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THE MERADCOM IN VITRO BIOSENSOR PROGRAM

by
Dr. Heinrich C. Egghart

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The feasibility of an enzymatic TNT detection concept was demonstrated involving a "sensor reaction" in which a TNT specific enzyme, the TNT reductase, catalyzes a reaction which is sensitively indicated by a light-emitting indicator reaction. The indicator reaction is the in vitro bacterial luciferase reaction whose light emission depends on the concentration of the biochemical reagent NADH. The NADH concentration is changed by the TNT specific sensor reaction.

To facilitate this TNT detection scheme, a hitherto unknown TNT reductase enzyme was "developed"; i.e., bacteria were induced to synthesize this enzyme. These bacteria were produced in quantities by a fermentation process and the enzyme was isolated from the cell contents of the bacteria and then purified. The enzymatic TNT detection scheme permitted the detection of as little as 2×10^{-14} mole of TNT in the liquid phase.

The feasibility of testing air containing TNT traces by direct interfacing with the liquid reagent system was demonstrated. Transfer of the TNT vapor molecules from the air into very small volumes of the liquid with satisfactory efficiency was accomplished. A continuous flow system was identified as the most promising configuration of a future TNT detector, and a breadboard model was fabricated.

An evaluation was performed of the parameters affecting the signal-to-noise ratio and thus the detection sensitivity. The measures were delineated which are necessary to accomplish further sensitivity increases. These include the elimination of an NADH oxidase activity which accompanies the TNT reductase, a reduction of a spontaneous light emission of the reagents of the indicator reaction, and an increase in the light efficiency of the indicator reaction.

> The new concept of light emission immunoassay was developed and applied for TNT detection. It involves interaction of free TNT and TNP-luciferase molecules with sites on immobilized TNP antibodies, isolated and prepared for this purpose. Subsequently, the light emission catalyzed by the TNP-luciferase bound to the immobilized antibody is a function of the free TNT concentration. This technique allowed the determination of 5×10^{-14} mole of TNT. ←

In an amplified light emission immunoassay technique also developed, free TNT and TNP-glucose-6-phosphate dehydrogenase molecules interact with the sites of the immobilized TNP antibodies. The TNP-glucose-6-phosphate dehydrogenase bonded to the TNP antibody catalyzes the formation NADH molecules at a high rate which results in an amplified light signal of the bacterial luciferase readout system. As above, the light signal is a function of the free TNT concentration. The detection of 10^{-17} mole of TNT was possible with this amplified light emission immunoassay technique.

CONTENTS

Section	Title	Page
	ILLUSTRATIONS	iv
	TABLES	v
I	INTRODUCTION	1
II	THE ENZYMATIC TECHNIQUE FOR TNT DETECTION	
	1. Background	3
	2. Enzymes as Analytical Reagents	4
	3. Synopsis of the Effort on Enzymatic TNT Detection	5
	4. Discussion of the Experimental Work	12
	5. Summary	38
III	IMMUNOCHEMICAL TECHNIQUES FOR TNT DETECTION	
	1. Preface	39
	2. Background	41
	3. Present Immunoassay Concepts	42
	4. Development of the Light Emission Immunoassay Concept	49
	5. Discussion of the Light Emitting Reactions Used	51
	6. Discussion of the Experimental Work	53
	7. Summary	61

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ILLUSTRATIONS

Figure	Title	Page
1	Change in Light Emission as a Function of TNT Quantities	8
2	Relationships Between Baseline Photon Flux B_A , Light Background B_L , and the TNT Induced Reduction in Photon Flux for a Signal-to-Noise Ratio of 10	10
3	Light Emission as a Function of NADH Levels	23
4	Light Emission as a Function of Low NADH Levels	24
5	Change in Light Emission Due to the TNT Reductase and NADH Oxidase Activities as a Function of Time	26
6	Relationship Between the Light Efficiency Product and the Multiplier Needed for Determining the Number of NADH Molecules Required for Attaining a Certain Light Level	29
7	Test Setup for Evaluation of the Air/Liquid Phase Interface Concept	33
8	Schematic Representation of the Continuous Flow Breadboard Model	35
9	Schematic Representation of the Phase Separator Used in the Continuous Flow Breadboard Model	36
10	Schematic Overview of the Enzymatic TNT Detection Scheme	40
11	Schematic Description of the Light Emission Immunoassay Technique	58
12	Light Emission due to the Antibody Bound TNP-Luciferase as a Function of Free TNT	59
13	Light Emission due to the Antibody Bound TNP-Luciferase as a Function of Free TNT	60
14	Schematic Description of the Amplified Light Emission Immunoassay Technique	62
15	Amplified Light Emission due to the Antibody Bound TNP-glucose-6-phosphate Dehydrogenase as a Function of Free TNT	63
16	Binding Curve of TNP-glucose-6-phosphate Dehydrogenase (G-6-PDH) to Antibody. Light Emission due to the Antibody Bound G-6-PDH After a 2-min and a 10-min Reaction Period	64

TABLES

Table	Title	Page
1	Optimal Media for Production of TNT Reductase by Bacterial Strain 96-3	16
2	Summary of TNT Reductase Purification	18
3	Substrate Specificity of Purified TNT Reductase	20

THE MERADCOM IN VITRO BIOSENSOR PROGRAM

I. INTRODUCTION

There are currently two primary approaches for solving the problem of mine and hidden explosive detection. One approach is detection via some form of electromagnetic radiation; the other is detection via the sensing of vapor molecules such as explosive vapors indicative of the presence of the explosive devices of interest. The techniques based on electromagnetic radiation are often unsatisfactory because of difficulties in distinguishing between the signatures of the explosive devices and other matter such as rocks and other anomalies. The physico/chemical techniques based on the sensing of indicative vapors suffer from insufficient sensitivities to detect the extremely small concentrations of these vapors in the air near mines and hidden explosive. The sensitivities of detectors such as the mass spectrometer have been increasing over the years, but so far no man-made instrument has reached a level of performance which promises success in mine detection. On the other hand, animals which are apparently vapor detectors are capable of astonishing feats of mine and hidden explosive detection.

In the past, technological development sometimes preceded biological knowledge; for instance, radar and sonar were developed before it was realized that these principles had been evolved by nature at a much earlier period. There is no reason why man and technology should not profit from biological knowledge and from detection techniques evolved by nature in the course of millions of years. The MERADCOM biosensor program was initiated with these considerations in mind and, since about 1974, two biosensor approaches have been pursued simultaneously. One is the *in vivo* approach using live animals as detectors, and the other is the *in vitro* approach using reagents provided by nature for explosive vapor detection.¹ While most physico-chemical detection techniques require heavy equipment, biosensors promise not only sensitivity and specificity but also portability and greater flexibility under field conditions.

In this report, the *in vitro* biosensor approach will be described beginning with the earlier efforts and completing the discussion with a view of work currently in progress.

¹ R. V. Nolan and D. L. Gravitt, MERADCOM Report 2217 (Sep 77).

The processes taking place in living organisms are distinguished by a remarkable specificity in recognizing and responding to minute quantities of substances. In general, this high specificity is due to the existence of receptors with specific sites which are related to the respective substances like key and keyhole. While most of these receptors function only in living organisms, there are some biochemical entities operating in this fashion which can be isolated and used *in vitro*. Prime examples are enzymes and antibodies. They provide specificity in enzymatic and immunochemical analysis and make it possible to detect and quantify the compound of interest in the presence of numerous other substances. Enzymes and antibodies are also capable of responding to very small concentrations. Thus, high sensitivities can be obtained with such biochemical analysis systems. These qualities prompted MERADCOM to apply these techniques to explosive detection. It is a novel approach. In the past such techniques were used only for the detection and quantitation of compounds found in biological systems.

For mine detection, sensing the explosive vapors emanating from buried mines requires extremely high detection sensitivities because explosives have low vapor pressures and their vapors must diffuse through the seals of mine casings and through the soil in which they are buried. The required high sensitivities cannot be expected from readout systems usually applied in biochemical analysis such as spectrophotometric or electrochemical techniques. Therefore, a new concept is used which is the coupling of light emitting biochemical indicator reactions to the enzymatic or immunochemical sensor reactions. Thus, the presence of the explosive molecules is indicated by a change in light emission which can be sensitively measured.

In 1973, the scientific community of the United States was asked for proposals to suggest which biosensor techniques were most promising for solving the problems of mine detection. The vapors emanating from military explosives such as TNT and DNT were recommended by MERADCOM to be the target vapors, because mines are made from various materials but almost all foreign and domestic mines contain TNT or mixtures of TNT with other explosives.² The concept promising the highest sensitivity was proposed by Beckman Instruments, Inc. This concept involved the use of an enzyme specifically responding to TNT, but this enzyme did not exist and required isolation. The enzymatic TNT detection program will be discussed in Section II of the report. An immunochemical approach to TNT detection, conceived at MERADCOM, will be discussed in Section III.

² Training Manuals, "Foreign Mine Warfare Equipment," TM5-280 and "Land Mines," TM9-1345-200.

II. THE ENZYMATIC TECHNIQUE FOR TNT DETECTION

1. Background. The effects of enzymes such as the transformation of juices into alcoholic beverages were already known to early man. In the 17th century, Lavoisier showed that in this fermentation process sugar was converted to carbon dioxide and alcohol.³ In the 17th century, the fermentation of sugar to alcohol was identified as a physiological act of the yeast cell which led Pasteur to the statement that life and fermentation were inseparable.⁴ In 1897, however, E. Buchner found that sugar is rapidly fermented by a cell-free extract made from yeast.⁵ This observation led to the formulation of the modern concept of enzymes and their eventual isolation and identification. Mere solubilization of the fermentation enzymes provided little information about their chemical nature, but in 1926, James B. Sumner obtained a crystalline protein from the jack bean and showed that urease activity was intimately associated with these crystals.⁶ Thus, the protein nature of the enzymes became established, a feat recognized by awarding a Nobel Prize to J. B. Sumner in 1947.

Now we know that enzymes are catalysts, synthesized by all living organisms, which accelerate the reactions upon which life depends. Unlike manmade catalysts, enzymes catalyze reactions under extremely mild conditions (room temperature) and in a highly specific manner. This specificity is perhaps best exemplified by the ability of enzymes to catalyze reactions with a single optical isomer of a given compound, but not with the other isomer of this compound. For instance, the enzyme glucose oxidase catalyzes exclusively the oxidation of β -D-glucose.

Enzymes are also efficient catalysts facilitating chemical reactions at rates which range from 10 thousand to 1 million moles of substrate per minute (turnover number of enzymes). Presently there are about 2000 enzymes known and several hundred have been isolated and are available commercially. The molecular weights of enzymes range from 10 thousand to 2 million. Enzymes cannot be made by man but are isolated from living organisms. However, by growing bacteria in the presence of the compound of interest it is possible to induce bacteria to synthesize a desired enzyme. This technique was of importance in the enzymatic TNT detection program and will be described in more detail later.

³ A. Lavoisier, *Traité Élémentaire de Chimie* (1789).

⁴ L. Pasteur, *Compt. Rend. Acad. Sci.*, *80*, 452 (1875).

⁵ E. Buchner, *Berichte*, *30*, 117 (1897).

⁶ J. B. Sumner, *J. Biol. Chem.*, *69*, 435 (1926).

2. Enzymes as Analytical Reagents. Enzymes are of great interest to analytical chemistry for their specificity and for their ability to catalyze the reactions of their substrates at low concentrations. These properties of enzymes make it possible to determine small concentrations of the molecule of interest in the presence of numerous other compounds. The concentration of the molecule of interest can be determined by measuring the change in the concentration of the end products or the starting materials caused by the enzymatic reaction. Many enzyme reactions require so-called cofactors, compounds which enter into the reaction and are thereby altered. A common cofactor in enzymatic reactions is nicotinamide-adenine dinucleotide, or more simply, NAD. This compound and its reduced form, the NADH, is the oxidant and reductant, respectively, in numerous enzymatic oxidation-reduction reactions. Fortunately, for the purpose of analysis, the light absorptions of NAD and NADH are different at 340 nm and thus provide a basis for spectrophotometric measurement. The determination of alcohol using the enzyme alcohol dehydrogenase, short ADH, is an example:



Such analysis schemes depending on the spectrophotometric or fluorimetric measurement of the formation or disappearance of NADH have a great importance in biochemical analysis. Numerous enzymes and metabolites have been measured with this technique since it was introduced in Warburg's Laboratory in the 1930s.⁷ Such techniques provide satisfactory sensitivities for many biochemical applications, but for the purpose of detecting molecules of explosives collected from the air near the explosive devices much higher sensitivities are required. Fortunately, a new concept for measuring low NADH concentrations was discovered in the early 1970s.⁸⁻¹⁰ It is an application of the reactions which enable bacteria to luminesce. This luminescence is based on two coupled enzymatic reactions. The first reaction is catalyzed by the NADH: FMN oxidoreductase (FMN reductase).



whereby FMN (flavin mononucleotide) is reduced by NADH (the reduced form of nicotinamide-adenine dinucleotide) to FMNH₂ (the reduced form of FMN). The FMNH₂ is being utilized in the second reaction which is catalyzed by the luciferase enzyme.



⁷ O. Warburg, *Wasserstoffübertragende Fermente*, Springer Verlag, Berlin (1948).

⁸ S. E. Brodin, F. Borglund, L. Tegner, and Wittermark, *Anal. Biochem.* **42**, 560 (1971).

⁹ D. J. D. Nicholas and Clarke, *Anal. Biochem.* **52**, 560 (1971).

¹⁰ R. E. Stanley, *Anal. Biochem.* **39**, 441 (1971).

In this reaction, FMNH₂ reduces molecular oxygen in the presence of a long chain aldehyde whereby FMN is formed and the aldehyde is oxidized to the corresponding acid. This reaction is accompanied by the emission of light (hv). In summary, NADH molecules are converted to photons which can be measured sensitively. This system was used as a sensitive indicator reaction in the enzymatic TNT detection program. The reagents and enzymes used in this indicator reaction system are available. The enzymes are isolated from luminescent marine bacteria which can be grown in sufficient quantities by fermentation processes. The purified enzymes are stable.

3. Synopsis of the Effort on Enzymatic TNT Detection. The effort on enzymatic TNT detection involved a considerable amount of experimental work. Before going into detail, it seems appropriate to provide the reader with a broader understanding of this program. For this purpose the whole effort is presented in this section in a concise format. The full detail will be given in a later section.

To be able to apply the highly sensitive light emitting reaction discussed above in an enzymatic TNT detection scheme, it was necessary to isolate a hitherto unknown, NADH-dependent, TNT specific enzyme. The existence of such an enzyme in nature could be postulated on the basis of some reports on TNT biodegradation.¹¹⁻¹⁴ The methods used to obtain it will be described in the next paragraph.

a. TNT Reductase Isolation. To obtain such an enzyme, bacteria from soil were grown in TNT-containing media. Thus, bacteria were induced to synthesize the desired enzyme. A large number of different bacterial strains were evaluated and the best TNT enzyme producing strain selected. Using fermentation techniques, kilogram quantities of the cells of this bacterial strain were obtained which served as raw material for enzyme isolation. To extract the enzyme, the cell walls were disrupted by sonication or expansion through the orifice of a pressure cell disruptor. After cell wall removal, the nucleic acids were precipitated with protamine sulfate and the crude extract fractionated by ammonium sulfate precipitation. The fraction containing the enzymatic activity was purified using several chromatographic techniques. Best results were obtained with DEAE (diethylaminoethyl) cellulose chromatography. The fermentation and purification procedures for isolating the TNT enzyme are now well established, and no problems are expected in scaling up these procedures to produce this enzyme in large quantities. It should be stressed that there is no reason to expect that similar enzymes for other explosives or for compounds usually accompanying explosives could not be isolated and produced in an analogous fashion.

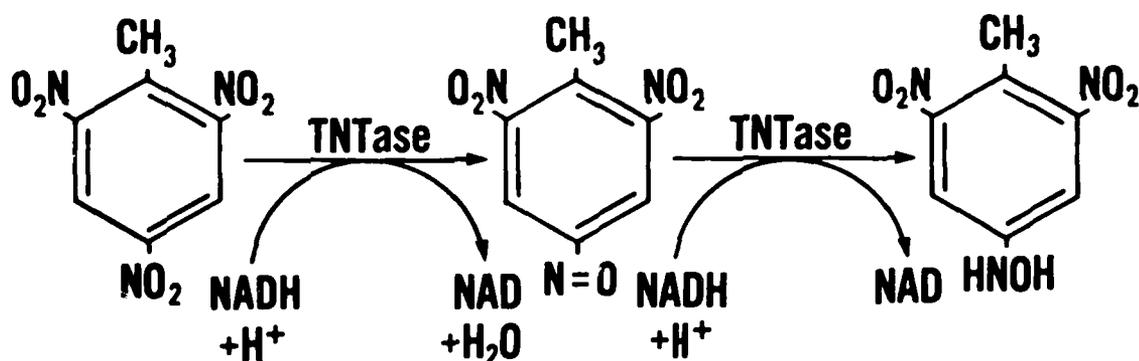
¹¹ Anon, US Army Natick Laboratories Posture Report FY73, "Microbial Transformation of α -TNT and Other Nitroaromatic Compounds," US Army Natick Laboratories, Natick, MA (1971).

¹² R. M. Enzinger, US Army Environmental Hygiene Agency Special Report No. 24-017-7071, "Special Study of the Effect of Alpha TNT on Microbial Systems and the Determination of the Biodegradability of Alpha TNT," US Army Environmental Hygiene Agency, Edgewood Arsenal, MD.

¹³ N. G. McCormick, "Microbial Degradation of Munitions Wastes," 74th Annual Meeting, Am. Soc. for Microbiol., Chicago, IL (1974).

¹⁴ J. L. Osmon and R. E. Klausmeier, *Developments in Industrial Microbiology* 14: 247-252 (1973).

b. Properties of the TNT Reductase. The TNT enzyme isolated is a TNT reductase, using NADH as cofactor; i.e., coupling of the enzymatic TNT reaction to the highly sensitive luciferase indicator reaction is possible. In the reaction catalyzed by this enzyme, two NADH molecules are consumed. For analytical purposes, this is all the information concerning the reaction mechanism which is required. Therefore, no rigorous study of the reaction mechanism was undertaken. However, all observations are compatible with the reaction mechanism proposed for TNT metabolism in animals and micro-organisms in which the para nitro group of TNT is reduced in a two-step reaction by two NADH molecules to a hydroxylamine, as shown below:¹⁵⁻¹⁷



The TNT reductase enzyme is primarily active for trinitroaromatic compounds. It has some activity for dinitrotoluene, an impurity always found in TNT. No enzymatic activity was found for mononitroaromatic compounds or any other aromatic or aliphatic compounds. The TNT reductase enzyme contains a residual NADH oxidase activity of about 0.1 percent, as measured at saturated substrate concentrations, which proved to be difficult to remove. The TNT reductase is a comparatively stable enzyme. It can be lyophilized and stored in this form for years. In solution, the enzyme could be kept at 30°C for 20 hours without a significant loss in activity.

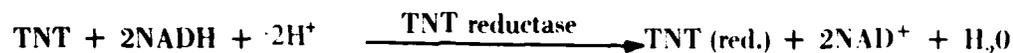
c. The Detection Concept and Feasibility Demonstration. The enzymatic TNT detection scheme is based on the chemical reactions shown below. The TNT reductase catalyzed reaction serves as sensor reaction; i.e., it provides the specificity. The light emitting indicator reaction (the bacterial luciferase reaction) provides the sensitivity. This indicator reaction appears complicated on paper, but in practice it is not. All the reagents involved in this reaction are contained in one solution and light is emitted at an intensity which is dependent on the NADH level present.

¹⁵ E. Breiding and N. Jolliffe, *J. Pharmacol. Exptl. Therap.* 88: 300-315 (1946).

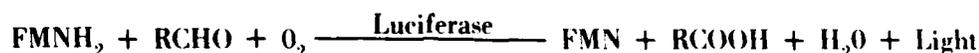
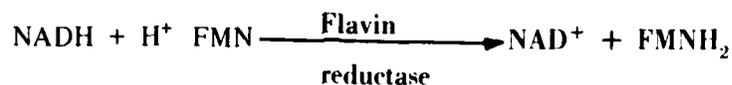
¹⁶ N. G. McCormick, E. E. Feebery, and H. S. Levinson, *Appl. and Environ. Microbiol.* 31: 949-958 (1976).

¹⁷ W. D. Wou, R. J. Heckly, D. J. Glover, and J. C. Hoffsommer, *Appl. Microbiol.* 27: 513-516 (1974).

SENSOR REACTION

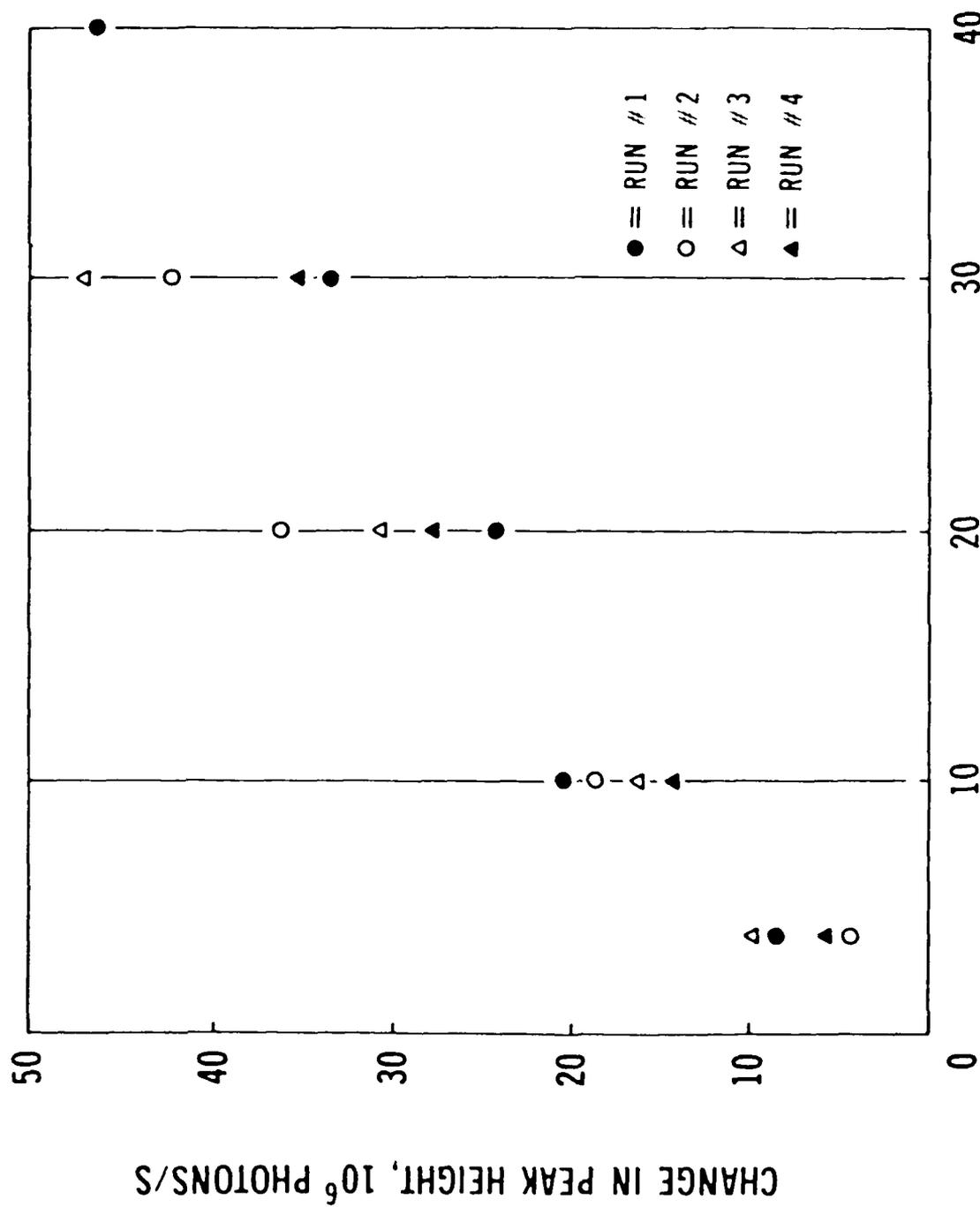


INDICATOR (LUCIFERASE) REACTION



The reagents and enzymes used in the indicator reaction are all available. FMN and its reduced form FMNH₂ is the flavin mononucleotide, a cofactor which is of considerable importance in many biochemical systems. RCHO is a long-chain aldehyde (usually tetradecanal) which is oxidized in the luciferase reaction to the corresponding acid. The luciferase and flavin reductase are obtained from marine organisms. Since the TNT specific enzyme is a reductase, NADH is consumed in the sensor reaction. Consequently, the presence of TNT is indicated by a smaller than expected light output of the indicator reaction. The reverse situation, namely an increase of light emission in the presence of TNT, would be desirable from the point of view of detection sensitivity. However, this is not possible because nature provided us with a TNT reductase.

For the feasibility demonstration of this enzymatic TNT detection scheme, a photometer was used in which the reaction vessel is positioned in front of a photomultiplier tube. The reagents could be injected without causing any light leaks. The photometer had a peak reading and integrating capability. A 10⁻⁶M TNT standard solution was prepared. Aliquots of this solution were subjected to the TNT reductase catalyzed reaction with NADH. The time necessary for the reaction is a function of the enzyme concentration. With a very high enzyme concentration, a reaction time of 15 seconds was possible but usually lower enzyme concentrations were used and longer reaction times were allowed. After termination of the TNT reductase catalyzed reaction, the whole solution was injected into the reaction vessel of the photometer containing the complete luciferase reagent solution. The luciferase indicator reaction reaches its peak light emission in about 2 seconds and is essentially completed after 10 seconds. The peak light output as well as the integrated light output were useful measures. The difference in the light output between TNT standard samples and control samples containing no TNT provided a measure for the quantity of TNT present. The light change due to the residual NADH-oxidase activity was experimentally determined and taken into account. The results are shown on Figure 1. The four runs displayed on this figure were conducted at different times within a period of half a year. The scatter of the data includes variations due to reagent activities and due to the manual injection procedures. The data displayed on Figure 1 demonstrate that TNT could be detected reliably at subpicomole quantities even with the unoptimized reaction scheme.



TNT QUANTITIES, 10^{-13} MOLES

Figure 1. Change in light emission as a function of TNT quantities.

Subsequently, the detection sensitivity was increased and 2×10^{-14} mole of TNT can now be detected. This was accomplished by working with lower NADH concentrations. On these efforts, it was observed that the luciferase reagent emits some light spontaneously independent of added NADH. All of this will be discussed in more detail in the next section.

d. Optimization Considerations. A system using a photomultiplier tube, which is otherwise perfect, will have a noise that is the square root of the photon flux.

In the enzymatic TNT detection scheme, the effect of TNT is to reduce the normal "base line" photon flux. The reduction of the photon flux, due to TNT, is the signal. The overall photon flux will be the sum of the base line photon flux, the background light emission mentioned above and the photomultiplier thermionic emission. According to the above definition the signal-to-noise ratio will be:

$$\frac{S}{N} = \frac{S}{\sqrt{B_T + B_L + B_A}} \quad (\text{Eq. 1})$$

S = change in photon flux due to the TNT.

B_T = thermionic emission (dark current) equivalent photon flux.

B_L = spontaneous light emission of the luciferase reagent, independent of NADH.

B_A = photon flux due to the NADH/luciferase/TNT reductase reaction, with no TNT present.

The above equation is correct for the "base line" photon flux. For the TNT-reduced "base line" photon flux, the signal-to-noise ratio will be

$$\frac{S}{N} = \frac{S}{\sqrt{B_T + B_L + B_A - S}} \quad (\text{Eq. 2})$$

The graphic representation of the above equation, shown in Figure 2, was obtained by solving this equation for an S/N ratio of 10. This figure illustrates the relationships between the "base line" photon flux " B_A ," the photon flux due to the spontaneous light emission of the luciferase reagent " B_L ," and the TNT induced change in photon flux " S " when a signal-to-noise ratio of 10 is demanded.

LUCIFERASE 'GLOW' EFFECT

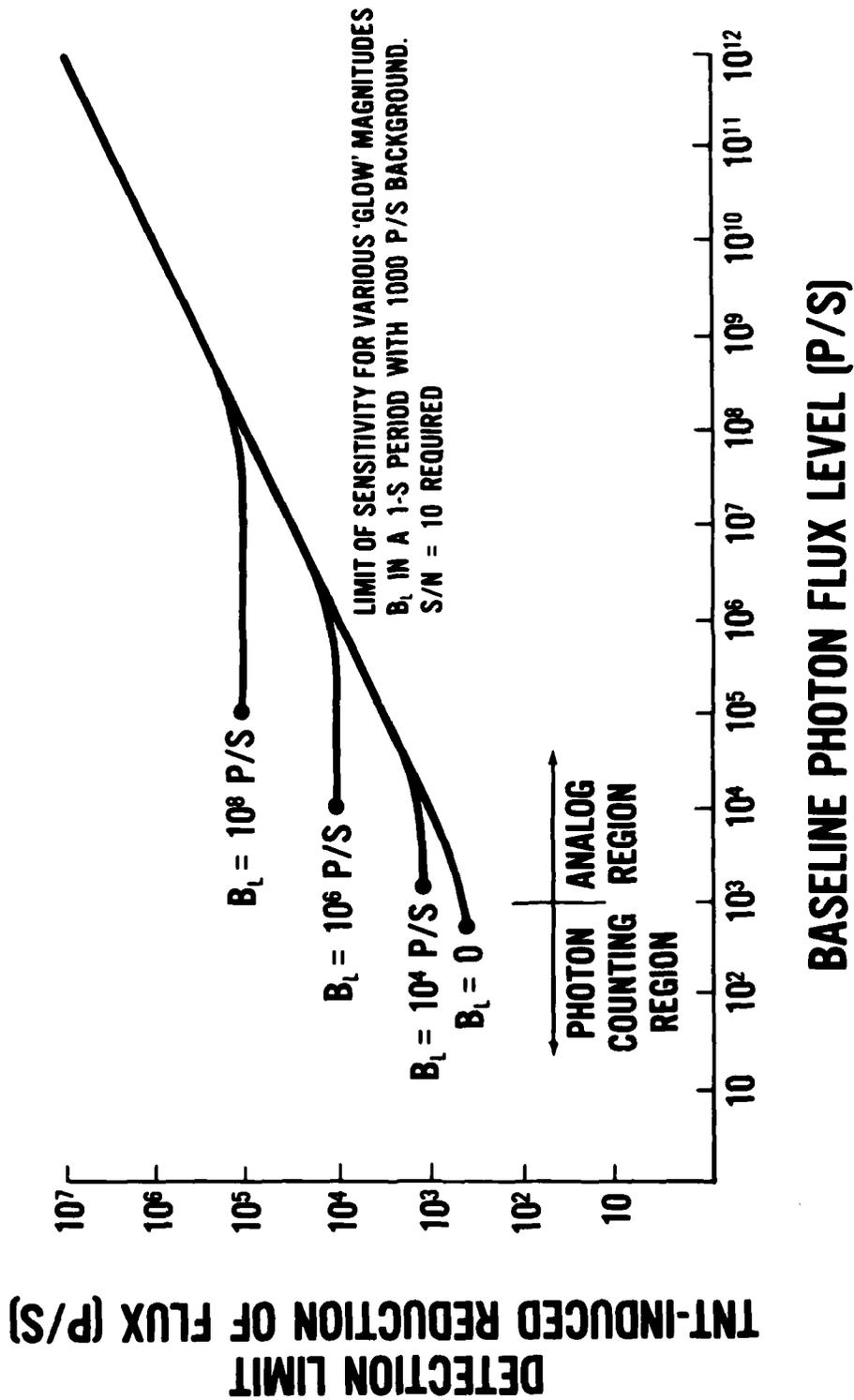


Figure 2. Relationships between baseline photon flux B_A , light background B_L , and the TNT induced reduction in photon flux for a signal-to-noise ratio of 10.

B_T was 10^9 photons per second with the best PMT tube, which is satisfactory. B_L depends on the concentration of the luciferase reagent and was usually 10^5 photons per second. B_A was in the range of 10^7 and 10^8 photons per second in the experiments with 2×10^{-6} molar NADH solutions, displayed on Figure 2. The use of lower NADH concentrations could be expected to result in a lower TNT detection limit because B_A would be smaller and thus S would become a larger portion of the "base line" photon flux; i.e., the signal-to-noise ratio would be improved. As expected with 6×10^{-7} molar NADH solutions, the detection limit was lower and 2×10^{-14} mole of TNT could be detected.

In order to optimize the "signal," that is, the TNT induced light flux reduction, the light efficiencies of the instrumentation and the reactions were evaluated. The overall light efficiency is the product of the efficiency of the PMT tube " E_{PMT} ," of the reaction cell " E_{cell} " and of the luciferase reaction " $E_{luciferase}$." E_{PMT} is about 0.2. E_{cell} was about 0.1 for the laboratory apparatus. To increase E_{cell} , a new cell was designed which is a glass spiral in which the complete indicator reaction is performed in front of the photomultiplier tube. $E_{luciferase}$ was found to be about 10^{-4} ; i.e., 10,000 NADH molecules are required to produce one photon whereas the other NADH molecules are oxidized in a dark reaction. These considerations showed that a significant gain in sensitivity would result from an increase in the light efficiency of the indicator reaction. The FMN reductase reaction was identified as being responsible for the low efficiency.

e. TNT Vapor Detection and Continuous Flow Breadboard Model. The objective of the enzymatic TNT detection effort was to develop a technique providing a mine detection capability via detecting TNT vapors emanating from such devices. For interfacing these vapors with the liquid reagent system of the enzymatic TNT detection scheme, techniques could be envisioned involving adsorption of the TNT vapors on solids and subsequent elution. A direct interfacing of the air to be tested with the liquid reagent system was, however, much more desirable. Direct interfacing could be considered because at the extremely low TNT concentrations of interest even aqueous solutions can be expected to be satisfactory solvents for TNT. In such an approach, it is necessary for obtaining high sensitivity that the air to be tested for the presence of TNT vapors is interfaced with the smallest possible volume of the liquid phase. These ideas were tested with an experimental setup in which a flowing film of the NADH/buffer solution was exposed to a stream of TNT saturated air. These tests indicated an efficiency of 35 percent in the transfer of TNT from the gas phase into the liquid phase using 1.25×10^{-6} torr as the TNT vapor pressure value in the calculations. More advanced air/liquid interface systems will be discussed later.

When planning the design of a chemical analysis or detection system, the question arises whether a batch system dealing with one sample at a time or a continuous flow system is preferable. An automated batch system could certainly be built which mimicks the steps of the laboratory procedure used in the feasibility demonstration of the enzymatic TNT detection scheme. Such a system would be sensitive but it would be complex and expensive because of the requirement of mechanical manipulation of precise aliquots of several reagents and the need for precise timing. Considerably simpler are continuous flow systems which have fewer moving parts and which are usually more reliable. A breadboard model of a continuous flow system for enzymatic TNT detection was built. It had an improved air/liquid interface system as well as a spiral shaped reaction cell positioned closely in front of the photomultiplier window. The side of this reaction cell not facing the photomultiplier window was aluminized for maximum light efficiency of the cell.

4. Discussion of the Experimental Work. In Section II, paragraph 3, an overview of the enzymatic TNT detection scheme was presented. For the sake of conciseness many phases of this effort were only briefly outlined. More detail will be given in this chapter. The enzymatic TNT detection concept is based on a TNT transforming enzyme whose existence could be postulated but which has never been isolated prior to this effort. In order to obtain this enzyme, bacteria were induced to synthesize this enzyme. This was done by growing bacteria in the presence of TNT and isolating the enzyme formed in the bacterial cells. To increase the probability of finding bacterial strains which produce a TNT transforming enzyme soils were chosen as source of the bacteria which had been exposed to TNT for years.

a. Microbial Strain Selection. Soil samples were obtained from several Army and Navy installations where TNT is produced or used. These soil samples as well as soil from the MERADCOM Test Area where a piece of TNT had been buried for several years served as sources of bacteria. Microbial isolates were obtained by direct plating of these soils on agar-filled petri dishes and by application of liquid enrichment techniques. A great variety of organisms were found to be in these soil samples but the use of TNT in the plating media or liquid enrichment media narrowed the populations isolated exclusively to gram-negative, motile, bacterial rods. Although most of these isolates shared the same cell morphology, many different colony types (i.e., different strains) were observed; 489 strains were isolated and evaluated by direct plating, and 275 with liquid enrichment techniques.

An important phase of these efforts was the development of screening techniques permitting the determination which isolates are most effective in metabolizing TNT. Quite attractive for this purpose appeared the reaction of TNT with alkali, allowing the screening of bacterial colonies on the plates.¹⁴ With this technique, the majority of the strains evaluated showed TNT degrading capabilities and it became obvious that a more discriminating technique was required. Several colorimetric procedures for TNT quantitation were investigated, but they suffered from media interference.¹⁸⁻¹⁹ A definitive classification of the bacterial strains according to their TNT degrading capabilities was accomplished with gas chromatography (G.C.). A Hewlett-Packard Model No. 7620 with a column containing 10 percent Silicone GE SE-30 on a 80-100 mesh Gas-Chrom S support was used. With this technique, the reduction of the TNT concentration by the bacteria was measured. For the identification of the products of the TNT biodegradation, a thin layer chromatographic technique was used. The system selected employed a non-polar solvent and a silica gel support with ultraviolet light and ethylene diamine used for visualizing.¹⁷⁻²⁰

In the first phase of the bacterial strain selection effort, four media formulations and three substrate concentrations were studied. These were very rich complex, rich complex, synthetic with glucose, and synthetic, all containing high (1000 p/m), intermediate (500 p/m), or low (100 p/m) levels of TNT. The results of these investigations indicated that rich complex enrichment media with high TNT concentrations were most effective in terms of the number and variety of organisms isolated. In the work with synthetic media, it was found that the viability of the bacteria was affected by the TNT and DNT. The resulting limited population growth led to a decrease in the rate and the extent of TNT degradation. The viability problem was circumvented by the development of a two-step, resting cell procedure. It consisted of growing large numbers of cells in rich complex media not containing any TNT, followed by washing the cells and placing them for one or two days in fresh synthetic media containing TNT. The substrate (TNT) remaining after two days was determined by G.C. for each strain studied. Nineteen percent of all strains depleted essentially all TNT and DNT, 33 percent depleted substantial amounts of these substrates, and 48 percent depleted little or none. As a major product of TNT degradation, 2,2',6,6'-tetranitro-4,4'-azoxytoluene was identified. Also found were 4-hydroxylamino-2,6-DNT; 2,6-diamino-4-nitrotoluene; 4-amino-2,6-DNT; 2-amino-4,6-DNT; and 2,2' 4,4'-tetranitro-6,6'-azoxytoluene. The formation of the azoxy compounds is postulated to result from the condensation of a nitroso and a hydroxylamino-TNT derivative.²¹⁻²²

¹⁴ J. L. Osmon and R. E. Klausmeier, *Developments in Industrial Microbiology* 14: 247-252 (1973).

¹⁷ W. D. Won, R. J. Heckly, D. J. Glover, and J. C. Hoff-somer, *Appl. Microbiol.* 27: 513-516 (1974).

¹⁸ J. M. Haines, 1955, Mason & Hanger-Silas Mason Co., Inc., "TNT Waste Water Disposal from Shell-loading Plants," Progress Report No. 1, "The Analysis of TNT in Water: Survey of Technical Literature," Mason & Hanger-Silas Mason Co., Inc., Iowa Ordnance Plant, Burlington, IA.

¹⁹ R. E. Klausmeier, J. L. Osmon, and D. R. Walls, *Dev. Ind. Microbiol.* 15: 309-317 (1974).

²⁰ C. D. Chandler, J. A. Kohlbeck, and W. T. Bolleter, *J. Chromat.* 61: 123 (1972).

²¹ B. B. Westfall, *J. Pharmacol. Exptl. Therap.* 78: 386 (1943).

²² S. Yamashima, *Bell Chem. Soc., Japan*, 27: 85 (1959).

The work discussed in this section led to the selection of 25 most promising bacterial strains. A strain, designated 96-3, isolated from Fort Belvoir soil, was chosen as a model organism because of its excellent growth and TNT degradation characteristics and its relatively high resistance against the toxic effects of TNT. Cells of this model strain were disrupted by sonification and the cell free extract tested for its enzymatic activity. It was found that the cell free extract contained an enzyme requiring NADH as a cofactor and catalyzing a reaction in which the para nitro group of TNT is reduced whereby two NADH molecules are oxidized. The extracts of three other strains (105-12, 130-3, and 190-6) which were morphologically similar to the 96-3 strain also showed significant TNT reductase activity. Some other strains provided extracts with little TNT reductase activity and the extracts of the remaining strains showed no activity. Since all 25 strains investigated had excellent TNT transforming capabilities in the live form, the lack of enzyme activity was attributed to the extraction procedure. The crude enzymes obtained from strains 96-3, 105-12, 130-3, 190-6 were tested for their specificity to establish an additional basis for selecting a best strain. All four enzymes were active for trinitro substituted derivatives of toluene, benzene, benzoic acid, and benzaldehyde. Activity for dinitro derivatives of the above aromatic compounds was comparatively low and there was no activity found for ortho, meta, and para mononitrotoluene. No activity was observed for compounds such as nitro methane, sodium nitrite, 3,4 dihydroxybenzoate, 2,4 dichlorotoluene, and benzyl alcohol. Since the specificities of these four crude enzymes did not differ significantly, no additional selection criterium was found and the strain 96-3 was chosen as the best strain.

b. Fermentation Optimization. Paragraph 4a describes the experimental approach used to select the bacterial strain 96-3 as the source for the TNT reductase. This section details the work which was required to establish the best conditions for growth of this micro-organism to provide enough enzyme for the purification work and for future needs.

The studies described here were conducted with 30-liter fermentors. In later phases of the work, 100-liter fermentors were used. First the best aeration rates were established. The growth rate increased slightly with increasing aeration rate, but since a practical limit of aeration in large fermentors is 1 volume of air per volume culture per minute, this level was used for all subsequent experiments. Fermentations were conducted at pH 6.6, pH 6.9, and pH 7.4. Although the same maximum growth was found at all three pH levels, the rate of growth was best at pH 6.6, and this pH value was chosen for future work.

TNT reductase synthesis by strains 96-3 was induced by cultivation in the presence of TNT. In the absence of TNT from the growth medium very little TNT reductase was synthesized. The TNT added disappeared rapidly. Consequently, it was necessary to add TNT throughout the fermentation to obtain optimal TNT reductase synthesis. The TNT level was maintained by adding intermittently or continuously solutions of TNT in acetone to the fermentation medium. Experiments in which the TNT level was held at 100 ppm and at 25 ppm indicated that more TNT reductase is obtained at the higher TNT level. The TNT level did not affect the amount of NADH oxidase produced, an enzymatic activity which was found to accompany the TNT reductase. Consequently the maintenance of the TNT level at 100 ppm resulted in a better ratio of TNT reductase to NADH oxidase. Experiments with a continuous feed of 0.2 g and 0.5 g TNT/l fermentation/hour showed that the higher TNT level depressed the per liter yield of TNT reductase because of reduced viability. The 0.05 g/l/h rate was found to be superior to all other rates studied and was chosen as optimal. Since TNT inhibits the growth, an attempt was made to enhance the TNT reductase yield by delaying the exposure of the growing cells to TNT. In this way, the cells would be cultured first in the absence of TNT to maximize cell growth and then in the presence of TNT to induce TNT reductase synthesis. Cells exposed to TNT late grew much faster, but the cells exposed to TNT early produced significantly more TNT reductase and, therefore, the two-phase culture method was not advantageous.

Flask culture experiments were undertaken to identify optimal nutrient parameters for enzyme production. A fermentation medium containing one percent autolyzed yeast extract and glucose was found to be superior to media with higher concentrations of these nutrients. Surfactants, often found to be beneficial in fermentation media containing sparingly soluble nutrients, did not improve enzyme yields. For this purpose, the surfactants Tween 20 and Tween 80 were tested in concentrations up to 0.20 percent. The best buffer and trace salt concentrations were also determined. While differences with different salt combinations were not significant, the trace salt concentrations listed in Table 1 appeared to give best results. As inorganic nitrogen source ammonium nitrate was best in combination with glucose. In combination with sucrose, more NADH oxidase was formed. Trinitrobenzoate was found to be a suitable TNT substitute for inducing the synthesis of TNT reductase.

Table 1 shows the optimal media for production of TNT reductase by bacterial strain 96-3. Other optimal conditions are: A TNT feed rate to the fermentor from the time of inoculation of 0.05 g TNT/liter fermentation/hour (acetone solution containing 5 g TNT/liter). If necessary, F-1 emulsion (Hodag Chemical Co.) can be added as antifoam agent. The pH is maintained throughout the fermentation by addition of KOH or H_3PO_3 as needed. Physical parameters for 100-liter fermentors, include an aeration rate at 1 volume air/volume fermentation volume/minute, an agitation rate of 60 r/min and a temperature of 32°C.

Table 1. Optimal Media for Production of TNT Reductase by Bacterial Strain 96-3

Component	Concentration (Percent Weight per Volume)
Autolyzed Yeast Extract	1
Glucose	1
Ammonium Nitrate	0.3
Magnesium Sulfate	0.2
Dibasic Potassium Phosphate (Trihydrate)	0.25
Potassium Chloride	0.025
Trace Salts	1 ml/l of a Solution Containing: (Percent Weight per Volume)
Ferrous Sulfate Heptahydrate	0.12
Zinc Sulfate Heptahydrate	0.12
Manganous Chloride Tetrahydrate	0.104
Sodium Chloride	0.5

During the work with 100-liter fermentors, a bacteriophage infection became apparent which had not been observed in the prior work performed with flasks. The bacteriophage problem manifested itself by characteristic declines in the growth rate and a concurrent loss in cellular TNT reductase activity. All observations indicated that there is a lysogenic relationship between the bacteriophage virus and the 96-3 bacterial strain. That is, the virus exists normally in symbiosis with the bacterial host and enters a lytic cycle only when the bacteria are exposed to certain stresses. Apparently, the conditions in the fermentors provided the stress to initiate the lytic cycle.

The first approach taken to solve the bacteriophage problem was an attempt to produce "cured" bacteria. For this purpose, the cells were exposed to a combination of mitomycin C (2 mg/ml) and acridine orange (20 mg/ml). Mitomycin C is an agent which induces the lytic cycle when a symbiotic virus host relationship exists. It promotes viral DNA replication independent of host DNA replication. Acridine orange, a potent mutagen, then causes damage to the viral genetic material. The damaged virus DNA is then lost through dilution among the progeny of bacterial cell division, giving rise to virus-free cells. After this treatment, the survivors were exposed again to mitomycin C. Lysis was found again to take place indicating that a "curing" had not been accomplished. Survivors of the mitomycin C treatment grew slower and produced very little TNT reductase. Alternate strains such as the strain 130-3 were investigated, and it was determined that they, too, carried the bacteriophage infection. The bacteriophage problem was finally circumvented by using mild fermentation conditions which do not provide the stress leading to the initiation of the lytic cycle. These are the conditions listed above for work with 100-liter fermentors. Subsequently, many successful fermentation runs were performed with 100-liter fermentors.

c. Enzyme Isolation and Purification. The first step in extraction of enzymes from microbial cells is the disruption of the cell walls. This may be accomplished in a variety of ways, including lysis effected by solvents, shaking with glass beads, or subjecting the cells to ultrasound or to high shear stress. The last method entails passing a cell suspension through a small orifice under high pressure. The performance of all these techniques for extracting the TNT reductase from the bacterial strain 96-3 were investigated. In these studies, the cells were suspended in 0.025 M potassium phosphate buffer, pH 7.0, and mercaptoethanol (1 m M) was present as stabilizing agent. Lysis, using toluene as solvent, resulted in the smallest release of enzymes from the cells. Shaking with glass beads in small tubes by a vortex mixer for 5 minutes provided the highest enzyme yield. However, this method was considered unsuitable for scaleup; therefore, sonication as well as expansion of cell suspensions through the orifice of a pressure cell disruptor were preferred. In the case of sonication, 3 minutes was sufficient to release all the enzyme obtainable by this method. Sonication beyond 7 minutes reduced the enzyme yield.

In enzyme purification, it is useful to remove the nucleic acids before conducting protein separation work. For this purpose, precipitation techniques were selected. The precipitation agents tested included manganous chloride, streptomycin sulfate, acridine orange, and protamine sulfate. The performances of these precipitation agents in reducing the nucleic acid content of the cell extracts were evaluated spectrophotometrically by comparing the absorptions of the supernatants at 280 nm and 260 nm. Manganous chloride was ineffective, streptomycin sulfate showed intermediate performance, and protamine sulfate was best.

As the first step in protein separation, ammonium sulfate precipitation was selected. Ninety-two percent of the TNT reductase of the crude extract was found to precipitate in the range between 35 percent and 60 percent ammonium sulfate saturation. Accordingly, ammonium sulfate was added to give a 35 percent saturation and the precipitated, unwanted proteins were removed by centrifugation. After the increase of the ammonium sulfate concentration to 60 percent, the TNT reductase containing precipitate was obtained and redissolved in 0.02-M potassium phosphate buffer, pH 6.5. With the protamine sulfate treated cell extracts, some of the NADH oxidase activity precipitated at a lower ammonium sulfate concentration than the TNT reductase activity. These two initial purification steps reduced the NADH oxidase activity from 38 percent of the TNT reductase activity found in the crude extract, to 2.7 percent. Subsequently, the enzyme preparation was dialyzed over night versus 30 volumes of 0.02 M potassium phosphate buffer and further purified by ion exchange chromatography with DEAE (diethylamino ethyl) cellulose. A linear gradient elution was used with 0.05 M potassium phosphate buffer initially, and the same buffer with 0.35 M potassium chloride added as the final eluent. The enzyme obtained had a specific activity of 2.68 U/mg and a NADH oxidase contamination level of 0.13 percent as determined near saturated substrate concentration. The purification data are summarized in Table 2.

Table 2. Summary of TNT Reductase Purification

Step	Specific Activity (IU/mg)	NADH Oxidase Activity (% of TNTase)
Crude Extract	0.07	38.3
Post Protamine Sulfate	0.58	2.2
Post Ammonium Sulfate	0.99	0.6
Post Dialysis	1.16	0.5
Post DEAE-Cellulose	2.68	0.13

The performance of other chromatographic columns for TNT reductase purification including CM (carboxymethyl)-cellulose and alumina C γ were tested but best results were obtained with DEAE-cellulose. An attempt was also made to apply affinity chromatography for additional TNT reductase purification. For this purpose, agarose beads were activated with cyanogenbromide as first described by Axen, et al.²³ and reacted with hexamethyldiamine (HMD). The agarose-HMD was then reacted with trinitrobenzenesulfonate. The performance of affinity chromatographic columns prepared in this manner was erratic. Their effectiveness was high at times but the recovery of the TNT reductase was inconsistent.

d. Properties of the Purified TNT Reductase. The purified enzyme was tested for stability. At 2°C to 5°C in 0.05 M potassium phosphate buffer and at a total protein concentration of 0.5 mg/ml, full activity was retained for a week. In thermal denaturation experiments, conducted at a later time, 20 hours at 30°C reduced the activity of the enzyme from 18.0 U/ml to 12.3 U/ml.

To characterize the TNT reductase, its properties were investigated spectrophotometrically, making use of the absorption of NADH at 340 nm. With this technique, the effects of the substrate concentrations on the TNT reductase reaction velocity were determined. Using Lineweaver Burk plots, these data provided the Michaelis-Menten constants (K_m) of the enzymes involved. K_m is the dissociation constant for the reversible reaction $E + S \rightleftharpoons ES$ in which E stands for enzyme and S for substrate. K_m is numerically equal to the substrate concentration providing one-half of the maximum (limiting) reaction velocity. The Michaelis-Menten constant of the TNT reductase was found to be 1.9×10^{-5} M for TNT and 1.6×10^{-4} M for DNT. These data illustrate the preference of the enzyme for trinitroaromatic compounds and indicate that at very low substrate concentrations where TNT is still observable, DNT would not be observed. The Michaelis-Menten constant of the TNT reductase for NADH as substrate is 3.5×10^{-5} M. These measurements were made with 0.05 M potassium phosphate buffer at pH 7, at a reaction temperature of 30°C and an enzyme concentration of 0.0055 U/ml. For the determination of the K_m for TNT and DNT, the NADH concentration was 10^{-4} M, for the determination of K_m for NADH the TNT concentration was 4.4×10^{-5} M. The Michaelis-Menten constant for the NADH oxidase impurity enzyme was determined to be 1×10^{-5} M.

In the course of these kinetic studies observations were made indicating that the TNT reductase is inhibited by NADH. With 10^{-4} M NADH 37 percent of the TNT reductase activity was lost in 1 minute and 77 percent in 3 minutes. The extent of inhibition was similar with 10^{-5} and 10^{-6} M NADH solutions but with 10^{-7} M solutions only a 20 percent inhibition occurred in the time span of 3 minutes. These tests were also conducted with solutions containing EDTA (ethylenediamine-tetra-acetic acid), dithiothreitol, mercaptoethanol, and catalase to determine if such agents could exert a protective effect on the enzyme. These agents did not protect the enzyme, but TNT does. In the presence of TNT, the NADH caused inhibition of the TNT reductase is not observed.

²³ R. Axen, J. Porath, and S. Ernback. *Nature*, 214, 1302 (1967).

The specificity of the purified TNT reductase was determined using the spectrophotometric assay technique. The results are shown in Table 3. TNT as well as the other compounds were tested at 4.4×10^{-5} M concentration. The unreactive compounds were also tested at 4.4×10^{-4} M concentration. Essentially the same specificity pattern was found as with the crude TNT reductase, That is, the TNT reductase is primarily active for trinitroaromatic compounds, has some activity for dinitroaromatics, and no activity for mononitroaromatic or other compounds.

Table 3. Substrate Specificity of Purified TNT Reductase

Substrate	Relative Activity (% of TNT at 4.4×10^{-5} M)	
	4.4×10^{-5} M Substrate	4.4×10^{-4} M Substrate
TNT	100	
Trinitrobenzene	96	—
Trinitrobenzoic Acid	76	—
Trinitrobenzaldehyde	87	
1-Chloro-2,4-Dinitrobenzene	29	
Benzylalcohol	0	0
Metanitrotoluene	0	0
Orthonitrotoluene	0	0
Paranitrotoluene	0	0
Dichlorotoluene	0	0
Nitromethane	0	0
DNT	0	14

Gel filtration chromatography and acrylamide gel electrophoresis indicated that the molecular weight of the TNT reductase is approximately 100,000 daltons. The TNT reductase activity peaks at pH 7, but this peak is broad and the activities are not much smaller at pH 6 and 8. At pH 5 and 9, the activities are considerably smaller. The activity of the NADH oxidase increases in the range from pH 5 to pH 9. However this difference in the pH dependances of the TNT reductase and NADH oxidase activities is not large enough to be useful for suppressing the NADH oxidase activity.

Attempts were made to immobilize the TNT reductase on solid supports. An immobilized TNT reductase was of considerable interest because in this form the enzyme could fulfill two functions in a TNT vapor detector. It could serve as the catalyst and thus facilitate the TNT specific sensor reaction and it could also function as the adsorbent for the TNT vapor molecules. Assuming a turnover number of the TNT reductase of 10,000, a conservative estimate, one can calculate that one unit of the enzyme contains 10^{10} moles of enzyme. Assuming that there is one active site per enzyme molecule, it follows that 1 unit represents 10^{13} active sites; i.e., TNT molecule adsorption sites. These considerations indicated that the immobilized enzyme could provide adequate adsorption capacity.

Agarose beads were selected as the support material. Agarose from Pharmacia Fine Chemicals (Sephacrose 4B) was activated with cyanogen bromide.²³ The activated, washed, agarose beads were mixed with 25 ml buffer and 10 ml of TNT reductase containing 10.3 U/ml and stirred overnight at 4°C. These procedures were conducted at pH 7.5 and pH 8. Although the enzyme is stable under these conditions and all activity had disappeared from the solution, the activity of the agarose beads was only 0.6 U/ml beads. This yield of immobilized TNT reductase was considered to be too small.

A second immobilization attempt was made with a commercially available activated agarose preparation (Affi-Gel 10 from Biorad Laboratories) which is an N-hydroxysuccinimide ester of a succinylated aminoalkyl agarose. This reagent usually couples with protein amino groups. No immobilization was accomplished with the TNT reductase.

e. The Luciferase Reagent. The luciferase enzyme complex containing both the FMN reductase and luciferase enzymes is derived from marine bacteria. The light yielding reactions catalyzed by these enzymes were discussed above. These reactions can serve as highly sensitive indicator reactions for NADH or all compounds and processes affecting NADH concentrations. Light is emitted in direct proportion to the quantity of NADH present.

²³ R. Aven, J. Porath, and S. Ernback, *Nature*, **211**, 1302 (1967).

Before formulation for luciferase reagent use, the enzyme complex contained 0.6 units of FMN reductase and 50,000 units of luciferase per milligram protein. The luciferase reagent normally contained 0.05 unit/ml FMN reductase, 7500 units/ml luciferase, 2.5×10^{-4} M FMN, and $10 \mu\text{l/ml}$ of a saturated alcoholic solution of tetradecanal, all dissolved in 0.2 M potassium phosphate buffer, pH 7. The response of this reagent to NADH was studied thoroughly. The results are shown in Figures 3 and 4. The experiments displayed on these figures show that the light response of the luciferase reagent is linear over a wide range of NADH quantities. The slopes of the curves on these two figures differ somewhat because different samples of luciferase were used which differed in activity.

Of interest with respect to the sensitivity to be obtained, was the efficiency of the luciferase reaction in terms of light emitted per NADH molecule. A number of experiments were conducted to determine the parameters limiting the light efficiency. First the light yield for a fixed quantity of NADH as a function of luciferase reagent concentration was determined. Considerable increases in light yields were found to be possible by increasing the luciferase reagent concentration. Experiments were also conducted to determine if any of the components of the luciferase reagent were limiting the light yield. The tetradecanal concentration could be reduced 2000 fold before a reduction in the light yield became noticeable. No light was obtained in the absence of FMN. With 1×10^{-5} M FMN present, one half of the maximal light yield was observed. Increasing the FMN concentration beyond 2.5×10^{-4} M did not increase the light yield. These experiments showed that the higher light yield at increased luciferase reagent concentrations is due to the increased enzyme concentration.

f. TNT Assay With the TNT Reductase/Luciferase System (Feasibility Demonstration). The chemical equations representing the enzymatic TNT detection scheme were shown in Section II, paragraph 3c. The TNT reductase catalyzes the specificity providing sensor reaction. The enzymes of the luciferase reagent catalyze the sensitivity providing, light emitting, indicator reaction. The feasibility of this scheme was demonstrated using the techniques discussed below. The photometer used allowed the injection of the reagents through a light-tight port into a reaction vessel positioned in front of a photomultiplier tube. The instrument has peak height and integrating capability. The experiments were conducted in the following way: A TNT reductase reaction mixture was prepared by adding approximately 0.22 unit of the TNT reductase to 1 ml of 0.05 M potassium phosphate buffer, pH 7. Immediately before use, a $20 \mu\text{l}$ aliquot of 10^{-4} M NADH was added, making the NADH concentration 2×10^{-6} M. A TNT standard solution was prepared by dissolving 1 mg of TNT in 1 ml of acetone and diluting with distilled water to give a 10^{-6} M TNT solution. $50 \mu\text{l}$ of the TNT reductase reaction mixture were added to aliquots of the TNT standard solution and allowed to react for 5 minutes. The reaction was stopped by adding $50 \mu\text{l}$ of 0.3 N sodium hydroxide; $20 \mu\text{l}$ of this stabilized reaction mix was injected into 1 ml of the luciferase reagent in the photometer reaction vessel and the resultant peak light emission was recorded. In addition to the TNT sample, two control samples not containing TNT were processed. One of these controls was allowed to react with the TNT reductase reagent for 5 minutes as were

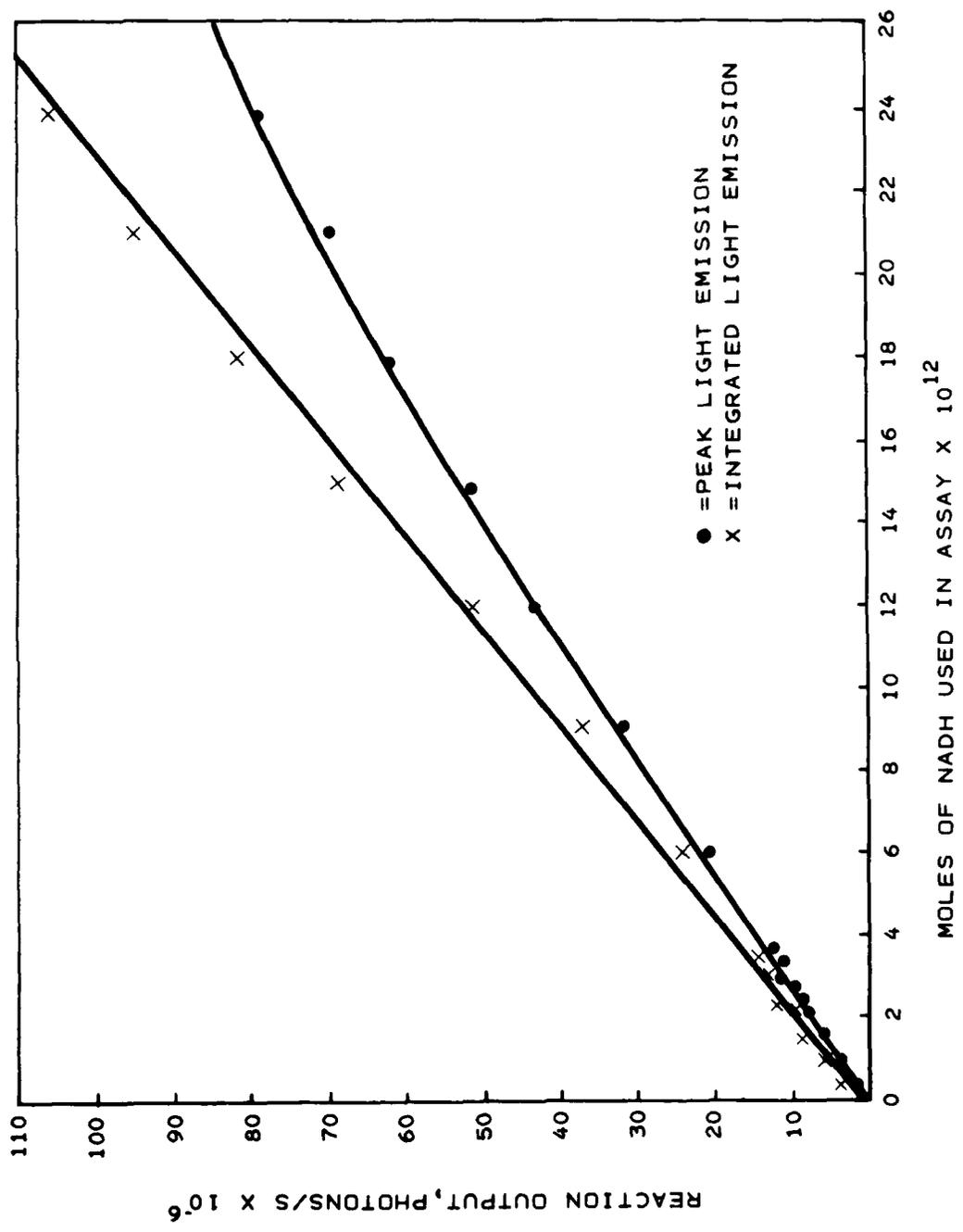


Figure 3. Light emission as a function of NADH levels.

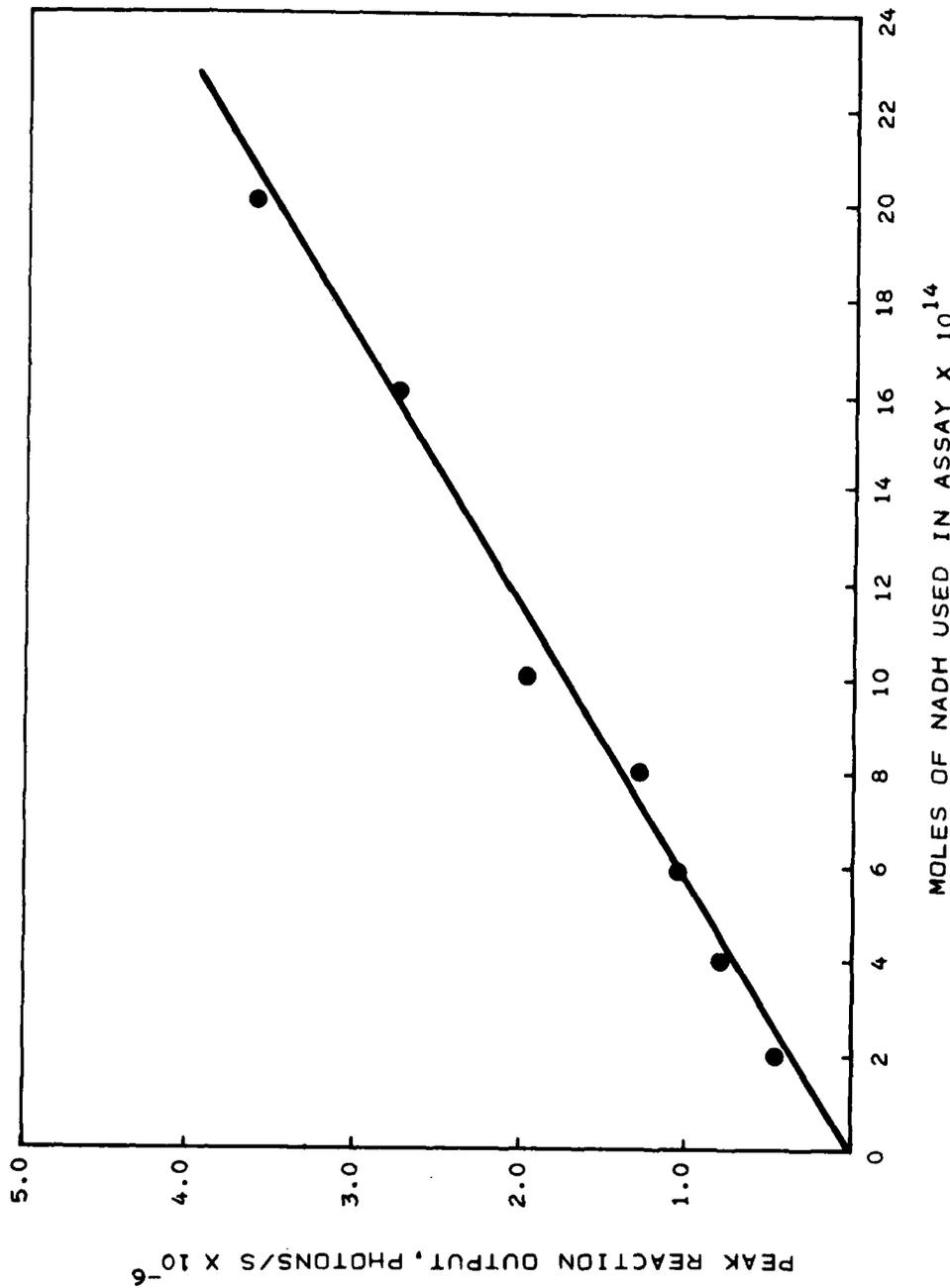


Figure 4. Light emission as a function of low NADH levels.

the TNT samples; the other was not. The NADH oxidase effect was characterized as the difference in light emission of the TNT reductase exposed and unexposed controls, while the TNT content was characterized as the difference between TNT reductase exposed controls and the TNT samples. The results are shown on Figure 1; 5×10^{-13} mole of TNT could be reliably detected under these operating conditions. These results were reproducible. In fact, the data of Figure 1 were collected over a 6-month period with different reagent and enzyme batches. The 5-minute reaction time was chosen for convenience. The reaction times are much shorter at higher enzyme concentrations. In one experiment, at a very high enzyme concentration, only a 15-second reaction time was required for completion of the TNT reductase reaction.

As discussed briefly before, a higher detection sensitivity can be expected when the NADH concentration is lower. As expected, a lower detection limit was obtained with 6×10^{-7} M NADH solutions. Injecting $20 \mu\text{l}$ of a 1 ml reaction mix initially being 6×10^{-7} M in NADH and 1×10^{-9} M in TNT and using 0.1 U/ml of TNT reductase, 2×10^{-14} mole of TNT could be detected. The success of these experiments led to work with 10^{-8} M NADH solutions. Unfortunately, it was found that in this concentration range the intensity of light emission is not linearly related to the NADH concentration. This behavior can be understood on the basis of the Michaelis-Menten theory applied to a situation where the substrate concentration is significantly smaller than the Michaelis-Menten constant.

In the work at low NADH concentrations, it was observed that the luciferase reagent emitted some light spontaneously. This background could be compensated by adjusting the zero calibration of the luminescence photometer, but nonetheless represented a noise producing component, as will be discussed in paragraph g.

Another observation made in the work with very low NADH concentrations was that the injection of the 0.3 N NaOH solution, used to terminate the reaction, caused a spurious light emission. This was easily corrected by eliminating this sodium hydroxide injection and terminating the reaction by injection into the luciferase reagent after accurately timed intervals. Figure 5 shows the decrease in light output due to the TNT reductase and NADH oxidase reaction as a function of reaction time. In the experiment displayed in this figure, 0.1 U/ml TNT reductase was used. The NADH concentration was 2×10^{-7} M, the TNT concentration 6×10^{-9} M. As Figure 5 shows, a 1-minute reaction time is sufficient to obtain the maximum difference between the sample and the control. Accordingly, 1-minute reaction times were used in all future work.

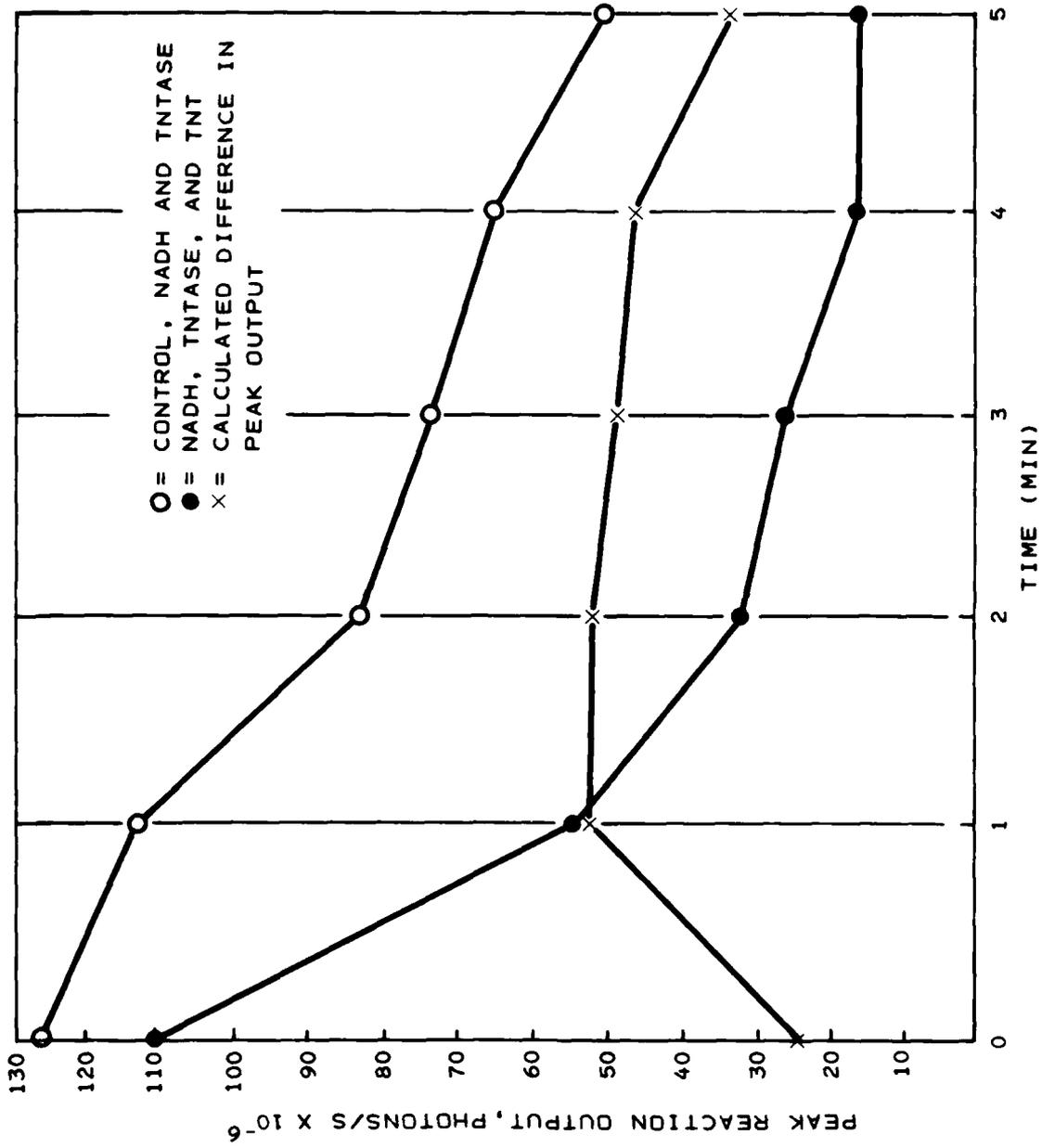


Figure 5. Change in light emission due to the TNT reductase and NADH oxidase activities as a function of time.

g. Systems Evaluation and Optimization Considerations. A system using a photomultiplier tube, which is otherwise perfect, will have a noise that is the square root of the photon flux. In the enzymatic TNT detection scheme, the effect of TNT is to reduce the normal "base line" photon flux. The reduction of the photon flux, due to TNT, is the signal. The overall photon flux will be the sum of the base line photon flux, the background light emission mentioned above, and the photomultiplier thermionic emission equivalent photon flux. According to the above definition, the signal-to-noise ratio will be:

$$\frac{S}{N} = \frac{S}{\sqrt{B_T + B_L + B_A}}$$

S = change in photon flux due to the TNT.

B_T = thermionic emission (dark current) equivalent photon flux.

B_L = spontaneous light emission of the luciferase reagent, independent of NADH.

B_A = photon flux due to the NADH/luciferase/TNT reductase reaction, with no TNT present.

The above equation is correct for the "base line" photon flux. For the TNT reduced "base line" photon flux, the signal-to-noise ratio will be:

$$\frac{S}{N} = \frac{S}{\sqrt{B_T + B_L + B_A - S}}$$

The graphic representation of the above equation, shown in Figure 2, was obtained by solving this equation for an S/N ratio of 10. This figure illustrates the relationships between the "base line" photon flux " B_A ," the photon flux due to the spontaneous light emission of the luciferase reagent " B_L ," and the TNT induced change in photon flux " S " when a signal-to-noise ratio of 10 is demanded.

As a consequence of the fact that the luciferase solution emits some light prior to the addition of NADH, there is a limitation on sensitivity which is set by the magnitude of this light background. Figure 2 is a plot emphasizing this effect. The ordinate and abscissa of this curve are in terms of detected photons (photoelectrons) at the output of the photomultiplier tube. The baseline photon flux level, the abscissa, is the photon flux, B_A , due to the NADH/luciferase/TNT reductase detection reaction. It is assumed that $B_T = 1000$ photons/second (p/s) and that a signal-to-noise ratio of 10 is required. The ordinate in Figure 2 is the TNT induced reduction in photon flux required to provide a signal-to-noise ratio of 10. B_L , the

photon count from the luciferase light background is used as a parameter to generate the family of curves. If the luciferase concentration is sufficiently low, making the background negligible, the limit of sensitivity occurs at very low NADH baseline photon flux levels, B_{λ} . In this case, B_{λ} can go as low as 10^3 photons per second (p/s), which equals the assumed thermionic emission, B_T . The photon flux change required for a signal-to-noise ratio of 10, is then $10(2 \times 10^3)^{1/2} = 450$ p/s.

At the baseline levels below approximately 10^6 p/s the system is inherently nonlinear because the signal produced by the TNT is an appreciable fraction of the total baseline level (Equation 7). For the lowest detection limits, therefore, doubling the amount of TNT does not double the system output signal. To determine whether TNT is present or not with ultimate sensitivity, one must measure dark noise, background light emission magnitude, baseline signal, and change in baseline signal as a result of the TNT. These four measurements would be used in solving a second-order equation to make the decision whether TNT is present or not. At levels between 10^4 and 10^6 p/s in the baseline, the nonlinearity could probably be adequately approximated with a logarithmic amplifier. Operation at baseline levels below 10^4 would probably require a microprocessor to achieve optimum sensitivity.

There are two general reasons for the use of photon-counting electronics instead of analog electronics. One is to obtain very high sensitivity and is applicable when the photon rates are less than about 1000 p/s. Relatively inexpensive analog electrometers are sensitive to approximately this level. The measurements made in the work on the enzymatic TNT detection scheme are in the photon rate range of 10^4 to 10^8 p/s. Thus, there is no inherent sensitivity advantage to photon counting electronics and present analog instrumentation is adequate. The other advantage of photon counting is system linearity and the ability to discriminate small changes in large signals. However, in the enzymatic TNT detection system, the best sensitivity is in the photon rate range below 10^6 p/s and the signal is an appreciable fraction of the baseline light levels. Measurements on the laboratory photometer showed that the analog electronics used is photon noise limited down to the dark noise of the photo tube; i.e., a factor of 2 increase in light causes a $\sqrt{2}$ increase in noise.

Important considerations, as far as the detection sensitivity is concerned, are the quantum efficiency and the light collection efficiency. In the laboratory photometer, the quantum efficiency of the photomultiplier tube is 0.2, the efficiency of the luciferase reagent in converting NADH molecules into light is approximately 10^{-4} , and the light collection efficiency of the photomultiplier tube when viewing the reaction cell is about 0.1. Consequently, the light efficiency product is approximately 2×10^{-6} , and the multiplier to arrive at the number of NADH molecules which must be present to obtain a certain light level is 5×10^5 . This relationship is illustrated in Figure 6. To achieve a light level of 10^5 p/s, about 5×10^{10} molecules of NADH must be in the reaction cell. For simplicity, it is assumed that all of the NADH reacts in 1 second and peak light output values are used in the calculations as if a 1-second rectangular pulse occurred. x

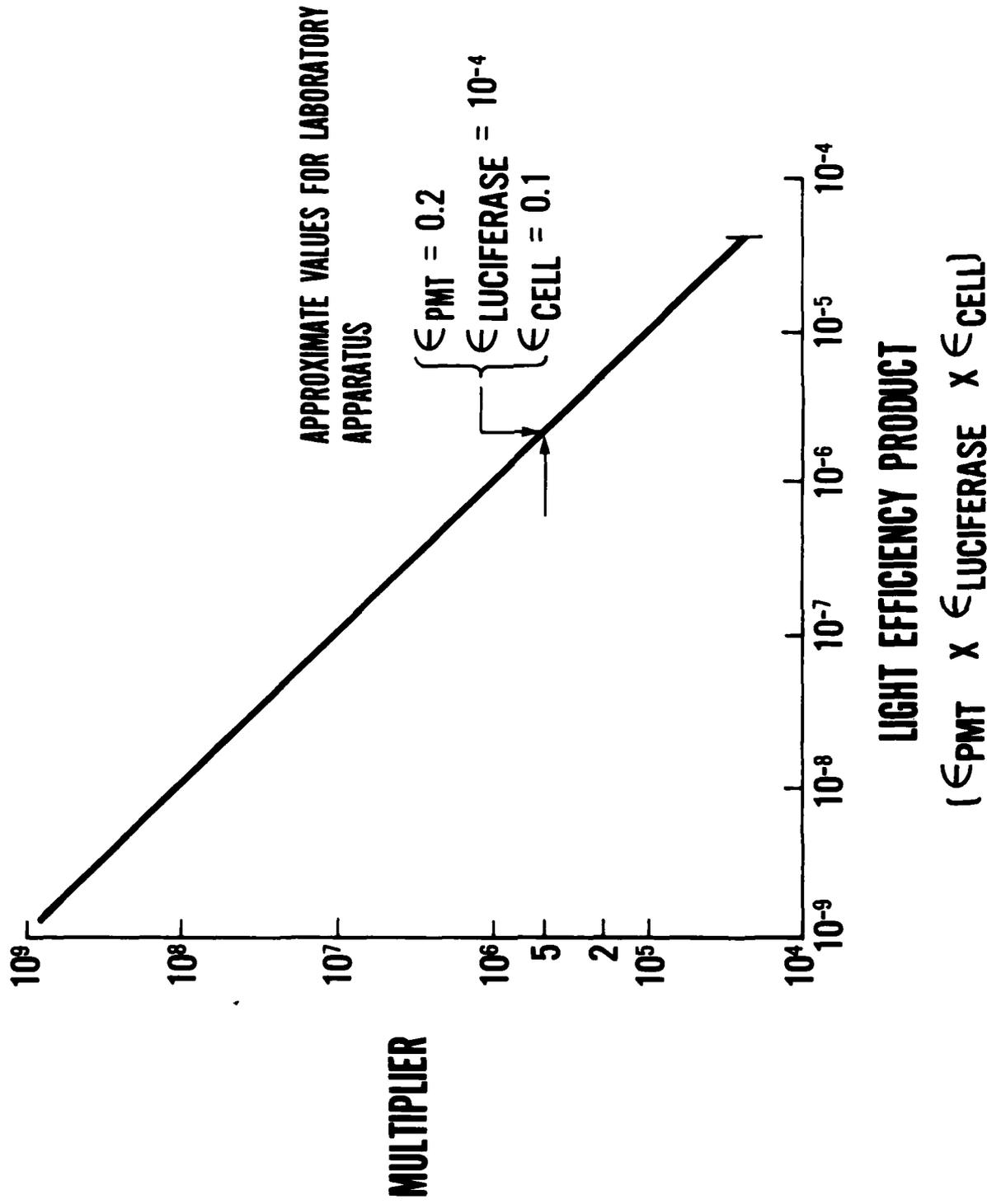


Figure 6. Relationship between the light efficiency product and the multiplier needed for determining the number of NADH molecules required for attaining a certain light level.

For a signal-to-noise ratio of 10 and a baseline photon flux (due to NADH) of 10^5 p/s, the reduction in photon flux due to the TNT reaction must be $10 \times (10^5)^{1/2} = 3160$ p/s. By simple proportion, this means that about 1.6×10^9 of the necessary 5×10^{10} molecules of NADH must be consumed by the reaction with TNT. These calculations are designed to shed light on the principles involved. In practice, the signal-to-noise ratio will be influenced also by the TNT reaction efficiency which will depend on the concentrations of TNT, NADH, and of the TNT reductase as well as on the reaction time chosen. The choice of these parameters will be affected by the presence of the NADH oxidase activity in the TNT reductase. For the gas phase detector the efficiency of interfacing the vapor molecules with the liquid reaction system will be important also.

The signal-to-noise ratio is a function of the square root of the light efficiency product. An increase in the light efficiency product from 2×10^{-6} to 2×10^{-4} would result in an improvement of the signal-to-noise ratio by a factor of 10. An improvement by a factor of 4 or 6 can be gained by improving the radiometric instrument design parameters ϵ_{PMT} and ϵ_{Cell} . The greatest gain would result from improving the efficiency of the luciferase reaction in converting NADH molecules to light. Extensive research will be required to determine whether or not the quantum efficiency of the indicator reaction in converting NADH molecules into light could be improved.

Before this section is concluded some data will be provided on parameters affecting the signal-to-noise ratio.

The dark current B_T of a selected photomultiplier tube was 10^3 p/s. This parameter sets the ultimate limit on sensitivity since B_A must be significantly larger than B_T . The B_T value for the tubes used in the experiments involving relatively high light levels was 3×10^4 p/s.

The reaction cell efficiency ϵ_c of the laboratory photometer used was about 0.1. For later work, a spiral shaped reaction cell was designed in which the light emitting reaction takes place closely in front of the photomultiplier tube.

The luciferase reagent emits light spontaneously. This light emission (B_l) was found to be proportional to the activity of the reagent. This means that reducing B_l would also reduce B_A , the detection reaction output. A luciferase reagent concentration was selected which sets the value of B_l at about 10^5 p/s. This provided a detection reaction output which peaks at about 2 seconds after the addition of NADH and which is 90 percent complete after about 10 seconds.

The injection of 20 μ l of 5×10^{-9} M NADH into 1 ml of luciferase reagent provided a light output of about 1.7×10^5 p/s. Since these 20 μ l of the NADH solution contain 10^{-13} mole NADH, which is about 6×10^{10} molecules, the reaction provided about 3 p/s for 10^6 NADH molecules. Consequently, the experimentally determined light efficiency product $\epsilon_p \times \epsilon_t \times \epsilon_l$ is 3×10^{-6} . Since ϵ_p and ϵ_t are about 0.2 and 0.1, respectively, the experimental ϵ_l is about 1.5×10^{-5} p/s emitted per NADH molecule. For the luciferase catalyzed reaction, quantum efficiencies from 0.1 to 0.3 were reported.²⁴ This suggests that the low value of ϵ_l is due to a low efficiency of the FMN reductase catalyzed reaction.

The reproducibility of the baseline light reaction was checked by injecting 20 μ l aliquots of a 3.34×10^{-9} M NADH solution (6.7×10^{14} mole) into 1 ml luciferase reagent solution. Twenty-nine replicas resulted in a mean light output of 5.5×10^4 p/s, with a standard deviation of 6.7 percent.

The evaluation discussed in this section showed that the sensitivity of the enzymatic TNT detection scheme depends on a number of parameters. To arrive at the optimum condition, a considerable amount of experimentation is required because some of these parameters are interdependent. For instance, high luciferase reagent concentrations result in high light yields per NADH. On the other hand, high luciferase reagent concentrations cause a high light background which is a noise component. High TNT reductase concentrations can facilitate the transformation of the TNT sample at such rates that the reaction is nearly completed after 15 seconds but the presence of the NADH oxidase activity does not allow the use of such high TNT reductase concentrations. Theoretically the enzymatic TNT detection concept would provide lower detection limits than presently found. To obtain a lower detection limit and to simplify the procedures it will be necessary to eliminate, or at least significantly reduce, the NADH oxidase activity. A significant impact on the detection limit would result also from an increase of the quantum efficiency of the indicator reaction and a decrease in its spontaneous light emission.

h. TNT Vapor Detection and Continuous Flow Breadboard Model

Air/Liquid Interface. The objective of the effort on enzymatic TNT detection was to develop a technique providing a mine detection capability via sensing TNT vapors emanating from such devices. For interfacing these vapors with the liquid reagent system of the enzymatic TNT detection scheme, a technique was considered involving adsorption of TNT vapors on solids and subsequent elution. Gold supported on alumina was found to be a particularly efficient TNT adsorbing medium. Such an interfacing system, however, would necessitate time-consuming manipulations, and a more direct method of interfacing the liquid and gaseous phases was desirable.

²⁴ W. J. Hastings and H. N. Neelson. *Am. Rev. Microbiol.* 31: 549 (1977).

Only about 10^{-3} mole of TNT are soluble in 1 liter of water; i.e., water is a poor solvent for TNT considering common concentration ranges. In the TNT detection program we are concerned, however, with solutions containing 10^{-9} mole of TNT per liter or less, and it was reasonable to expect that aqueous media would have considerable dissolving power for such small amounts of TNT. This idea was proven by bubbling TNT saturated air through the aqueous buffer solution used in the enzymatic TNT detection scheme. That experiment demonstrated the feasibility of using the medium of the enzymatic TNT detection scheme for TNT vapor absorption. For high sensitivity, interfacing the gaseous phase with the smallest possible volume of the liquid phase is necessary. To develop such a capability a simple configuration of an air/liquid phase contactor was investigated first. It was a straight vertical glass tube the walls of which were continuously wetted by the NADH/buffer solution. The test setup is shown in Figure 7. Known amounts of TNT-saturated air were passed through the contactor for specific periods and the efficiency of the contactor was expressed as the ratio of the detected amount to the amount passed through the contactor. The TNT saturator was a 5-liter vessel filled with TNT-coated Raschig rings. The TNT quantities absorbed in the liquid phase were determined with the enzymatic TNT detection system. Calibration was performed with TNT standard solutions. The flow rate of TNT-saturated air was varied from experiment to experiment from 0 and 1 liter per minute and a sage pump was set to deliver 0.27 ml of liquid per minute. The air flow through the contactor was well within the turbulent region. Taking the vapor pressure of TNT as 1.25×10^{-6} torr, a contactor efficiency of approximately 35 percent was obtained.

For use in a continuous flow model for enzymatic TNT vapor detection, a contactor was built consisting of an 8-mm-diameter glass tube of 60 cm length into which a helical glass coil was fitted with a turnspacing of 6 mm. The minimum practical liquid flow was 1 ml/min, and air flows of up to 4 l/min were possible. For the future, an air/liquid interface system was envisioned in which the liquid is being nebulized into the air stream. Liquid aerosol generators using ultrasonically driven plates containing fine orifices are known and could be useful for this purpose.

Continuous Flow Breadboard Model. In the planning of the design of a chemical analysis or detection system, the question arises whether a batch system, dealing with one sample at a time, or a continuous flow system would be preferable. A mechanized batch system, patterned after the techniques used for the feasibility demonstration of the enzymatic TNT detection concept, certainly could be built. Such an automated system is essentially a robot mimicking the steps of a laboratory procedure. Sample and reagents can be metered into a reaction vessel by motor-driven syringes, valves can be activated by solenoids, and computer control could be applied. Such an automated system would be complicated and expensive due to the requirement of mechanical manipulation of precise aliquots of several reagents and the need for precise timing. Considerably simpler are continuous flow systems which have fewer moving parts and are usually more reliable. In continuous flow systems, it is important to maintain constant, very small liquid flow rates. For enzymatic TNT detection, the flow of four reagent streams must be maintained. These are a stream of the NADH/buffer used in the contactor, and the streams of the TNT reductase/buffer, of the luciferase reagent/buffer, and of a tetradecanal solution which must meet the luciferase reagent briefly before the light reaction is taking place.

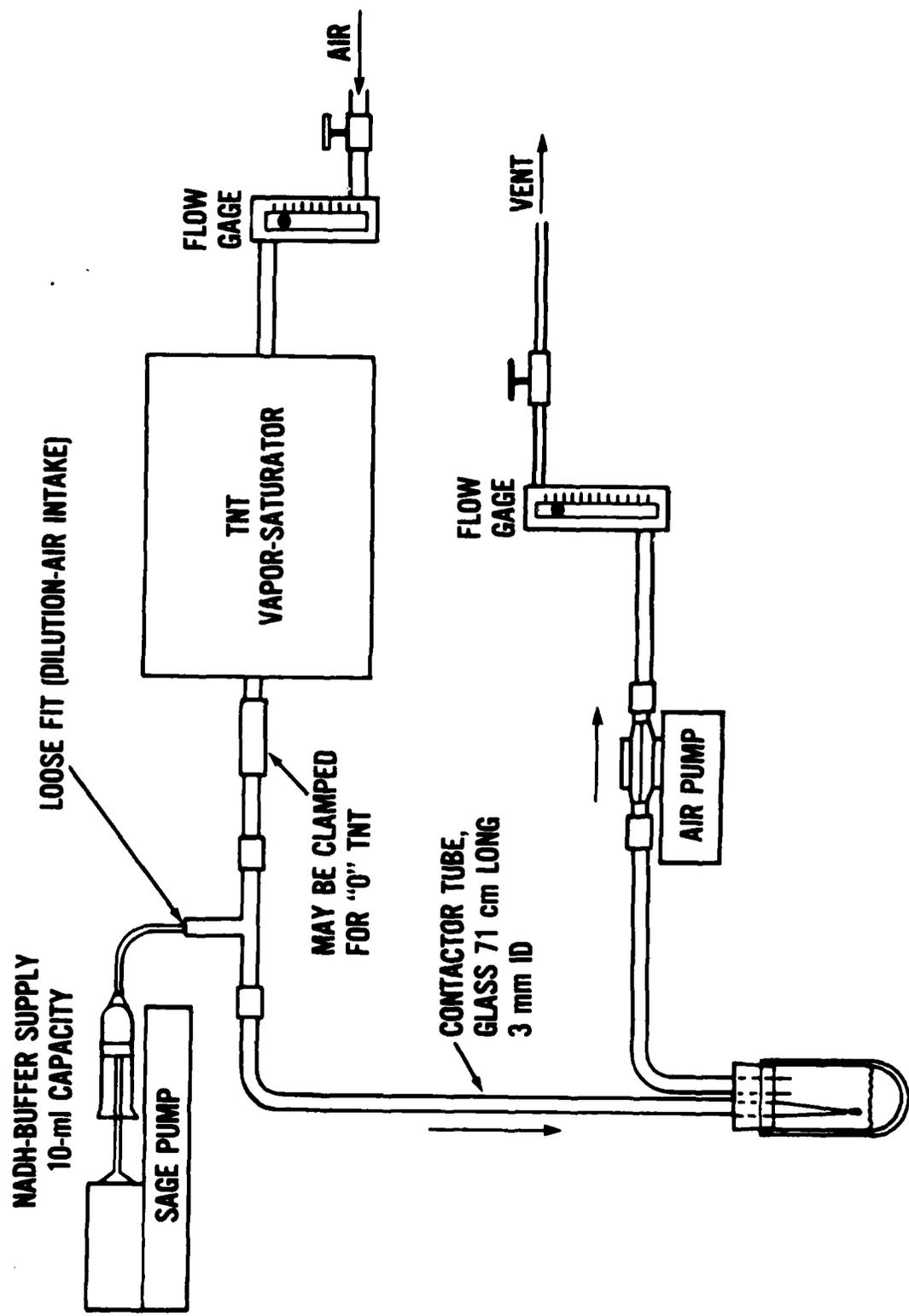


Figure 7. Test setup for evaluation of the air/liquid phase interface concept.

A breadboard model of a continuous flow system for enzymatic TNT detection was built and will be described here. This system was constructed near the end of the program and little time was left for testing. A schematic representation of this model is shown in Figure 8. Entering the top of the contactor is the air sample stream and a liquid stream of NADH/buffer. Pump 1, feeding this liquid stream and pump 2 are of peristaltic type. The air pump drawing in the air sample via the contactor and the phase separator is of vibrating-diaphragm type. For testing purposes, argon from a pressurized container is routed through the TNT saturator, the contactor, and the phase separator. The TNT saturator is a tube containing TNT-coated Chromosorb G as described originally by Pella.²⁵ The design of the phase separator can be seen in Figure 9. Its large internal diameter, compared with that of the contactor tube allows the air stream to slow down, preventing carryover of droplets into the air flow regulator. The liquid effluent from the phase separator joins a stream of the TNT reductase/buffer solution in mixer 1. Reaction of the NADH with TNT begins in this mixer and continues in the delay element. The latter is designed to allow the reaction to continue for 30 seconds to 1 minute (as in the bench-top procedure) before the next reagent addition. The unit referred to in Figure 8 as mixer 2 is designed to introduce, separately, the reaction mix from the delay element and the combined luciferase reagent and tetradecanal solution to the center of a glass spiral (photo spiral) which serves as reaction cell positioned closely in front of the window of the photomultiplier tube. This configuration was chosen because, for maximum sensitivity, it is important that the mixing of these two reagent streams takes place within full view of the photomultiplier. The photospiral consists of 17 cm of tubing with an inside diameter of 1 mm. The side of the photospiral facing away from the photomultiplier window is aluminized to increase the efficiency of light collection. The signal from the photomultiplier is transmitted to an amplifier and a recorder.

i. **Enzyme Research.** The evaluation discussed above showed which approaches could lead to an enhanced sensitivity of the enzymatic TNT detection scheme. Clearly, the greatest benefit as far as sensitivity and practicality is concerned could be expected from an elimination or significant reduction of the NADH oxidase activity which accompanies the TNT reductase. With this objective in mind, research was performed following two lines of investigation. These were efforts to separate the NADH oxidase from the TNT reductase using advanced protein separation techniques and characterization of the enzyme including efforts to selectively inhibit or denature the NADH oxidase.

Efforts on Physical Separation of the TNT Reductase and NADH Oxidase—Separation on the basis of molecular size (gel filtration) using sephadex G-150—was attempted. The peaks of both enzyme activities were found in the same fractions indicating that this principle does not separate the TNT reductase and NADH oxidase activities.

²⁵ D. A. Pella, *Analytical Chemistry*, 48 1632 (1976).

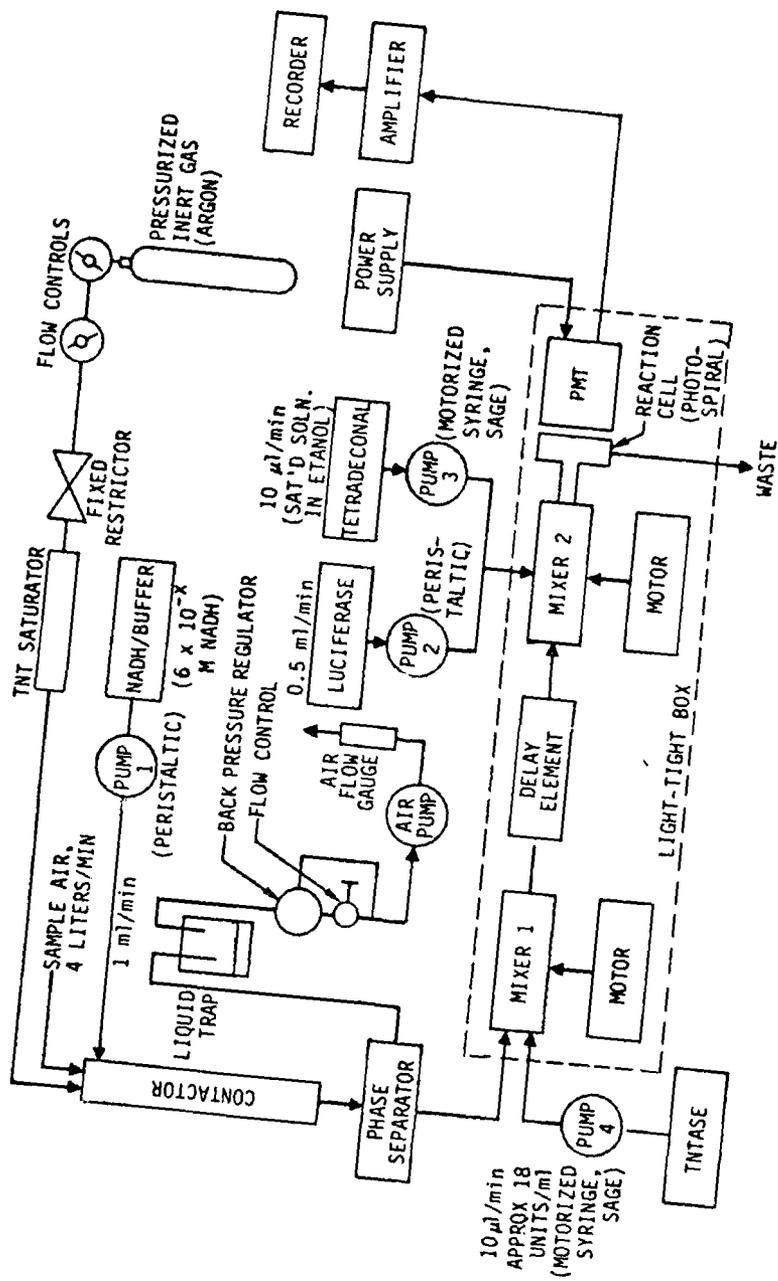


Figure 8. Schematic representation of the continuous flow breadboard model.

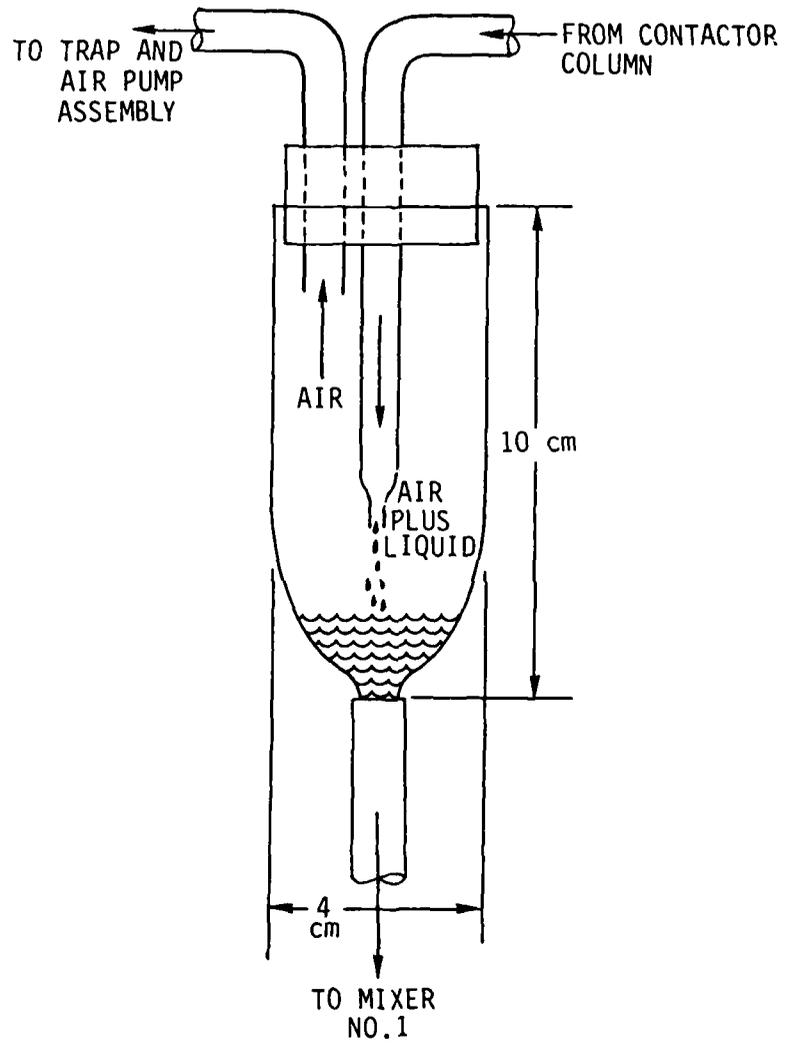


Figure 9. Schematic representation of the phase separator used in the continuous flow breadboard model.

A precise protein separation technique is polyacrylamide gel electrophoresis.²⁶ TNT reductase (600 μ g/experiment) was used and a current of 1/mA/tube was applied. Coomassie Blue served for staining purposes. Several bands appeared. The TNT reductase and NADH oxidase activities were found in the same band.

Isoelectric focussing allows the separation of proteins differing in their isoelectric point by as little as a 0.02 pH value.²⁷ Application of this technique did not result in the separation because both enzymes have apparent isoelectric points of 4.5 and are being denatured at this pH. The experiments were performed in sucrose density gradients using an LKB 8100 ampholine electrofocusing apparatus. The temperature was maintained at 4°C and the power input was 4 to 6 watts for all runs.

Another technique of separation by charge is DEAE-cellulose chromatography. A DE-52 Diethylaminoethyl cellulose anion exchange column (1.0 x 16 cm) was prepared using 1g DE-52 resin/15 mg total protein. The protein was eluted with a linear gradient of 0-0.4 M KCl in 0.05 M potassium phosphate buffer, pH 7.3. A total of 82 fractions were collected and assayed for TNT reductase and NADH oxidase activities. The peak TNT reductase activity eluted with 0.12 M KCl whereas the peak NADH oxidase activity eluted with 0.14 M KCl. These results were encouraging but a purification to less than 0.1 percent of NADH oxidase contamination (measured near saturated substrate concentration) was not accomplished.

Enzyme Characterization. The lack of success in physical separation of the TNT reductase and NADH oxidase activities led to the expectation that both activities could be exerted by the same enzyme or even the same enzyme site. Experiments involving prolonged incubation of the TNT-reductase showed, however, that NADH exerts a concentration dependent inhibition effect on the TNT reductase activity but not on the NADH oxidase activity indicating that the two activities are not due to the same catalytic site. TNT protects the site of the TNT reductase and, thus, slows the inhibition process. This type of experiment was also performed with the NADH analogs α -NADH (the usual NADH is the β form), nicotinamide hypoxanthine dinucleotide and 3-acetylpyridine NADH. Also these analogs inhibited the TNT reductase activity and not the NADH oxidase activity. It turned out also that the α -NADH was not a substrate for the TNT reductase reaction. With NADPH the enzymatic activities as well as the inhibition effect were similar to those observed with NADH. Incubation in the presence of catalase which destroys hydrogen peroxide and in the presence of N-methyl-L-tryptophan which reacts with active forms of oxygen were performed to test the idea that reactive forms of oxygen are responsible for the inactivation of the TNT reductase by NADH. No protection effect was observed. The effects of group specific reagents were also studied. N-ethylmaleide, p-chloromercuribenzoate, phenylmethylsulfonylfluoride, iodoacetic acid, and 3-bromopyruvic

²⁶ B. J. Davis, *Am. N. Y. Acad. Sci.*, 121, 409 (1964).

²⁷ H. Haglund, *Science Tools*, 11, No. 2, 17 (1967).

acid affected neither the TNT-reductase nor the NADH oxidase activity. This may suggest that free sulfhydryl groups as well as serine residues are not present in the active sites providing these activities. N-bromosuccinimide inhibits both the TNT-reductase and NADH oxidase proportionately, possibly indicating that tryptophan, tyrosyl, and/or histidyl residues contribute to the enzymatic activities in the active sites. Experiments were also performed directed at making use of different rates of thermal inactivation of the enzymatic activities. The results showed that the loss of TNT reductase activity was closely paralleled by a corresponding loss of NADH oxidase activity.

Molecular oxygen serves as the electron acceptor for some NADH oxidases. If this were the case in the NADH oxidase of interest, elimination of the molecular oxygen from the solution would affect the enzymes activity. To explore this possibility, the solutions were first bubbled with pure nitrogen and the TNT reductase reaction performed in the presence of glucose and glucose oxidase for scavenging of any remaining molecular oxygen. These measures did not affect the NADH oxidase activity.

The work discussed in this section did not result in physical separation of the TNT reductase and the NADH oxidase activities nor in a selective inhibition of the NADH oxidase activity. The results indicated that these activities are not due to the same catalytic site but they led to the hypothesis that both activities are exerted by the same enzyme.

5. Summary. The feasibility of an enzymatic TNT detection concept was demonstrated involving a "sensor reaction" in which a TNT specific enzyme, the TNT reductase, catalyzes a reaction which is sensitively indicated by a light emitting indicator reaction. As indicator reaction serves the *in vitro* bacterial luciferase reaction whose light emission depends on the concentration of the biochemical reagent NADH, the concentration of which is changed by the TNT specific sensor reaction.

To make this TNT detection scheme possible, a hitherto unknown TNT reductase enzyme was "developed"; i.e., bacteria were induced to synthesize this enzyme. These bacteria were produced in quantities by a fermentation process and the enzyme was isolated from the cell contents of the bacteria and then purified.

The feasibility of the detection concept outlined above was demonstrated using a special laboratory photometer and standard TNT solutions; 2×10^{-14} mole of TNT in the liquid phase could be detected.

The feasibility of TNT vapor detection was demonstrated using a setup in which the air to be checked for the presence of TNT is interfaced directly with the aqueous reagent system of the enzymatic TNT detection scheme. Transfer of the TNT vapors from the air into very small volumes of the liquid, with satisfactory efficiency, was accomplished.

A continuous flow system was identified as the most promising configuration of a future detector and a breadboard model was constructed.

An evaluation was performed of the parameters affecting the signal-to-noise ratio and herewith the detection sensitivity. The measures were delineated which are necessary to accomplish further sensitivity increases. These include the elimination of a NADH oxidase activity which accompanies the TNT reductase, a reduction of a spontaneous light emission of the reagents of the indicator reaction and an increase in the light efficiency of the indicator reaction.

Research was performed aimed at separating the TNT reductase and NADH oxidase activities or to selectively inhibit the NADH oxidase activity. This was not accomplished. The results led to the hypothesis that both activities are exerted by the same enzyme.

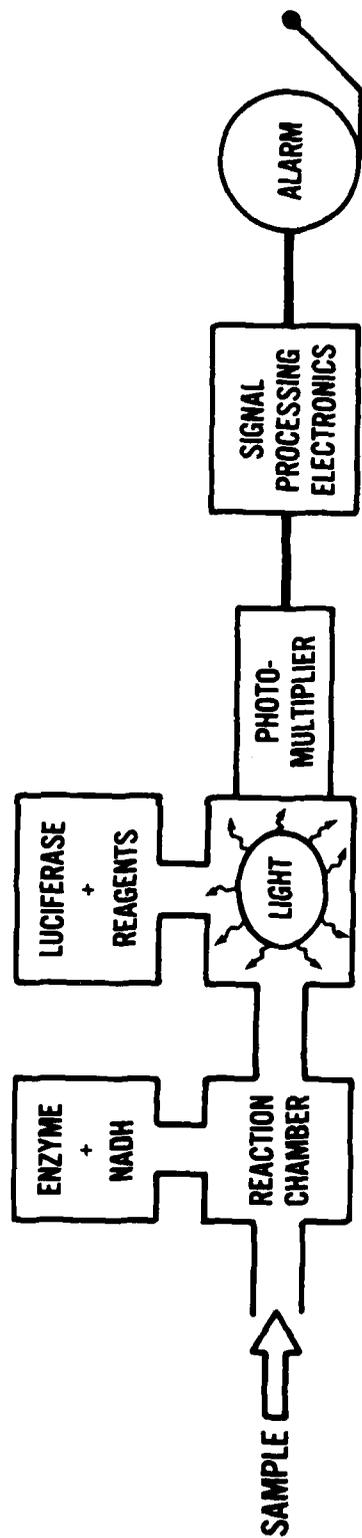
The enzymatic TNT detection scheme, in its present stage of development, represents an extremely sensitive method for detection and quantitation of TNT in solution. Much work will be required in the development of a practical TNT vapor detector but no new technologies are required to reach this goal. A TNT reductase enzyme without any NADH oxidase activity will be advantageous. It is expected that enzymes can be developed for explosives other than TNT or for compounds indicative of the presence of explosives. Detectors incorporating several enzymes providing the capability to detect several compounds simultaneously can be envisioned. Figure 10 provides an overview of the existing enzymatic TNT detection concept.

III. IMMUNOCHEMICAL TECHNIQUES FOR TNT DETECTION

1. Preface. In Section II of this report, an enzymatic technique for TNT detection was discussed in which specificity was provided by a TNT reductase enzyme and sensitivity by a light emitting reaction. A lower detection limit of 2×10^{-14} mole of TNT was achieved, but it did not seem likely that significant further increases in sensitivity could be achieved without embarking on an extensive and time-consuming research effort. It seemed more promising, therefore, to explore the field of immunoassay, a discipline in which outstanding specificities and sensitivities are found. The most developed and sensitive immunoassay technique utilizes radioactive isotopes for readout. Light can be measured with similar sensitivity as radioactivity, which led to the formulation of a new concept which we termed light emission immunoassay. In this concept, specificity is provided by antibodies and sensitivities by a light emission readout. The concept of light emission immunoassay for TNT was conceived at MERADCOM; the experimental work is being performed at the University of California, San Diego. The work is a low level, incrementally funded 6.1 research effort which began in October 1979.

ENZYMATIC DETECTION OF EXPLOSIVES VAPORS

- PRINCIPLE: ENZYMES CATALYZE REACTIONS WITH SPECIFIC MOLECULES
- DESCRIPTION
 - EXPLOSIVE VAPOR PUT IN SOLUTION
 - EXPLOSIVE MOLECULES REDUCED IN PRESENCE OF ENZYME, CONSUMING NADH
 - CHANGED NADH CONCENTRATION RESULTS IN A CHANGED LEVEL OF LIGHT EMISSION, THUS INDICATING THE PRESENCE OF EXPLOSIVE MOLECULES.



- STATUS
 - ENZYME FOR TNT PRODUCED
 - CONCEPT FEASIBILITY ESTABLISHED
 - SENSITIVITY TO 10^{-14} MOLE OF TNT DEMONSTRATED

Figure 10. Schematic overview of the enzymatic TNT detection scheme.

2. Background. When an animal receives one or more injections of certain foreign substances, molecules which possess the unique property of reacting with the material injected appear in the serum within a few days. These substances are called antibodies, or immunoglobulins, and the materials which stimulated their production are called antigens. An animal which has formed antibodies is said to be immunized. Formerly only proteins were believed to stimulate antibody production, but it is now known that also other foreign macromolecules such as polysaccharides, nucleic acids and lipids can be antigenic. Antibody molecules are made by nature to interact specifically with the antigen which caused their production, whereby antigen-antibody complexes are formed. If the antigen was red blood cells of a foreign species, the antibody antigen interaction will result in clumping of the cells. This phenomenon is called agglutination. If a soluble antigen such as, for instance, egg albumin is mixed in suitable proportions with its immune serum, a precipitate of the antigen-antibody complex forms. This process is called the precipitin reaction which was useful for a long time for the quantitative analysis of many antigens.

Antibodies are proteins and belong to the class of globulins. There are several classes of immunoglobulins of which the so-called IgG globulins are the most common and the best understood. They have a molecular weight of about 150,000 and consist of four peptide chains. A major portion of these chains have a constant amino acid sequence while the amino acid sequence is variable near the ends of these chains. Each IgG globulin molecule has two antigen binding sites.

The specificity of antibodies—that is, their capacity to react only with the substances used to produce them (i.e., the antigen) or with substances showing a very close chemical relationship with the antigen—has been responsible for their tremendous importance to the chemist, since it has made possible not only the detection but also the quantitative determination of antigens in mixtures which could not be resolved otherwise. The usefulness of this technique was greatly increased when it was discovered that small non-antigenic molecules could be chemically coupled to proteins and, in this form, elicit the formation of antibodies possessing specificity toward the respective small molecule.^{28 29 30} The proteins function as carriers for the molecules of interest and render them antigenic. In the language of immunochemistry, small molecules which can be made antigenic in this fashion are termed haptens. Of prime importance was the observation that the antibody will not only react with the complete antigen (hapten-carrier-conjugate) but also with the hapten alone. This principle has been used to develop sensitive and specific analysis techniques for various small molecules as well as for larger molecules which themselves are complete antigens.

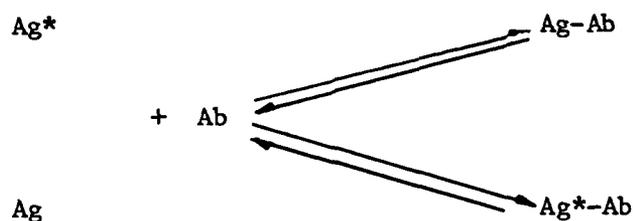
²⁸ R. Obermayer and E. P. Pick, *Wien. Klin. Wochenschr.* 19, 327 (1905).

²⁹ K. Landsteiner, *The Specificity of Serological Reactions*, Harvard Univ. Press, Boston, MA (1945).

³⁰ K. Landsteiner and J. van der Scheer, *J. Exp. Med.*, 63, 325 (1936).

3. Present Immunoassay Concept. Antibodies have been used for a long time for the determination of various antigens, but the sensitivities of the older techniques, such as the precipitin reaction were too small to be useful in the study of, for instance, hormones. In the late 1950s and during the 1960s, however, highly sensitive immunoassay techniques became known which employ labeled antigens or antibodies. The most developed of these techniques is radioimmunoassay. This technique was developed to a significant extent by R. S. Yalow and S. Berson which was recognized by awarding the 1977 Nobel Prize in Physiology and Medicine to R. S. Yalow.³¹⁻³⁵

R. S. Yalow and S. Berson observed that radioactive insulin disappeared more slowly from the plasma of patients who had received insulin previously than from subjects never treated with insulin.³⁶ These investigators interpreted the retarded rate of insulin disappearance as a consequence of the presence of insulin antibodies which had formed in response to the administration of exogenous insulin. This explanation appeared at first unacceptable to the immunologists of that time, but Yalow and Berson proved their results, all of which resulted in the development of radioimmunoassay (RIA) and competitive protein binding assay in general. This technique is simple in principle as can be seen from the reaction equation given below. The unlabeled and the radioactively labeled molecules compete for a limited number of sites on a specific binding protein such as an antibody. Once equilibrium has been reached, the fraction of the labeled compound which is bound is inversely proportional to the concentration of the unlabeled molecule, i.e., the concentration of the substance to be measured. After separation of bound and free fractions, the ratio of the bound and free label can be determined. This ratio is compared with the bound versus free label ratio obtained with standard samples and thus the concentrations of the unknown sample can be determined.



Ag* = labeled antigen
 Ag = unlabeled antigen
 Ab = antibody

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- 31 S. A. Berson and R. S. Yalow, *J. Clin. Invest.* 36, 873 (1957).
 32 S. A. Berson and R. S. Yalow, *Advan. Biol. Med. Phys.* 6, 349 (1958).
 33 S. A. Berson and R. S. Yalow, *Ann. N. Y. Acad. Sci.* 82, 338 (1959).
 34 R. S. Yalow and S. A. Berson, *Nature*, 184, 1648 (1959).
 35 R. S. Yalow and S. A. Berson, *Principles of Competitive Protein-Binding Assays*, W. D. Odell and W. D. Daughaday (eds), J. B. Lippincott Co., Philadelphia, PA, p. 1 (1971).
 36 S. A. Berson, R. S. Yalow, A. Bauman, M. A. Rothschild, and K. Newerly, *J. Clin. Invest.* 35, 170 (1956).

In principle, any radioactive atom which can be incorporated into the molecule of interest could serve as tracer, but gamma emitting radionuclides are preferable. Unfortunately, the isotopes of carbon and hydrogen, the most common elements in organic molecules, do not include gamma radiation emitting isotopes. ^{14}C and ^3H being beta emitters, necessitate liquid scintillation counting. Therefore, the gamma-emitting radionuclides ^{125}I and ^{131}I have become the labels of choice even though their half lives are only 60 and 8.1 days. The iodine isotopes can be coupled to the amino acids, tyrosine and histidine, commonly present in proteins, and thus these radionuclides have become the predominant taggants for RIA of proteins. There are several methods of iodinating such molecules, but the chloramine T oxidation-sodium metabisulfite method is most widely used.^{37 38} Some molecules, such as steroids, have also molecular arrangements which allow the incorporation of ^{125}I . Unfortunately, however, since the iodine atom is approximately 25 percent as big as a steroid, the incorporation of iodine significantly distorts the molecule and alters its geometric "fit" with the antibody binding site; therefore, immunoreactivity is affected.

A great variety of techniques have been developed for the separation of the bound and free labeled compounds. One of the most widely used separation methods employs solid phase adsorbents which preferentially adsorb the free compound. Examples are charcoal (Norit), silica, talc, ion exchange resins, cellulose, and sephadex. Another category of separation techniques involves the precipitation of the soluble complexes with reagents such as salts, ethanol, methanol, acetone, or polyethylene glycol. Far more elegant than these chemical precipitation techniques is immunoprecipitation employing a second antibody directed against the first antibody. Thus, specific precipitation of the soluble primary reaction product $\text{Ag}^*\text{-Ab}$, as insoluble $\text{Ag}^*\text{-Ab}_1\text{-Ab}_2$ occurs. The first antibodies are often obtained from rabbits; the second antibody, from larger animals such as goats or sheep. The second antibody, of course, can be used for the precipitation of any antibody produced by another species. The advantage of the second or double antibody technique is its specificity resulting in precipitation of only the first antibody leading to a clean separation of free and bound label. Another method, gaining quickly in importance, is the use of antibodies immobilized to solid supports. The separation of bound and free molecules can be accomplished in this case by simple decanting of the liquid phase.³⁹ Chromatoelectrophoresis is also used for separation of bound and free labels.

³⁵ R. S. Yalow and S. A. Berson, *Principles of Competitive Protein-Binding Assays*, W. D. Odell and W. D. Daughaday, (eds), J. B. Lippincott Co., Philadelphia, PA, p. 1 (1971).

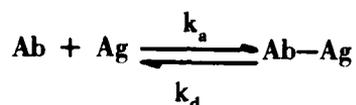
³⁷ W. M. Hunter and F. C. Greenwood, *Nature (London)* 194, 495 (1962).

³⁸ F. C. Greenwood, W. M. Hunter, and J. S. Glover, *Biochem. J.* 89, 114 (1963).

³⁹ K. Catt and G. W. Tregear, *Science*, 158, 1570 (1967).

⁴⁰ S. A. Berson and R. S. Yalow, *The Hormones, IV*, G. Pincus, K. V. Thimann, and F. B. Astwood (eds), Academic Press, New York, p. 557 (1964).

The introduction of radiotracer techniques into the field of immunoassay increased the sensitivity by many orders of magnitudes, and detection and analysis in the pico and femto mole range became possible. This performance prompted theoretical and experimental evaluation of the factors affecting the lowest detection limit of competitive binding assays. It was recognized quite early that the ultimate limitation of competitive binding assays is the affinity of the antibody for the antigen.^{35 40} The interaction of the antibody and antigen at equilibrium may be expressed in the following way:



where Ab represents the free antibody, Ag the free antigen, Ab-Ag the antibody-antigen complex, and k_a and k_d the associations and dissociation constants, respectively.

Applying the law of mass action to this interaction

$$k_a(\text{Ab})(\text{Ag}) = k_d(\text{Ab-Ag})$$

thus the equilibrium or affinity constant (K) may be calculated

$$\frac{k_a}{k_d} = K = \frac{(\text{Ab-Ag})}{(\text{Ab})(\text{Ag})}$$

According to the deductions of Berson and Yalow, the minimal detection limit of competitive binding assays such as RIA is $0.1125/K$.^{35 40} This result was tested more recently and the agreement between the theoretical prediction and the experimental observation was excellent.⁴¹ Thus for the development of sensitive competitive binding assays one would hope to have the highest affinity antibody possible as well as an antibody of consistent affinity.

³⁵ R. S. Yalow and S. A. Berson, *Principles of Competitive Protein-Binding Assays*, W. D. Odell and W. D. Daughaday (eds), J. B. Lippincott Co., Philadelphia, PA, p. 1 (1971).

⁴⁰ S. A. Berson and R. S. Yalow, *The Hormones*, IV, G. Pincus, K. V. Thimann, and E. B. Astwood (eds), Academic Press, New York, p. 557 (1964).

⁴¹ J. H. Schuurman and C. L. deLigny, *Anal. Chem.*, 51, 2 (1978).

The response of animals to antigens is unfortunately not consistent and considerable variation in the quality of antibodies produced is commonly found.⁴²⁻⁴³ This variability in the antibody response is by now fairly well understood. Every animal has a great number of lymphocytes (antibody producing cells) which are capable of responding to a given immunogenic stimulus.⁴⁴ Upon stimulation by the immunogen, all of these proliferate and release antibodies into the bloodstream. Thus a family of antibodies with a broad spectrum of affinities is produced. As the stimulating antigen diminishes, only those lymphocytes producing the highest affinity antibody continue to proliferate so that the average affinity of the antibody increases with time.⁴³⁻⁴⁵⁻⁴⁶ This phenomenon has been termed the maturation of the immune response.⁴³ There is also evidence for genetic control of antibody affinity.⁴⁷ The various factors affecting antibody affinity were discussed in detail by M. H. Steward.⁴⁸ The effect of booster injections on antibody affinity was investigated.⁴⁹

The variation in affinity of antibody preparations has significant implications with regard to reaction kinetics. It has been known for some time that antigen-antibody reactions have high rate constants, and it is this speed of reaction which has made kinetic studies exceedingly difficult when traditional kinetic methods are employed.⁵⁰ Of course, the actual speed of antigen-antibody reactions, being bimolecular reactions, depends not only on the rate constants but also on the concentrations of the reactants and will be low at very low concentrations. The use of techniques for the study of fast reactions such as the temperature jump method and stopped-flow technique in combination with sensitive spectrophotometric methods have provided an opportunity to make meaningful kinetic measurements of the antigen-antibody reaction.⁵¹⁻⁵⁴ Such techniques were applied for the determination of the rate constants of antigen-antibody association (k_a) and the rate constants of antigen-antibody dissociation k_d .⁵⁵ It was found that the rate constants for association are quite similar for all antigens investigated but large differences were found in the rate constants of dissociation. Corroborating results were obtained by other investigators and all these findings were compiled and tabulated by M. W. Steward.⁴⁸ The data indicate that the large variation in the affinity of antibodies for antigens; i.e., in the stability of antibody-antigen complexes is mainly governed by the dissociation rate constants.

42 F. Karush, *Adv. Immunol.*, **2**, 1 (1962).

43 G. W. Siskind and B. Benacerraf, *Adv. Immunol.*, **10**, 1 (1969).

44 H. N. Eisen and G. W. Siskind, *Biochemistry*, **3**, 996 (1964).

45 L. A. Steiner and H. N. Eisen, *J. Exp. Med.*, **126**, 1161 (1967).

46 F. E. Watz and M. W. Steward, *Immunology*, **29**, 543 (1975).

47 M. W. Steward, *Immunochemistry: An Advanced Textbook*, L. E. Glynn and M. W. Steward (eds), Wiley, NY 233, (1977).

48 Young Tai Kim, D. Greenbaum, P. Davis, Sr., A. Fink, Th. P. Werblin, and G. W. Siskind, *The Journal of Immunology*, **114**, 1302 (1975).

49 B. S. Hooker and W. C. Boyd, *J. Gen. Physiol.*, **19**, 373 (1935).

50 M. Eigen and L. DeMaeyer (in), *Techniques of Organic Chemistry*, **8**, Part II, Interscience, New York (1963).

51 B. Chance (in), *Techniques of Organic Chemistry*, **8**, Part II, Interscience, New York (1963).

52 A. Froese and A. H. Schon, *Immunochemistry*, **2**, 135 (1965).

53 A. Froese and A. H. Schon (in), *Methods in Immunology and Immunochemistry*, Vol III, M. Chase and C. William (eds), Academic Press, New York, London, p. 412.

54 A. Froese, *Immunochemistry*, **5**, 253 (1968).

The antibody affinity does not only determine the theoretical lower detection limit of immunoassays, but affects also the incubation times required to achieve equilibrium conditions. It is, therefore, fortunate that recently a method was discovered and developed permitting the production of antibodies which are absolutely uniform in affinity, kinetic parameters, and chemical composition. This method produces a single antibody type, a monospecific antibody. In this procedure, spleen cells from an immunized mouse are fused with myeloma cells from the same strain of mouse. Myeloma cells can be grown in the laboratory by *in vitro* tissue culture methods. When the spleen cell and myeloma cell are fused, a hybrid forms which not only can be grown *in vitro* but which also secretes into the culture medium a single species of antibody directed against the stimulating immunogen. In other words, a single antibody-producing spleen cell can be immortalized by fusing it with a myeloma (tumor) cell and the descendants can be grown in a laboratory culture and produce the original single antibody.⁵⁶⁻⁵⁸ The availability of these completely homogenous antibodies, referred to as monoclonal antibodies has far-reaching implication for immunochemistry and for medicine.

The development of radioimmunoassay has revolutionized clinical chemistry and for the first time made it possible to quantitate accurately physiological agents such as hormones. The high sensitivity of this technique also allowed the elucidation of the principles of competitive binding assay and the evaluation of the sensitivities which can be expected. Radioimmunoassay, however, also has its drawbacks. It involves radioactive isotopes; consequently, only licensed laboratories with appropriately trained personnel can use this technique. To avoid the use of complex scintillation counters which are necessary if ^{14}C or ^3H are used, the radioimmunoassay technique is mainly applied for compounds which can be tagged with gamma emitting ^{125}I and ^{131}I . The ^{14}C and ^3H isotopes have long half lives but comparatively low specific activities, and hence they provide lower assay sensitivities. ^{125}I and ^{131}I have high specific activities but their half lives are only 60 days and 8.1 days, respectively, resulting in shelf life problems. Furthermore, not all molecules can be tagged with iodine isotopes and small molecules are often modified too much in their immunochemical properties when tagged with the rather large iodine isotopes. For these reasons there is much research underway to apply the principles discovered in the work on radioimmunoassay but to use nonradioactive labels. These efforts led to a variety of new immunoassay concepts which have not reached the prominence of radioimmunoassay but which have already replaced immunoassay in some applications. Some of these will be discussed in the next paragraphs.

⁵⁶ G. Kohler and C. Milstein, *Nature*, 256, 495 (1975).

⁵⁷ G. Kohler and C. Milstein, *Eur. J. Immunol.* 6, 511 (1976).

⁵⁸ H. Koprowski, W. Gerhard, and C. M. Croce, *Proc. Natl. Acad. Sci. USA* 74, No. 7 2985 (1977).

One of the new techniques developed is spin label immunoassay.⁵⁹⁻⁶² In this technique known amounts of antibodies are mixed with an analog of the molecule to be detected that has been labeled with the stable radical nitroxide (spin label). The molecule to be detected will compete for sites on the antibody and some of the spin labeled molecules will be displaced from the antibody. The presence of the free, spin labeled, molecules is signaled by the appearance of a sharp three-line electron spin resonance spectrum superimposed on the broad spectrum produced by the bound label. The signal intensity is a direct measure of the concentration of the molecule of interest. In this technique some of the disadvantages of radioimmunoassay are avoided. No radioactive isotopes and health hazards are involved. It is a homogeneous assay; i.e., separation of free and bound labels is not required. Spin label immunoassay, however, is not as sensitive as radioimmunoassay. It also requires heavy and expensive equipment and therefore can presently not be considered for applications requiring a portable instrument.

Already in the 1960s, the quenching of the natural fluorescence of proteins played a significant role in the study of hapten-antibody reactions. The change in fluorescence of antibodies when they combine with haptens such as dinitrophenyl-lysine was measured and led to one of the first accurate determinations of the equilibrium constant of an antibody-hapten reaction.^{63 64} Analytical application of fluorescence quenching was not considered, however, prior to the establishment of the principles of competitive protein binding assay. Now, the use of fluorescent molecules as labels is one of the avenues for the development of immunoassays not depending on radioactive isotopes. Highly fluorescent molecules such as fluorescein are employed for labeling and rhodamine is often used as a quenching molecule. The fluorescein and the rhodamine are bonded to either the antibody or the hapten. Binding of the hapten to the antibody brings the fluorescent label and the quenching molecule into proximity, resulting in a decrease in fluorescence intensity. The molecule to be quantitated will also compete for sites on the antibody and will, consequently affect the change in fluorescence.⁶⁵ The concept described is a homogeneous immunoassay; i.e., separation of bound and free label is not required. The first assay systems based on this principle did not provide high sensitivity but more sophisticated concepts under development promise high sensitivity.⁶⁶

59 R. Leute, E. Ullman, A. Goldstein, and L. Herzenberg. *Nature, New Biol.*, 236, 93 (1972).

60 R. Schneider, R. Bastioni, R. Leute, et al., *Immunoassay for Drugs Subject to Abuse*, S. Mule, I. Sunshine, M. Brande, and R. Willette (eds), CRC Press, Cleveland, Ohio, p 45 (1974).

61 M. Montgomery, J. Holtzman, R. Leute, et al., *Clin. Chem.* 21, 221 (1975).

62 R. Leute, E. Ullman, A. Goldstein, *J. Am. Med. Association*, 221, no. 11 p. 1231, (1972).

63 S. F. Velick, C. W. Parker, and H. N. Eisen, *Proc. Natl. Acad. Sci., USA*, 46, 1470 (1960).

64 J. R. Little and H. N. Eisen, *Biochemistry*, 5, 3385 (1966).

65 E. F. Ullman, M. Schwarzenberg, and K. E. Rubenstein, *The J. of Biol. Chem.* 251, 4172 (1976).

66 T. Hirschfeld, Lawrence Livermore Laboratory.

Another technique applied for the study of immunochemical reactions is fluorescence polarization.⁶⁷⁻⁶⁸ This technique is based on the principle that fluorescence from stationary molecules will be polarized when initiated by polarized light, whereas the fluorescence from rapidly rotating molecules will not be polarized. Since antibodies have molecular weights of the order of 10^5 daltons, their angular velocities of rotation are much smaller than those of smaller molecules. Therefore, when a relatively low molecular weight fluorescein-labeled organic compound which ordinarily exhibits very little fluorescence polarization becomes bonded to an antibody, a marked increase in fluorescence polarization takes place.⁶⁹ This concept provides fairly good sensitivity, but the equipment required is substantial in size. If a portable instrument is desired the use of the fluorescence polarization and to a lesser extent fluorescence intensity methods are not advisable.

Probably the most significant new development is the use of enzymes as labels. In different versions of this technique, either the antigen or the antibody or even a second antibody can be labeled with enzymes. The development of enzymeimmunoassay was made possible by a long line of advances in the methods of protein chemistry. One of the most important advances was the development of techniques to couple enzymes to antibodies or antigens.⁷⁰⁻⁷¹ The use of enzymes as labels of antigens, haptens, and antibodies in quantitative immunoassay was originally explored independently by two groups of investigators.⁷²⁻⁷⁸ Their efforts led to enzymeimmunoassays which are closely related to radioimmunoassay, except that an enzymatic reaction catalyzed by the enzyme, serving as label, provides the readout. Separation of bound and free label is required and can be accomplished by a second antibody as precipitant or, more simply, with immunoreactants being immobilized to solid supports. Such analysis systems are now well known under the term Enzyme Linked Immunosorbent Assay, or short ELISA. In these assays interference by background is dependent on the perfection of the separation technique and very high sensitivities can be obtained. This type of enzymeimmunoassay, where separation is required, is often referred to as heterogeneous immunoassay. Another group of investigators succeeded in the development of an enzymeimmunoassay technique which does not necessitate a separation step and is referred to as homogeneous enzyme immunoassay.⁷⁹⁻⁸⁰ It involves the preparation of a conjugate consisting of the molecule

67 W. B. Dandliker and V. A. de Saussure, *Immunochemistry*, 7, 799 (1970).

68 W. B. Dandliker and S. A. Levison, *Immunochemistry*, 5, 171 (1968).

69 H. R. Lukens, C. B. Williams, S. A. Levison, W. B. Dandliker, and D. Muryama, U.S. Environmental Agency Report, EPA 650/1-75-004 (1975).

70 S. Avrameas, *Immunochem.*, 5, 43 (1969).

71 S. Avrameas, T. Ternynck, and J. L. Guesdon, *Scand. J. Immunol.* 8, Suppl. 7, 7 (1978).

72 E. Engvall and P. Perlmann, *Protides of the Biological Fluids*, Proceedings of the Nineteenth Colloquium Brugge (1971).

73 E. Engvall and P. Perlmann, *Immunochemistry* 8, 871-4 (1971).

74 E. Engvall, K. Jonsson, and P. Perlmann, *Biochem. Biophys. Acta* 251, 427 (1971).

75 E. Engvall and P. Perlmann, *J. Immunol.* 109, 129-135 (1972).

76 B. K. Van Weeman and A. H. W. M. Schuurs, *FEBS Lett.* 15, 232 (1971).

77 B. K. Van Weeman and A. H. W. M. Schuurs, *FEBS Lett.* 24, 77-81 (1972).

78 B. K. Van Weeman and A. H. W. M. Schuurs, *FEBS Lett.* 43, 215-218 (1974).

79 K. E. Rubenstein, R. S. Schneider, and E. F. Ullman, *Biochem. Biophys. Res. Commun.* 47, 846-851 (1972).

80 G. L. Rowley, K. E. Rubenstein, J. Huisden, and E. F. Ullman, *J. Biol. Chem.* 250, 3759 (1975).

of interest and a chosen enzyme. When such a conjugate is exposed to the antibody of the molecule of interest complexation occurs and the activity of the enzyme will be modified. If the free molecule to be detected is also present, competition for antibody sites will take place between the free molecule and the conjugate; consequently, some of the conjugate molecules will not become bonded to the antibody. Since the free and bonded conjugates have different enzymatic activities, the concentration of the molecule of interest can be determined. The quantity actually measured is the product of the enzymatic reaction catalyzed by the enzyme chosen. Spectrophotometric readout is often used, but fluorescence or other physical properties of the product formed can be used for readout. Since there is no need for a separation step, the homogeneous enzyme immunoassay technique is simpler and requires less time than the other immunoassay system. Because of these advantages, homogeneous immunoassay is already introduced into the clinical laboratory under the name EMIT (Enzyme Multiplied Immunoassay Technique). This technique was developed by Syva Corporation which has the basic patents. The EMIT system is used to determine the concentration of drugs such as morphine and other small molecules. This system is mainly applicable for the analysis of small molecules since the catalytic activity of a conjugate consisting of a large molecule and an enzyme would not be affected sufficiently by being bonded to an antibody. Today's homogeneous immunoassays are not highly sensitive, mainly because the changes in enzyme activities due to bonding to an antibody are not big enough. For many applications, the sensitivity is high enough, however, even though spectrophotometric readout is usually employed.

The choice of enzyme to be used in any enzyme immunoassay depends on several criteria: The enzyme should have a high turnover number so that the product being measured and serving as indicator is produced at a high rate; the product formed in the enzymatic reaction should be detectable with a very sensitive method. The enzyme should be readily available and should possess reactive groups facilitating the linking with other molecules without significantly impairing enzymatic activity.

4. Development of the Light Emission Immunoassay Concept. The idea to develop new immunoassay techniques for the purpose of TNT detection was not only prompted by the extremely high sensitivities provided by immunoassay but also by the fact that aromatic nitro compounds were favored model compounds for basic research in immunochem-

istry.^{45 49 55 63 64 81-88} There are several reasons for this: Firstly, well-known laboratory techniques are available to couple aromatic nitro compounds to proteins, a step necessary for raising antibodies, since small molecules are not antigenic. Examples are the reactions of proteins with dinitrofluorobenzene or dinitrobenzenesulfonate to prepare DNP-protein conjugates or the reaction with trinitrobenzenesulfonate for the preparation of TNP-protein conjugates.^{64 89 90} Secondly, the di- and trinitrophenylated proteins are excellent antigens. Thirdly, the fluorescence quenching capabilities of aromatic nitrocompounds permitted interesting studies of antigen-antibody reactions.^{63 64}

On the basis of these studies, it could be expected that the prospects were good for developing a satisfactory antibody for TNT capable of providing the specificity in an immunochemical TNT detection scheme. The second requirement to be fulfilled before a specific and sensitive immunoassay for TNT could be considered was the availability of a new and highly sensitive readout mechanism. The use of radioactive isotopes for readout purposes is not desirable for concepts to be applied ultimately by soldiers in the field. Fortunately, not only radiation emitted from radioactive isotopes can be measured with great sensitivity but also light. This thought led to the idea of developing immunoassay techniques utilizing light emission for readout. As described in an earlier section of this report, bioluminescent reactions provide very high sensitivities. These light emitting reactions are catalyzed by enzymes called luciferases. Since enzymes are proteins, it should be possible using reagents such as mentioned in the previous paragraph to prepare conjugates consisting of luciferase enzymes and nitro aromatic compounds. It is obvious that such conjugates, comprising a light emission catalyzing enzyme, could be used for a light emission immunoassay like radioactive isotopes are used in radioimmunoassay. Both the bacterial and firefly luciferase enzymes could be considered. Detection schemes could also be envisioned in which the luciferase enzyme is coupled to the antibody. TNP groups bonded to surfaces of solids would be used to trap such luciferase antibody conjugates. Free TNT could effect the release of such conjugates and the light produced by the released conjugate would be a sensitive indication for the presence of the free TNT. Alternatively, detection schemes could be imagined in which specific antibodies control the light emission from luciferase reactions, thus providing a basis for a homogeneous immunoassay.

⁴⁵ H. N. Eisen and G. W. Siskind, *Biochemistry* 3, 996 (1964).

⁴⁹ Young Tai Kim, D. Greenbaum, P. Davis, Sr., A. Fink, Th. P. Werblin, and G. W. Siskind, *The J. of Immunology*, 114, 1302 (1975).

⁵⁵ A. Froese, *Immunochemistry* 5, 253 (1968).

⁶³ S. F. Velick, C. W. Parker, and H. N. Eisen, *Proc. Natl. Acad. Sci.*, 46, 1470 (1960).

⁶⁴ J. R. Little and H. N. Eisen, *Biochemistry*, 5, 3385 (1966).

⁸¹ L. A. Day, J. M. Sturtevant, and S. J. Singer, *Ann N. Y. Acad. Sci.* 103, 611 (1963).

⁸² E. D. Day, *Advanced Immunochemistry*, William and Wilkins, Baltimore (1972).

⁸³ B. G. Barisas, S. J. Singer, and J. M. Sturtevant, *Immunochemistry*, 12, 411 (1975).

⁸⁴ K. A. Kelly, A. H. Schon, and A. Froese, *Immunochemistry* 8, 613 (1971).

⁸⁵ I. Pecht, D. Givol, and M. Sela, *J. Mol. Biol.* 68, 241 (1972).

⁸⁶ J. R. Little and H. N. Eisen, *Biochemistry* 6, 3119 (1967).

⁸⁷ D. M. Jacobs and D. C. Morrison, *The J. of Immunology* 114, 360 (1975).

⁸⁸ J. L. Winkelhake and E. W. Voss, *Biochemistry* 9, 1845 (1970).

⁸⁹ H. N. Eisen, S. Belman, and M. E. Carsten, *J. Am. Chem. Soc.* 75, 4583 (1953).

⁹⁰ T. Okuyama and K. Satake, *J. Biochem (Tokyo)* 47, 454 (1960).

Of particular interest was the potential for signal amplification provided by the bacterial luciferase reaction system. A main feature of this system is that a biochemical agent called NADH is transformed into photons. It also happens that there are many dehydrogenase enzymes, also referred to as NAD oxidoreductase enzymes, which catalyze reactions in which NADH is formed. Potentially, all these dehydrogenase enzymes could be linked with the TNP groups and then used, in conjunction with an antibody for TNT and the bacterial luciferase system, to design light emission immunoassay systems for TNT. The important consideration, here, is that some of these dehydrogenase enzymes produce 10^5 NADH molecules per minute, thus a fabulous amplification of the signal could be expected to take place.

For the realization of the ideas outlined above a laboratory was desirable, possessing experience and expertise in the area of the light emitting biochemical reactions (luciferase reactions), in the area of immunology and in protein chemistry in general. There are many laboratories with experience in protein chemistry as well as immunology but only a few which have experience with light emitting biochemical reactions. It was fortunate, therefore, that one of the most experienced and respected laboratories in this field was interested in developing the light emission immunoassay concept using nitro aromatic compounds as model compounds. It is the laboratory of Professor M. A. DeLuca at the University of California at San Diego, LaJolla which continues the pioneering work of W. D. McElroy (now Chancellor of the University of California, San Diego) who was a leader in this field for decades. It is also advantageous that Professor DeLuca and co-workers had succeeded in immobilizing the enzymes involved in the bacterial luciferase reaction on porous glass beads which are then bonded to small glass rods.^{91 92} In this form, these enzymes can be reused many times and provide a convenient and inexpensive method of detecting, via light emission, any substance which causes a change in NADH concentration.

The efforts to develop a light emission immunoassay technique for TNT detection, as performed in the laboratory of Professor DeLuca, will be discussed below. This discussion will have a preliminary character because the work is ongoing at the present time.

5. Discussion of the Light Emitting Reactions Used. Before proceeding to the discussion of the development of light emission immunoassay, it is advisable to present a brief description of the luciferase reaction systems used.

⁹¹ E. Jablonski and M. DeLuca, Proc. Natl. Acad. Sci., USA, 73, 3848 (1976).

⁹² E. Jablonski and M. DeLuca in "Methods in Enzymology," Vol. LVII, (ed), p. 202 (1978).

The bacterial luciferase system referred to repeatedly in this report consists of two reactions. The first reaction is catalyzed by the NADH: FMN oxidoreductase (FMN reductase)

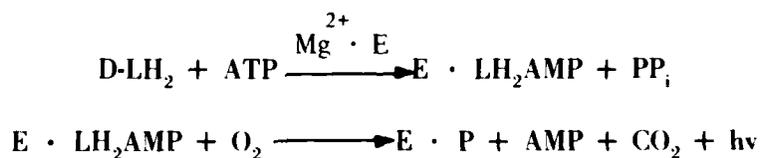


whereby FMN (flavin mononucleotide) is reduced by NADH (nicotinaemide-adenine dinucleotide) to FMNH₂ (the reduced form of FMN). The latter is being utilized in the second reaction, catalyzed by the luciferase enzyme.



In this reaction, FMNH₂ reduces molecular oxygen in the presence of a long chain aldehyde. This reaction is accompanied by the emission of light (hν). Much research on these reactions was performed by J. W. Hastings who wrote exhaustive reviews on the subject.^{93 94} Both enzymes are isolated from marine bacteria which can be obtained in sufficient quantities by fermentation processes. The purified enzyme is very stable.

The other luciferase system utilized in the work on light emission immunoassay is the firefly luciferase system. The firefly luciferase catalyzes, in the presence of a compound referred to as luciferin (D-LH₂), ATP (adenosine triphosphate) and molecular oxygen the following reactions:



⁹³ W. J. Hastings and K. H. Neelson. *Ann. Rev. Microbiol.* 31, 549 (1977).

⁹⁴ W. J. Hastings in "Methods in Enzymology," Vol LVII, M. A. DeLuca (ed.), page 125 (1978).

The first reaction leads to the formation of enzyme bound luciferyl adenylate and pyrophosphate. The second reaction requires a stoichiometric amount of oxygen and leads to the formation of an enzyme bound excited product which subsequently decomposes which is accompanied by the emission of light.⁹⁵⁻⁹⁷ Also formed are adenosine monophosphate and carbon dioxide.

The important fact from the viewpoint of analysis and detection is that the light emitted depends on the ATP concentration. ATP is of great importance in the energy household of living organisms and is either produced or consumed by many enzymatic reactions. Consequently, the firefly luciferase reaction provides a highly sensitive means of detecting and analyzing many important compounds with great sensitivity. Usually, the injection of the complete luciferase reagent into an ATP containing solution results in a flash of light whose intensity provides a sensitive measure for the ATP concentration. In 1976, using a purified luciferase preparation, Lundin et al. defined conditions resulting in an almost constant light emission with a negligible decay rate at ATP concentrations less than $1 \mu\text{m}$.⁹⁸ This development has further increased the great value of the firefly luciferase system to the analytical chemist. The factors affecting the kinetics of light emission from crude and purified firefly luciferase were elucidated by DeLuca et al.⁹⁹ The luciferin required for this reaction can be synthesized but the luciferase enzyme must be isolated from fireflies. This enzyme was first purified by Green and McElroy.¹⁰⁰ The purified enzyme can be stored indefinitely at 10-12 mg/ml in 10 percent ammonium sulfate solution at pH 7.8 and $+ 4^{\circ}\text{C}$.

6. Discussion of the Experimental Work. Work on the development of light emission immunoassays and their application for the detection of low levels of TNT will be discussed in this paragraph. It is a preliminary account because the work is still in progress.

a. Antibody Preparation. Since the immunosystem does not respond to small molecules such as TNT, the first step to be taken was the preparation of a protein-TNP conjugate which could serve as an antigen.

⁹⁵ W. D. McElroy and M. DeLuca in *Chemiluminescence and Bioluminescence*, M. J. Cormier, D. M. Hercules, and J. Lee (eds), p. 285, Plenum, NY (1973).

⁹⁶ M. DeLuca, *Adv. Enzymol.* 44, 37 (1976).

⁹⁷ M. DeLuca and W. D. McElroy in *Methods in Enzymology*, Vol LVII, M. A. DeLuca (ed.).

⁹⁸ A. Lundin, A. Richardson, and A. Thore, *Anal. Biochem.* 75, 611 (1976).

⁹⁹ M. DeLuca, J. Wannlund, and W. D. McElroy, *Anal. Biochem.* 95, 194 (1979).

¹⁰⁰ A. A. Green and W. D. McElroy, *Biochem. Biophys. Acta*, 20, 170 (1956).

(1) Antigen Preparation. Bovine serum albumin (BSA) was selected as the carrier protein. For the synthesis of the complete antigen the BSA was trinitrophenylated by reaction with 2,4,6 trinitrobenzenesulfonate (TNBS) in 0.2 M sodium borate buffer at pH 9.2. The reaction was allowed to proceed for 8 hours at 40°C. Then the reaction product was purified by dialysis and chromatography with a G-25 sephadex column. The degree of derivatization was determined spectrophotometrically at 350 nm where the molar extinction coefficient A_{TNP} is 15,400. Only BSA-TNP conjugates having at least 35 TNP groups per BSA molecule were used as antigen.

(2) Immunization. Both rabbits and goats were immunized with the BSA-TNP antigen having a minimum of 35 TNP groups per BSA molecule. The rabbits were injected in the lymph nodes in the case of the primary injection. After 4 weeks, booster injections were given using 1 mg BSA-TNP mixed with incomplete Freund's Adjuvant; i.e., water-oil emulsions. Ten days later, 50 ml blood was taken from the rabbits. After four days, the boosting and bleeding schedule was repeated as described above. The goats were injected at 6 sites, 2 intramuscular and 4 subcutaneous, and were subjected to the same boosting schedule as the rabbits but with 15 mg of the BSA-TNP per injection.

(3) Antibody Isolation, Purification, and Immobilization. The blood of the animals was freed of the red blood cells by centrifugation and the resulting serum was purified by fractionating ammonium sulfate precipitation. For further purification of the antibodies an affinity chromatographic column was prepared by linking TNP groups to Sepharose 4B using 1,6 hexanediamine as spacer arm. The trinitrophenylated sepharose 4B was poured into a column and served for affinity chromatographic purification of the TNP antibody.

Antibodies are easier to handle and more useful for analysis purposes when immobilized on solids. For immobilization of the TNP antibody, sepharose 4B was activated with CNBr and the antibody reacted with the activated sepharose. The immobilization procedure results in 80 percent of the antibody being bound to the sepharose whereby 24 mg of protein is bound per gram of sepharose. For storage the sepharose-antibody is kept in PBS buffer (phosphate buffered saline) at 1 gm sepharose-antibody per ml of buffer of pH 7.4. Sodium azide (0.02 percent) is added to prevent bacterial growth. During a 6-month period no loss in binding activity was observed.

Antibodies were also immobilized on sepharose CL 4B. With this support material less nonspecific adsorption took place which was important in the work on amplified light immunoassay.

Some difficulties were encountered at the beginning of the efforts on TNP antibody preparation. It appeared that the serum of the injected animals did not contain the antibody until it was discovered that washing with 1 N acetic acid provides the missing binding activity. It became clear that the TNP antibody possesses so much affinity that the more conventional, gentler washing procedures did not remove the BSA-TNP used for stimulation of the antibody formation. Washing with 1 N acetic acid of all immobilized TNP antibody preparations became a standard procedure.

Antibodies for DNP (the dinitrophenyl group) were obtained commercially and were immobilized in the same manner as discussed above.

b. Preparation of Enzyme-Antigen Conjugates. In the chapter on Development of the Light Emission Immunoassay Concept it was described how luciferase enzymes and dehydrogenase enzymes could be used as labels to provide a basis for the development of light emission immunoassay systems. The preparation of the required enzyme TNP and enzyme DNP conjugates will be discussed in this paragraph.

To accomplish the linking of TNP groups to the luciferase enzymes the reaction with TNBS (trinitrobenzenesulfonate) was used. The trinitrophenylation reaction was performed at luciferase to TNBS ratios ranging from 1:1 to 1:300. At the high ratio, up to 35 TNP groups were coupled to the luciferase, which resulted in the loss of the enzymatic activity. Fortunately, high trinitrophenylation is not required for providing a specific response to the antibody and the objective was to link 1-2 TNP groups to the luciferase enzymes. This was accomplished using a luciferase to TNBS ratio of 1 to 5. At this degree of trinitrophenylation the bacterial luciferase enzyme was 60 to 70 percent as active as in its natural state. The firefly luciferase lost more of its activity but by protecting the active site with substrate (ATP-Mg), adding 10^{-4} M DTT (Dithiothreitol) for sulfhydryl group protection as well as the addition of 10^{-3} M EDTA (ethylenediaminetetraacetic acid) for chelating impurity cations, it was possible to prepare a trinitrophenylated luciferase possessing 80 percent of activity of the enzyme in its natural condition.

DNP-luciferase conjugates were prepared using the FDNB (dinitrofluorobenzene) reagent. The results obtained were analogous to those discussed above for the TNP-luciferase conjugate.

Glucose-6-phosphate dehydrogenase is a well-known commercially available, very stable enzyme catalyzing the oxidation of d-glucose-6-phosphate by NAD whereby NADH is formed. In addition to the desirable properties already listed, this enzyme has a turnover number of 70,000; i.e., one mole of enzyme catalyzes the formation of 70,000 mole of NADH, resulting in a much enhanced light signal when the bacterial luciferase reaction is used for readout. To make use of this amplification system in an immunoassay for TNT detection, the

glucose-6-phosphate dehydrogenase enzyme had to be linked with TNP groups. For this purpose, the TNP and DNP conjugates of glucose-6-phosphate dehydrogenase were prepared using the TNBS and FDNB reagents. Great excesses of these reagents were required to prepare the conjugates at pH 7.8, but at pH 9 a 10 fold excess was sufficient to couple one TNP or DNP group to one enzyme molecule. Such conjugates had 80 percent of the activity of the enzyme in its natural state. They were stable in 0.1 M phosphate buffer at pH 7.8 and 4°C for 6 months without any loss in activity.

c. Assay Development. The objective of this effort was to determine the feasibility of the light emission immunoassay concept and to achieve the lowest possible detection limit for TNT. In accordance with this objective the conventional laboratory techniques of immunochemistry were applied and no attempts were made to date to simplify the procedures and to shorten the assay times. Light emission assays using TNP-luciferase conjugates were developed as well as a light emission assay using the amplification provided by the use of the TNP-glucose-6-phosphate dehydrogenase conjugate. The preparation of the necessary reagents was discussed in earlier paragraphs of this section; their use and the results obtained will be discussed here.

The idea of a homogeneous light emission immunoassay was mentioned in a previous paragraph. This idea was prompted by reports in the literature that the luciferase enzyme of the light emitting marine ostracod crustacean *Cypridina Hilgendorffii* is inhibited when it becomes bound to its antibody.¹⁰¹⁻¹⁰³ In analogy, it could be expected that luciferase-antigen conjugates such as TNP-luciferase would be inhibited by TNP antibodies. Such an inhibited TNP-luciferase could serve as the reagent of a homogeneous immunoassay because competition for antibody sites would take place in the presence of free TNT, resulting in liberating some of the TNP-luciferase molecules from the antibody. This would restore their catalytic activity and result in an increase in light emission indicating the presence of free TNT. Initial experiments showed this expectation to be true, but the inhibition of the luciferase activity by the antibody was too small to promise high sensitivity. Consequently, emphasis was placed on the development of other light emission immunoassay techniques. It may be worthwhile, however, to resume these efforts using a technique which permits linking the TNP groups at a location to the enzyme which is in the neighborhood of the catalytically active site. A drastic inhibition of the enzyme by the antibody may be expected in this case.

101 F. I. Tsuji and D. L. Davis, *J. of Immunology*, **82**, 153 (1959).

102 F. I. Tsuji, D. L. Davis, and E. M. Gindler, *J. of Immunology*, **88**, 83 (1962).

103 F. I. Tsuji, D. L. Davis, and D. H. Donald, *J. of Immunology*, **96**, 614 (1966).

A light emission immunoassay utilizing the immobilized antibody was developed. It requires three steps. In the first step, the immobilized antibody, the TNP-luciferase, and the free TNT, whose concentration is of interest, are incubated together; i.e., they are made to react with each other. This reaction is slow and requires about 1 hour to reach equilibrium. For detection purposes, however, it will not be necessary to wait for equilibrium conditions, and shorter incubation periods will be possible. In addition, decreasing of the incubation time could be accomplished by incubating the antibody first with the TNP-luciferase and allowing the free TNT to displace the bound TNP-luciferase. This procedure is expected to be faster since small molecules such as TNT require less incubation time than large molecules such as the TNP-luciferase. The antibody-TNP-luciferase complex will constitute, in this case, a preprepared reagent. The second step in the current technique is the washing of the immobilized antibody to remove the free TNT and, in particular, the free TNP-luciferase molecules. In the third step the light emission, catalyzed by the TNP-luciferase bound to the immobilized antibody, is measured in the presence of the appropriate reagent. The light emitted is a function of the concentration of the free TNT because of the competition of the free TNT and the TNP-luciferase for antibody binding sites. The lower detection limit was found to be 50 femto mole of TNT. This detection scheme is represented on Figure 11. The experimental results are displayed on Figures 12 and 13 which show the change in light emission as a function of the TNT quantities present. The concept described was also tested in work directed toward DNT detection. Somewhat higher detection limits were obtained.

Some work was also performed on a immunoassay in which antibodies (non immobilized) were incubated with TNP-luciferase and the free TNT, followed by precipitation with a second antibody. After a washing procedure, the light emission catalyzed by the precipitate is measured and provides a measure for the concentration of free TNT. This double antibody technique promises high sensitivity but requires more steps than the technique with the immobilized antibodies and is less promising as far as automation is concerned.

The most promising concept from the points of view of detection limit as well as potential for automation is the amplified light emission immunoassay, briefly outlined in the paragraph on concept development as well as in paragraph 6b. The glucose-6-phosphate dehydrogenase enzyme was chosen since it is a well characterized, commercially available, very stable enzyme whose turnover number of 70,000 promised a great amplification effect. Glucose-6-phosphate dehydrogenase multiplies the number of NADH molecules resulting in a much enhanced light signal of the bacterial luciferase system. Due to this amplification effect, as little as 10^{-10} mole of glucose-6-phosphate dehydrogenase can be detected. Therefore, it was to be expected that the detection limit for TNT could be lowered with such an amplifying system until the final limitation, given by the affinity constant of the antibody, is reached.

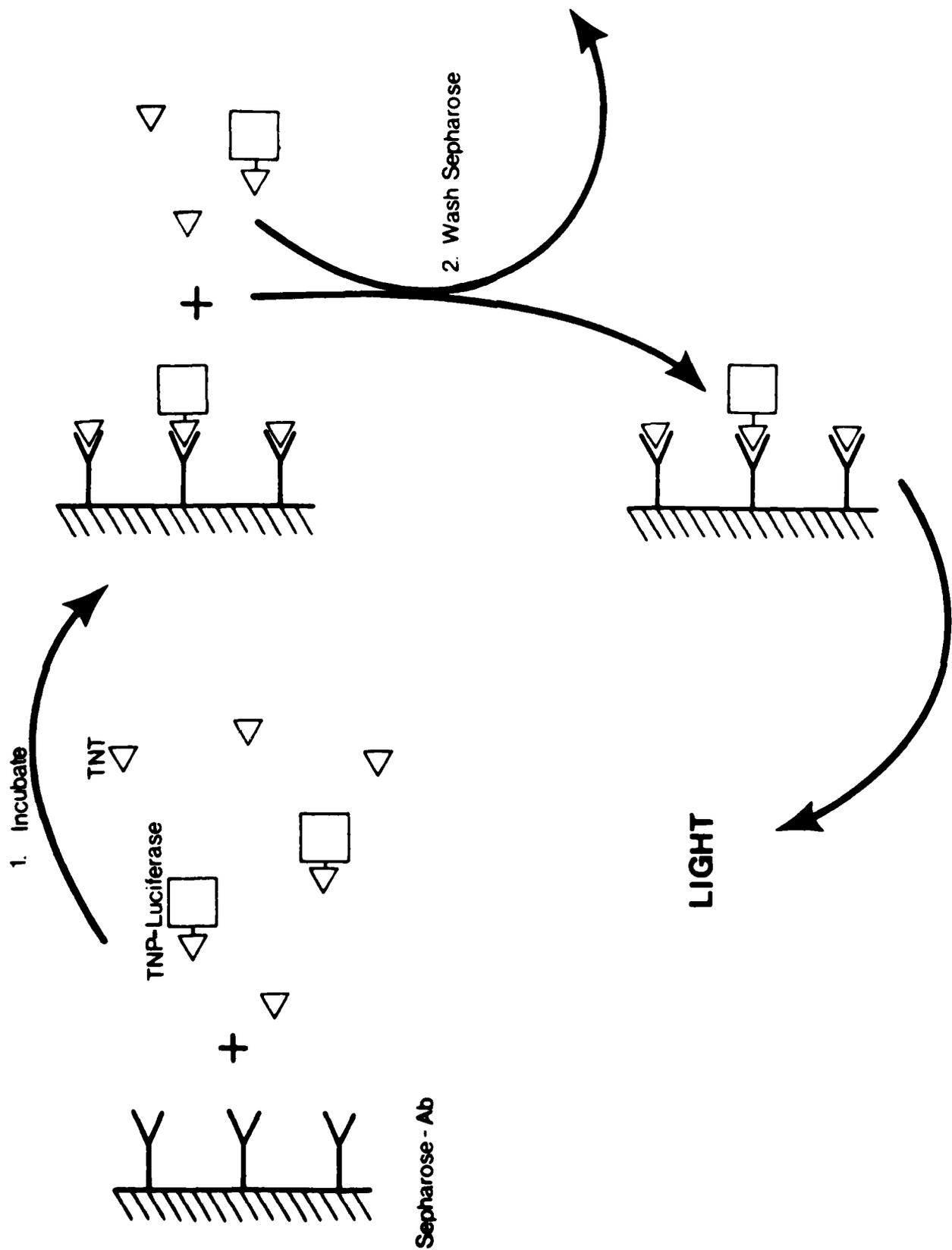


Figure 11. Schematic description of the light emission immunoassay technique.

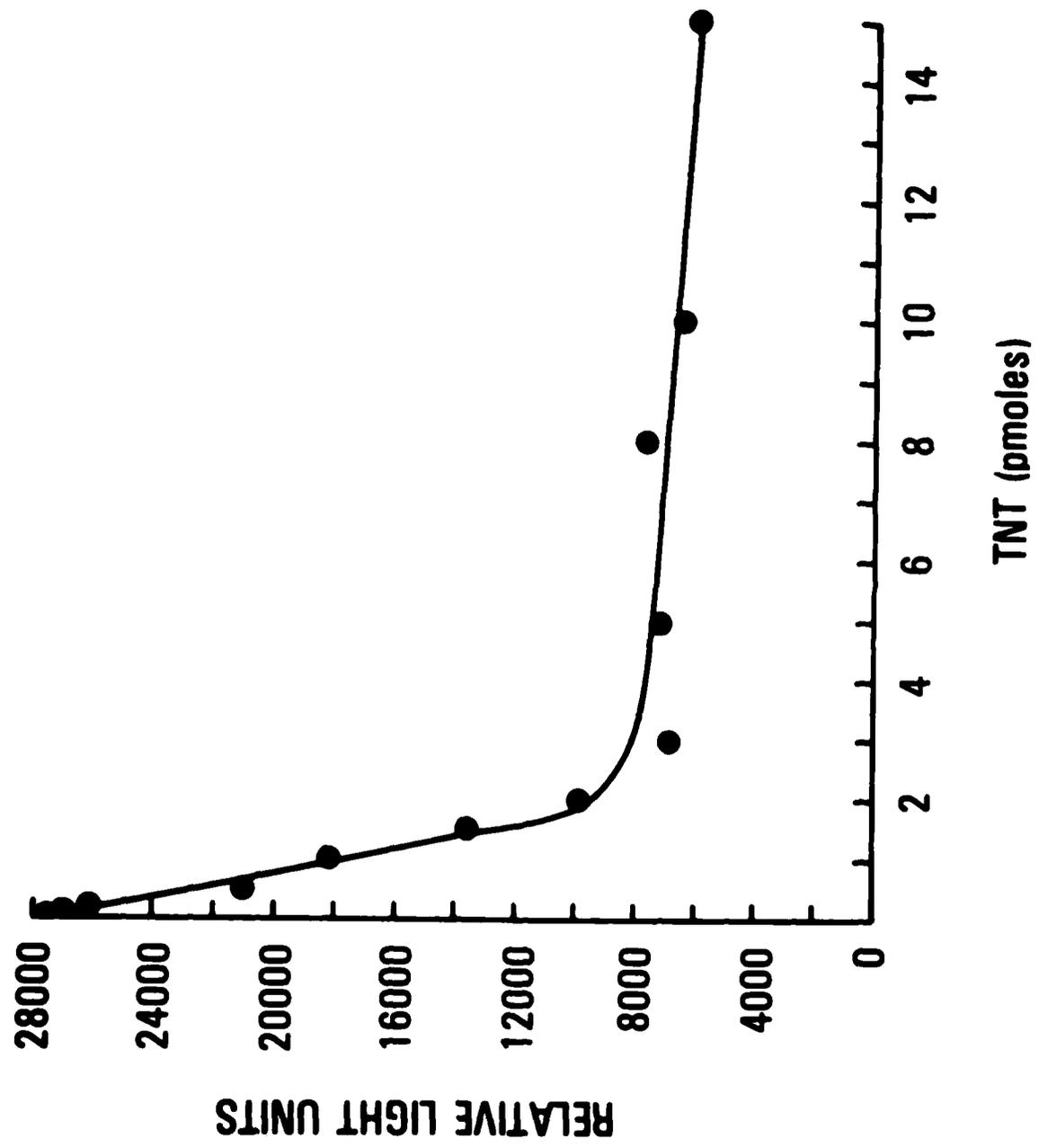


Figure 12. Light emission due to the antibody bound TNP-luciferase as a function of free TNT.

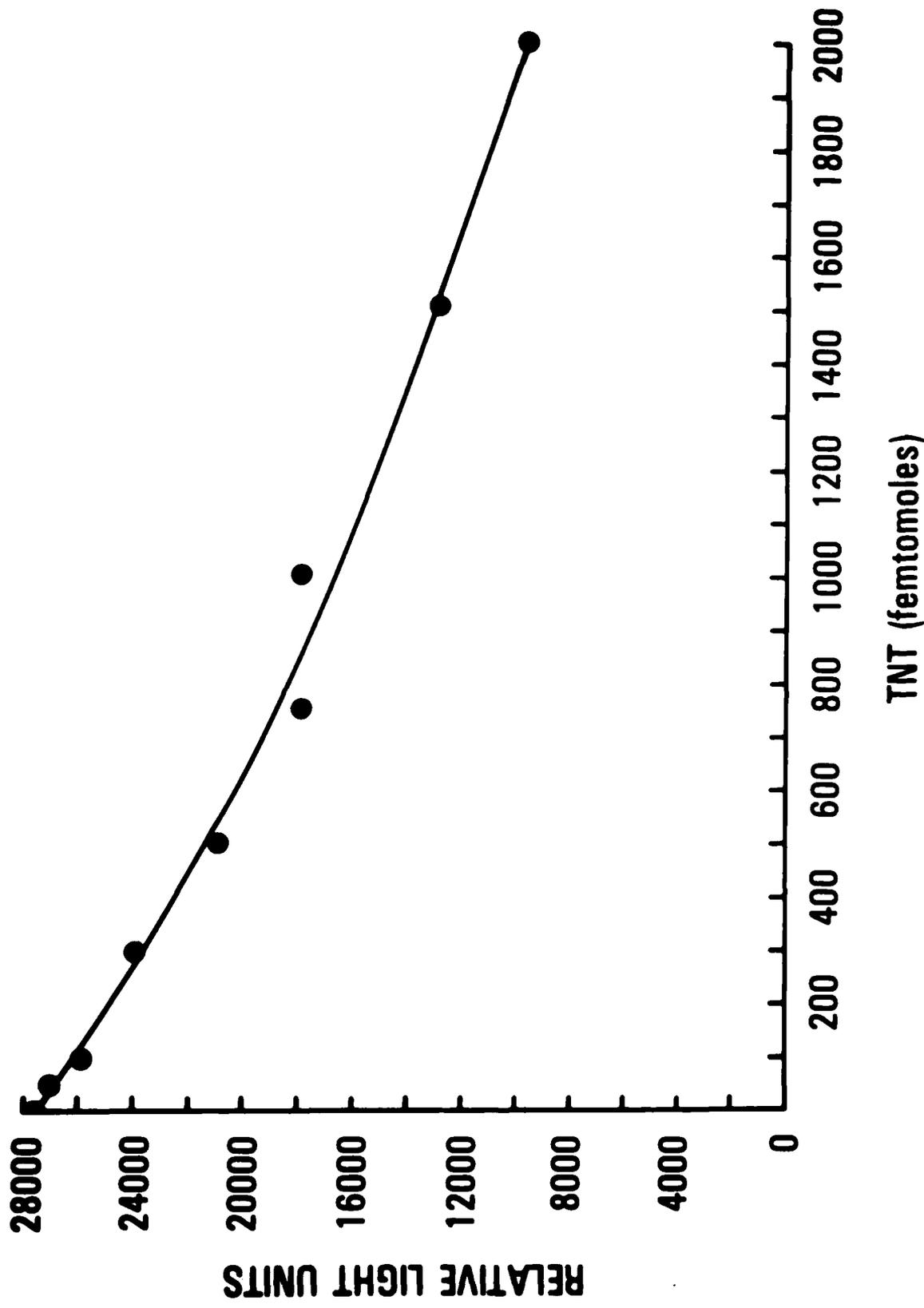


Figure 13. Light emission due to the antibody bound TNP-luciferase as a function of free TNT.

The amplified light emission immunoassay concept is schematically shown on Figure 14. The TNT molecules interact first with the immobilized antibodies, followed by the interaction of these antibodies with the TNP-glucose-6-phosphate dehydrogenase conjugate molecules and a washing step. This sequential reaction procedure promotes the utilization of the highest affinity antibody sites by the TNT. In the next reaction step the antibody bound TNT-glucose-6-phosphate dehydrogenase is given the opportunity to do its work, mainly to catalyze the reaction:



In accordance with the turnover number of the glucose-6-phosphate dehydrogenase, 70,000 NADH molecules are formed per enzyme molecule per minute. Due to this amplification effect, it was possible to detect as little as 10^{17} mole of TNT. The experimental results are displayed on Figure 15 where the change in light emission as a function of TNT quantities is shown. Figure 16 illustrates the linear increase of the amplification effect with time. Before concluding this section it should be added that, using tritium labeled compounds, it was established that the antibody provides the same number of sites for the TNT as for the TNP-glucose-6-phosphate dehydrogenase molecules.

7. Summary. A new immunoassay technique for TNT detection was developed using light emitting reactions as a highly sensitive readout. The concept was conceived at MERAD-COM, the experimental work is being performed at the University of California, San Diego, and is still in progress. The standard light emission immunoassay developed allows the detection of 50 femto mole of TNT. The amplified light emission immunoassay technique developed is capable of detecting 10^{17} mole of TNT, a detection sensitivity not nearly matched by any other technique. Immunoassay techniques are based on processes requiring time and, therefore, a future detector, based on such techniques, will be best suited for scenarios where speed is not important. Both the standard and, in particular, the amplified light emission immunoassay technique have the potential for being developed into a practical, continuous flow detection system.

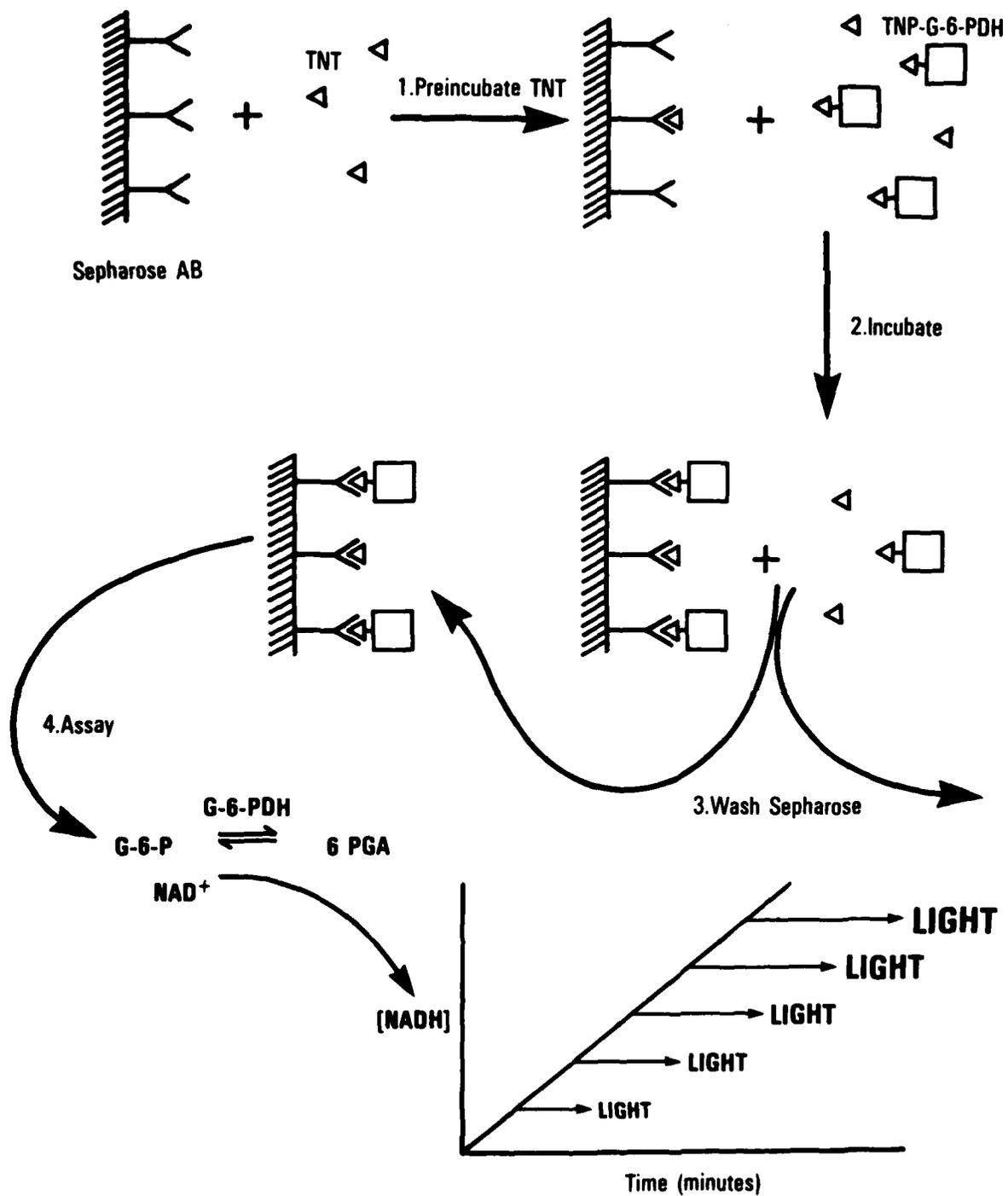


Figure 14. Schematic description of the amplified light emission immunoassay technique.

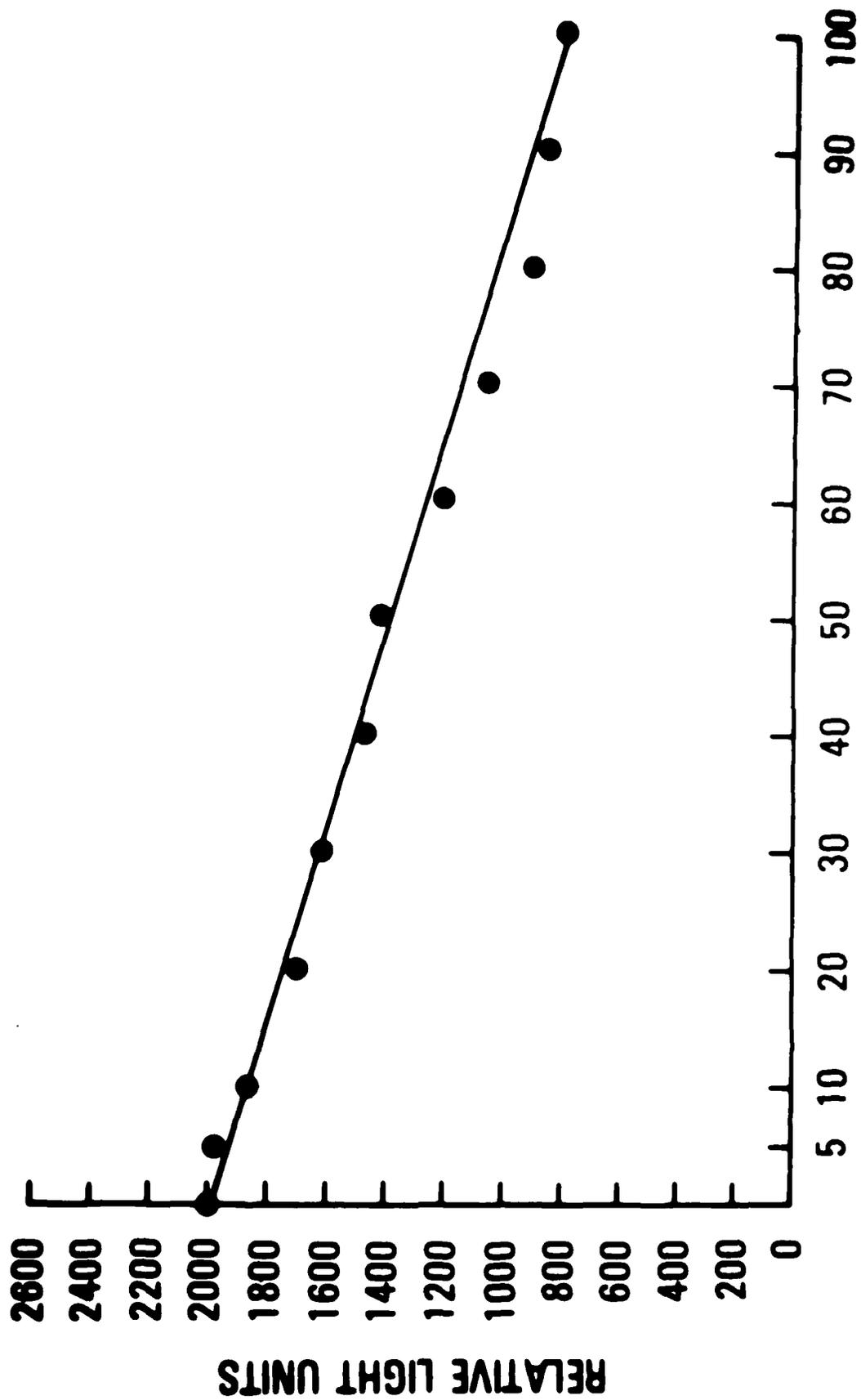
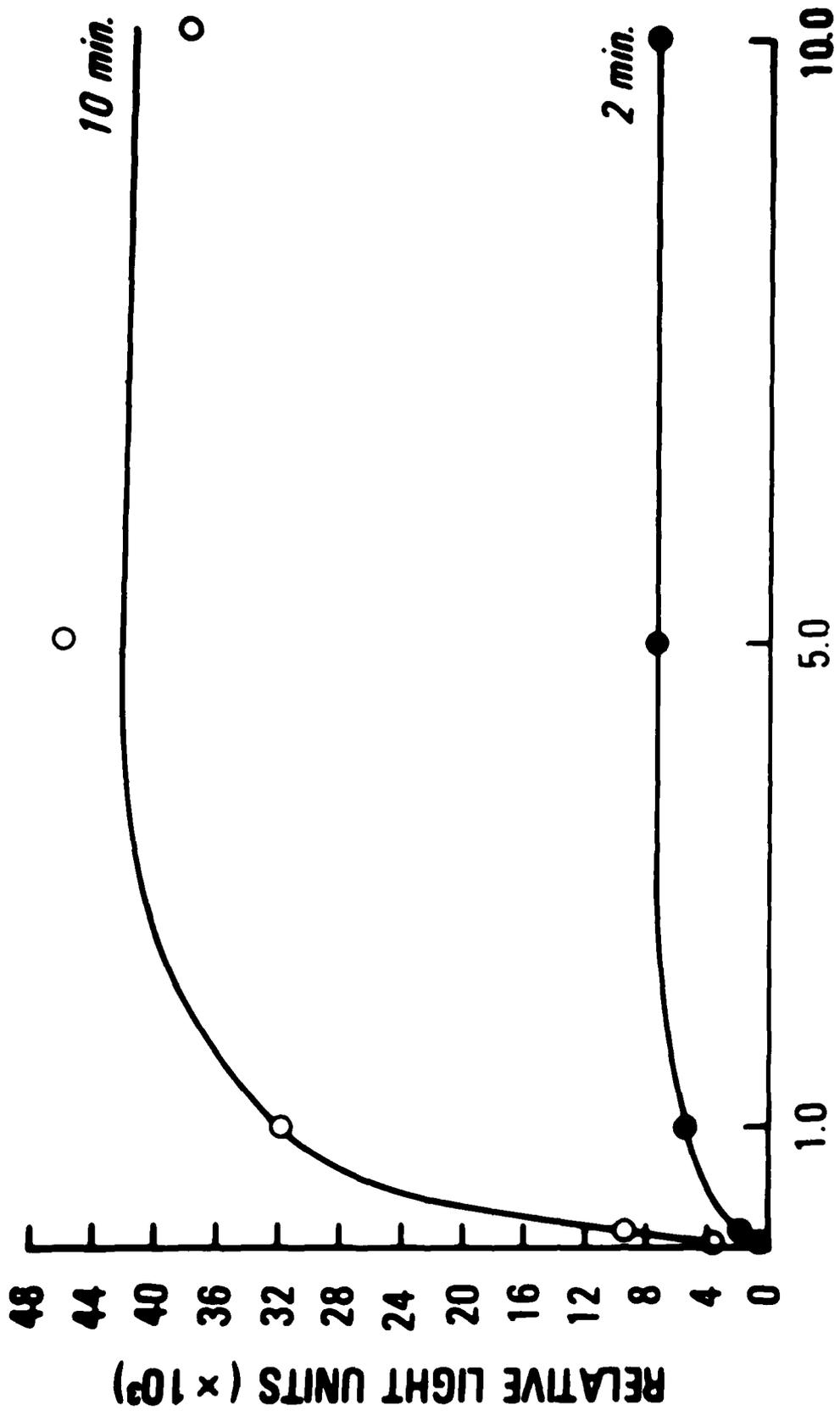


Figure 15. Amplified light emission due to the antibody bound TNP-glucose-6-phosphate dehydrogenase as a function of free TNT.



G-6-PDH-TNP (pmoles)

Figure 16. Binding curve of TNP-glucose-6-phosphate dehydrogenase (G-6-PDH) to antibody. Light emission due to the antibody bound G-6-PDH after a 2-min and a 10-min reaction period.

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