 9, 15, 17, 37, 40, 41
	12L	7, 9, 23, 25, 28, 33, 37, 39, 44
	13L	9, 17, 19, 28, 32, 37, 39, 45
	14 Control	1, 8, 9, 17, 37, 39
Dried Via Vacuum Oven at 54C For Two Hours	15L	3, 5, 9, 13, 19, 22, 29, 30, 31, 32, 33, 37, 39, 44
	16L	3, 8, 9, 11, 13, 16, 17, 18, 19, 22, 29, 31, 33, 37, 39, 45
	17H	3, 5, 8, 9, 13, 17, 18, 29, 31, 33, 37, 39
	18H	8, 9, 12, 13, 22, 29, 31, 33, 37, 39, 41, 42, 43
	19L	8, 14, 22, 29, 31, 33, 34, 35, 37, 39
	20L	4, 5, 12, 13, 27, 33, 36, 37, 39, 41
	21 Control	1, 3, 5, 9, 13, 18, 19, 22, 29, 31, 33, 37, 39, 44

TABLE XII

METHODS OF SAMPLE EVAPORATION

COMPARISON OF DUPLICATE ANALYSES

INDICATING THE ECD PEAKS BY WHICH THE DUPLICATE SAMPLES DIFFER

Notes	Samples	Peak Samples
1	8L	17, 43, 44, 45
1	9L	15, 39, 40, 41
1	10H	24, 28, 38
1	11H	41
1	*12L	7, 17, 23, 25, 33, 44
1	*13L	19, 32, 45
2	15L	18, 30, 32
2	16L	5, 8, 11, 16, 17, 44, 45
2	17H	17, 22
2	18H	3, 5, 12, 18, 41, 42, 43
2	*19L	5, 8, 13, 14, 34, 35
2	*20L	4, 12, 22, 27, 29, 31, 36, 41
3 1	14 Cont	8, 17
3 2	21 Cont	3, 5, 13, 18, 19, 22, 29, 31, 33, 44

Notes: 1 Dried via vacuum oven at 50C overnight
2 Dried via vacuum oven at 54C for 2 hours
3 14 & 15 are control samples

L Low concentration of amino acids
H High concentration of amino acids
* Samples were not extracted with ether prior to silylation

1.2 CONCLUSIONS AND SUMMARY

These experiments represent both a notable failure and an equally notable success in our attempts to obtain more information from the infected cells using GC techniques. Both are worth some discussion since they bear strongly on the direction to be taken by future research in the program.

The failure is the realization that present methods of ether extraction and silylation of metabolites from virus-infected cells are both quantitatively and qualitatively non-reproducible. The experimental observations concerning the performance of the acid ether extraction system are as follows.

1. A serious attempt has been made to determine the factors in the ether extraction process which result in variations of peak height and occasionally in position and shape of peaks. Certainly the ether-pH 2 extraction is anything but routine, despite the amount of experimental work performed with it. The efficiency of the extraction procedure is variable for reasons which are still unknown to us and the relative instability of stored ethyl ether extracts makes it difficult to achieve reliable data unless large numbers of samples are assayed. Removal of protein-lipid emulsions from the ether extracts by centrifugation, filtration and addition of surfactive agents failed to improve the reproducibility of the peaks.

2. The most consistent features reported in ether extracts of maintenance media in these experiments have been the disappearance of media components, not the appearance of new peaks. Some differences between infected and uninfected cells can be observed on this basis. Nevertheless there seems little likelihood that this tenuous data can be expanded into a practical technique for identifying unknown viruses. There would be little support for a system of classification based on substances which infected cells could not utilize. It appears that we must (1) search for materials which will accumulate rather than become incorporated into structural components or (2) develop means of analyzing the polymeric substances which accumulate, i.e., nucleic acids, proteins, phospholipids.

The alternative to the accumulation method is to analyze infected cells directly for viral DNA or RNA, proteins or phospholipids. Although purine and pyrimidine bases have been analyzed recently by GC, the base ratios of the nucleic acids would be of minor value as a taxonomic characteristic. Early virus enzymes, virus structural proteins, and membrane proteins cannot be analyzed in a definitive fashion by GC analysis. Only the phospholipids (PL) have been successfully handled by gas chromatography. Unfortunately the methodology requires preliminary purification of the PL on silicic acid which is followed by acid hydrolysis. However, there is reasonable speculation (4) that the PL content of cell membranes is not controlled entirely by the PL levels in the milieu but by the virus structures as well. This replacement has not been adequately studied by isotope dilution or by double labeling experiments to determine whether or not the insertion of PL into plasma and nuclear membranes is specifically under virus control. Fractionation of cells into these two components is easily performed by simple centrifugation and it should be possible to follow passive and active incorporation of various PL.

So far, silylations of dried culture supernatants have not provided significantly greater information than the acid-ether extracts. The aqueous phase remaining after acid ether extraction of the maintenance medium was dried in various ways and silylated after dry pyridine extraction. The assumption was made that many polar nonvolatile compounds would be converted to volatile derivations. It was a surprising aspect of the research that silylation provided fewer peaks than acid-ether extracts. Furthermore, in the glucose supplementation experiments the wide variation in peak locations and the frequent disappearance of control peaks from Sindbis supernatants suggest that silylation as practiced is a poorly controlled process.

On the basis of these considerations one concludes a) that maintenance media do not provide sufficient substrates or metabolites to give a wide variety of products in the supernatant or b) that the acid ether extraction and silylation techniques cannot concentrate reasonable quantities of organics from small populations of cells and variation remains high throughout the extraction system.

The experience to date, however, suggests that accumulation of appropriate substrates after virus infection may be more sensitive than direct analysis of the cell structural components or the cell metabolic pool.

In contrast to the disappointing work with direct acid ether extraction and silylation of metabolites, the use of halogenated organic acids as media supplements offers real promise of being a useful technique. These acids fullfil the need for effective "tracer" compounds which are degraded only slightly and which will accumulate in the supernatant of infected cells as an altered byproduct.

The data in Table V show that halogenated acids and perhaps some of their contaminants disappeared from the media. More important perhaps was the appearance of at least one new peak with eight of the ten halogenated acids chosen. The peaks with each acid were distinctive, having elution times which indicated that the compounds produced were unique to the parent compound. It may even be possible to combine the extracts of supernatants with different halogenated acids and to chromatograph two or more extracts simultaneously.

The obvious inference from these data is that responses to a battery of halogenated organic compounds may be the solution to our difficulties with extraction of natural metabolites from the cell. The response to each acid could be scored in much the same manner that fermentation tests are scored in determinative bacteriology.

This enrichment technique still requires answers for several important questions. Can better halogenated compounds be found? These ten were chosen simply on the basis that they were relatively simple and readily available.

How important is the concentration of the acid? Of the virus? Of supplements such as glucose or serum? These survey experiments were conducted at a level of 0.1% in the maintenance medium, a concentration which was clearly toxic in at least two cases. Considerable work will probably be required on this point alone.

Is productive yield of virus required? Apparently not in all cases. Dichloropropionic and bromocaprylic acids, for example, yielded neither hemagglutinin or PFU but produced unique peaks. This may be a function of the multiplicity of infection.

Are there clear-cut differences between viruses in the patterns of halogenated acid uptake? Does group or strain differentiation appear?

Can the accumulation of the halogenated product be accelerated by elevating the temperature, modifying the pH or varying the maintenance medium?

These and other questions must be answered before these experiments are claimed as even modest successes.

2.0 PART II - BACTERIA

Work is reported in the following areas: (1) early detection of bacterial infection by gas chromatographic analysis of blood sera and (2) experiments to increase the complexity of gas chromatographic signatures of variation of the culture conditions and the extraction procedure.

2.1 MATERIALS AND METHODS

Bacterial strains. Stock cultures of Aerobacter aerogenes, Escherichia coli B, Escherichia coli K-12, Proteus vulgaris, Serratia marcescens, Salmonella typhimurium, Bacillus cereus, and Bacillus subtilis (ATCC 6633) were maintained on Nutrient Agar slants. Klebsiella-Aerobacter group # 3065 was isolated from a clinical specimen in the New York Hospital and was maintained under identical conditions.

Gas chromatographic analysis of the growth medium. For subsequent extraction and gas chromatographic analysis for metabolites, 5 ml of Proom & Knight (P & K) medium was inoculated and incubated for 16-18 hours at 30°. The cells were removed by centrifugation and the supernatant was extracted in one of the following ways:

(1) Acid extraction. The supernatant was acidified by adding 0.2 ml 5 N hydrochloric acid and 1 ml 0.2 M KCl-HCl buffer, pH 2.0, and extracted three times, each time with 10 ml of diethyl ether. The combined ether phases (30 ml) were acidified, concentrated to 2 ml and dried over sodium sulfate; μl samples of ether extract were injected for gas chromatographic analysis.

(2) Alkaline extraction. The supernatant was alkalinized by adding 0.2 ml 5 N sodium hydroxide and 1 ml 0.2 M sodium bicarbonate-sodium carbonate buffer, pH 10.5, and extracted three times, each time with 10 ml diethyl ether. The ether phases (30 ml) were combined, concentrated to 2 ml and dried over sodium sulfate; 1-3 μl samples were analyzed in the GC.

A Varian Aerograph dual channel gas chromatograph was used in the studies. It was equipped with a flame ionization detector (FID) and an electron capture detector (ECD), the responses of which were recorded by a dual channel recorder at a chart speed of 40"/hour. Nitrogen was used as carrier gas. The conditions for gas chromatography are given in the lengends of the tables. Retention times were measured as distance in mm from the origin (time of injection).

2.2 RESULTS

2.2.1 Early Detection of Bacterial Infection by Gas Chromatographic Analysis of Blood Sera

This portion of the report constitutes a joint effort with the Cornell Medical School, New York Hospital, New York, N. Y., where the animal experiments have been conducted.

Approximately 10^6 cell of Klebsiella-Aerobacter strain #3065 were injected into rabbits (N. Y. Hospital strain designation). One half ml serum samples were drawn at 1, 3, 5 and 29 hours after injection and a control 1 hour prior to injection. The blood samples were frozen and stored at -20°C until they were analyzed. Duplicate animals were infected.

To avoid the formation of emulsions when serum is extracted with ether, the samples were treated to remove proteins. Two methods were employed: (1) To 5 volumes of serum, one volume each of 5% zinc sulfate solution and 0.3 N barium hydroxide solution was added and the suspension mixed. The precipitate was removed by centrifugation, and the supernatant was extracted with ether. (2) One volume of serum was diluted with water, and one volume each of sodium tungstate and 0.67 N sulfuric acid was added. After mixing and removing the precipitate by centrifugation, the supernatant was extracted with ether (acid extraction procedure, see Materials and Methods). Both treatments resulted in easily extractable supernatants. The first procedure was used to prepare the extracts for analysis.

At no time after infection of the animals were peaks detected which were not present in the control sample taken prior to inoculation. Several reasons could be responsible for these negative results: (1) the blood sample volume of 0.5 ml was too small; (2) the inoculum was too small to generate a sufficiently high concentration of metabolites in the blood; (3) the injected bacteria were inactivated in the blood quickly and did not generate metabolites; and (4) the metabolites present were adsorbed to the protein precipitate and removed from the sample prior to extraction. Negative results from blood cultures inoculated with serum samples at the times of collection support the third suggestion. The first three possibilities require further testing. The fourth hypothesis was investigated in a model experiment.

Klebsiella-Aerobacter strain #3065 was grown in P and K medium, and the cells were removed by centrifugation. Duplicate supernatants were divided into equal parts, one of which was extracted directly, while the second part was mixed with an equal amount of serum. In these samples, protein was precipitated and the supernatant was extracted under identical conditions. The concentrated ether extracts were analyzed, and the areas of the peaks not observed in the controls were compared in the two sets of samples.

The results are given in Tables XIII and XIV. Though the FID response after direct analysis is in good agreement in duplicate samples,

TABLE XIII

INFLUENCE OF PROTEIN PRECIPITATION ON THE RECOVERY OF METABOLITES BY ETHER EXTRACTION (TRIAL 1)

Sample	Peak	FID Peak area mm^2	FID Percent*	ECD Peak area mm^2	ECD Percent*
I Direct Analysis	A	188	--	--	--
	B	3,248	--	6,958	--
After protein precipitation	A	132	70	--	--
	B	960	30	4,090	59
II Direct Analysis	A	190	--	23,300	--
	B	3,342	--	--	--
After protein precipitation	A	52	27	6,656	30
	B	512	15	--	--

* Percent of peak area before protein precipitation

Conditions for GC analysis: Column Porapak Q, 3', 1/8"
Temperatures: Injector 230°, detector 210°, column 180°
N_2 flow rate: 50 ml/min.

TABLE XIV

INFLUENCE OF PROTEIN PRECIPITATION ON THE RECOVERY OF METABOLITES BY ETHER EXTRACTION (TRIAL 2)

	Sample	Peak	FID Peak area mm^2	FID Percent	ECD Peak area mm^2	ECD Percent
I	Direct analysis	C	--	--	6760	--
		D	162	--	4560	--
	After protein precipitation	C	--	--	4930	73
		D	104	64	3950	76
II	Direct analysis	C	--	--	7350	--
		D	1501	--	4090	--
	After protein precipitation	C	--	--	3850	52
		D	51	34	1570	38

Conditions for GC analysis: Column Porapak Q, 6', 1/8"

Temperatures as in Table V.

N_2 flow rate: 61 ml/min

addition and precipitation of protein affects the recovery of metabolites appreciably. The percentage recovered is different for different metabolites and not consistent in duplicate samples for the same metabolite. The precipitation of protein has to be modified or replaced by other means before blood samples can be directly extracted with ether.

2.2.2 Experiments to Increase the Complexity of Signatures

The conditions of incubation, extraction and GC analysis and the pH value of the medium were varied to obtain more complex signatures. The results are summarized in Tables XV and XVI. P and K medium was used throughout the study, and it was buffered and adjusted either to a pH of 7.0 or 6.2. It was inoculated and incubated 16-18 hours at 30^{0} using 5 ml of medium in a 20 ml screw capped test tube under aerobic and anaerobic conditions For isolating the metabolites, the acid extraction procedure was used, but in the case of anaerobic incubation at pH 6.2 the alkaline extraction was applied to obtain basic metabolites which are known to be often excreted in acidic media. For GC analysis of the ether extracts, two columns of different length were employed with the same solid support. With the longer column, compounds with short retention times were easily separated (Table XV). Studies using the short column, which yielded peaks having retention times about one-third of those obtained with the long column, were designed to show slow-moving compounds (Table XVI). Comparing the retention times of the peaks listed in Table XV, it can be seen that at pH 7.0 and under anaerobic conditions of incubation with subsequent acid extraction, a completely new set of metabolites was recovered by ether extraction, with one exception (275 mm). After anaerobic incubation at pH 6.2 followed by acid extraction, four new peaks appeared with retention times of 180, 190, 205, 300 mm. After alkaline extraction, three more peaks appeared, while five were missing but were evident in the acid extract. The results listed in Table XVI are in agreement with those of Table XV. It can be concluded that the number of peaks which were not present in uninoculated controls is increased by varying the culture conditions and by modifying the extraction procedure.

TABLE XV

GC ANALYSIS OF GROWTH MEDIUM AFTER INCUBATION AND EXTRACTION UNDER VARIOUS CONDITIONS
(Trial 1)

	Aerobic, pH 7.0, acid extract								Anaerobic, pH 7.0, acid extract								
	FID			ECD													
Retention times (mm)	G	92	32	112	132	164	270	382	G	21	36	100	120	143	275	320	545
Bacillus cereus	·	(·)				+			+								
Bacillus subtilis	(·)								–	·							
E. coli B	·	·	·						+		+						
E. coli K-12	·	·	+						+		+						
Aerob. aerogenes	··	(·)	·		·		+	(+)	··		+	+			+		+
Serratia marcesc.	·	·	·	·	·		·		+			·	·	·	+		
Proteus vulgaris	·	(·)				·			·		+					+	
S. typhimurium	·	·				·			··		+						

Conditions for GC: Column: Porapak Q, 6', 1/8"
Temperatures: Column 180°, detector 210°, injector 230°
Carrier gas: N_2: 45 ml min

G growth: – no visible growth
(·) slight growth
· good growth
·· heavy growth

GC response: + peak
(+) small peak or shoulder

TABLE XV - continued

Retention times (mn)	Anaerobic, pH 6.2, acid extract										Anaerobic, pH 6.2, alkaline extract								
	G	ECD									G	FID		ECD					
		33	39	105	125	150	180	190	205	300		36	49	36	49	110	180	190	220
Bacillus cereus	-										-								
Bacillus subtilis	-										-								
E. coli B	+		+	+					+		+	+	+	+	+	+		(+)	
E. coli K-12	(+)	+		+			+				(+)	+	+	+	+	+			
Aerob. aerogenes	+		+	+	(+)	(+)				+	+	+	+	+	+			++	(+)
Serratia marcesc.	(+)			+			(+)			(+)	(+)	+	+	+	+	+	+		(+)
Proteus vulgaris	(+)							+			(+)		+		+	+		(+)	
S. typhimurium	+		+	+			(+)				+	+	+		+	+		+	

TABLE XVI

GC ANALYSIS OF GROWTH MEDIUM AFTER INCUBATION AND EXTRACTION UNDER VARIOUS CONDITIONS (TRIAL 2)

	Aerobic, pH 7.0, acid extract					Anaerobic, pH 7.0, acid extract									
		ECD					FID			ECD					
Retention times (mm)	G	42	71	102	190	G	100	120	190	58	100	190	200	350	650
Bacillus cereus	+	+	+			+					+				
Bacillus subtilis	(+)					-									
E. coli B	+	+				+					+		+		
E. coli K-12	+	+				+									
Aerob. aerogenes	++	+		+	+	++		(+)	(+)	(+)	+	+			
Serratia marcesc.	-	+	(+)	+		+	(+)				+		+		
Proteus vulgaris	+	+	(+)			+					+			+	+
S. typhimurium	+	+	(+)			++					(+)		(-)		

Conditions for GC: Column: Porapak Q, 3-, 1/8"
Temperatures: Column 180°, detector 210°, injector 230°
Carrier gas: N_2 50 ml/min

G = growth: - no visible growth
(+) slight growth
+ good growth
++ heavy growth

GC response: + peak
(+) small peak or shoulder

TABLE XVI continued

Retention times (mm)	Anaerobic, pH 6.2, acid extract													Anaerobic, pH 6.2, alkaline extract							
		FID		ECD											FID		ECD				
	G	120	190	13	45	57	100	160	180	190	240	290	340	G	110	180	42	56	95	180	190
Bacillus cereus	-										(+)			-							
Bacillus subtilis	-													-							
E. coli B	+			+								(+)		+					+		
E. coli K-12	(+)			+					(+)					(+)							
Aerob. aerogenes	+	+	+	+		+	+			+				+	+	+	(+)	+	+	+	
Serratia marcesc.	(+)				+		+							(+)			(+)	(+)			+
Proteus vulgaris	(+)							+					+	(+)							
S. typhimurium	(+)			+										+							

3.0 PART III - HEAD SPACE

3.1 EVALUATION OF HEAD SPACE SAMPLING

Head space sampling refers to the collection and analysis of the vapor phase above a media in a closed vessel. In concept this sampling technique will allow direct analysis of the vapor above a complex media: When this vapor constains uniquely necessary metabolic products, the head space sample will characterize the causative organism.

The advantage of head space sampling over other techniques are several: 1) head space sampling can be non-perturbing, allowing continued growth in the media during sampling, 2) it can be most rapid since only volatile constituents are sampled, and 3) it is simpler to implement than systems employing solvent extraction and/or silylation techniques.

Components of the head space sample will vary from those of the media according to selection processes based primarily on the amount and vapor pressure of each constituent, on the nature of the media containing the constituents, and on temperature. The purpose of the following is to present the rationale of head space analysis, the experimental procedures, the experimental results, and conclusions.

3.1.1 Mathematical Model

A physical/mathematical analysis of head space sampling requires development of a partitioning model of the metabolic products between the media (liquid phase) and the head space (gas phase) within a full range of volume variations for any practical system. The symbols used in this analysis are defined in Table XVII. Following is the rationale and selected applications.

Rationale

The weight of any sample component in the total headspace above a media can be written as

$$W_G = (P_G V_G M_G)/(760 V_I) \qquad (1)$$

To relate the weight of the same sample component in the liquid phase to that in the gas phase we shall state Raoult's law, which relates vapor pressure of dilutions to the pure sample vapor pressure, as

$$P_G = \left[P_O(W_L/M_L)\right] / \left[(W_S/M_S) + (W_L/M_L)\right] \qquad (2)$$

TABLE XVII

TABLE OF SYMBOLS

C_G	Concentration of component in gaseous phase
C_L	Concentration of component in liquid phase
D_S	Density of solvent
ΔH_V	Heat of vaporization
K	Trouton's constant, 22 cal $mole^{-1}$ deg^{-1}
M_G	Molecular weight of component in gaseous phase
M_L	Molecular weight of component in liquid phase
M_S	Molecular weight of the solvent
P	Partial pressure
P_G	Partial pressure of component in dilution
P_O	Partial pressure of component in pure form
R	Gas Constant, 1.99 cal $mole^{-1}$ deg^{-1}
T	Temperature, deg K
T_b	Boiling point, deg K
V_G	Volume of head space (gaseous phase)
V_I	Volume of one mole of an ideal gas
V_L	Volume of liquid phase
W_G	Total weight of component in gaseous phase
W_L	Total weight of component in liquid phase
W_T	Total weight of component in entire system

Since we will always be dealing with dilute solutions in which $(W_S/M_S) \gg (W_L/M_L)$, we may use the approximation

$$P_G = (P_O W_L M_S)/(M_L W_S) \tag{3}$$

When Eq. (3) is substituted into Eq. (1), remembering that $M_G = M_L$, we obtain

$$W_G = [(P_O V_G M_S)/(760 V_I W_S)] W_L \tag{4}$$

which defines the partitioning of a small sample in a solvent between the liquid and gaseous phase above that solvent. Since the total sample size can be stated as

$$W_T = W_L + W_G \tag{5}$$

we can combine this with Eq. (4) to yield

$$W_G = [A/(1 + A)] W_T \tag{6}$$

Where A is $(P_O V_G M_S)/(760 V_I W_S)$

If expressions containing concentrations are desired we note that $C_G = W_G/V_G$ and that $C_L = W_L D_S/W_S$ so that, for example, Eq. (4) becomes

$$C_G = [(P_O M_S)/(760 V_I D_S)] C_L \tag{7}$$

Also, when considering different volume ratios, Eq. (4) can be written as

$$W_G = [(P_O V_G M_S)/(760 V_I V_L D_S)] W_L \tag{8}$$

The vapor pressure, P_O, is strongly dependent on temperature. The thermodynamic dependence is given by the Clausius-Clapeyron Equation which states

$$\log P = -[\Delta H_V/(2.303RT)] + C \tag{9}$$

where log P is to the base 10 and C is a constant. When experimental data is available, the following form is best used.

$$\log P_2 - \log P_1 = [\Delta H_V(T_2 - T_1)] / [2.303RT_1T_2] \quad (10)$$

When no prior data exists an approximation, Trouton's Rule, which can be used states

$$\Delta H_V \approx K\, T_b \quad (11)$$

Where $K \approx 22$ cal mole^{-1} deg^{-1}

and T_b is the boiling point at 760 mm Hg.

Writing Eq. (10) in the exponential form and substituting Eq. (11) to eliminate ΔH_V (using normal atmospheric pressure boiling points), we obtain

$$P_O = 760 \exp [(K/R)(1 - \{T_b/T\})] \quad (12)$$

where exp [ ] indicates the exponent of e.

This equation can be applied to a number of situations, e.g., Eq. (7) such that

$$C_G = [M_S/(V_I D_S)] \exp [(K/R)(1 - \{T_b/T\})] C_L \quad (13)$$

3.1.2 Selected Applications

The vapor pressure of a component in a dilute solution can be obtained from Eq. (7), when the ratio of the concentration of the gas to the liquid is measured via chromatography, i.e.,

$$P_O = [(760V_I D_S)/M_S] [C_G/C_L] \quad (14)$$

Note that only the ratio is required: absolute values are not needed. As an example, assume the following:

Solvent is water, i.e., $D_S = 1$ g/cc, $M_S = 18$ g/mole

Gas sample size is 1cc, peak height (or area) = 27 units

Liquid sample size is 1 μl (10^{-3}cc), peak height (or area) = 9 units

V_I is approximately 2.24×10^4 cc/mole.

Eq. (14) becomes

$$P_O = [(760 \times 2.24 \times 10^4 \times 1)/18] [27/(9 \times 10^3)]$$

$$P_O = 283 \text{ mm Hg}$$

This is the apparent vapor pressure of the pure material as measured in a water dilution.

The partitioning of a small sample between the head space and liquid is defined by Eqs. (6) and (8). For example using Eq. (8) assume we wish to find the vapor pressure at which $W_G = (1/1000)\, W_L$. Also assume

$$V_G = 2 \text{ cc}$$

$$V_L = 0.2 \text{ cc}$$

$$M_S = 18 \text{ g mole}$$

$$D_S = 1 \text{ g cc}$$

since $W_L > W_G$ we may say

$$W_G / W_L = 1/1000 = [(P_O \times 2 \times 18)/(760 \times 2.24 \times 10^4 \times 0.2 \times 1)]$$

$$P_O = 94.5$$

So that under these conditions when $P_O > 94.5$, $W_G / W_L > 1/1000$, when $P_O < 94.5$, $W_G / W_L < 1/1000$ and when $P_O = 94.5$, $W_G / W_L = 1/1000$.

The selection of solvents to maximize head space concentration is easily seen from Eq. (7). Assuming all factors, including P_O, are head constant, then

$$C_G = B(M_S / D_S) C_L$$

where B is a constant.

For example, consider water and ether as two solvents.

$$M_{Water} = 18 \text{ g mole}$$

$$M_{Ether} = 74 \text{ g mole}$$

$$D_{Water} = 1.0 \text{ g cc}$$

$$D_{Ether} = 0.71 \text{ g cc}$$

then

$$[C_G(\text{Ether})] / [C_G(\text{Water})] = 5.78$$

In this example, with equally dilute solutions of a component in water and in ether, the ether system will produce approximately six times the headspace concentration as the water system.

Calculations of vapor pressure dependence on temperature from tabulated data can be made directly from Eq. (10) using the two point equation for a straight line.

Similarly, estimation of vapor pressure as a function of temperature is achieved directly from Eq. (12), and the total relation of head space concentration is given in Eq. (13). As a final illustration of application of the head space rationale, consider the temperature increase necessary to increase the concentration in one head space sample by a factor of two. We have from Eq. (13)

$$C_{GT_1} = J \exp\left[(K/R)\left(1 - \left\{T_b/T_1\right\}\right)\right]$$

$$C_{GT_2} = J \exp\left[(K/R)\left(1 - \left\{T_b/T_2\right\}\right)\right]$$

we set

$$C_{GT_2} = 2\, C_{GT_1}$$

$$T_1 = 300^{\circ}K$$

$$T_b = 400^{\circ}K$$

and J is considered a constant when $V_{l1} \approx V_{l2}$.

Taking the ratio of C_{GT_1} C_{GT_2} we have

$$\ln 0.5 = \left[(KT_b)/R\right]\left[(1/T_2) - (1/300)\right]$$

$$\text{or } T_2 = 315^{\circ}K$$

Thus, for the conditions specified, a $15^{\circ}C$ rise in temperature is necessary to double the head space concentration.

3.1.3 Experimental Procedures

Sample bottles for head space analysis consist of 180 ml glass milk dilution bottles which have 5/16" holes bored in the plastic caps: cap liners are replaced with chromatography grade silicone rubber septum material cut to size. Bottles and separated, modified caps are washed, rinsed, and vacuum baked at $150^{\circ}C$ for two hours to remove trapped gases.

The chromatography system used is a Varian Aerograph Model 1520 equipped with tandum electron capture and flame ionization detector. A 1/8" × 10' Chromosorb 101 column was selected for use, since it runs water well without degradation and effects the necessary separations in a reasonable time.

Serial dilutions are used to produce the desired standard concentration, with dilutions continuing until the sample component is undetectable; a 50 ml liquid volume was standard for all experiments. The head space samples from the selected organisms were obtained through the septum after culturing the stated times at 37C.

All liquid samples were taken through the septum with a 10 μl Hamilton syringe; all gaseous samples were taken with a Hamilton 5 ml gas syringe.

3.1.4 Experimental Results

Serial dilutions were made to determine the vapor pressure and approximate limit of detection for a number of compounds using a Chromosorb 101 column. The detection limits are listed in Table XVIII.

TABLE XVIII

DETECTION LIMITS

Material	Detected at	Detector
Acetic Acid	3×10^{-8} grams*	ECD, FID
Trichloroacetic Acid	2×10^{-8} grams*	ECD, ---
Butyric Acid	5×10^{-7} grams*	ECD, FID
Diacetyl	1×10^{-9} grams**	ECD, ---
Ethyl Alcohol	4×10^{-9} grams	---, FID
Carbon Tetrachloride	1×10^{-12} grams	ECD, ---

* Dependent on immediate prior history
** After sensitization

The detection limits are for aqueous dilutions, except for carbon tetrachloride, which is a gaseous dilution, and are not necessarily the lowest values; however, dilutions of 10:1 from those listed could not be detected. A number of materials appear to react within or on the column in a non-reproducible manner. These reactions, or anomalies, are noted only with ultrasensitive detectors and would not be apparent on an average flame ionization detector or on a thermal conductivity detector. In the case of acetic acid and trichloroacetic acid the dependency on prior history is very strong. Initial injections at the listed detection limit were completely lost; only reaction products were observed. Butyric acid did not produce any discernible reaction products; however, sensitization is required after

repeated large water injections (1μl or more). The initial diacetyl injections were completely absorbed at the 5×10^{-8} gram level. After sensitization, 1×10^{-9} grams could be seen. Diacetyl is normally seen at the 10^{-12} level, as is carbon tetrachloride: the detection of carbon tetrachloride at 10^{-12} grams indicates that the column packing was reacting with and/or absorbing diacetyl.

Since a significant number of anomalies were observed with head space samples, with direct water injections, and with a combination of air/water injections, the vapor pressure data obtained is questionable and is not presented. However, vapor pressures of non-reactive materials appear to be within one order of magnitude of theoretical calculations, even at extreme dilutions and with other systems' absorptions (syringe, dilution flask, etc.) not taken into account.

Even though anomalies were observed with the Chromosorb 101 column, it proved to be the best available column tested to handle the comparison of head space, direct injection, and ether extracts of organisms. (The Porapak series, other Chromosorb products, and selected silicones, e.g., QF-1, DC-200, and SE-30 were tried.) To avoid display of anomalous effects, the output of both the flame ionization and electron capture detectors were attenuated to a point where previous column history showed no effect; this also removed the need of sensitization since the detectors in effect were no longer "ultrasensitive".

Two organisms, Aerobacter aerogenes and Aerobacter liquifaciens, were used to inoculate 50 ml of modified Proom and Knight medium to a level of 10^9 organisms/ml; 130 ml of sterile air head space was sealed above the media. Head space samples were drawn from un-inoculated medium controls and from the inoculated bottles at the end of the day indicated, i.e., day 2 is a 48 hour sample. Direct injections of the media plus organisms were also made at the end of the growth periods prior to taking an ether extract of the samples. A detailed chromatographic comparison of the three sampling techniques is shown in Table XIX. An abstract of this table showing signatures of significant peaks as a function of the different sampling techniques is given in Table XX.

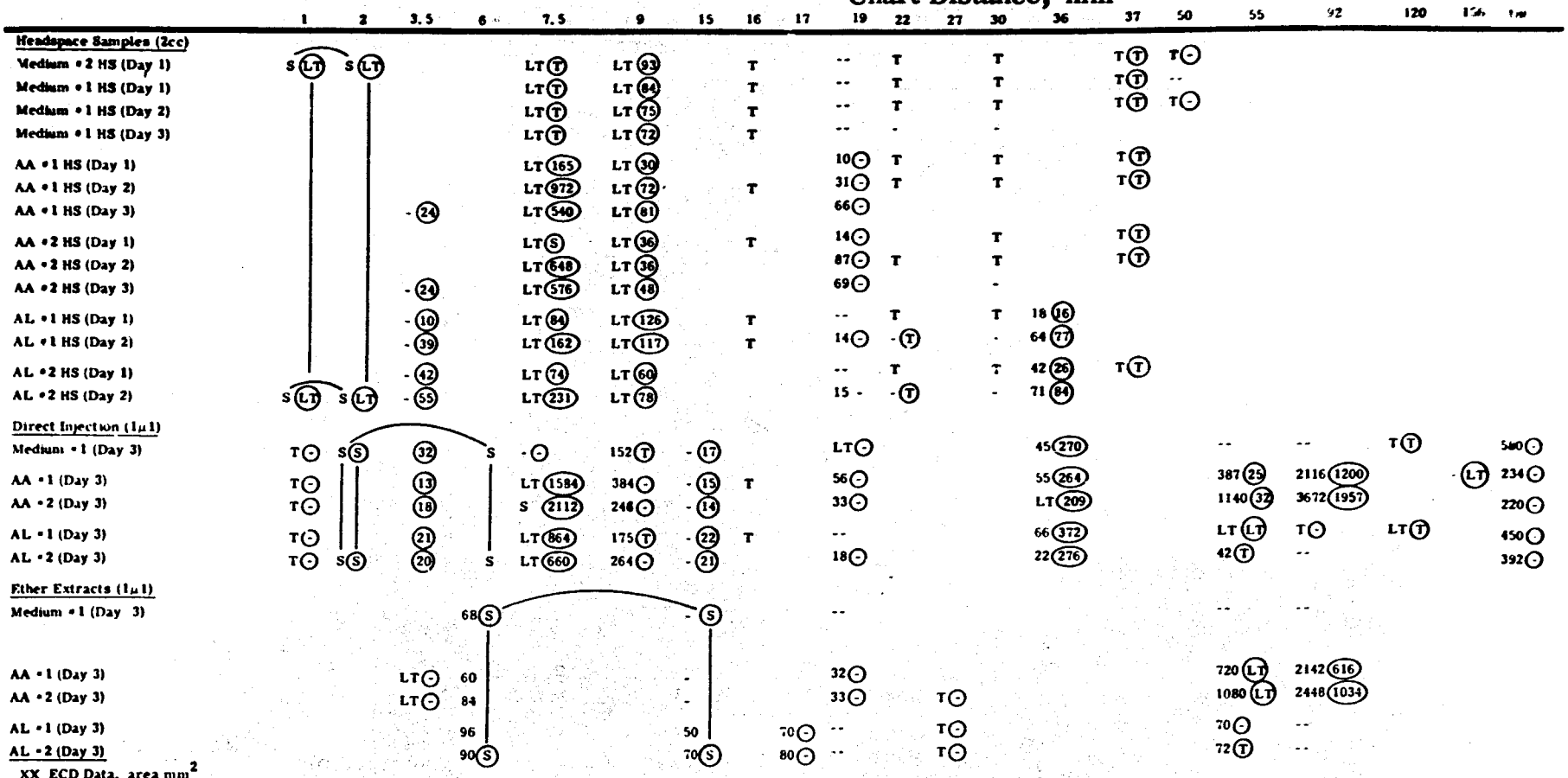
The detailed chromatograph comparison of Table XIX clearly illustrates advantages and disadvantages of each sampling method. The head space samples show the variation of the volatile metabolic product mixture with time. The absence of all late peaks (assumed to be the less volatile metabolic products) allows rapid evaluation with little likelihood of system contamination; when less volatile products are necessary for characterization, head space techniques are not indicated. This is not the case for practical systems to date.

Direct injection of the media (containing live organisms) offers a wealth of information, some of which tends to obscure characterizing data. A number of unwanted peaks occur from media components and from injection temperature pyrolysis of cells. Many late peaks occur and column contamination through repeated injections could become a serious problem.

TABLE XIX

COMPARISON OF SAMPLING TECHNIQUES

(Aerobacter Liquifaciens & Aerobacter Aerogenes)



XX ECD Data, area mm^2

XX Flame Data, area mm^2

AA Aerobacter aerogenes

AL Aerobacter liquifaciens

Medium Proom and Knight (Modified)

T Trace

LT Less than 10 mm^2

S Saturated

TABLE XX

SIGNATURES OF SIGNIFICANT PEAKS AS A FUNCTION OF DIFFERENT SAMPLING TECHNIQUES

		Flame Signature	Electron Capture Signature
Head Space of:	AA	----	B
	AL	C	C B
Direct Injection of:	AA	E D*	E D B
	AL	d*	d b
Ether Extract of:	AA	E d	E D B
	AL	d	A d

Legend:

Peak Designation	A	B	C	D	E
Retention Time, mm	17	19	36	55	92

AA Aerogacter Aerogenes

AL Aerogacter Liquifaciens

Small letters are trace quantities or are significantly smaller (10:1) than comparative peak

*Peak D present in Medium AA and AL with direct injection

One type of obscuring effect is noted with the 36 mm peak: this peak is significant in the head space sample but not with direct injection. This strongly suggests that the direct injection sample has two or more materials with the same retention time (36 mm) with one component, as produced by Aerobacter liquifaciens, having a high vapor pressure such that it is characteristic only of the head space sample of Aerobacter liquifaciens and not Aerobacter aerogenes. This also illustrates the selectivity of head space sampling over direct injection.

The ether extract sample involves a hydrochloric acid acidification step (to 6 N) prior to ether extraction. The appearance of peaks such as those at 17 and 27 mm suggest chemical changes resulting from acidification. Other than changes as suggested above, the ether extraction methods offers a good range of information through its extraction selectivity. The large flame peak (from ether) does obscure some data. Column contamination risks lie between the head space and the direct injection methods.

Table XX summarizes significant peaks as a function of different sampling techniques, by arranging these peaks in decreasing order of peak area. It is to be noted that signatures obtained by all methods, while not identical, clearly characterize the presence of each organism.

3.1.5 Conclusions

Preliminary experiments indicate that application of a conventional physical chemistry rationale to the head space sampling technique is satisfactory. The state-of-the-art of GLC columns designed for simultaneously handling large amounts of water while maintaining stability for ultrasensitive detectors is not adequate. However, either objective is readily obtained without the other.

The head space concept was demonstrated with two organisms. Distinctions between each organism and the control were evident with head space sampling, with direct injection, and with ether extracts. As theoretically predicted, the head space produced its distinctions early in the chromatogram, which characteristically had no late peaks. The richness of information in the direct injection at the sampling time used proved to be a detriment as well as an advantage. The ether extract, through its selectivity, provides clearer distinctions, and again lacked the extremely late peaks of the direct injections.

In general, head space sampling can be a valuable tool where nonperturbing samples of volatile products will identify the causative organisms.

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13 ABSTRACT

The objective of this program is to assess the feasibility of rapidly detecting and identifying pathogens when present in small populations. Gas chromatography is the methodology used.

It was established that present methods of ether extraction and silylation of tissue culture supernatants and lysates are not conducive to obtaining reproducible results.

Use of halogenated organic acids as media supplements offers promise of being a useful technique.

Various methods of ether extraction of blood sera for indication of infection proved to be unsuccessful.

Variation in incubation and extraction procedures was effective in altering the signature of bacteria.

The effectiveness of the head space technique for obtaining microbial signatures was tested.

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