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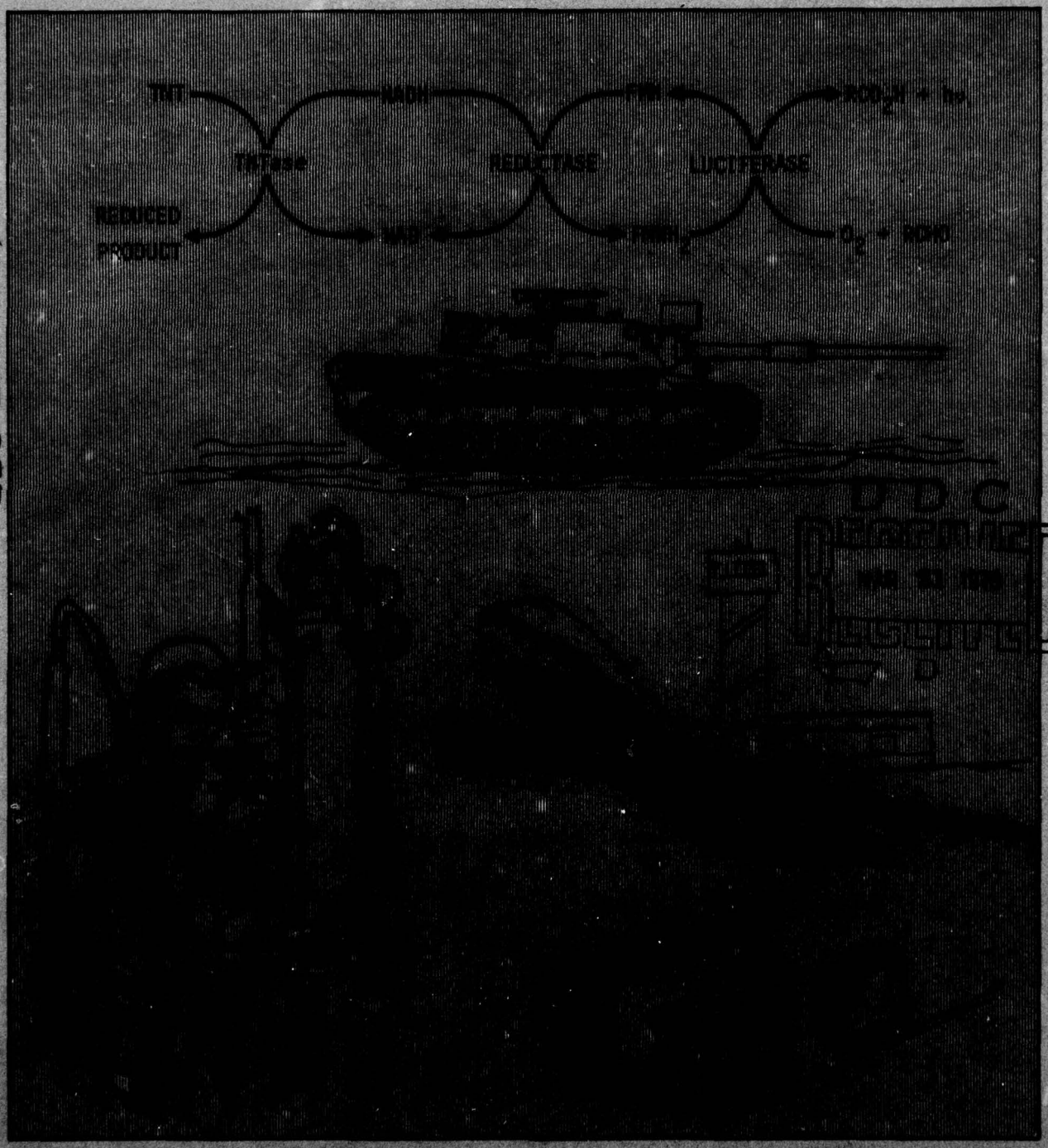

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DETECTION OF EXPLOSIVE VAPORS BY ENZYMATIC METHODS

FINAL TECHNICAL REPORT

By

A. Strickler and M. Greene

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1.0 INTRODUCTION

The work reported here was a continuation of the program of the previous three years (September 1973 through September 1976) documented in Beckman/Advanced Technology Operations (ATO) Interim Technical Reports numbered IR-2696-102 (for 1975-1976) and IR-2696-103 (for 1974-1975).

The principal aim of the effort now reported was to demonstrate the feasibility of vapor phase TNT recovery and analysis with a speed and sensitivity at least equal to that previously demonstrated for detection of TNT in liquid standards. Three major objectives were:

a) Determine the feasibility of recovering TNT vapor from air using a simple, thin-film gas-liquid contactor.

b) Fabricate a continuous flow system to permit evaluation of possible advantages compared with the batch-type system previously employed.

c) Refine the continuous flow system sufficiently to permit the detection of 10^{-14} mole of TNT contained in one liter of air within two minutes.

The first objective is essentially independent of the other two, since an efficient means of TNT vapor recovery is an essential part of a practical TNT detection system, whether it be based upon continuous flow or batch-type chemistry. The second and third objectives were included to provide some direct experience with a continuous system, which has some attractive potential advantages over a batch-type system.

2.0 INVESTIGATION

2.1 Investigation of NADH Oxidase/NADH Reaction Kinetics

At the conclusion of the prior effort it was apparent that one of the major factors limiting TNT sensitivity was the presence of a nonspecific oxidase in the TNT reductase (TNTase). This caused a significant loss of NADH in the absence of TNT. Experimental evidence indicated that the amount of NADH remaining after one-minute incubation with 0.1 unit/mℓ of TNTase was an exponential function of the initial NADH concentration. (See Report No. IR-2696-102, Figure 3-12.) In addition, in a test performed at Beckman/Advanced Technology Operations, it had appeared that the initial rapid loss of NADH was followed by a relatively slow decay. The two results implied that the effect of the NADH oxidase might be circumvented simply by using a higher initial NADH

concentration, and waiting several minutes for completion of the rapid decay. Accordingly, this possibility was investigated before proceeding to the major objectives cited above.

Using a fixed TNTase activity of 0.18 unit/mℓ, and varying the initial NADH concentration from 6×10^{-8} M to 6×10^{-7} M, it was found that the NADH oxidase behaves as an ordinary enzyme, and that the Michaelis-Menten equation is applicable. The prior ATO test result, indicating a slow loss of NADH following the initial rapid loss, must have been erroneous. It could not be repeated. Re-evaluation of the data (Report No. IR-2696-800, Figures 3-11 and 3-12) suggests that those prior results were not, in fact, inconsistent with Michaelis-Menten theory. (Section 3.0, "Discussion," in this report, includes a further explanation of the equations used here.) When the Michaelis-Menten equation applies, the residual NADH concentration, C, is related to the initial concentration, C_o, as follows:

$$C = C_o - K_1 t \quad \text{for high concentrations of NADH}$$

or

$$C = C_o e^{-K_1 t / K_m} \quad \text{for low concentrations of NADH}$$

The value of t was fixed at one minute in the experiment resulting in the data of interest. The relationships above are then reducible to:

$$C = C_o - A \quad \text{for high concentrations of NADH}$$

or

$$C = B\, C_o \quad \text{for low concentrations of NADH}$$

Also, it should be noted that the NADH concentrations are zero or positive, that the constants A and B are positive, and that in the transition region of initial NADH concentrations there is a smooth transition from one function to the other. The data points plotted in Report IR-2696-102, Figure 3-11, are consistent with the equations above (although additional data points at higher C_o are needed to verify high range behavior) if $A = K_1 t$ corresponds to about 10^6 p/s. The intermediate concentration range lies between 7×10^{-8} M and 1.2×10^{-7} M (or higher) NADH. The low concentration range (for which $C = B\, C_o$ is valid) is zero to 7×10^{-8} M NADH. The value of B associated with residual NADH corresponds to about 10^{12} p/s per molar initial NADH concentration for the one-minute incubation period employed.

Tests on residual NADH (luciferase reaction peak light output) versus incubation time were performed using the laboratory photometer and batch technique. Reagents were mixed anew and combined after various incubation times. The scatter in the data was too great to permit determination of the NADH loss rate with reasonable precision.

A technique was then developed for obtaining the NADH loss rate upon adding TNTase (containing NADH oxidase) in which 20 μℓ quantities of the reaction mix were repeatedly injected into a single vial of luciferase reagent at 15-second intervals. Although NADH sensitivity of the luciferase reagent changed with both time and repeated injections, scatter of the data was significantly less than had been obtained when new luciferase reagent vials and reaction mixes were prepared for each test of a different incubation time.

2.2 Investigation of Continuous Flow Concepts

Analysis indicated that the basic requirements of a continuous flow system were the following:

- Air-liquid contactor for recovery of TNT vapor by solution in the liquid reagent(s).
- Gas-liquid phase separator, to permit delivery of bubble-free liquid to the next reagent addition stage.
- Delay line, to permit a predetermined TNT reaction mix incubation time.
- Light output reaction cell having flow-through capability and also having the maximum possible photon collection efficiency.
- Air flow control system.
- Liquid reagent flow control system.
- Syphon breakers, compensators for air-flow-induced differential pressure, etc., as required to achieve steady air and liquid flow rates.

Tests were performed to establish the minimum number of liquid flow controls that would be required. The following was ascertained to be true, or probably true, as noted in each case:

- Tetradecanal is not sufficiently soluble in the luciferase solution, or at least not rapidly soluble. It must be predissolved (as in alcohol).
- Tetradecanal must be added to the luciferase reagent less than about five minutes before use in the detection reaction.
- The luciferase reagent, reconstituted and diluted to normal strength, is stable for more than eight hours if tetradecanal is not added.
- The ratio of luciferase glow to light output sensitivity (for the NADH reaction) appears to decrease (which is desirable) when the luciferase solution is aged overnight. This initial test result was not confirmed by additional testing.

- NADH in pH 7 buffer is stable for more than eight hours at concentrations of 10^{-7} M and higher. (It may also be stable at lower concentrations.)

- 10^{-7} NADH in pH 7 buffer can probably be used in the air-liquid contactor.

- The enzyme TNTase should probably be added after gas-liquid phase separation, based upon the characteristics of enzymes in general. (There are no test results to prove or disprove this supposition.)

- The TNTase can probably be held in diluted form for at least eight hours, using pH 7 buffer as diluent. Reduced temperature (below 10° or 15°C) may be necessary. Present test data are incomplete in this regard.

In summary, it was determined that at least four liquid reagent flows must be controlled. These are: 1) NADH plus buffer, 2) TNTase plus buffer, 3) luciferase reagent plus buffer, and 4) tetradecanal-saturated ethanol (or equivalent). It may also be necessary, in general, to add water to the NADH/buffer, which should be introduced at the inlet of the gas-liquid contactor. In the worst case, a controlled water flow may be required to replace the amount evaporating into the sample air. The TNTase should be added after gas-liquid phase separation, at the inlet of a "delay line" leading to the reaction cell inlet.

The pH 7 buffer-diluted luciferase should be blended with the tetradecanal, with a delay in passage after the junction of about 1/2 to 5 minutes before this reagent stream joins the TNT/NADH/TNTase reaction mix stream. Final confluence of these reagents must occur within the field of view of the photocathode of the photomultiplier tube, in the reaction cell. These features are incorporated in the Flow Schematic Diagram (Figure 2-1).

In Figure 2-1 the gas pump is at the outlet end of the system and draws the sample air through the contactor. This prevents loss of TNT and/or sampling delay which might be caused by sorption on inlet system surfaces if the pump were at the inlet end. The liquid enters the contactor as close to the air inlet as possible, for the same reason. The gas flow rate and contactor diameter must be of values that assure turbulent gas flow. This promotes gas-liquid contact and rapid movement of the liquid through the contactor.

Glass was selected as the material of construction for critical parts of the breadboard test system. Glass is probably one of the better available materials in respect to low TNT sorption and probably as good as any for handling reagents at micromolar concentration. Glass also permits visual observation of contactor and phase separator performance. This facilitates evaluation and subsequent improvement of design. Unfortunately, glass acts as a "light pipe," making it necessary to enclose the entire apparatus in a light-tight housing when the PMT is in use. For preliminary evaluation, the glass components were butted together and joined with silicone rubber and/or Tygon tubing to minimize exposure of the reagents to these elastomers.

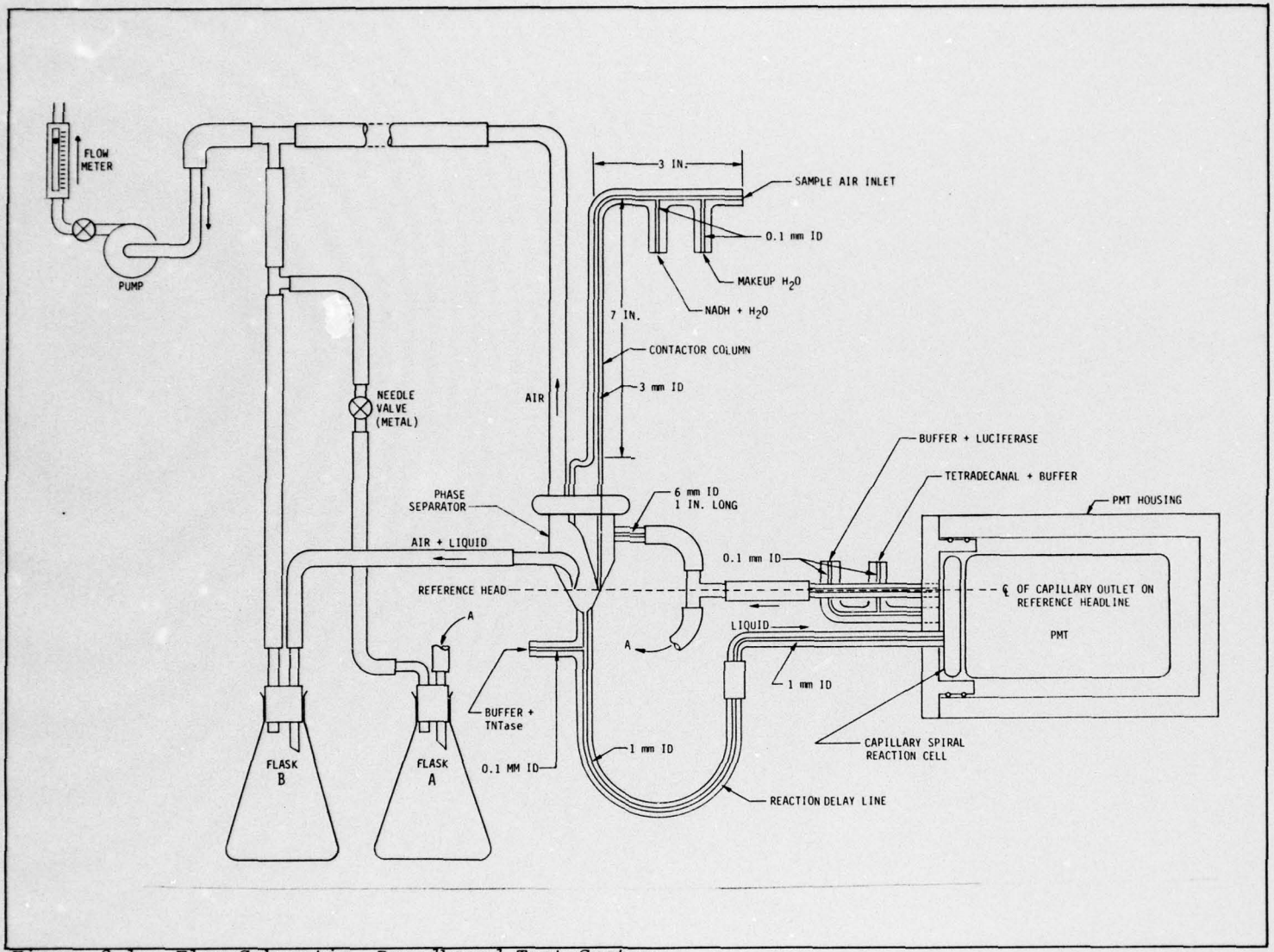

Figure 2-1. Flow Schematic, Breadboard Test System

The phase separator (Figure 2-1) provides a continuous glass path for the liquid to flow over. This avoids drop-wise delivery of the liquid to the lower region of the separator and thereafter to the delay line. Most of the air drawn out the top of the phase separator goes directly to the suction pump. A small amount of air is drawn through two capillary outlets from the phase separator to perform specific functions. Air drawn through the upper capillary provides the differential pressure to drive the reaction mix through the delay line and reaction cell. The necessary pressure drop is obtained by adjusting air flow through flask A using a needle valve. The driving force for the flow through flask A is the pressure drop due to the main air flow. This also drives the air flow through the lower capillary on the phase separator. The flow restrictor shown in the main air flow line will ultimately be sized to provide a pressure drop of about 10 cm of water at the desired main air flow rate.

Flow through the lower capillary assures that the liquid level will not exceed that defined by the capillary tip. Excess liquid is "sipped" out of the system by this air flow, avoiding the problem of matching the liquid flow rate precisely to the rate at which liquid exits the contactor. The excess liquid is transported to flask B by this air flow.

The orientation of the spiral cell shown in Figure 2-1 is such that the center line of the discharge capillary is at the same level as the sipper in the phase separator. In the absence of an air flow through flask A, there will be no liquid flow through the reaction cell. Increasing the air flow through flask A increases the pressure drop across the reaction cell, increasing the liquid flow rate. The reaction cell stream joins the air flow at a tee of large enough cross section to serve as a syphon breaker. A photograph (Figure 2-2) shows the appearance of the spiral reaction cell.

The choice of liquid flow rates was based on the practicalities of controlling very low flows. The smallest possible flow is desirable from the standpoint of achieving maximum TNT concentration for a given amount of air sample. A flow rate of 10 $\mu\ell$/s for each reagent was chosen as a design target. This provides 20 $\mu\ell$/s of the TNT/NADH/TNTase reaction mixture. Assuming 100%-efficient TNT recovery, and an air flow of 1.0 ℓ/s, the intake of a liter of air containing 10^{-14} moles TNT (the lower detection limit goal) would provide a 20-$\mu\ell$ volume of reaction mix having the same reagent concentrations and half the minimum TNT concentration detected in prior work with batch sampling. The time-width of this TNT-enriched sample traversing the system will, of course, be only one second in this case, assuming no lagging of the reagent occurs (due, for example, to a slow-moving layer at the walls), as it passes through the contactor, phase separator, and delay line. To the extent that the one-second TNT peak is broadened by such lag, the TNT concentration will be reduced, and the detection limit goal not achieved. Wall sorption effects will similarly cause peak broadening and loss of sensitivity. Finally, the detection reaction light output pulse in the luciferase reagent-NADH reaction is about 10 seconds long, peaking in about two seconds. The electronics output is filtered with about a one-half-second time constant to provide good tracking of this peak while suppressing higher frequency noise. If the TNT peak

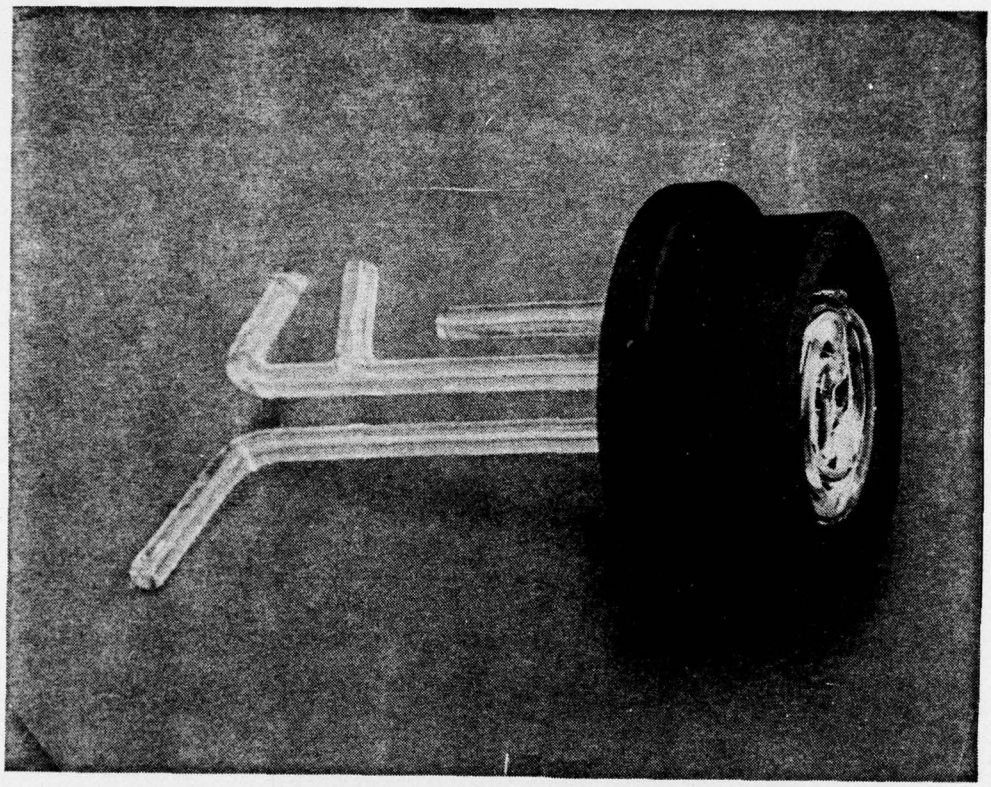

Figure 2-2. Spiral Reaction Cell

broadens by a factor of two or more (to two or more seconds for a one-second input pulse of TNT vapor) the existing circuit will provide good tracking. It is very improbable that a one-second TNT peak will not be broadened at least two- to four-fold. In any event it is a simple matter to increase the band-pass of the amplifier if necessary.

It was decided that, for the initial model, liquid flows would be controlled by capillary restrictors combined with a hydraulic head. It may be better in future models to utilize positive feed devices, e.g., dual motor-driven syringes. For initial evaluation of the present system, deionized water was used in measuring flow rate vs. head. Thus, the head for each reservoir was selected to provide roughly equal flow for each of the four reagents. (An inlet capillary for makeup water was included in the design, but was not used in the preliminary tests.) The liquid flow rate obtained with a maximum head of 123 cm water was about 2.5 μℓ/s, only about one-fourth of the nominal design

value. Since the lowered flow rate would increase the TNT concentration in the reaction mix for a given set of conditions, this flow rate was considered to be acceptable for initial evaluation.

2.3 Initial Effort to Operate the Complete Continuous Flow System

The entire system was assembled as shown in Figure 2-1. (Reservoirs and "standpipes" are not shown.) Deionized water was added to each reservoir in turn to verify that flow in each case was as expected. The air flow rate was varied between about 0.1 ℓ/s and 1.0 ℓ/s to determine the acceptable rate for liquid flow into and out of the phase separator. It appeared that an air flow of about 0.3 ℓ/s would provide good phase separation.

The deionized water in each reservoir was replaced by the proper reagents in the sequence NADH, TNTase, luciferase, and tetradecanal. The PMT output was expected to increase due to the luciferase glow when the tetradecanal flow was added, but this was not observed. The flow control capillaries for the TNTase, luciferase, and tetradecanal were found to clog very rapidly. These were 0.1 mm in diameter and about 2 cm long. The effort to operate the system as a whole was then deferred in favor of a systematic evaluation of its main subsystems. This started with an evaluation of the TNT recovery efficiency of the contactor. It was concluded that if the complete system were again to be evaluated while retaining the restrictors, the reagents would have to be prefiltered. A 10-μm absolute filter would suffice to prevent clogging of the 100-μm-diameter capillaries.

2.4 Evaluation of Contactor Efficiency

Experimental Setup. In approaching an evaluation of the contactor *per se*, it was felt best to test first the simplest of designs--a straight vertical tube as used in the continuous breadboard (Figure 2-1). In order to introduce a minimum of new variables, the continuous-action contactor was combined with discrete testing of the effluent, as established for use in previous bench-top tests. This procedure used the photometer, recorder, and TNT vapor generator as described in Section 2.0 and Figure 2-1 of Report IR-2696-102.

The test setup arrived at after testing other approaches is shown in Figure 2-3. A diaphragm pump (Thomas Industries, Model 107CA050) at the output end of the air stream draws room air in part through the TNT vapor saturator, and in part through a leak at the NADH inlet. If the gage following the air pump is set at a higher volume rate than that indicated on the inlet gage to the saturator, the difference is made up by flow at the NADH inlet. The NADH buffer solution was fed by a Sage variable speed motor-driven syringe (10 mℓ). The path from the TNT saturator through the contactor was Pyrex glass, except for butt-end connections of silicone rubber and a silicone rubber tube at the pinch clamp position.

Experiment Design. The object was to pass a known amount of TNT vapor through the contactor tube during a selected time interval, measure the amount detectable in the liquid effluent of the contactor, and to express the contactor

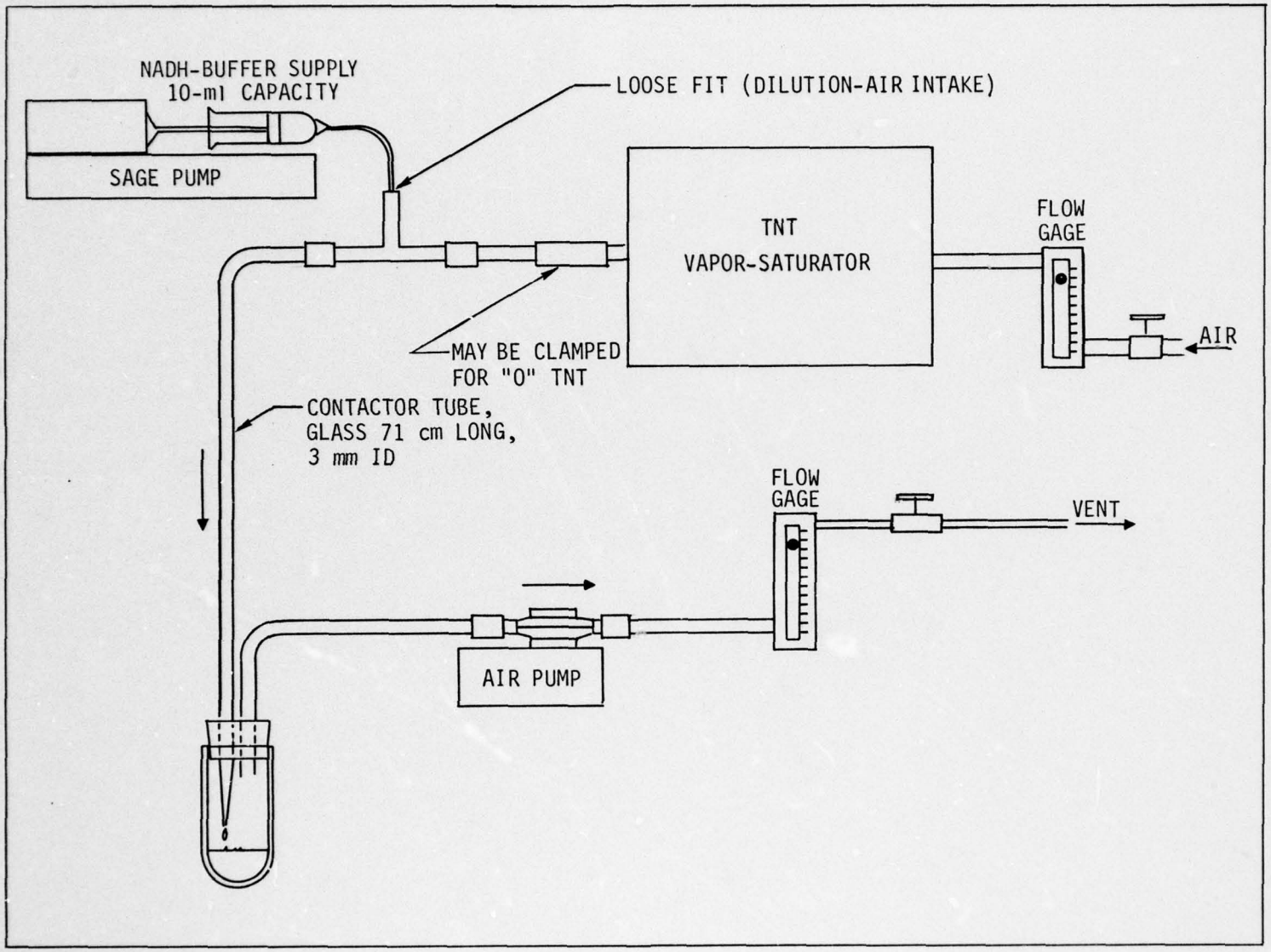

Figure 2-3. Air-Liquid Contactor Setup

efficiency as the ratio of detected amount to the amount passed through the column. (The quantitative uncertainties in this procedure and the reasons for the presently selected approach are dealt with in "Discussion," paragraph 3.3.) It was of interest, in these preliminary tests, first to define a useful quantitation technique for contactor efficiency, then to apply it to progressively more efficient contactor designs while exploring variables such as air sample volume rate, absorber-liquid flow rate, column cross section, etc.

For our earlier contactor tests, we attempted to use the chemistry which in the standard bench-top test maximized the sensitivity TNT detectability at 2×10^{-14} mol. This corresponded to the scheme shown in Report No. IR-2696-102, Figure 3-1, which was the basis also of operating values selected for the continuous breadboard. The scheme was to inject known volumes of TNT-saturated air via a syringe at the top of the contactor, measure TNT in the effluent, and compare the latter values against a TNT calibration using aqueous TNT added directly to the effluent. It was found, however, that the TNT signal decrements ("Δ's," or TNTase minus TNT signals) were so erratic that no useful calibration could be obtained.

We recognized that contactor efficiency is independent of the concentration of the vapor in the gas phase (so long as the absorbing liquid does not approach saturation, a condition easily satisfied here). That is, the quantity of vapor absorbed is proportional to the concentration in the vapor phase, such that the ratio of concentrations attained in the two phases is constant.

Accordingly, it seemed appropriate to work with higher concentrations of TNT where the contributions of baseline noise could be proportionately smaller. In an earlier procedure (Report No. IR-2696-102, paragraph 3.2.3.1), TNT had been tested at higher levels. The results of those tests, Figure 3-9 of that report, showed useful calibrations in four runs, made over a six-month period, in the range of 0 to 4×10^{-12} moles of TNT. The chemistry of this procedure was adapted to our continuous flow contactor tests. At the same time, we decided to increase the contactor column length from 7 inches (the dimension used in the continuous flow breadboard) to 28 inches (the 3-mm ID being unchanged), and to inject the TNT vapor continuously from the TNT vapor saturator as shown in Figure 2-3.

Operating conditions were the following:

- The diaphragm air pump was operated at its maximum volume rate, drawing one liter per minute of total air/vapor mixture through the contactor.
- The contactor liquid was 8.33×10^{-7} M NADH in standard Beckman buffer, pH 7. This was fed to the column by the Sage pump at 0.27 mℓ per minute.
- Flow of room air through the TNT vapor saturator was variable, as set by the valve and gage at the saturator intake. At the maximum TNT mass transfer rate included in our data, flow through the saturator was 625 mℓ per minute.

As mentioned earlier, the flow rate of dilution-air from the room into the leak around the NADH/buffer inlet was always the difference between the diaphragm pump rate and the TNT saturator intake rate.

To conduct a run, the air pump was turned on, and adjustment made of both the total flow and flow through the TNT vapor saturator. The Sage pump was then started. After allowing sufficient time for complete wetting of the contactor tube, the container for collecting the column effluent was temporarily removed, emptied, and promptly remounted. The effluent (approximately 0.5 mℓ volume) was then collected over a two-minute interval. The effluent collection tube was removed, 5 μℓ of TNTase (0.055 unit of activity) were added, and after one-minute incubation at room temperature, 20 μℓ of the mixture was injected into one mℓ of the usual luciferase working solution (LWS) in the photometer.

Under the selected operating conditions, air flow through the contactor (Reynolds number approximately 3×10^4) was well within the turbulent region. This helped assure maximum vapor absorption for the given column geometry and operating conditions. Evaporation loss of water from the NADH/buffer solution into the contacting air stream was deemed negligible, being only about 5% if the contacting air was assumed to have an RH of 50 percent.

Controls and Calibration. Reference "blanks," in which NADH was added to the luciferase working solution without TNT or TNTase, were made prior to each day's work and at intervals during the day. The concentration of 10^{-7} M working NADH/buffer solution was adjusted as necessary with a spectrophotometer to give a standard response on the recorder upon addition of the NADH to the LWS. These blank values were the photometer peak readings less the luciferase glow level.

Separate tests indicated that a given amount of NADH, whether added to the LWS directly, or by flow through the contactor column (carrying air at one liter per minute) gave the same reading. The NADH therefore did not seem to be affected by flow through the contactor *per se*. Further, the photometer reading was the same even when no air flowed through the contactor during passage of the NADH. Accordingly, the "air effect" which had appeared in prior low-level TNT experiments was not observable.

For calibration of the system, we needed a curve of "delta"--the difference between blank peak heights and TNT peak heights--versus known quantities of aqueous TNT added to 0.5 mℓ volume of effluent. The test values provided the data points for the calibration curve shown in Figure 2-4.

The Δ TNT for this curve was obtained by subtracting the average of replicate TNT peak values (at each of the indicated concentrations) from the average of all the blank values. The blanks ranged from 98.5 to 105.7 ($\overline{X}$ = 100.6). The calibration curve proved useful, although in an automated continuous flow system, it would probably be more accurate, if possible, to make alternate blank and test readings, with automatic electronic data correction.

TNT Vapor Contact Tests. Having acquired a calibration curve, we next investigated the efficiency of TNT vapor absorption, measuring the total absorbed by 0.5 mℓ of NADH/buffer flowing through the contactor.

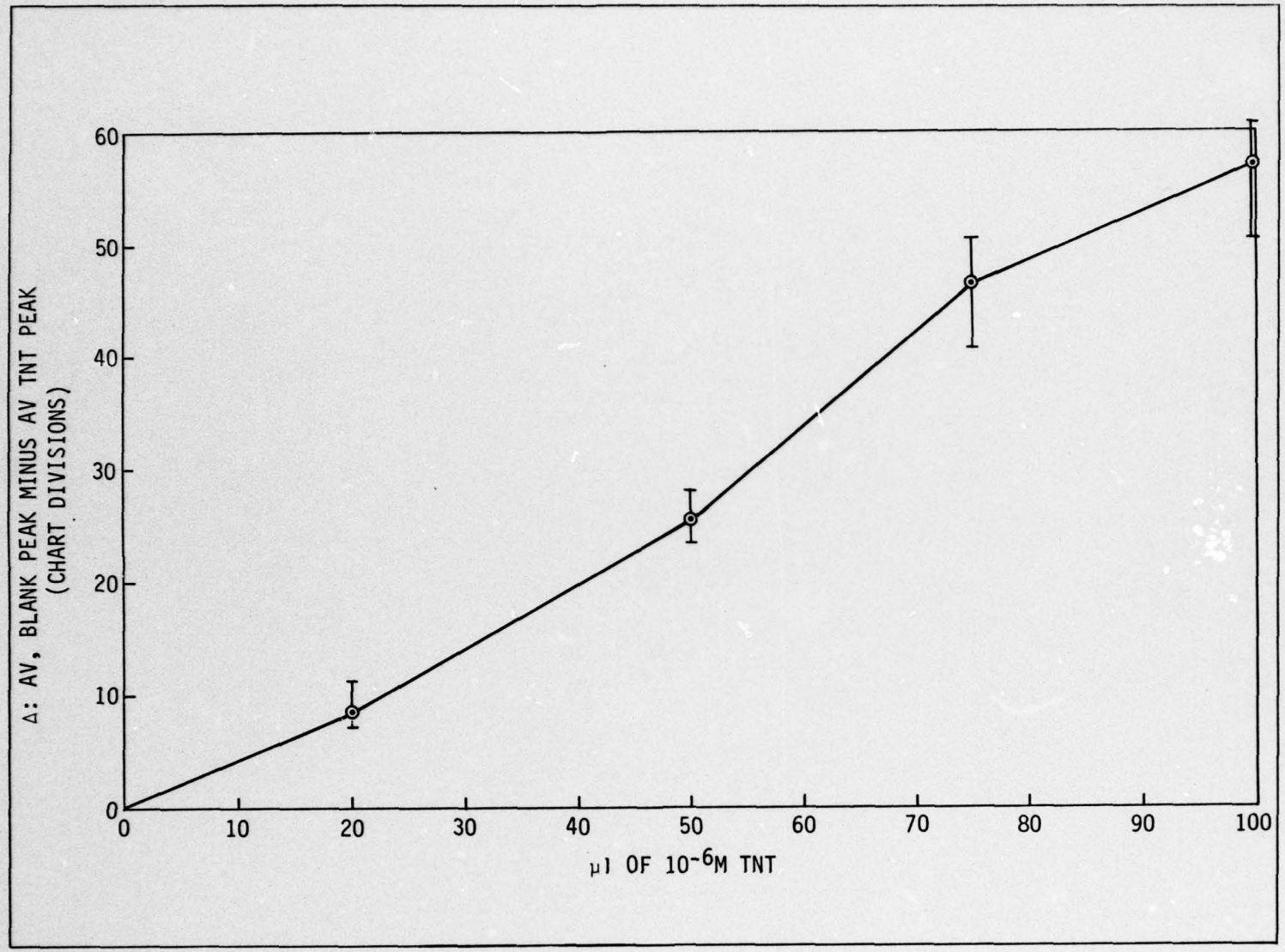

Figure 2-4. Calibration Curve for Aqueous TNT

The Sage pump was set to deliver 0.27 mℓ/min, hence we collected approximately 0.5 mℓ during a two-minute period. A diaphragm pump sucked air, or air mixed with TNT-saturated vapor, through the contactor tube at a total rate of one ℓ/min. The TNT vapor flow rate was varied between zero and one ℓ/min. Each collected fraction was then treated in the routine way. A plot of the results is presented in Figure 2-5. Low flow rates for TNT vapor (6.0 mℓ/min and below) gave erratic results.

If the flow rate of saturated TNT vapor from the TNT Vapor Generator (the "bomb") is set at 624 mℓ/min, and sustained for two minutes, then assuming the TNT vapor pressure at saturation (room temperature, 23°C) is 1.25×10^{-6} torr, the amount of TNT in the 1248 mℓ (for example) is equivalent to 83 μℓ of 10^{-6} M TNT (in water solution). From the calibration curve, Figure 2-3, this concentration of TNT may be expected to have a $\Delta = 50$. Our vapor test gave a $\Delta = 17$. This represents a contactor efficiency of 34%. The equivalent μℓ 10^{-6} M TNT_{aq} for other volumes of TNT vapor tested were calculated and the results for both aqueous TNT and vapor TNT, related to a common abscissa scale, are plotted in Figure 2-6.

Due to the variability in the data, it is difficult to extract an "average" contact efficiency (i.e., response ratio for aqueous TNT vs. vapor TNT) from the values shown in Figure 2-5. It does not appear possible to draw a straight line through the origin for the TNT vapor response. This may reflect inherent nonlinearity at low levels. If an average efficiency must be stated, it would be in the neighborhood of 35%.

However, another large qualifier must apply to these efficiency values. This relates to the vapor pressure of TNT at room temperature (nominal temperature 23°C). According to a relatively recent reference (Pella, P.A., *Analytical Chemistry, 48,* 1632, September 1976), the value may be seven-fold larger than that assumed during the study program heretofore (1.25×10^{-6} torr). This would make the observed contactor efficiencies smaller by the same factor. This is commented on further in Section 3.0 of this report.

3.0 DISCUSSION

3.1 NADH Oxidase Reaction Kinetics

At the outset of the work covered by the present report, it was believed that the NADH oxidase problem could be circumvented by using excess NADH and a reasonable reaction time (one to several minutes) to allow the NADA loss rate to decrease to an acceptable level. The initial results showed that this was not the case. The rate of loss of NADH was found to be constant (for fixed TNTase activity) at high levels of NADH, and logarithmic at lower levels of NADH. The question of whether the rate of loss of NADH is constant or a logarithmic function of NADH concentration is of considerable theoretical interest. In its simplest form the Michaelis-Menten equation (where applicable) predicts that the rate will be

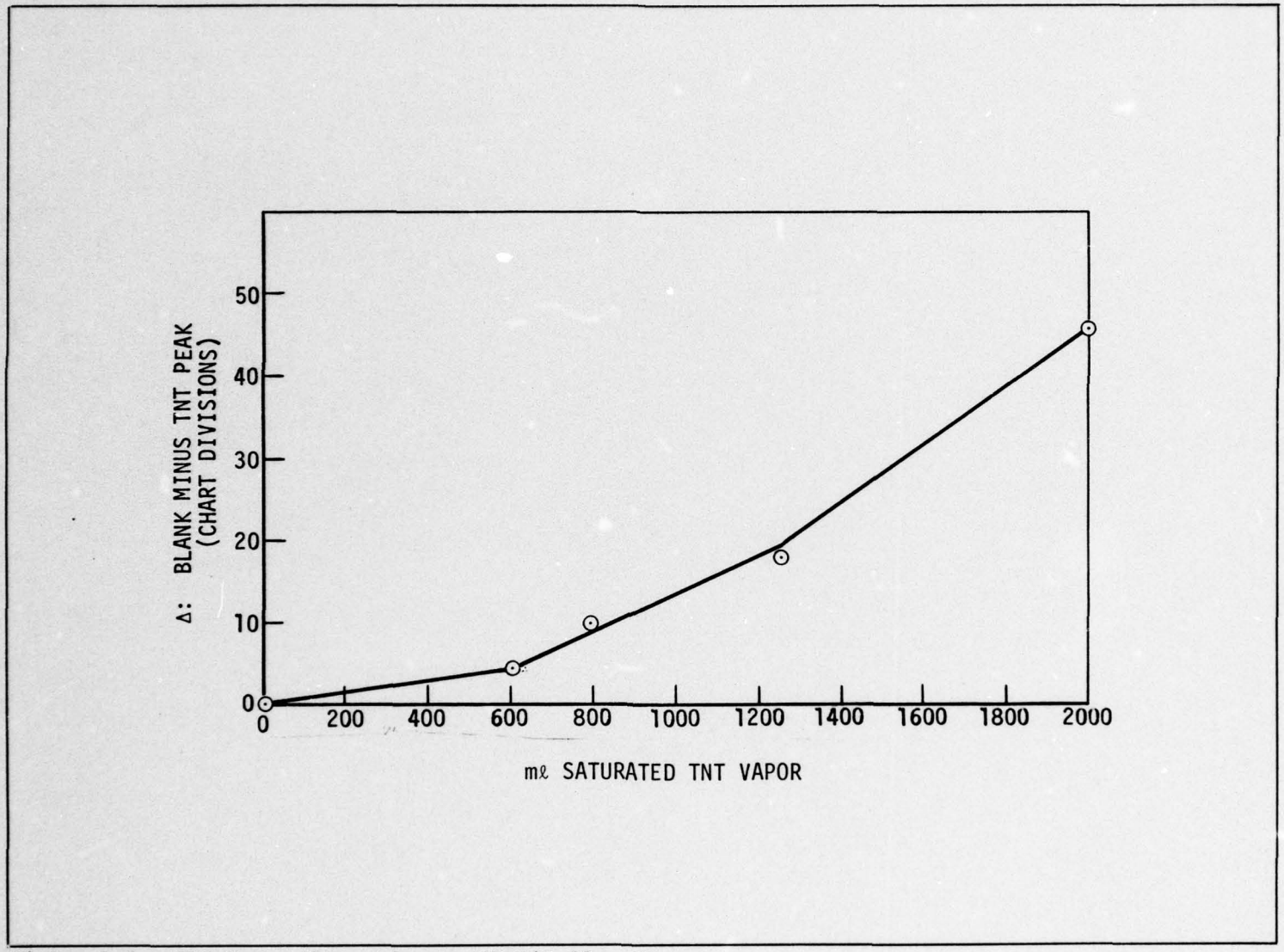

Figure 2-5. TNT Vapor Absorption by NADH/Buffer Stream

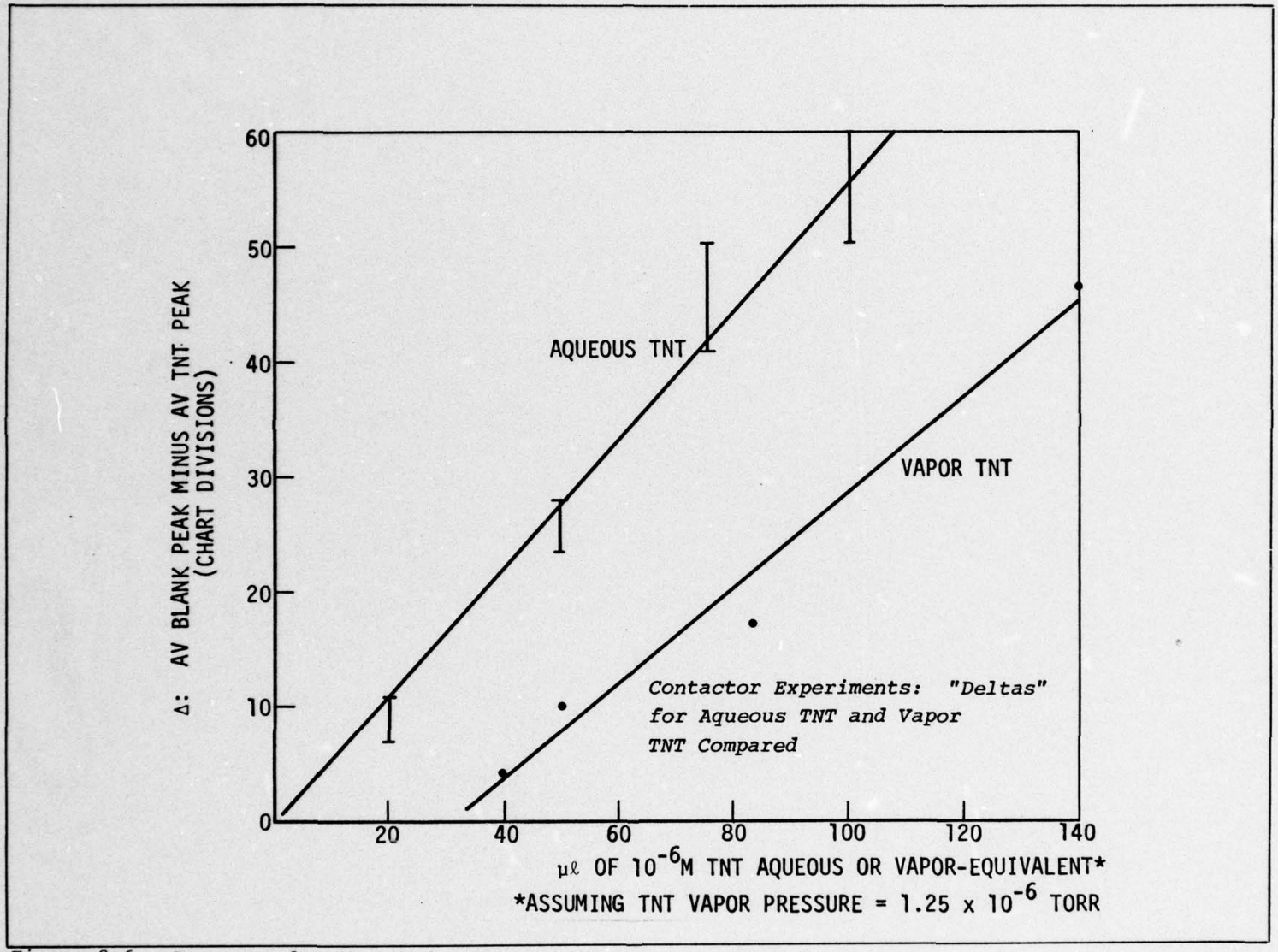

Figure 2-6. Response for Absorbed TNT Vapor Vs. Aqueous TNT References

$$\frac{dC}{dt} = \frac{-K_1 C}{K_m + C} \qquad (3\text{-}1)$$

where

K_1 is a constant including TNTase concentration (actually, the NADH oxidase concentration) as a factor.

C is the concentration of the substrate (NADH in this case).

K_m is the Michaelis constant.

t is the time after combining the reagents.

It follows from Equation (3-1) that when C is negligible compared to K_m the rate is a logarithmic function of C. That is, when

$$\frac{1}{C}\frac{dC}{dt} = \frac{-K_1}{K_m} \qquad (3\text{-}2)$$

then

$$\log (C/C_o) = \frac{-K_1}{K_m} t \qquad (3\text{-}3)$$

In this equation C_o denotes the NADH concentration at zero time. When C is much larger than K_m in Equation (3-1), however, C cancels out. In this case

$$\frac{dC}{dt} = -K_1 \qquad (3\text{-}4)$$

and

$$C = C_o - K_1 t \qquad (3\text{-}5)$$

A change in the decay function from one showing a linear decrement (Equation 3-5) to one showing a logarithmic relationship (Equation 3-3) is expected for reactions obeying the Michaelis-Menten equation. It appears that the oxidation of NADH by a component of TNTase is such a reaction.

A comparison was made of results obtained in April and May, 1977, with those obtained in the FY 1976 effort. The agreement with regard to photometer (luciferase reaction) sensitivity to NADH was good. In addition, a log decay of NADH, at least for the lower concentrations tested, was observed in a series of tests, the results of which are plotted in part in Figures 3-11 and 3-12 of IR-2696-102. This is consistent with the recent findings for comparable concentrations.

In reviewing the prior data from which an approximate NADH oxidase Michaelis constant, K_m, was determined (Report No. IR-2696-102, Figure 3-5), it was found that a much better linear plot is obtained if the square root of the NADH concentration is used rather than the concentration. The rates for these tests were obtained at high NADH and TNTase concentrations, using the absorbance at 340 nm to monitor the decrease in NADH concentration. The data are, therefore, relatively precise, and are independent of the luciferase reaction assay (required for the NADH concentrations of interest). The square-root law can be accounted for by assuming that NADH is largely associated as $(NADH)_2$, while the reactive species is NADH. This mechanism would mean that C should be replaced by the square root of the NADH concentration in Equation (3-1). As noted above, the plot of $(dC/dt)^{-1}$ vs. $(C)^{-1/2}$ is very linear, with negligible data scatter. The intercept with the $(C)^{-1/2}$ axis represents K_m in the normal sense, multiplied by the square root of twice the dissociation constant, K_D, for 2 NADH $\rightleftharpoons$ $(NADH)_2$ equilibrium. (This assumes, of course, that such NADH association is in fact the cause of the observed rate function.)

It is probable that many other mechanisms (mostly more complicated) could explain the observed square-root law. Furthermore, it is not clear that this relationship holds for the lower NADH concentrations of interest. In fact, when $K_D\ C \ll 1$ the expression derived from the assumption of NADH association as $(NADH)_2$ reduces (approximately) to the normal Michaelis-Menten equation, and the square-root dependence is not discernible. Under these conditions, the reciprocal of the Michaelis constant would be given by minus twice the intercept with the 1/C axis when dt/dC is plotted against 1/C. This could provide a convenient further test of the NADH association concept and/or the validity of the inequality $K_D\ C \ll 1$ in the concentration range of interest. This determination of K_m would be independent of the dissociation constant, K_D, and, could permit determination of K_D by combining this result with the result from the $(C)^{-1/2}$ plot.

3.2 TNT Reaction Effect

The TNT reaction effect is small, compared with the NADH oxidase effect, for the concentrations of major interest. Initial attempts to determine the rate of the NADH/NADH-oxidase reaction using the ordinary batch sampling technique were disappointing. Particularly troublesome in the use of batch-sample technique for studying reaction mechanisms has been the variability of replicates. A variation of more than $\pm 5\%$ for replicates has been typical. This is one of the reasons for trying a continuous flow system. It was decided that a series of reaction mix aliquots (20 $\mu\ell$) should be injected into a given luciferase reagent mix. The results of tests performed with this technique in 1976 appeared surprisingly reproducible, although the luciferase reagent slowly lost sensitivity with time and/or repeated usage.

The test results using repeated injections into the same luciferase solution are shown in Figure 3-1. Several features of the curves in Figure 3-1 suggested that further tests would be worthwhile. These may be summarized as follows:

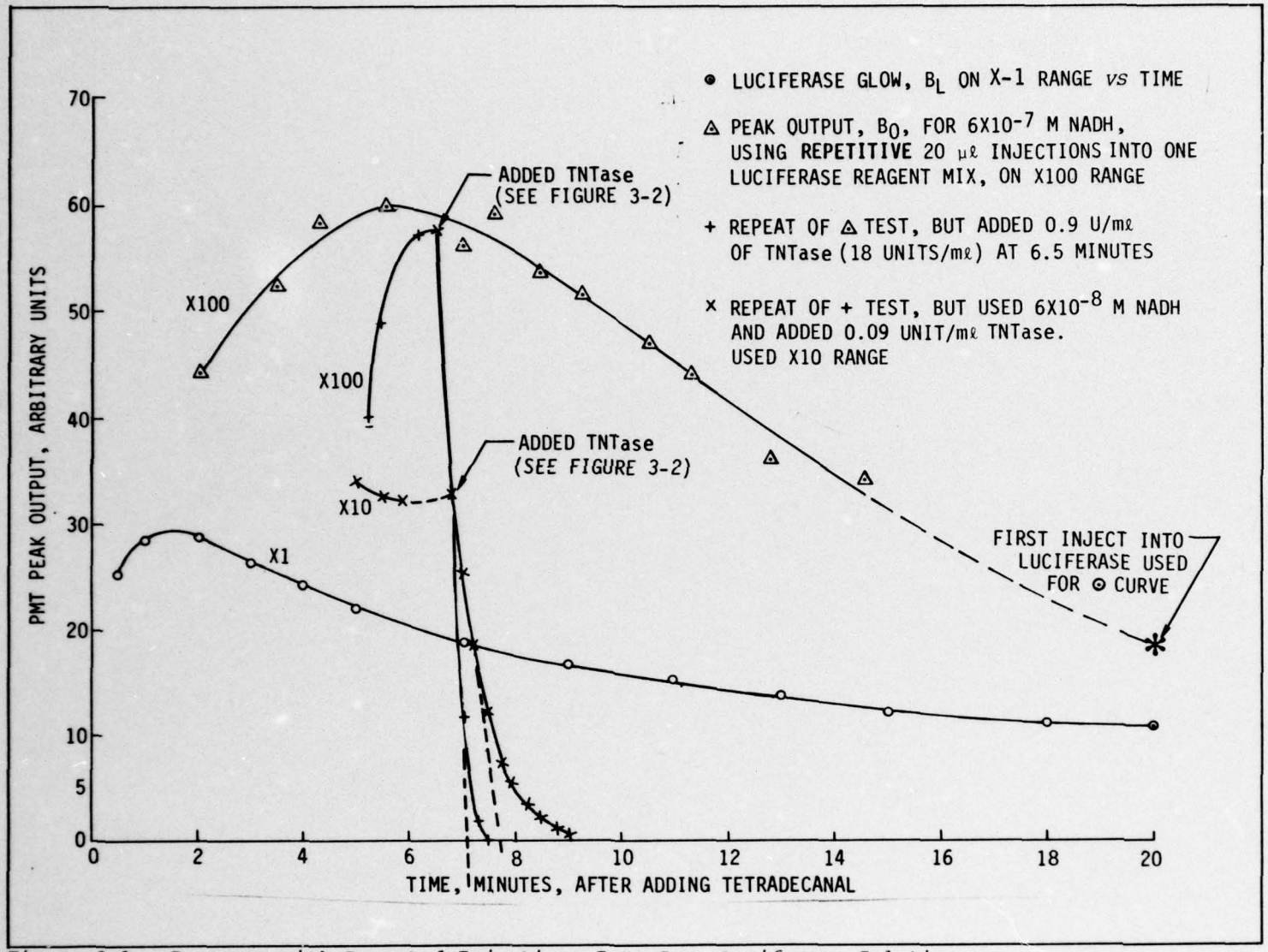

Figure 3-1. Response with Repeated Injections Into Same Luciferase Solution

- The luciferase glow curve is typical, but the detection reaction output, B_O, does not appear simply proportional to B_L for 6 x 10^{-7} M NADH. It may be for 6 x 10^{-8} M NADH.
- The B_O curve (triangles) suggests that the peak sensitivity may occur about five minutes after the tetradecanal is added to the luciferase reagent. However, in view of the second B_O data (+ points, which became B_A data when TNTase was added) it is more probable that the sensitivity increases for the first three injections.
- The maximum output for 6 x 10^{-7} M NADH is about 20 times that for 6 x 10^{-8} M NADH, while that for the first injection is much closer to ten times. The previously demonstrated linearity of peak light output vs. NADH concentration was based upon single usage of the luciferase.
- There is no apparent increase in sensitivity for the first few injections for 6 x 10^{-8} M NADH (the X curve) as there is for 6 x 10^{-7} M NADH (the triangle and + curves).
- If a linear decrement applies, the initial slopes of the decay curves after TNTase (and NADH oxidase) addition should be in the ratio of ten to one for the (+) and (X) data, respectively, corresponding to the ratio of TNTase added. The ratio was found to be about thirty to one. The enhanced response to 6 x 10^{-7} M NADH after the first three injections accounts for a twenty-to-one ratio. The additional 50% higher slope for the higher concentration suggests that 6 x 10^{-8} M NADH is in the range of transition from linear to log decay. This is consistent with all prior results for this concentration.

The two curves of Figure 3-1 for which TNTase was added are replotted in Figure 3-2 with more suitable scaling. The nonlinearity of the luciferase light output response to NADH (after several injections) is apparent, since the data for 6 x 10^{-8} M NADH are plotted on a scale (ordinate only) which is ten times more sensitive than that for the 6 x 10^{-7} M NADH. In addition, there is a dashed line in Figure 3-2 which represents the initial slope of the 6 x 10^{-7} M NADH and 0.9 unit/mℓ TNTase data with the sensitivity normalized to that of the (+) curve. The higher slope of this normalized curve probably indicates that the transition from a linear to a log decrement begins below 6 x 10^{-7} M, but above 6 x 10^{-8} M NADH.

The final test of this series involved addition of TNT. Soon after the data of Figures 3-1 and 3-2 were obtained on 6 x 10^{-8} M NADH with 0.09 unit/mℓ TNTase, the test was repeated, except that 5 μℓ of 10^{-6} M TNT were added with the NADH. This made the reaction mix 6 x 10^{-8} M in NADH and 5 x 10^{-9} M in TNT. The TNTase was added last, as before, after obtaining several initial peaks. The results are summarized in Figure 3-3, which shows the output as a function of time with and without TNT added. While there was a slight offset in the initial (no TNTase) outputs, it is clear that the difference increased

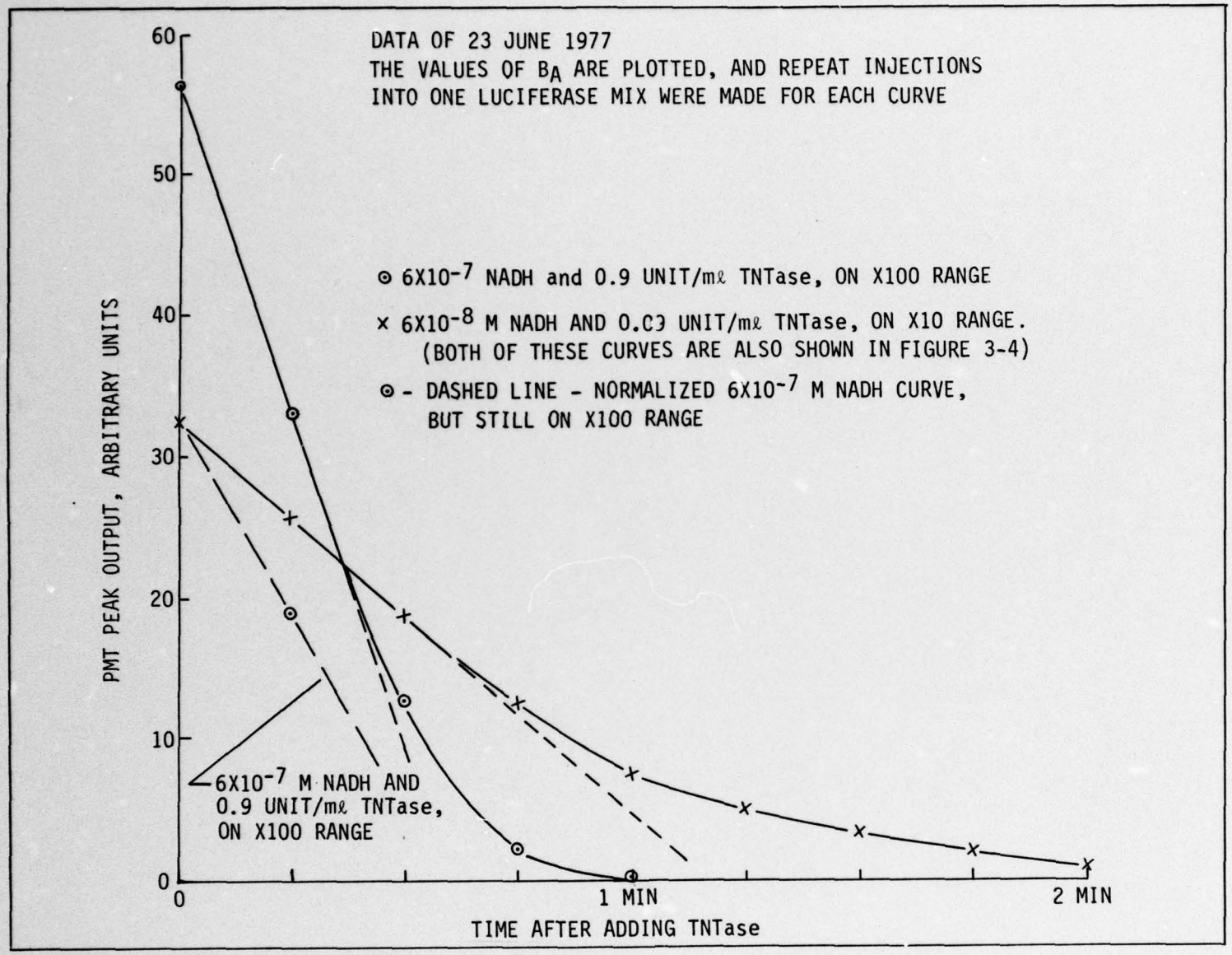

Figure 3-2. Decay Curves for Various Concentrations of NADH and TNTase

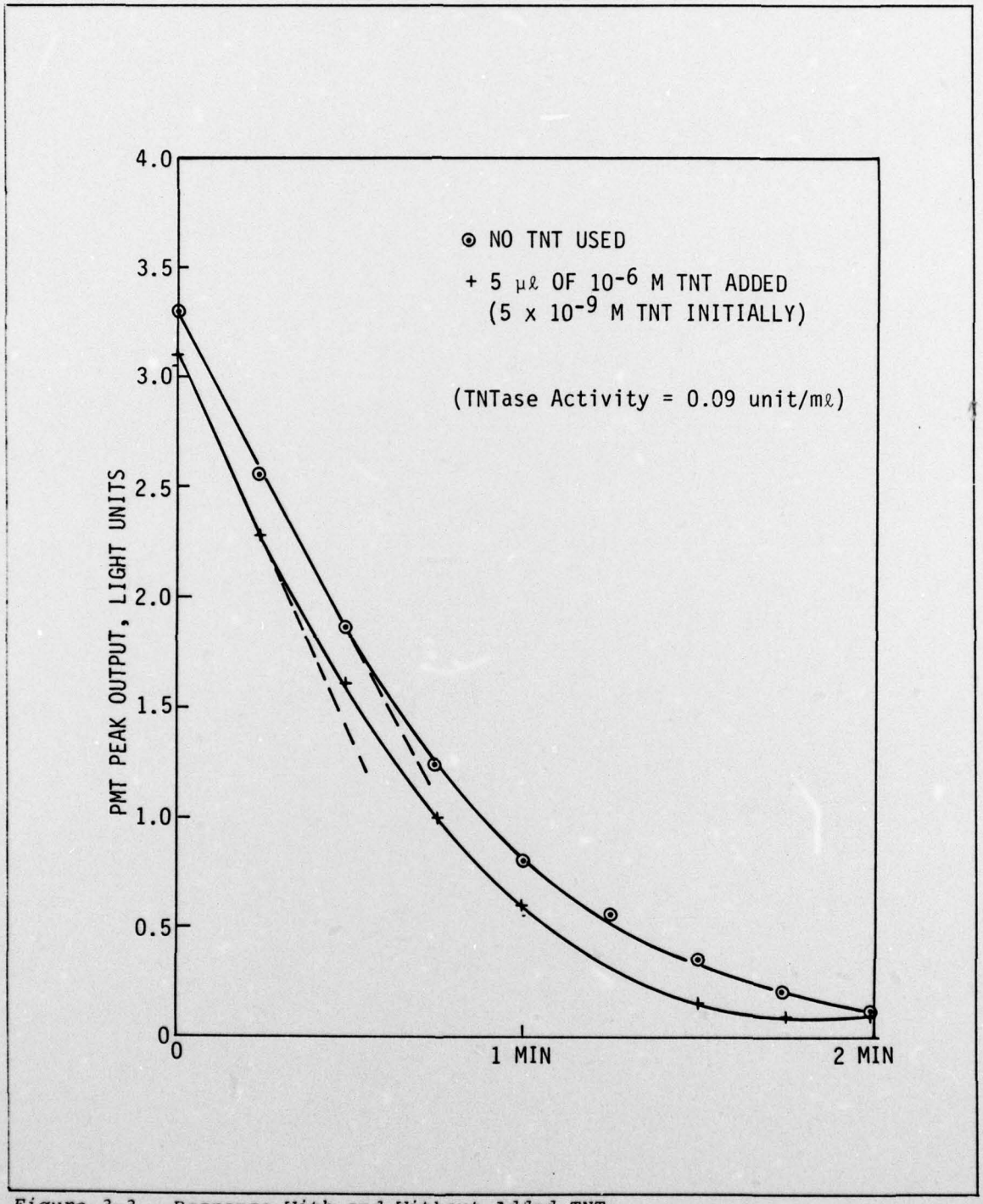

Figure 3-3. Response With and Without Added TNT

during the initial minute of reaction. It is also apparent that the initial slope for the TNT-containing reaction is about 15% greater than that for which TNT was not present.

The data indicate that at about one-half minute the TNT reaction has reduced the NADH concentration by about 6.6×10^{-10} molar. For two molecules of NADH per TNT, this means that the TNT concentration was reduced by about 3.3×10^{-10} molar in the first half minute. This corresponds to reaction of about 6.5% of the TNT in the first half minute. This is in reasonable agreement with 1976 data (estimated in the same way) for comparable TNT concentrations with twice as much TNTase, for which about 12% of the TNT reacted in the first minute.

Taking a different approach, the initial slopes of the two curves are 5.22×10^{-8} M/min (no TNT) and 6.15×10^{-8} M/min (with 5×10^{-9} M TNT). The difference in slopes, which is the rate of loss of NADH due to the presence of 5×10^{-9} M TNT, is 9.4×10^{-9} M NADH/min. Assuming that two NADH molecules react with each TNT molecule, about one minute at the initial rate would consume all of the TNT (5×10^{-9} M TNT). From this standpoint it appears that about 63% of the TNT may have been consumed in the first minute. This method of calculation is more direct and seems more reliable.

Additional tests were performed with the technique of repeated, timed, injections of a given reaction mix into a given luciferase reagent vial. This series of tests indicated the following:

- The sensitivity of the luciferase reagent to 6×10^{-8} M NADH as well as to 6×10^{-7} M NADH increases with repeated injections. The peak sensitivity occurs after 7 to 10 injections (20 µℓ each) for the lower concentrations, and for the third injection for the higher concentration. The enhancement in sensitivity may be related to the concentration of NAD in the luciferase, but there is also a time factor. That is, for 6×10^{-8} M NADH the sensitivity decreases with time after several repeat injections are made, but partial recovery occurs with additional injections. The new peak is normally achieved at about the third injection. It appeared that bubbles on the luciferase surface, which are known to form when the reaction mix is injected, might be partially responsible for the enhancement in sensitivity with repeated injections. Such bubbles could scatter light to the PMT which would otherwise be lost.

- The light output sensitivity of the luciferase is roughly proportional to the luciferase glow level. However, when reconstituted luciferase is held overnight before diluting 20 to one with pH 7 buffer and adding tetradecanal, the NADH sensitivity-to-luciferase glow ratio is higher by a factor of about three. This suggests that an effective reduction in luciferase glow as a noise generating component may be obtained by "aging" the reagent.

- The peak luciferase response to NADH for repeated injections of NADH is apparently a linear function of NADH concentration in the 6×10^{-8}

to 6 x 10^{-7} M NADH range. The fact that it takes more injections to reach peak output at the lower concentration probably accounts for the apparent nonlinearity observed in the earlier test. This also indicates that surface bubbles are not the major cause of sensitivity change.

- Several tests performed with NADH and TNT only verified that there is no detectable loss of NADH due to direct reaction with TNT in the absence of TNTase.

The NADH and TNTase concentrations were varied over a narrow range to give usable decay rates from the viewpoint of providing enough data points. The TNT concentration was varied more widely in an effort to determine the effect of TNT concentration upon the rate of loss of NADH. Preliminary analysis of the analog recorder charts revealed several factors of interest, but nothing clearly indicating a preferred set of operating conditions for a continuous flow system. The major tentative conclusions are:

- There are probably significant deviations in the first two points (zero time and fifteen seconds) after TNTase is added due to variable mixing of the TNTase with the NADH solution. This may be a significant problem in the preliminary continuous flow system, since no means of mixing other than diffusion was included in the design. The intended use of a relatively large volume (flow rate) of diluted TNTase will help, but slow and erratic mixing may be a s source of baseline noise.

- The scatter tendency for the first few data points (in time) (probably due to TNTase concentration gradients when mixing) makes it difficult to detect the increase in NADH oxidation rate due to low concentrations of TNT (5 x 10^{-10} M TNT). The results are reliable for 5 x 10^{-9} M to 1.5 x 10^{-8} M TNT.

- Using the analog recorder chart and a straight edge, it appears that the reaction rate is well approximated by a series of straight lines with decreasing slopes. With TNT present, the initial slope is higher than it is when there is no TNT, and the high slope continues longer when more TNT is present. The second-line segment when TNT is present approximates the first slope when TNT is not present for comparable residual NADH levels. (See Figure 3-4 for replots of recorder data illustrating these points.)

- It appears probable that the maximum TNT effect for 5 x 10^{-9} M TNT occurs within about 30 seconds after adding only 0.036 unit/mℓ of TNTase (one-fifth the concentration normally used).

- Using less than usual TNTase may be beneficial in the continuous flow system. It also appears that this could be beneficial with the batch system if a reaction time of one minute or more is allowed.

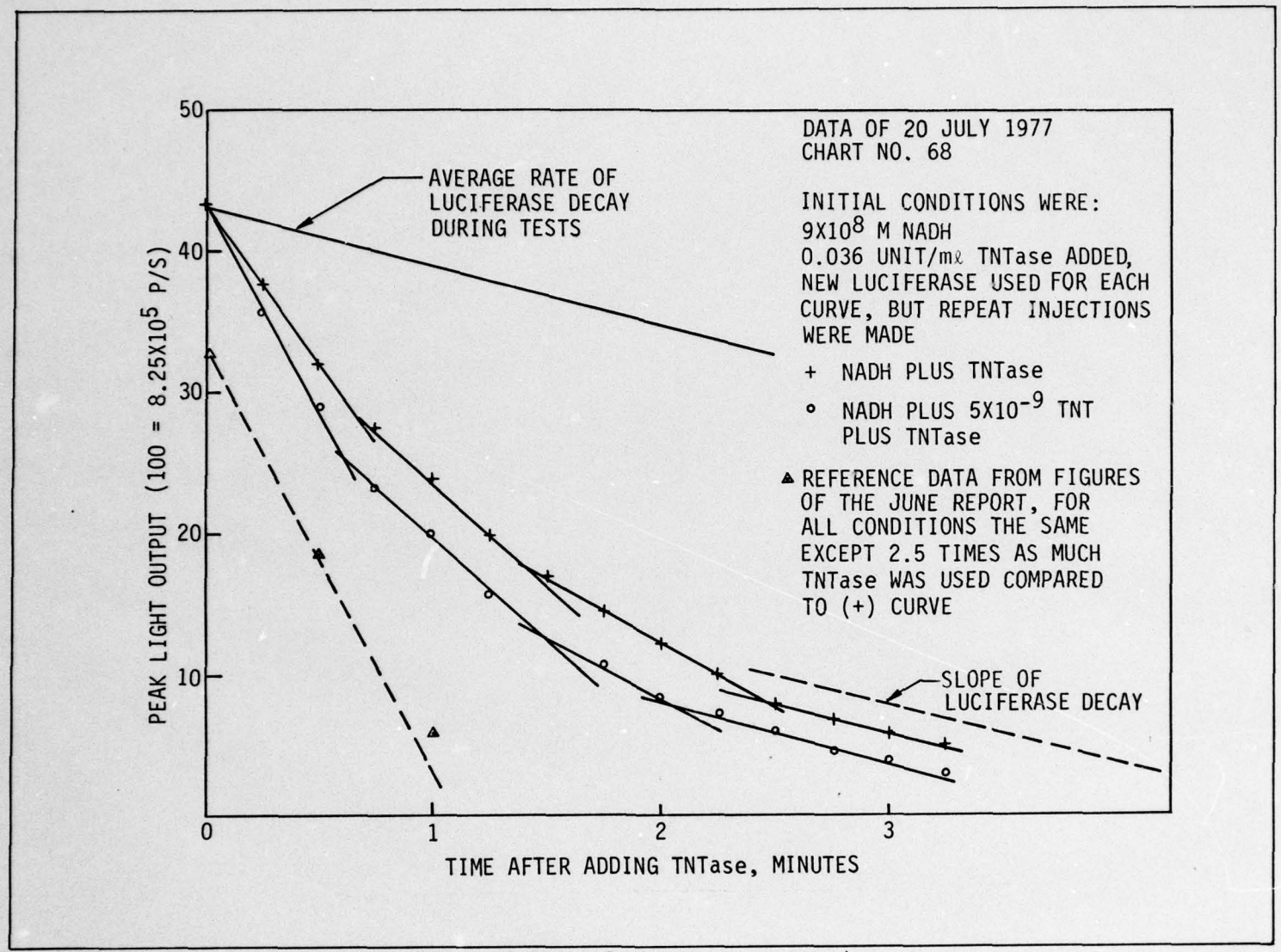

Figure 3-4. Responses Showing Effect of Reduced TNTase Concentration

The average rate of luciferase decay during the tests is also shown in Figure 3-4. The NADH reaction curves are plotted without correction for this luciferase sensitivity decay. The loss of sensitivity was determined by taking an aliquot of the NADH solution before adding TNT and another before adding TNTase. After the peak light output decayed to a level below that plotted in Figure 3-4, repeat injections of the reference solutions (no TNTase) were made to establish the loss of luciferase sensitivity which occurred during the NADH/TNTase (or NADH/TNT/TNTase) test.

It should be noted that in each case the last "linear" decay shown in Figure 3-4 has essentially the slope of the luciferase sensitivity decay, suggesting that the NADH/NADH oxidase reaction rate is very slow for NADH concentrations below about 2×10^{-8} M NADH with 0.036 unit/mℓ TNTase activity. It is believed that the NADH concentration actually decayed to zero, and that the readings are due to a "zero error" arising from technique. Specifically, a signal equivalent to about 2×10^{-8} M NADH is obtained when the steel needle of the Hamilton syringe is inserted into the luciferase reagent. The photometer had been modified to prevent the needle from reaching the luciferase surface if the cuvette contained no more than 1.8 mℓ, but in the repeated injection tests illustrated in Figure 3-4 the total volume exceeded this amount before the tests were over.

Finally, it should be noted that comparison of the data of Figure 3-4 with those of Figure 3-3 is of interest. The only intentional difference is that 2.5 times as much TNTase was used for the test of Figure 3-3 as for the test of Figure 3-4. The initial slope of the curve of Figure 3-3 is very nearly 2.5 times that of the *second* slope of Figure 3-4. The initial slope of the curve of Figure 3-4 is so similar to that of Figure 3-3 that one could infer that TNTase concentration is unimportant in regard to the initial rate of loss of NADH. Similar observations in 1976 led to the false expectation that NADH oxidase could be "titrated" as a workaround. The data from another test, in which 1.5×10^{-8} M TNT was reacted with 9×10^{-8} M NADH and 0.09 unit/mℓ TNTase, superimposes on the (+) plot of Figure 3-4 when normalized (initial three points only). It is evident that the NADH/NADH oxidase/TNT/TNTase reaction kinetics are complicated, but it also appears that the effect of TNT on initial reaction rates is roughly the same for the two TNTase concentrations. As noted above, it appears that a reduction in TNTase concentration could improve the differential signal due to TNT.

3.3 Comments on Continuous Flow System Testing

The initial effort to make the assembled continuous flow system operational was unsuccessful. The immediate cause of failure was clogging of the capillaries, apparently due to particulate matter in the TNTase and luciferase reagents. In-line filters are certainly feasible, but the necessary time and funding were not available when the problem was recognized. It is expected that additional improvements will be required when the assembled system is again tested. Meanwhile, it was decided that the effort should be redirected toward a more thorough evaluation of the subsystems, particularly an evaluation of TNT recovery efficiency in the gas-liquid contactor.

3.4 Comments on Contactor Efficiency Testing and Data Variability

It should be noted that the tests reported here are preliminary. Their principal intent was to define a quantitation technique. Certain limitations in the present technique have been made evident. The large variability in data, even at higher TNT concentrations, appears to stem principally from the magnitude of the NADH oxidase effect. It is hoped that future work in the Beckman Microbics Division will alleviate this problem. Another source of variability observed during our recent work is the imperfect mixing of the luciferase reaction mix (1 mℓ) following injection of the (20 μℓ) TNT/TNTase/NADH mix. In a bench-top experiment, injection of 20 μℓ of a colored solution into one mℓ of clear liquid in a vessel similar to that used in the photometer gives visibly incomplete and variable mixing. Stirring the luciferase mix continuously, or injecting a several-fold larger volume of TNT reaction mix, could reduce the variability considerably.

Contactor Design

The design of vapor/liquid contactors is an established part of chemical engineering practice, since contactors are frequently used in chemical production processes.* Although some fundamental theory has been developed, the large number of critical variables has resulted in design approaches based to a large extent on experience, intuition, and trial and error. The present problem is particularly difficult in that very large volumes of vapor containing air must be extracted at maximum speed by very small volumes of contactor liquid

In the further systematic pursuit of the present program, a study must be made of relevant chemical engineering practices. One of the alternatives to be considered is the atomization (or "nebulization") of the contactor liquid in the column for a large increase of effective contact area, coupled with means for rapid condensation of the mist.

A variable to be studied in contactor performance is "trailing" of sample in the contactor. This could be evaluated by following a test run (using TNT vapor as described) with a run in which the TNT vapor is omitted but only an air-stream is used. This could help quantitate the magnitude and duration of the trailing.

Saturation Vapor Pressure of TNT

It has been accepted during the TNT enzymatic detection study that the saturation vapor pressure of TNT at nominal room temperature is about 1.25×10^{-6} torr. That value is now placed in question by a relatively recent

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*Maddox, R.N., "Gas Absorption," in R.H. Perry and C.H. Chilton, *Chemical Engineers' Handbook,* 5th ed., Sec. 14 (1973), McGraw-Hill.

publication.* The work appears to have been very carefully done, although we have not yet studied it critically. From interpolation of data in Pella's Table II, with conversion of equilibrium vapor concentration to vapor pressure, the equilibrium pressure for TNT at 23°C becomes about 6.1×10^{-6} torr, or about five-fold higher than we have assumed. Accordingly, if this value is accurate, the calculated TNT collection efficiency that we derived in our tests must be reduced by a similar factor. Various aspects of this discrepancy will require further study.

4.0 RECOMMENDATION

It is recommended that the next stage of work in pursuit of the present program's objective comprise the following:

- A survey of the state-of-the-art of vapor/liquid contactor systems, with recommendation of the best approach to use in an improved TNT vapor collector. Design, fabrication, and testing of the contactor, with documentation of observed collection efficiencies at various TNT vapor concentrations.

- An investigation of reaction parameters important for the design of the TNT enzyme reaction loop of a continuous flow TNT detector system. Design, fabrication, and testing of an optimized reaction loop, with documentation of test results.

- Integration of the vapor/liquid contactor and reaction loop, with testing of the integrated system at various TNT vapor concentrations.

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*Pella, P.A., *Analytical Chemistry, 48,* 1632, September 1976.

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This Final Report covers the work performed in the period Oct. 1976, through Dec. 1977. The previously submitted Interim Reports (IR-2696-102 and IR-2696-103), when added to this report, comprise the "Total Final Report."

The biological approach to mine detection selected for development (in 1973) is based on two sequential chemical reactions. In the first stage, TNT reacts with NADH in a reaction which is catalyzed by the specific enzyme TNT reductase, or "TNTase." In the second stage, residual NADH is used in another enzyme

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catalyzed reaction to reduce FMN to $FMNH_2^*$. The $FMNH_2^*$ further reacts with dissolved oxygen and an aldehyde (tetradecanal) to produce photons (having a peak at a wavelength of 492 nm) and other reaction products. It is this final reaction that provides the very high sensitivity of the method. It is conducted in the field of view of a PMT, which is capable of sensing the loss of 10^9 (or less) molecules of NADH due to the first-stage TNT reaction.

The second-stage detection reaction had been previously developed by Beckman Instruments, Inc., and was available at the inception of this program. The specific enzyme TNTase was identified, and techniques for the production of intermediate quantities were early developments of this contract.

The Interim Report for the preceding period (October 1975-1976) contains the results of preliminary investigations of the TNT reaction kinetics using the optical absorbance of NADH at 340 nm to monitor the progress of the reaction. Investigations at lower NADH concentrations utilized the enzymatic reaction only. A systems analysis was presented. The analysis served as the basis for a Test Plan designed to provide maximum efficiency of effort during system optimization. The Test Plan and the results of several cycles through the Plan were discussed in the Interim Report. The factors limiting sensitivity of the method at the end of the reporting period were identified, and recommendations made for further development. The sensitivity to TNT (liquid standard) was found to be 0.02 picomole (2×10^{-14} moles). It was anticipated that the vapor sampling capability could be advanced to the point where 0.01 picomole of TNT contained in one liter of air could be detected within two minutes. With the recommended further improvement of the chemistry, it was anticipated that another order of magnitude or more improvement in sensitivity could be achieved. In addition, it appeared virtually certain that specific enzymes for detection of other explosive vapors could be found.

The present document discusses the work performed at Beckman-ATO in 1976-1977. It presents the results of investigation of the NADH/NADH oxidase reaction kinetics, an initial design of a continuous flow TNT vapor detector system, and the results of tests on the efficiency of a simple thin-film gas-liquid contactor for the recovery of TNT vapor.

The investigation of NADH/NADH-oxidase reaction kinetics indicated that the NADH oxidase is an enzyme with a fast recovery rate, and that the Michaelis-Menten equation applies if the square root of the NADH concentration is used instead of the concentration. Evidence that the concentration of TNTase can be reduced without reducing the TNT sensitivity is presented. This suggests that reduction of the amount of TNTase used may improve the relative rates of TNT- and non-specific oxidase consumptions of NADH.

A conceptual design of a continuous flow TNT vapor detection system was developed. A breadboard system was fabricated, but preliminary attempts at performing system tests were unsuccessful due to persistent clogging of reagent flow control capillaries. The remaining time and funds were then re-directed toward evaluation of the thin-film gas-liquid contactor as a simple means of recovering TNT vapor directly in a buffer-NADH solution. Test results are given which indicate that the TNT recovery efficiency is either 35% or 7%, depending upon whether the vapor pressure of TNT at 23°C is 1.25×10^{-6} torr, as previously assumed, or about five times this high (as recently reported by Pella, P.A., *Analytical Chemistry*, 48, 1632, Sept., 1976).