DEVELOPMENT OF SENSITIVE ASSAYS FOR BIOINDICATORS IN GROUND WATER

By

JEFFREY LANE HOWARD

Bachelor of Science

Northwestern Oklahoma State University

Alva, Oklahoma

1975

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1980

DEVELOPMENT OF SENSITIVE ASSAYS FOR

BIOINDICATORS IN GROUND WATER

Thesis Approved:

Franklin R. Leach
Thesis Advisor

Ulrich K. Melcher

Margaret K Essenberg

Norman N Durham

Norman N Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

The author wihses to express his sincere appreciation to his major advisor, Dr. Franklin R. Leach, for his guidance and friendship during the time spent in his laboratory. The advice and help of the other committee members, Dr. Ulrich K. Melcher, Dr. Margaret K. Essenberg, and Dr. Norman N. Durham, are also acknowledged.

The help and encouragement of JoAnn J. Webster, Dr. Jenq C. Chang and Phyllis M. Taylor is sincerely appreciated. Their technical assistance and sympathetic ears were always available when they were needed. A special thanks is given to Jerri Jackson and Vivian Curtis for preparation of the figures shown in this study.

Financial assistance provided by the Department of Biochemistry at Oklahoma State University and the Environmental Protection Agency (Grant No. R804613) is gratefully acknowledged. The author also commends the faculty of the Department of Biochemistry for providing a high standard of research and academic achievement which stimulates those who want to learn.

Most of all, I want to acknowledge the unfailing encouragement and support of my wife, Susan, whose love and understanding made the effort and achievement of graduate school meaningful and satisfying.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AMP - Adenosine Monophosphate

ADP - Adenosine Diphosphate

ATP - Adenosine Triphosphate

E. coli - Escherichia coli Crookes strain, American Type Culture Collection No. 8739

EDTA - Ethylenediaminetetraacetic Acid

FMN - Flavin Mononucleotide

Hb - Hemoglobin

INT(F) - 2-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride (formazan)

I(ΔI) - Fluorescence intensity or change in fluorescence intensity

Luminol - 5-amino-2,3-dihydro-1,4-phthalazinedione

M-9 - Isotonic salt solution used as a minimal medium for E. coli

NAD(H) - Nicotinamide adenine dinucleotide (reduced)

NADP(H) - Nicotinamide adenine dinucleotide phosphate (reduced)

ng - Nanogram, 10^{-9} gram

nmol - Nanomole, 10^{-9} mole

Pk. Ht. - Peak Height

pg - Picogram, 10^{-12} gram

pmol - Picomole, 10^{-12} mole

TTC - 2,3,5-triphenyltetrazolium chloride

TPF - Triphenyl formazan

Tris - 2-amino-2-(hydroxymethyl)-1,3-propanediol

CHAPTER I

INTRODUCTION

Like any other organism, man is inextricably dependent upon the resources of the earth. Until recent years, management or conservation of resources - water, energy, minerals, forests, and crops - was considered unnecessary since the supply of resources far exceeded man's demand for them. However, the rapid depletion and frequently callous use of resources has led to the realization that man must manage these resources so they will be available for future use.

Of the resources named, water is perhaps the most important since it is required for life itself. Water comprises over 70% of the weight of an Escherichia coli cell and its physical properties - heat of vaporization, specific heat, surface tension, polarity, and degree of ionization - are important factors in determining the structure and biological properties of proteins, nucleic acids, membranes, and many other cell components. In addition to being the solvent for almost all biological molecules, water is often a reactant in biochemical reactions, acting as proton donor or acceptor in many reactions or affecting the ionization or polarity of another reactant. Because of its physical and biochemical properties, water has shaped the ways in which biological processes occur in cells and organisms.

As water is a vital component of biological systems, it is also a vital component of the health and economy of the United States. Water

resources consist of surface waters - lakes, rivers, and streams - and subsurface water - ground water and water from unsaturated soil zones. Ground water resources (water at or below the level of the subsurface water table) become more important as surface water supplies are used and abused. Approximately 20% of all water used and 50% of all drinking water in the United States comes from ground water supplies. About 95% of the nation's fresh water reserves are ground water (1). These reserves must be protected and conserved if America is to maintain her growth and prosperity.

The location of ground water beneath a zone of unsaturated soil and water movement through soil is both an aid and a problem in protecting it from pollution. Ground water is protected from the introduction of pollutants by the requirement that they percolate through the soil before reaching the water table (except for the practice of injecting wastes into wells). The soil and its associated biological activity act upon and usually detoxify wastes before they reach the ground water. On the other hand pollutants that do enter a ground water supply are cleared very slowly because of the slow movement of ground water through soil, taking years in some cases to be washed or diluted out. Once a ground water supply is contaminated, it is almost impossible to clean up in any way but the slow dilution or degradation of the contaminants. For this reason it is important to detect and, if possible, correct the potential pollution of ground water before it is contaminated and lost to use for years or decades.

The fate of pollutants percolating through the unsaturated zone of soil and in ground water depends upon the physical properties of the soil and the amount of biological activity in the soil and ground water. The

soil can react with pollutants through sorptive and chemical processes. The biological activity (enzymes and microorganisms) in the soil and ground water may act upon and change pollutants. These pollutants may also modify the biological activity, increasing or decreasing it or changing the type of activity. Detection and quantitation of these modifications may provide an early signal of pollution of ground water.

Biological Indicators

Because pollutants can modulate the amounts of biochemicals, enzymes, or the number of organisms present in the subsurface environment, variations in these entities can be indicative of the appearance of pollution in the environment. These components of the biological activity of soil and ground water are called biological indicators or bioindicators.

To be useful in detecting and quantitating pollution, a bioindicator must have the following properties:

1. specifically required for biological activity
2. constant amount per unit of living material
3. rapidly degraded or inactivated after cessation of biological activity
4. present in measurable amounts in all living material
5. amenable to specific and sensitive determination.

A living organism is composed of many potential bioindicators - proteins and enzymatic activities, nucleic acids, membrane and cell wall components, metabolites, and cofactors. In addition the number and types of organisms present can be a bioindicator. The approximate composition of an E. coli cell is shown in Table I (2).

TABLE I

APPROXIMATE CHEMICAL COMPOSITION OF AN *E. COLI* CELL

Component	Percent of total cell weight	Number of molecules per cell	Number of different kinds
H_2O	70	4×10^{10}	1
Inorganic ions	1	2.5×10^{8}	20
Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Cl^-, PO_4^{3-}, SO_4^{2-}, etc.			
Carbohydrates and precursors	1	2×10^{8}	200
Amino acids and precursors	0.4	3×10^{7}	100
Nucleotides and precursors	0.4	1.2×10^{7}	200
Lipids and precursors	2	2.5×10^{7}	50
Other small molecules	0.2	1.5×10^{7}	250
Proteins	15		$2\text{-}3 \times 10^{3}$
Nucleic acids			
DNA	1	4	1
RNA	6		
16S rRNA		3×10^{4}	
23S rRNA		3×10^{4}	
tRNA		4×10^{5}	1
mRNA		1×10^{3}	1000

The effect of a pollutant on the amounts of bioindicators in soil or ground water depends upon the nature of the pollutant. A toxic material could decrease the biological activity and the amounts of bioindicators; a nutrient could cause an increase in bioindicators. A pollutant could also cause a turnover or redistribution of the types of microorganisms present in the subsurface environment. However, it is believed that the infiltration of pollutants will perturb the steady state amounts of bioindicators and can be detected by these changes (1).

Analytical Chemistry of Bioindicators

The utility of a bioindicator as a means of detecting pollution depends upon the specificity and sensitivity of the analytical technique used to measure changes in the amounts of the bioindicators. Methods of determination of bioindicators include stoichiometric methods - spectrophotometry, fluorimetry, light scattering, luminescence; chemical methods - specific reactions; enzymatic methods - use of enzymes to measure bioindicators and measurements of enzymatic activities; and biochemical methods - measurement of cell growth, protein synthesis, and gas formations. These methods can be combined or coupled so that many cell components can be measured (Table II) (3).

In order to measure the amount of bioindicator in a soil or water sample it may be necessary to extract the bioindicator from soil or release it from cells. Concentration or some form of amplification of the bioindicator may also be necessary. Extraction procedures involve release of the bioindicator from soil or cells by organic solvents, detergents, EDTA, acids or alkali, or enzyme treatment. The procedure used depends upon the bioindicator desired and no one procedure can be

TABLE II

SENSITIVITIES OF CURRENT BIOCHEMICAL ANALYTICAL METHODS

Procedure	Quantity Measured		Reference
General Procedures			
Stoichiometric Procedures			
Gasometry			
Warburg Apparatus	300	µg	4
Cartesian Diver	0.1	ng	4, 5
Spectrophotometry			
Conventional 1 ml Cuvette	2	µg	4, 5, 6
Microcuvette	1	µg	4, 5, 6
Capillary tubes	1	ng	4, 5, 6
Luminescence	0.1	ng	7
Radioisotopic [^{14}C] Counting	1	ng	7
Fluorometry			
Standard 4 ml Cuvette	2	µg	7
Microcuvette	20	ng	7
Microscopic	0.5	pg	8
Cycling Procedures			
One-Enzyme System	1	µg	5, 7
Two-Enzyme System	0.1	ng	5, 7
Double Cycling	5	fg	5, 7
Gas Chromatographic Methods			
Flame Ionization	0.1	ng	5
Electron Capture	1	pg	5
Mass Spectrometry	5	ng	G. R. Waller*
Specific Procedures			
Amino Acids and Proteins			
Ion Exchange Microcolumn and Ninhydrin	10	µg	T. H. Liao*
Lowry Method	10	ng	F. R. Leach*
Spectrophotometry-Coomassie Brilliant Blue G-250	50	ng	F. R. Leach*
[^{3}H]Dansylation and Counting	50	ng	9
Fluorometry			
Orthophthalaldehyde	50	ng	F. R. Leach*

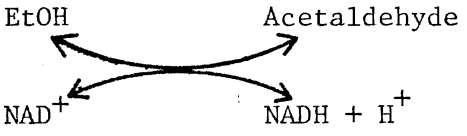
TABLE II (Continued)

Procedure	Quantity Measured	Reference
Hydrolysis and Orthophthalaldehyde	10 ng	F. R. Leach*
Fluorescamine	50 ng	10
ATP		
Spectrophotometry	0.1 µg	6
Luminescence	50 fg	F. R. Leach*
Fluorometry	50 pg	7
Double Cycling	5 fg	7
Pyridine Nucleotides		
UV Spectrophotometry	3.3 µg	7
Microspectrophotometry	0.6 ng	6
Fluorometry	60 ng	7
Enzymatic Cycling	0.6 pg	7
Double Cycling	0.06 fg	7
DNA		
Spectrophotometry		
Direct	1 µg	11
Diphenylamine	1 µg	F. R. Leach*
Fluorometry		
Diaminobenzoic Acid	0.2 µg	F. R. Leach*
Ethidium Bromide	0.4 ng	M. G. Rockley*
Diamidino-2-phenylindole	0.5 ng	F. R. Leach*
RNA		
Spectrophotometry		
Direct	1 ug	11
Orcinol	2.5 µg	F. R. Leach*
Fluorometry		
Ethidium Bromide	5 ng	12

* Personal Communication

used for all bioindicators. Concentration techniques include evaporation of extractant, dialysis, membrane filtration, and flocculation of bacteria. Again the method chosen depends on the determinations to be made.

It is possible in some instances to increase greatly the sensitivity of a determination by amplifying the bioindicator being measured. Amplification is an outcome of catalysis and is most easily understood using an enzyme model. Enzymes, lactate dehydrogenase (LDH) for example, act catalytically and produce many molecules of product per molecule of enzyme in a given length of time. If the product can then be measured with the same limit of detection as that of the enzyme itself, then the amount of enzymatic activity which can be detected has been reduced. For example, alcohol dehydrogenase (ADH) (Equation 1) reduces NAD^+ to



(1)

NADH in the presence of ethanol at a rate of 1000 molecules of NADH/min^{-1} molecule ADH^{-1}. After one minute there will be 1000 molecules of NADH per molecule of ADH. Since the fluorometric limit of detection for NADH and the protein ADH is approximately the same (60 ng, from Table II), measurement of NADH increases the sensitivity of detection of ADH 1000-fold.

It is also possible to amplify a bioindicator by changing it to something that can be measured with greater sensitivity. Measurement of pyridine nucleotides NAD^+ and $NADP^+$ can be amplified 10-fold by treatment with 10 N NaOH, which produces a product that is 10-times more fluorescent

than the native molecules. Firefly luciferase can be used to convert stoichiometrically ATP to photons which can be detected with a 1000-fold greater sensitivity than direct fluorometric assay of ATP.

Lowry and coworkers introduced an amplification technique in 1961 called enzymatic cycling (13). This technique uses two enzymatic reactions which have a common substrate, $NADP^+$, to generate products of the two reactions which can be quantitated. Enzymatic cycling can produce a 20,000-fold amplification of the amount of $NADP^+$ that can be detected (Figure 1). ATP can be measured using enzymatic cycling, either by coupling it to the NADP cycle (Figure 2) or directly using enzymes which interconvert ATP and ADP (Figure 3).

If two enzymatic cycles are coupled together, the 20,000-fold amplification of one $NADP^+$ cycle is multiplied by the 20,000-fold amplification of a second $NADP^+$ cycle (Figure 4) to produce a final amplification of 400 million-fold over the amount originally present. Because of the potential for such increases in the sensitivity of detection of bioindicators by enzymatic cycling, it will be considered in some detail in this study.

Selected Bioindicators

Bioindicators (and potential bioindicators) can be divided into four groups:

1. enzymatic activities - dehydrogenases, phosphatases, urease, glycosidic hydrolases, catalase, etc.
2. macromolecules - nucleic acids, proteins, cell wall polysaccharides.
3. metabolic intermediates - ATP, pyridine nucleotides, organic

Figure 1. Reaction Scheme for Enzymatic Cycling of $NADP^+$.

$NADP^+$ is reduced and reoxidized by the two competing enzymes, glucose-6-phosphate dehydrogenase and glutamate dehydrogenase, producing many molecules of product for each molecule of $NADP^+$ being cycled. The amount of product, 6-phosphogluconate, is determined by another enzymatic reaction, 6-phosphogluconate dehydrogenase.

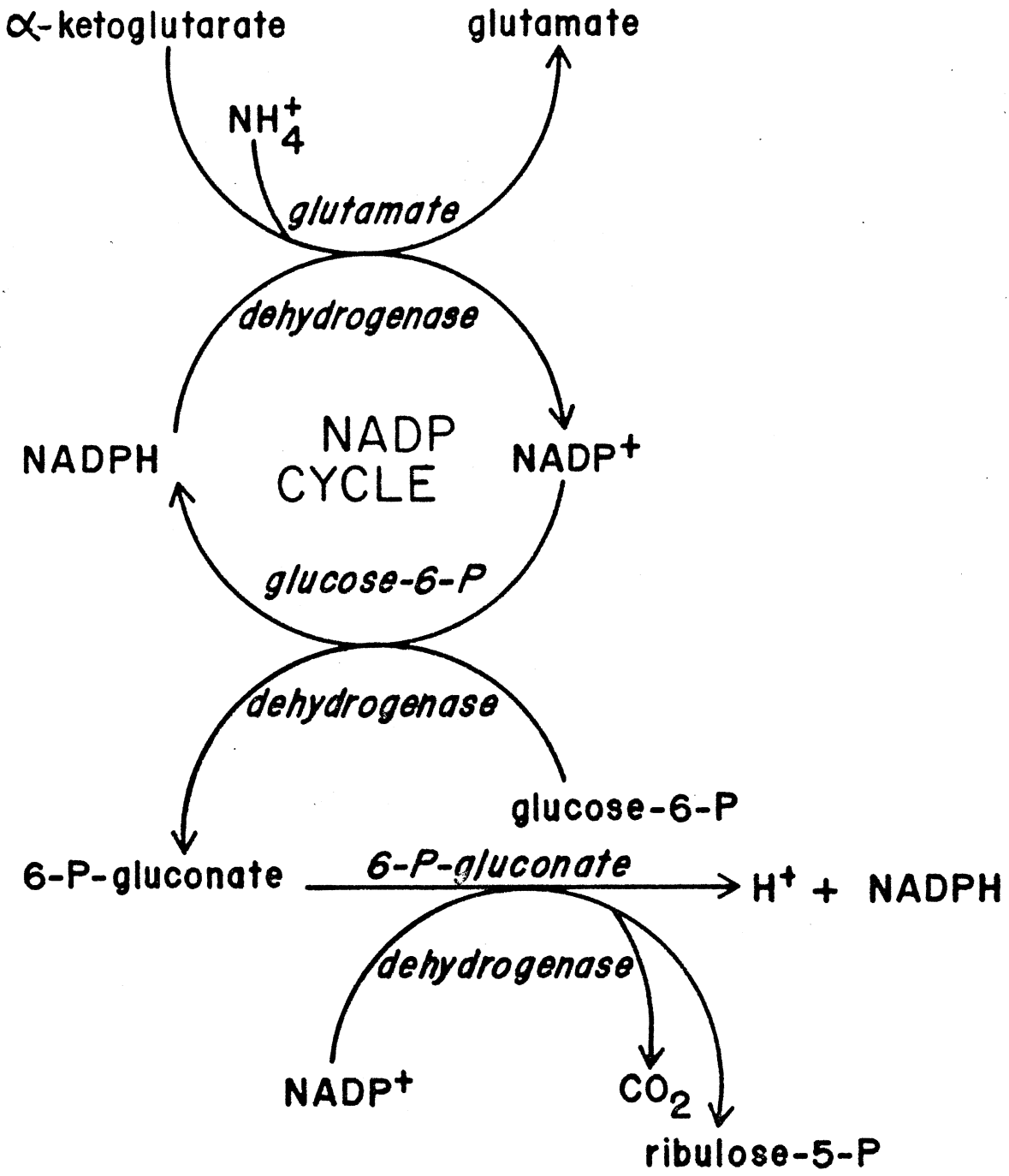

α-ketoglutarate
glutamate
NH4+
glutamate
dehydrogenase
NADPH
NADP
CYCLE
NADP+
glucose-6-P
dehydrogenase
glucose-6-P
6-P-gluconate
6-P-gluconate
H+ + NADPH
dehydrogenase
NADP+
CO2
ribulose-5-P

Figure 2. Reaction Scheme for Measurement of ATP Using Enzymatic Cycling of $NADP^+$.

NADPH is quantitatively formed by the reactions of hexokinase and glucose-6-phosphate dehydrogenase. The NADPH is then cycled to increase the sensitivity of its measurement (Figure 1).

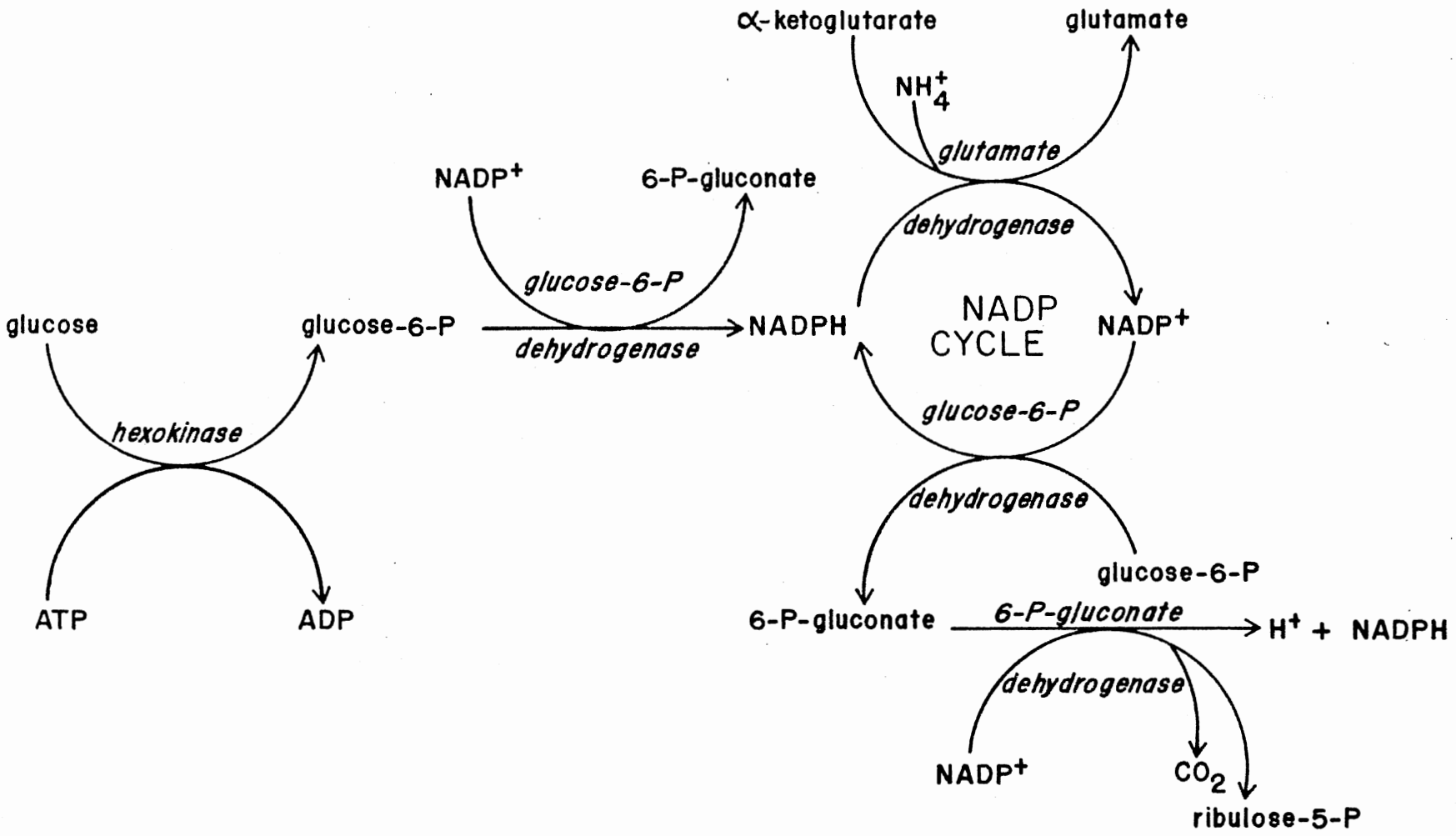

α-ketoglutarate
glutamate
NH4+
glutamate
dehydrogenase
NADP+
6-P-gluconate
glucose-6-P
dehydrogenase
glucose
glucose-6-P
NADPH
NADP
CYCLE
NADP+
glucose-6-P
hexokinase
dehydrogenase
glucose-6-P
ATP
ADP
6-P-gluconate
6-P-gluconate
H+ + NADPH
dehydrogenase
NADP+
CO2
ribulose-5-P

Figure 3. Reaction Scheme for Enzymatic Cycling of ATP.

Competing enzyme reactions can be used to cycle ATP as well as $NADP^+$. Hexokinase and pyruvate kinase repetitively dephosphorylate and phosphorylate ATP generating reaction products which can then be measured and related to the amount of ATP being cycled.

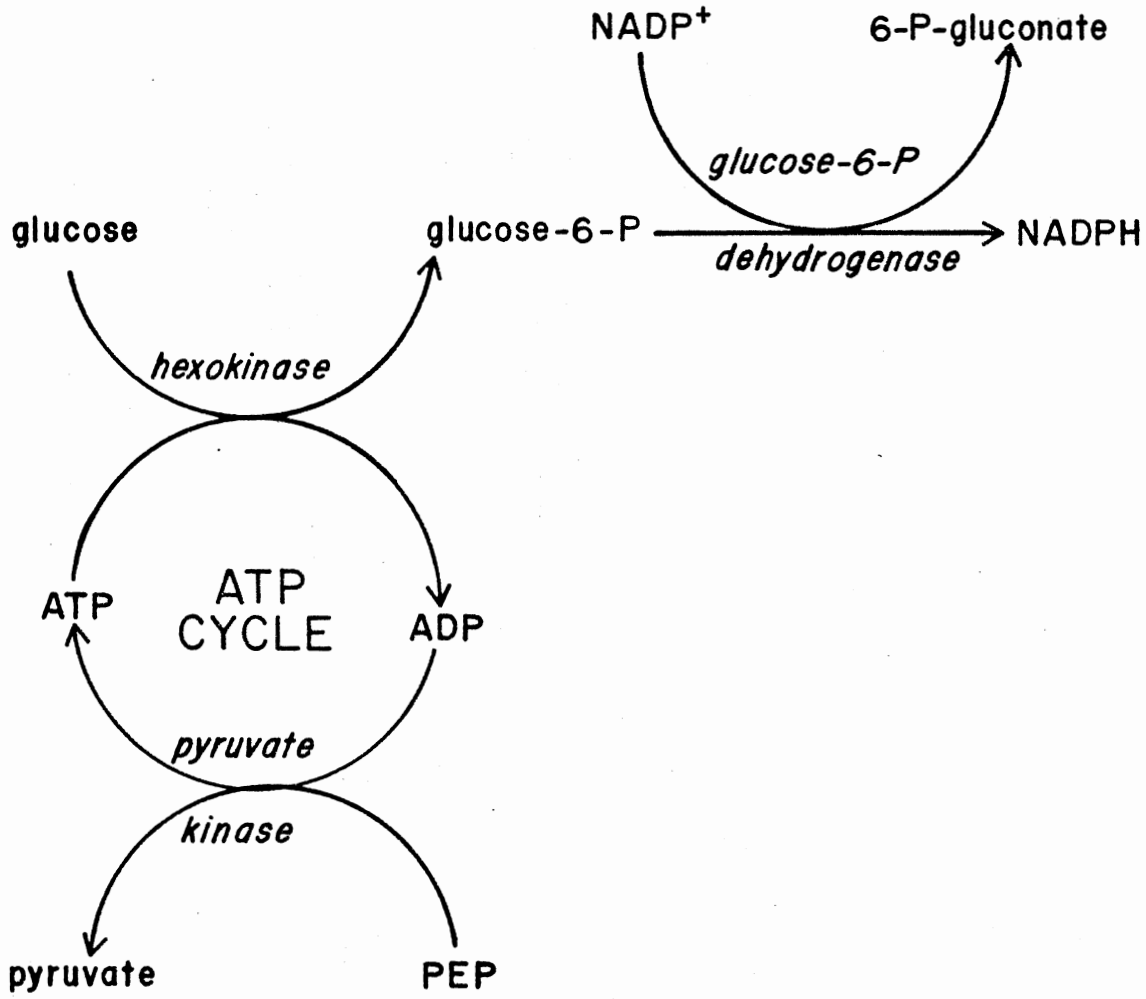
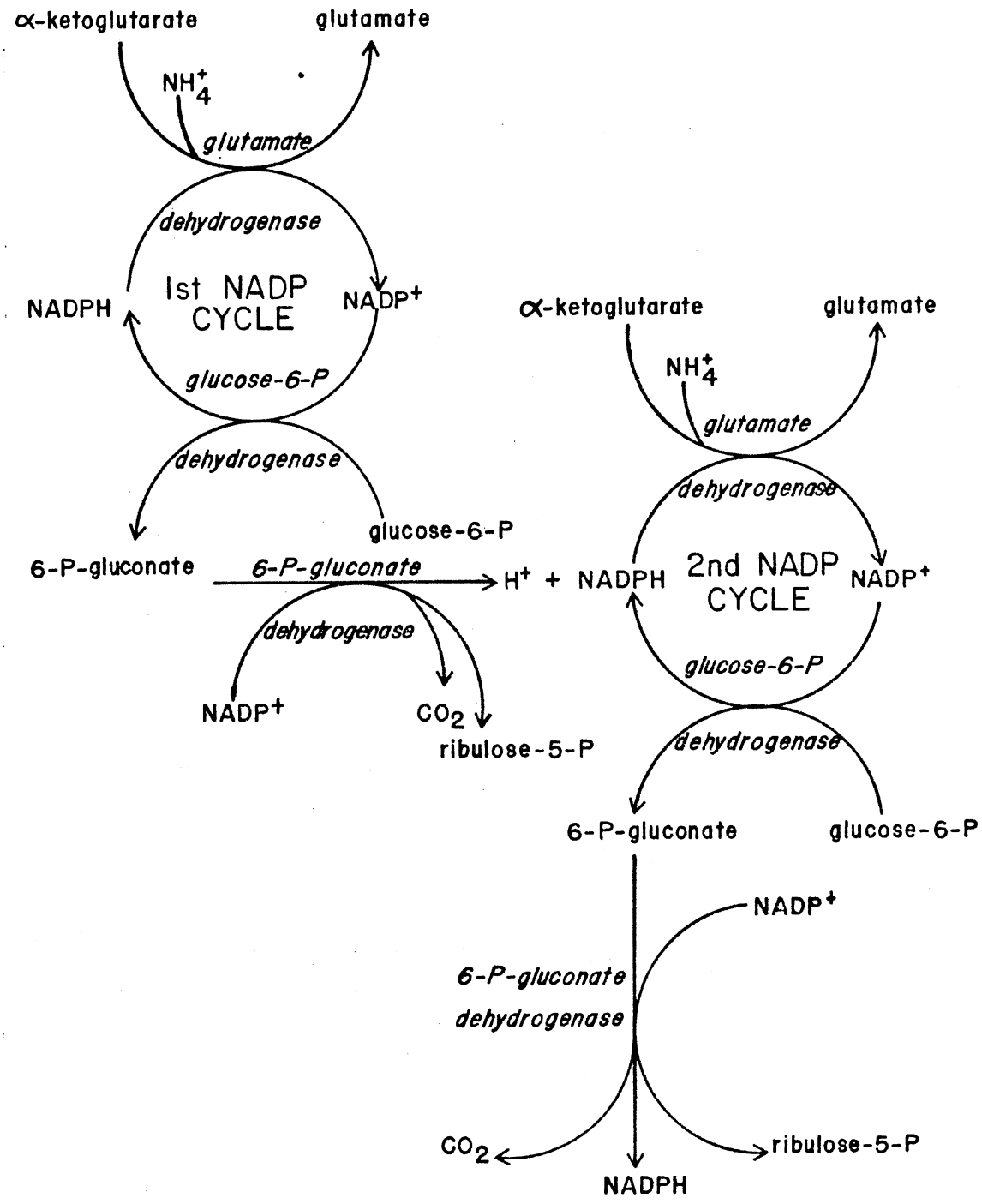

NADP+
6-P-gluconate
glucose-6-P
dehydrogenase
glucose
glucose-6-P
NADPH
hexokinase
ATP
ATP
CYCLE
ADP
pyruvate
kinase
pyruvate
PEP

Figure 4. Reaction Scheme for Double Cycling of $NADP^+$.

The NADPH produced by one enzymatic cycling sequence (Figure 1) can be cycled in turn to increase the sensitivity of detection of the original $NADP^+$ sample by the product of the two cycling rates. If the first cycle amplifies the response 20,000 times and the second cycle also amplifies 20,000, then the total amplification is 400 million ($2 \times 10^4 \times 2 \times 10^4$).


α-ketoglutarate
glutamate
NH4+
glutamate
dehydrogenase
1st NADP CYCLE
NADPH
NADP+
glucose-6-P
dehydrogenase
6-P-gluconate
6-P-gluconate
glucose-6-P
H+ + NADPH
dehydrogenase
NADP+
CO2
ribulose-5-P
α-ketoglutarate
glutamate
NH4+
glutamate
dehydrogenase
2nd NADP CYCLE
NADP+
glucose-6-P
dehydrogenase
6-P-gluconate
glucose-6-P
NADP+
6-P-gluconate dehydrogenase
CO2
ribulose-5-P
NADPH

phsophates, amino acids, iron porphyrins.

4. whole organisms - gas production (metabolic activity), changes in turbidity, plate counts.

Each group contains bioindicators which have been extensively studied and potential bioindicators whose correlations with biological activity is not known. Bioindicators have been selected from each group and studied in this laboratory in order to develop sensitive methods for determining ground water quality. The bioindicators selected for this study are the enzymatic activities lactate dehydrogenase, catalase, alkaline phosphatase, and adenylate kinase, and the metabolic cofactors associated with cellular respiration - ATP, pyridine nucleotides, FMN, and iron porphyrins.

Enzymatic Activities

The catalytic nature of enzymes make them amenable to the sensitive quantitation required of bioindicators. Most work has been done on the enzymatic activities found in soils and has been reviewed (14, 15). Much less is known about enzymatic activities in ground water (16).

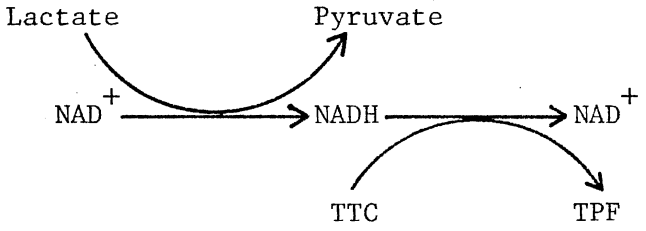
Lactate Dehydrogenase (E. C. No. 1.1.1.27). Most determinations of dehydrogenase activity in environmental samples have in general been performed using endogenous substrates. However, if a specific substrate is added, conditions may be such that one dehydrogenase will predominate. Reviews on dehydrogenases in soil (17) and in water (18) cover variations in the methodology of dehydrogenase determination. The reaction of lactate dehydrogenase is given in Equation 2. Lactate dehydrogenase acts metabolically as a terminus of glycolysis and in maintaining the

ratio of reduced and oxidized pyridine nucleotides.

(2)

Lactate dehydrogenase has several characteristics which make it suitable for study as a bioindicator. It is fragile and does not persist in the absence of viable organisms (19). Extracellular dehydrogenases are negligible so no pretreatment of water samples is required (20). Water samples may be treated with surfactants, ultrasound, or organic solvents to release the enzymatic activity, but no treatment may be necessary for soil samples as soils adsorb and stabilize proteins (21).

Most determinations of dehydrogenase activity in soil and water use an artificial electron acceptor, either directly or coupled to the dehydrogenase by another enzymatic reaction. Triphenyltetrazolium chloride (TTC; 2,3,5-triphenyl-2H-tetrazolium chloride) is reduced by dehydrogenase activity to the water-insoluble compound triphenyl formazan (TPF) (Equation 3). The TPF is extracted from the sample into



(3)

an organic solvent and quantitated spectrophotometrically by its absorbance at 490 nm. Analogs of TTC, such as tetrazolium blue or INT (2-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride) have also been used in the determination of dehydrogenase (22) with some preference

for INT in water samples (23). The optimal assay conditions depend upon the specific dehydrogenase being measured and the properties of the sample (soil pH, temperature) with incubation time varied to suit the activity of the sample (24).

Catalase (E. C. No. 1.11.1.6). The determination of catalase activity in soil has a long history and several reviews are available (25, 26). The metabolic function of catalase is to scavage and destroy hydrogen peroxide formed by oxidases in the cell. The reaction is shown in Equation 4.

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2 \qquad (4)$$

Determination of catalase activity may be made by measuring the oxygen produced or the hydrogen peroxide remaining at the end of the incubation. Oxygen can be quantitated by manometry (27) or volumetric techniques or by use of an oxygen electrode (28). Hydrogen peroxide quantitation can be done spectrophotometrically or titrimetrically (29, 30). The best method for any catalase determination is dependent upon the amount of catalase activity and interferences - turbidity, reducing or oxidizing agents - present in the sample.

Catalase is extensively adsorbed and stabilized by soils, causing errors in the catalase activity of viable organisms measured in soil samples (31). It is probably a better indicator of biological activity in water than in soils (32).

Alkaline Phosphatase (E. C. No. 3.1.3.1). The phosphatases of interest as bioindicators are nearly all monoesterases. Alkaline phosphatase and acid phosphatase are nonspecific monoesterases which hydrolyze

a number of organic phosphates, but at different pH's. The reaction is shown in Equation 5. The function of alkaline phosphatase in cells is

$$R\text{-}OPO_3^{2-} + H_2O \longrightarrow R\text{-}OH + HOPO_3^{2-} \qquad (5)$$

R = aliphatic or aromatic group

the salvage of phosphate from degraded nucleotides and removal of terminal phosphate groups from deoxyribo- and ribonucleotides.

Phosphatase activity in soil and water is affected by the methods used for sample collection and storage (33, 34). The half-life of alkaline phosphatase in lake water at 18^{o} is 3.2 days (35). The stability of phosphatase in soil depends on the type of soil (36).

Because alkaline phosphatase is a nonspecific enzyme, many substrates have been used in the determination of enzyme activity. Some researchers add no substrate and measure the increase in free phosphate from substrate native to the sample being incubated (37). Others use natural substrates like glycerolphosphate or ribulose-5-phosphate and measure release of phosphate or alcohol (38). Difficulties are sometimes encountered due to adsorption of reaction products onto clay particles leading to a reduction of measured phosphatase activity. Phenolic phosphates are the most widely used substitutes for determination of phosphatase activity because they yield highly-colored compounds which can easily be quantitated spectrophotometrically. Phenol phosphate, *p*-nitrophenyl phosphate, and 2-naphthyl phosphate have all been used successfully (38).

Adenylate Kinase (E. C. No. 2.7.4.3). ATP occupies a very important position in cellular metabolism, that of major energy carrier and coupler

of different metabolic pathways. The energy charge of the cell, shown by Equation 6, determines the overall regulation of cellular metabolism,

$$\text{Energy Charge} = \frac{(\text{ATP}) + \frac{1}{2}(\text{ADP})}{(\text{ATP}) + (\text{ADP}) + (\text{AMP})} \qquad (6)$$

making the concentrations of the three adenine nucleotides of critical importance to cell growth and reproduction. An important enzyme involved in maintaining the optimum ratio of adenine nucleotides is adenylate kinase (Equation 7) (39).

$$\text{ADP} + \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \qquad (7)$$

No work has been reported on the amounts or persistence of adenylate kinase in soil or water. It has been chosen for study for two reasons:

1. adenylate kinase is an ubiquitous enzyme which regulates the relative concentration of the three adenine nucleotides - ATP, ADP and AMP, and
2. techniques are available in the laboratory to quantitate ATP with very high sensitivity using firefly luciferase and enzymatic cycling, permitting detection of small amounts of adenylate kinase activity.

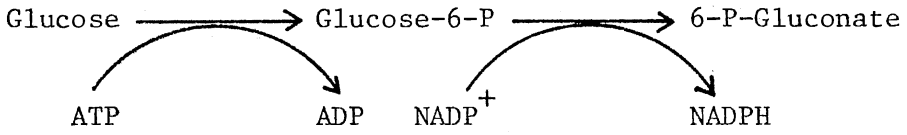
Respiratory Cofactors

All living organisms require energy to grow and reproduce. This energy may be derived from sunlight (autotrophs), or from the oxidation of inorganic (chemotrophs) or organic compounds (heterotrophs). Organisms have metabolic pathways which channel energy using certain compounds and cofactors - high energy phosphate bonds: ATP, GTP, PEP; and oxidation-reduction pairs: NAD^+-NADH, $NADP^+$-NADPH, FAD-$FADH_2$, and oxidized-

reduced cytochromes. Several of these energy-related compounds have been selected for study as bioindicators of subsurface pollution: ATP, pyridine nucleotides ($NAD(P)^+$ and NAD(P)H), flavin mononucleotide (FMN), and protein-bound iron or heme of cytochromes.

Adenosine Triphosphate (ATP). ATP is the principle energy carrier in all living organisms. The ATP contents of most cell types are similar and rapidly decreases when exposed to microbial activity. This has led to the proposal that ATP be used to quantitate viable biomass in activated sludge and seawater (40, 41). Studies in recent years have produced a tremendous body of literature on the quantitation of ATP using firefly (Photinus pyralis) luciferase. Work has been done in this laboratory on the optimization, range, and sensitivity of the firefly luciferase-ATP determination (42).

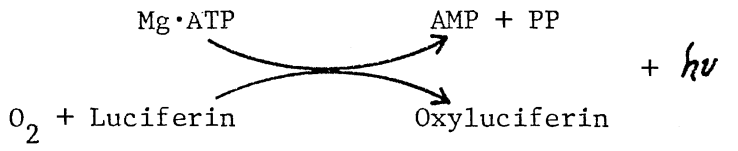
ATP is commonly quantitated by one of three methods, each of which utilizes enzymatic reactions. The first is a simple coupled assay in which ATP is reacted with hexokinase and glucose-6-phosphate dehydrogenase to form NADPH, which is measured spectrophotometrically or fluorometrically (43). The reaction is shown below:



(8)

The second method utilizes firefly luciferase to measure ATP. The current standard method is based on the work of McElroy and colleagues (44-46). When ATP is added to luciferase and the cofactor luciferin in a buffer containing Mg^{2+}, light is produced with an intensity and duration that is proportional to the amount of ATP (Equation 9). Variations

in the methodologies for using the luciferase reaction have been reviewed (47).



(9)

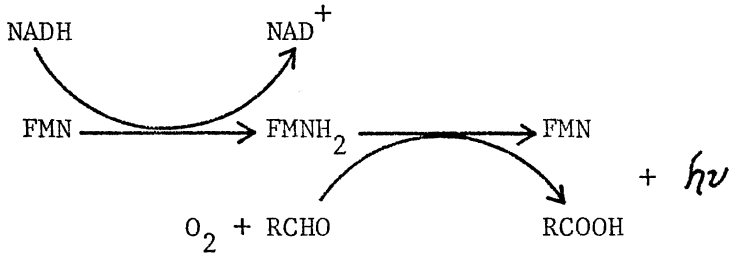
Enzymatic cycling has also been used to quantitate ATP (48) (See Figure 3). This method has not been used in soil and ground water studies due to the relative simplicity and sensitivity of the alternative firefly luciferase method. However, luciferase is inhibited by metal ions which are co-extracted with ATP from soil (49). Inhibitions and interferences can be more easily compensated for in the enzymatic cycling procedure, where enzyme concentration, substrate concentration and incubation time can be varied to accomodate changes in the cycling rate caused by inhibitors.

Pyridine Nucleotides. The pyridine nucleotides, NAD^+ and $NADP^+$, and their reduced forms, NADH and NADPH, are the principal electron carriers of intermediate metabolism in all organisms. They provide reducing power for carbon fixation in plants and carry the energy of oxidation to ATP through electron transport in aerobic organisms. The amounts or persistence of pyridine nucleotides in soil and water have not been studied but their ubiquitous presence in cells make them potential bioindicators.

Pyridine nucleotides can be quantitated by several methods. The reduced pyridine nucleotides have a strong absorbance at 340 nm and a strong fluorescence at 455 nm and these provide the basis for most determinations of pyridine nucleotides. NAD(P)H can be measured directly

and $NAD(P)^+$ can be determined by an enzymatic reduction (lactate and LDH, for example) and spectrophotometric or fluorometric measurement. Use of microcells or capillaries improve the sensitivity of these determinations (Table II).

It is also possible to measure NAD(P)H using the enzymes NAD(P)H: FMN oxidoreductase and bacterial luciferase from Photobacter fisheri (50). The luciferase produces light proportional to the NAD(P)H present during a reaction involving FMN, oxygen and an aliphatic aldehyde (51). Specificity of the enzymes for NADH or NADPH depends upon the strain used for enzyme purification. Bacterial luciferase has been used to detect as little as 1 pg NADH (52) (Equation 10).



(10)

R = Long-chain aliphatic group

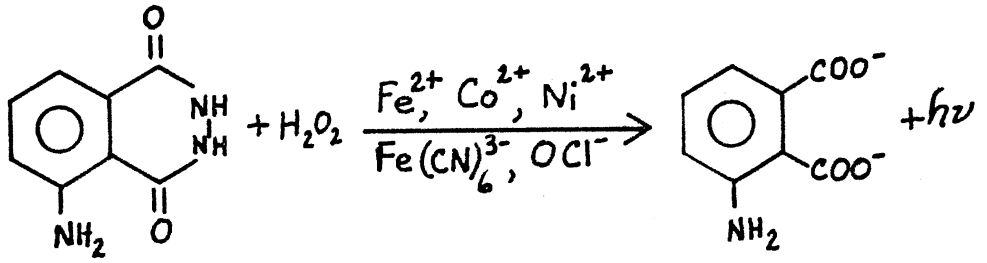
As was stated earlier and shown in Figure 1, enzymatic cycling can be used to quantitate $NAD(P)^+$ by amplifying the amount present in a sample. Enzymatic cycling permits the determination of any compound or enzymatic activity which can be coupled to pyridine nucleotide oxidation or reduction. As little as 10 ng $NADP^+$ can be detected by enzymatic cycling (7).

Flavin Nucleotides. Like the pyridine nucleotides, the flavin nucleotides, FMN and FAD, are electron carriers in cellular metabolism and are ubiquitous in living organisms (53). Flavins can be extracted

from cells by cold perchloric acid (54) and FAD converted to FMN by boiling the perchloric acid extract (55). FMN can then be quantitated using bacterial luciferase (50). The actual substrate for bacterial luciferase is $FMNH_2$, so FMN is reduced enzymatically or chemically prior to luminescence measurements with bacterial luciferase (56).

Iron Porphyrins. Protein-bound iron atoms act as electron carriers, as in cytochromes, or as catalytic sites in enzymes, such as catalase and horseradish peroxidase. Quantitation of iron porphyrins has been used to measure biomass in an effluent stream (57) and comparisons of the heme contents of different species of bacteria have been made (58).

Iron porphyrins can be quantitated by the chemiluminescent oxidation of luminol (5-amino-2,3-dihydro-1,4-phthazinedione). Luminol is oxidized by peroxides or oxygen with a quantum yield of 0.0124 under a variety of conditions (59). Iron porphyrins catalyze the oxidation, making the intensity of light proportional to the amount of catalyst present. In addition to iron porphyrins, Co^{2+}, Fe^{2+}, $Fe(CN_6)^{3-}$ and Cu^{2+} also act as catalysts in the oxidation of luminol (57) (Equation 11).



(11)

Extraction of iron porphyrins from cells is not necessary because introduction of bacterial samples into the pH 12.0 medium used for the chemiluminescent determination lyses cells and releases the porphyrins (57). A good correleation exists between iron porphyrins and bacterial

cell numbers (58) but a poor correlation between luminescence and viable cells (60).

Statement of Goals

The goals of this study may be stated as follows:

1. develop sensitive assays for the enzymes
   a. lactate dehydrogenase
   b. catalase
   c. alkaline phosphatase
   d. adenylate kinase
2. develop sensitive assays for the respiratory cofactors:
   a. ATP
   b. pyridine nucleotides NAD^+ and $NADP^+$ and flavin mononucleotide
   c. iron porphyrins
3. apply the sensitivity obtained through enzymatic cycling to the assay of selected bioindicators.

CHAPTER II

EXPERIMENTAL PROCEDURES

Materials

Hexokinase, lactate dehydrogenase, alkaline phosphatase, catalase, adenylate kinase, glucose-6-phosphate dehydrogenase (baker's, Torula and Leuconostoc), glutamate dehydrogenase, diaphorase and 6-phosphogluconate dehydrogenase were purchased from Sigma Chemical Company. Firefly luciferase used to quantitate ATP was obtained from DuPont Chemical Company. Adenosine triphosphate, adenosine diphosphate, $NADP^+$, NAD^+, 6-phosphogluconate, p-nitrophenyl phosphate, pyruvic acid, glucose, INT, INTF and hemoglobin were obtained from Sigma Chemical Company, and glucose-6-phosphate and NADH were purchased from Boehringer Mannhein Company. Lactic acid, hydrogen peroxide and ethylenediaminetetraacetic acid (EDTA) were from Fisher Scientific. 2-Amino-2-(hydroxy-methyl)-1,3-propanediol (TRIS) was obtained from Coleman, Matheson and Bell. All other chemicals used were of reagent grade and were obtained from various sources.

Methods

Instrumentation

Fluorometric measurements were made using an Aminco SPF-125 spectrofluorometer equipped with a thermostatted sample chamber, integrator-

timer, auto printer and Houston chart recorder. Two spectrophotometers were used in the study: a Zeiss Model PM6 with temperature regulated cuvette holder, automatic sample changer, printer and chart recorder; and a Gilford Model 2000 with auto printer and a Haake circulating water bath.

Three instruments were used for luminescence determinations. Most ATP and luminol determinations were made using an SAI Model 3000 integrating photometer with sample injector and Houston chart recorder. An Aminco Chem-Glow sample holder and phototube were connected to the Aminco SPF-125 high voltage supply and used for some measurements. Some chemiluminescence determinations were made using a Packard Pico-Lite Model 6100 luminometer with a Houston chart recorder.

A Brinkmann Model 5211-5232 Dilutor-Dispenser was used to facilitate addition and improve mixing of reagents for the enzymatic cycling procedures.

Growth of E. coli

Bacteria used in the experiments were E. coli Crookes (ATCC No. 8739) grown from cultures maintained on Difco nutrient agar plates kept at room temperature. Difco nutrient broth (20-50 ml) was inoculated from the plates and incubated overnight on a rotary shaker at 37° C. Cells were either harvested after the overnight incubation or diluted 1:4 with fresh nutrient broth and incubated 1-2 hr on a rotary shaker at 37° C before harvesting. Cells were harvested by 20 min centrifugation at 27,000 x g. The pellet was washed with 10 ml of M-9 medium (61) and suspended in 10 ml of M-9 or distilled water. Serial dilutions were made and turbidity (A_{630}) and plate counts were used to quantitate bacteria.

Pyridine Nucleotides

Reduced pyridine nucleotides (NADH and NADPH) were measured either spectrophotometrically at 340 nm or fluorometrically with excitation at 340 nm, 1 mm slit and emission at 455 nm, 2 mm slit. Instrument sensitivity (high voltage) on the spectrofluorometer was set at maximum for all measurements. Standard NADH solutions and 5 mM quinine sulfate were used to monitor variations in lamp intensity.

Enzymatic Cycling

Amplification of $NADP^+$ and NAD^+ by enzymatic cycling was carried out using the procedure of Lowry and Passonneau (13) as modified by Chi et al. (62). The procedure has two steps: First, NAD(P) is cycled for some time, usually one hour, after which the reaction is stopped by heat; and second, the product of one of the cycling enzymes, 6-phosphogluconate, is quantitated. The cycling reagent consists of 5 mM α-ketoglutarate, 10 mM ammonium acetate, 0.1 mM 5'ADP, 1.0 mM glucose-6-phosphate, 0.072 U glutamate dehydrogenase, and 0.5 U glucose-6-phosphate dehydrogenase in 100 mM Tris acetate pH 8.0. The amounts of enzymes GDH and G6PDH used depended upon the specific activities of the enzyme preparations used. Optimal amounts of enzymes to be used in the cycling reagent were determined by the procedure of Lowry and Passonneau (7). The gluconate reagent contains 0.1 mM EDTA, 30 mM ammonium acetate, 5 mM $MgCl_2$, 30-100 μM $NADP^+$, and 25-100 mU 6-phosphogluconate dehydrogenase in 40 mM Tris-HCl pH 8.1 (Figure 1).

The procedure for enzymatic cycling of pyridine nucleotides was as follows. To a 50 μl sample in a 1.5 ml plastic tube was added 100 μl

cycling reagent with a Brinkmann Dilutor Dispenser. The samples were incubated 30-120 min, depending on the amounts of pyridine nucleotides in the sample, at 37° C in a circulating water bath. The reaction was stopped by placing the tubes in a 100° C water bath for two minutes. The tubes were cooled 10 minutes at 4° C and 1.0 ml gluconate reagent was added. After a 30 minute incubation at 37° C the fluorescence intensity of NADPH was determined in an Aminco SPF-125 spectrofluorometer (excitation 340 nm, emission 455 nm).

Adenosine Triphosphate

Coupled-Enzyme Assay. ATP was determined using a coupled enzymes system where ATP was reacted with hexokinase-glucose-6-phosphate dehydrogenase and the fluorescence of the NADPH produced was quantitated (63). The reagent consisted of 6 mM glucose, 0.6 U glucose-6-phsophate dehydrogenase, 12 U hexokinase and 300 μM $NADP^+$ in 0.1 M Tris-acetate buffer pH 8.0. The $NADP^+$ and glucose concentrations were varied to suit the range of ATP to be measured. Hexokinase reagent (0.5 ml) was mixed with 1.0 ml sample in 3.0 ml plastic vials and incubated 15 minutes at 37° C. NADPH was quantitated spectrofluorometrically and related to ATP in the sample.

Firefly Luciferase Assay. ATP was measured by a procedure developed in this laboratory utilizing firefly luciferase (42). A 1 ml assay volume was used and contained 5 mM $MgSO_4$, 0.5 mM EDTA and 0.5 mM dithiothreitol in 0.025 M Tricine pH 7.8, 0.1 ml sample and 0.01-0.1 ml luciferase (depending on the preparation). The SAI Model 3000 Photometer was used with the following settings: sensitivity 700, delay 15 sec. and count 60 sec.

Enzymatic Cycling. The NADPH produced by the coupled-enzyme assay was cycled in order to increase the sensitivity of the assay. The same reagent as that used in the coupled-enzyme assay was used to generate NADPH for cycling. Hexokinase reagent (10 µl) was mixed with 20 µl sample in a 1.5 ml plastic tube and incubated 10 min in a 37° C water bath.

Excess $NADP^+$ was destroyed by adding 10 µl 1.0 N NaOH and incubating 1-2 hr at 65° C. The samples were cooled 10 min in an ice water bath and neutralized with 10 ul 1.0 N HCl. The samples were then treated according to the procedure for enzymatic cycling of pyridine nucleotides already discussed. The final fluorescence was measured and related to ATP in the samples using ATP and $NADP^+$ standards.

Bacterial Luciferase

NADH Measurement. A NADH test kit from Lumac Co. was used for luminescence determinations of NADH. The luciferase reagent contained 9.7 nmol FMN, 10 µg of an unspecified aldehyde, and an unknown amount of enzyme preparation in 0.1 M phosphate buffer, pH 6.9. The sample (0.1 ml) was injected into 0.4 ml reagent and the luminescence integrated over five minutes using the Packard Pico-Lite Model 6100 luminometer.

FMN Measurement. A similar assay was used to measure FMN by luminescence. NADH was used to provide reducing power for production of $FMNH_2$, the true substrate of the bacterial luciferase (Equation 10). FMN samples (0.2 ml) were mixed with luciferase reagent containing 0.2 mg crude luciferase preparation, 20 µg NADH and 1.5 µg dodecyl aldehyde in 0.4 M Tris HCl, pH 6.4. The SAI Model 3000 Photometer with settings of 10 sec delay, 30 sec count and 400 sensitivity was used for the measurements.

Lactate Dehydrogenase

Kinetic Assay. Lactate dehydrogenase activity was measured by the rate of decrease in absorbance or fluorescence of the substrate NADH as it was oxidized to NAD^+. The reagent used consisted of 4 mM NADH and 10 mM pyruvate in 30 mM phosphate buffer pH 7.4. A 0.1 ml sample was added to 0.9 ml reagent in a 1 ml cuvette in a 37° sample chamber and the ΔA_{340} or ΔI was measured on a chart recorder.

Incubation Assay. Smaller amounts of lactate dehydrogenase were measured by an incubation-type assay which uses a dye as the final electron acceptor of the reaction. The reagent contained 0.14 mM NAD^+, 50 mM lactate, 40 µg/ml INT, and 1 U/ml Diaphorase in 0.2 M Tris-acetate pH 8.0. The 0.1 ml sample was added to 0.9 ml reagent in a 3 ml capped vial and incubated 1 hr at 37° C. The INTF formed (Equation 3) was extracted into 2 ml 1.5:1 tetrachloroethylene:acetone and quantitated by its A_{490} in a spectrophotometer. The incubation time was varied to measure different ranges of enzymatic activity.

Determination of LDH Using Enzymatic Cycling. Extremely small amounts (1 µU - 1 mU) of LDH were detected using enzymatic cycling to quantitate the NAD^+ produced by the reaction when pyruvate and NADH were substrates. The reagent was made up of 30 mM pyruvate and 10 mM NADH in 30 mM phosphate buffer pH 7.4. LDH reagent (10 µl) was mixed with 20 µl samples in 1.5 ml microfuge tubes and incubated 1 hr in a 37° C water bath. The reaction was stopped and excess NADH destroyed by adding 10 µl 1.0 N HCl. After 10 min the samples were neutralized with 10 µl 1.0 N NaOH. The enzymatic cycling procedure already discussed was used to amplify and quantitate the NAD^+ produced by LDH activity.

Alkaline Phosphatase

Kinetic Assay. The continuous or kinetic assay of alkaline phosphatase was performed using the method of Malany and Horecker (64). The reagent was 1.0 mM *p*-nitrophenyl phosphate in 1.0 M Tris-HCl pH 8.0. 0.1 ml sample was added to 0.9 ml reagent in a 1.0 ml cuvette in a 37° C sample holder of a Zeiss or Gilford spectrophotometer and the change in absorbance at 410 nm was measured using a chart recorder. The minimum rate detectable was 0.005/min and activities were reported as ΔA/min.

Incubation Assay. Two incubation assays were used. The first was a one hour incubation using 6.6 mM *p*-nitrophenyl phosphate in 0.6 M Tris-HCl pH 8.2. A 0.1 ml sample was mixed with 0.9 ml reagent and incubated in a 38° C water bath for one hour. The alkaline phosphatase was measured by the increase in absorbance at 410 nm caused by the *p*-nitrophenol released by the reaction. A long incubation assay was used to measure very small amounts of enzyme activity. *p*-Nitrophenyl phosphate (0.0135 M in 1.0 M Tris-HCl pH 8.0) was mixed 1:1 with sample (0.5 ml of each) and incubated in a walk-in incubator at 37° C for up to four days. Enzyme activity was determined spectrophotometrically as stated for the one hour incubation assay (65).

Catalase

Kinetic Assay. Catalase activity was measured by the disappearance of hydrogen peroxide, which was quantitated spectrophotometrically at 240 nm using a chart recorder. The samples (2.0 ml) were mixed with 1.0 ml of 0.053 M H_2O_2 in 0.01 M phosphate buffer pH 7.0 in a 3 ml cuvette and placed in a spectrophotometer whose sample chamber was kept at 37° C and

the ΔA_{240} was measured.

Incubation Assay. Samples (0.1 ml) were mixed with 0.9 ml of 0.018 M H_2O_2 in 0.01 M phosphate buffer pH 7.0 in 1.5 ml plastic tubes and placed in a 37° C water bath for 1 hr. The A_{240} of samples and catalase standards were measured and the activities of samples were read from a standard curve of known catalase samples.

Adenylate Kinase

Adenylate kinase activity was measured by quantitation of ATP produced and so the procedures used for ATP measurement were applicable to the determination of adenylate kinase activity. ATP generated by incubation of ADP with enzyme samples was measured using the coupled-enzyme assay, firefly luciferase, and enzymatic cycling. The adenylate kinase reagent contained 0.5 mM ADP and 5 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.5.

Coupled-Enzyme Assay. Adenylate kinase was measured using both kinetic and incubation assays where the reagent components for the coupled-enzyme assay of ATP were added to the adenylate kinase reagent so that NADPH production was equivalent to the ATP generated by the adenylate kinase. The combined reagent (0.9 ml) was added to 0.1 ml samples and kinetic determinations were made spectrophotometrically at 37° C using a chart recorder. Alternatively the mixture was incubated one hour at 37° C and NADPH quantitated fluorometrically as discussed for pyridine nucleotide measurements.

Iron Porphyrins

Determination of Hemoglobin. Luminol chemiluminescence was used to quantitate hemoglobin using injection of 0.2 ml samples into 0.5 ml of luminol reagent containing 0.25 mM luminol, 10 mM EDTA, and 290 mM hydrogen peroxide in 50 mM phosphate buffer, pH 11.6. The SAI Model 3000 Photometer was set for 0.5 sec delay and 10 sec count with the sensitivity set on 700. The light intensity of the assay was attenuated using aluminum disks with different-sized holes so that the range of hemoglobin concentrations measured could be extended.

Quantitation of Biomass. The chemiluminescent oxidation of luminol in the presence of bacterial iron porphyrins was used to quantitate bacteria using the method of Thomas et al. (57). The reagent consisted of 0.25 mM luminol, 6.3 mM EDTA, and 29 mM hydrogen peroxide in 0.75 N sodium hydroxide. Stock solutions of 10-fold concentration of luminol and EDTA were prepared and working solutions were made from these daily. Hydrogen peroxide was diluted from a 30% stock solution. Luminescence was measured using an SAI Model 3000 Photometer with a 0.2 ml injector and chart recorder. The sensitivity, delay and count times of the instrument and proportions of reagent and sample were varied to optimize the light production. Most determinations were made using 0.5 ml sample mixed with 0.5 ml reagent and a 5 sec delay and 30 sec count time. Other determinations were made by injectiong 0.2 ml sample into 0.5 ml or 0.8 ml luminol reagent with 0.5 sec delay and 10 sec or 30 sec count time. Pure cultures of E. coli were used as standard bacteria and light production was related to viable cell numbers by plate counts on nutrient agar.

Measurement of Bioindicators in E. coli

Extraction of ATP and Pyridine Nucleotides from E. coli. E. coli cells were grown and harvested using the method already discussed except 0.05 M Tricine pH 7.7 was used to wash and suspend cells. Cell concentrations were determined by nutrient agar plate counts and turbidity (A_{630}) measurements.

Culture tubes (18 mm) containing 4.5 ml extraction buffer (10 mM $MgSO_4$ and 2 mM EDTA in 50 mM Tricine pH 7.7) were heated to boiling in a water bath. Cell samples (0.5 ml) were added and disrupted by the boiling buffer. The tubes were then put on ice ten minutes and then frozen in dry ice/acetone for storage. If samples were to be assayed immediately after extraction, aliquots of the extract were taken after cooling on ice ten minutes.

Toluene Treatment of E. coli. Enzyme activities were measured in E. coli after the cells were treated with 0.1% toluene to make the membranes permeable to substrates and products. Toluene (1 µl per ml of sample) was added to samples and the samples were vortexed 10 sec. Aliquots were than taken from the heated cell samples for the enzyme assays.

CHAPTER III

SELECTED ENZYMATIC ACTIVITIES

Enzymatic activities are measured by product formation or substrate utilization. This may be done by continuous assays, where the rate or velocity of the enzymatic reaction is measured, or by determination of total product at the end of an incubation of enzyme with substrate. The sensitivity of an enzyme assay is determined by the sensitivity of the analytical technique or method used to measure an increase in product or decrease in substrate concentraiton in the assay.

The enzymes, lactate dehydrogenase, alkaline phosphatase, catalase, and adenylate kinase were measured using several assay methods. The sensitivity of each assay was determined so that an assay method with high sensitivity could be selected and used to determine the amount of enzyme in bacterial samples. E. coli was used as a model to test the selected enzyme assays for sensitivity and to determine cell numbers required for measurable enzymatic activity.

Lactate Dehydrogenase

The glycolytic enzyme lactate dehydrogenase (E. C. No. 1.1.1.27) reversibly catalyzes the reduction of pyruvate to lactate and oxidation of NADH to NAD^+ (Equation 2). Lactate dehydrogenase from some microbes can use other electron donors such as cytochrome c, ferricyanide, and quinone in addition to NADH (66), but only the most common substrates,

NAD^+ and NADH, were used in the study. In incubation assays, lactate dehydrogenase was coupled to the formation of the reduced dye, INTF, by diaphorase, a nonspecific enzyme which uses NADH as substrate.

Kinetic or Continuous Assay

Initial velocity measurements of lactate dehydrogenase were made spectrophotometrically using a chart recorder to monitor changes in absorbance (Figure 5). The limit of detection was 0.1 μg of lactate dehydrogenase or 1.5 nmol NADH oxidized/min and the range 0.1-15 μg of lactate dehydrogenase.

Incubation Assay

A one-hour incubation assay using lactate and NAD^+ as substrate was used to measure lactate dehydrogenase. Activity was determined by a coupled enzyme reaction where NADH was oxidized with concommitant reduction of the dye INT by diaphorase. The use of a dye such as INT or TTC is preferred for many determinations because the product to be quantitated can be extracted from turbid or soil samples with organic solvents, improving the sensitivity of the measurement.

INTF formation was measured, both before and after extraction with tetrachloroethylene:acetone (Figure 6). The limit of detection was 1.5 ng and 1.0 ng of lactate dehydrogenase for the unextracted and extracted samples. The increased response of the extracts even though the INTF concentration was one-half that of the unextracted samples was the result of complete solvation of INTF in the extract. Precipitation of the water-insoluble INTF was observed in the samples prior to extraction which reduced the response for each sample.

Figure 5. Continuous Assay of Lactate Dehydrogenase.

Absorbance measurements (O) were made at 340 nm. The chart recorder was calibrated so that a full-scale deflection was 1 absorbance unit. The chart speed was 5 cm/min and the minimum change detected was 0.005 A/min. The sample holder on the spectrophotometer was maintained at 30° C for the assays.

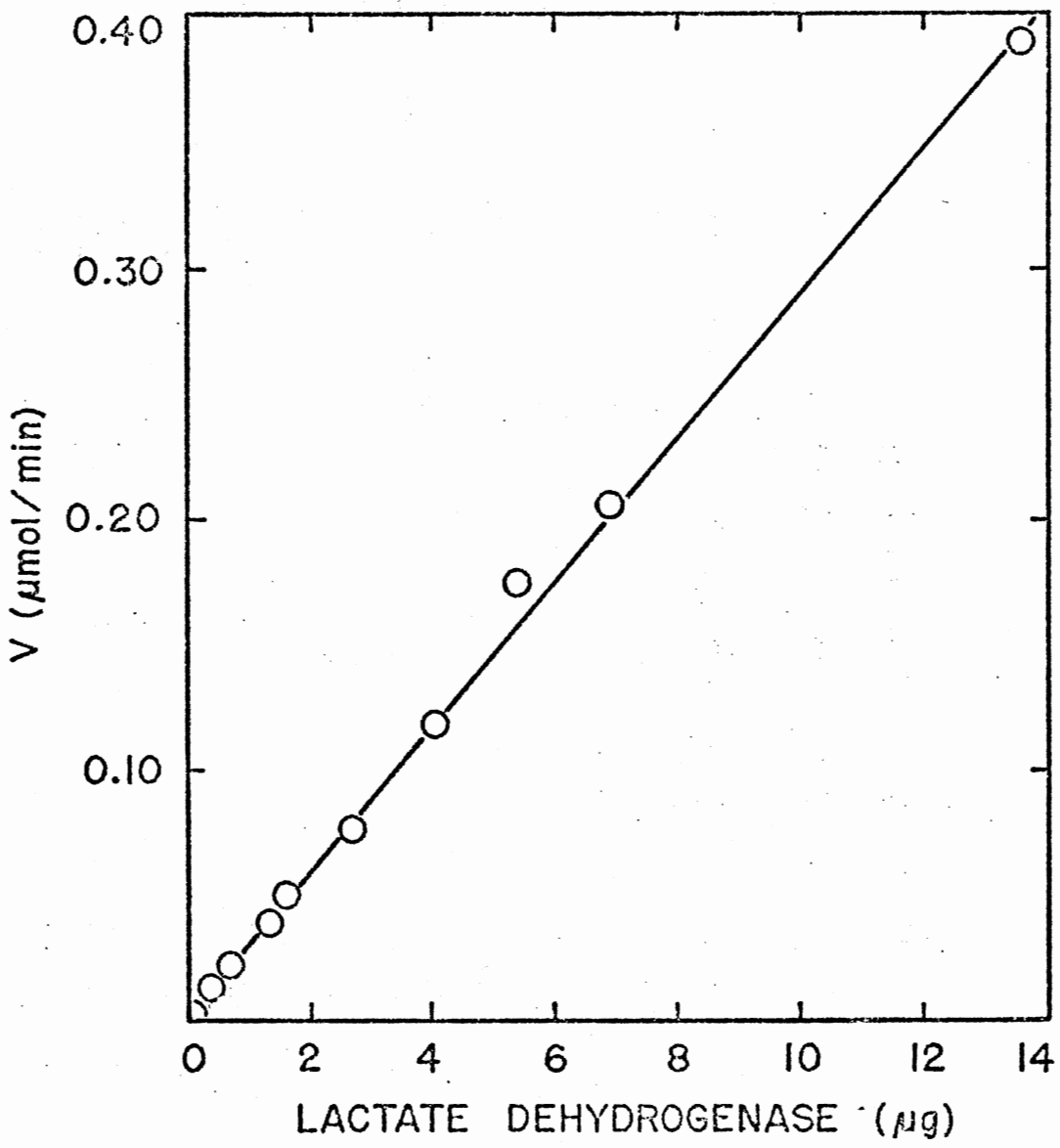
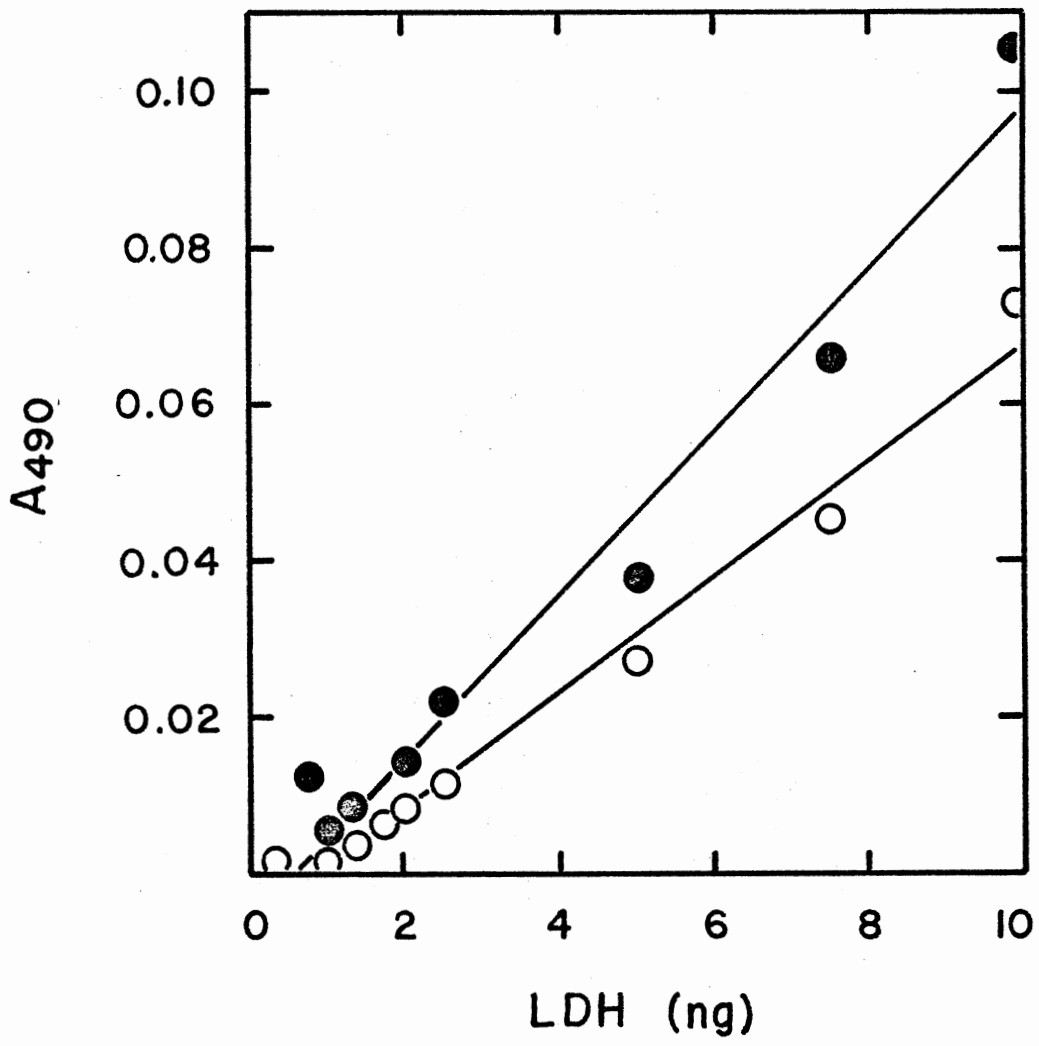

0.40
0.30
0.20
0.10
V (μmol/min)
0
2
4
6
8
10
12
14
LACTATE DEHYDROGENASE (μg)

Figure 6. Incubation Assay of Lactate Dehydrogenase.

INTF formation was measured spectrophotometrically at 490 nm before (O) and after (●) extraction with 2 ml tetrachloroethylene:acetone (1.5:1). Samples (0.1 ml) were mixed with 0.9 ml reagent and incubated one hour at 37° C.


0.10
0.08
0.06
0.04
0.02
A490
0
2
4
6
8
10
LDH (ng)

Measurement of Lactate Dehydrogenase in E. coli

Lactate dehydrogenase was measured in toluene-treated E. coli using the incubation assay. Cells (10^6-10^9/ml or 10^5-10^8/assay) were treated with 0.1% toluene prior to addition of 0.9 ml of the reagent used for the incubation assay. Samples were incubated one hour and the INTF produced was extracted with tetrachloroethylene:acetone and read spectrophotometrically at 490 nm. Lactate dehydrogenase activity was observed in all samples with the limit of detection being 10^6 cells/ml. This corresponds to the INTF produced by a one hour incubation of 1.0 ng lactate dehydrogenase using the standard curve for extracted samples in Figure 6.

Alkaline Phosphatase

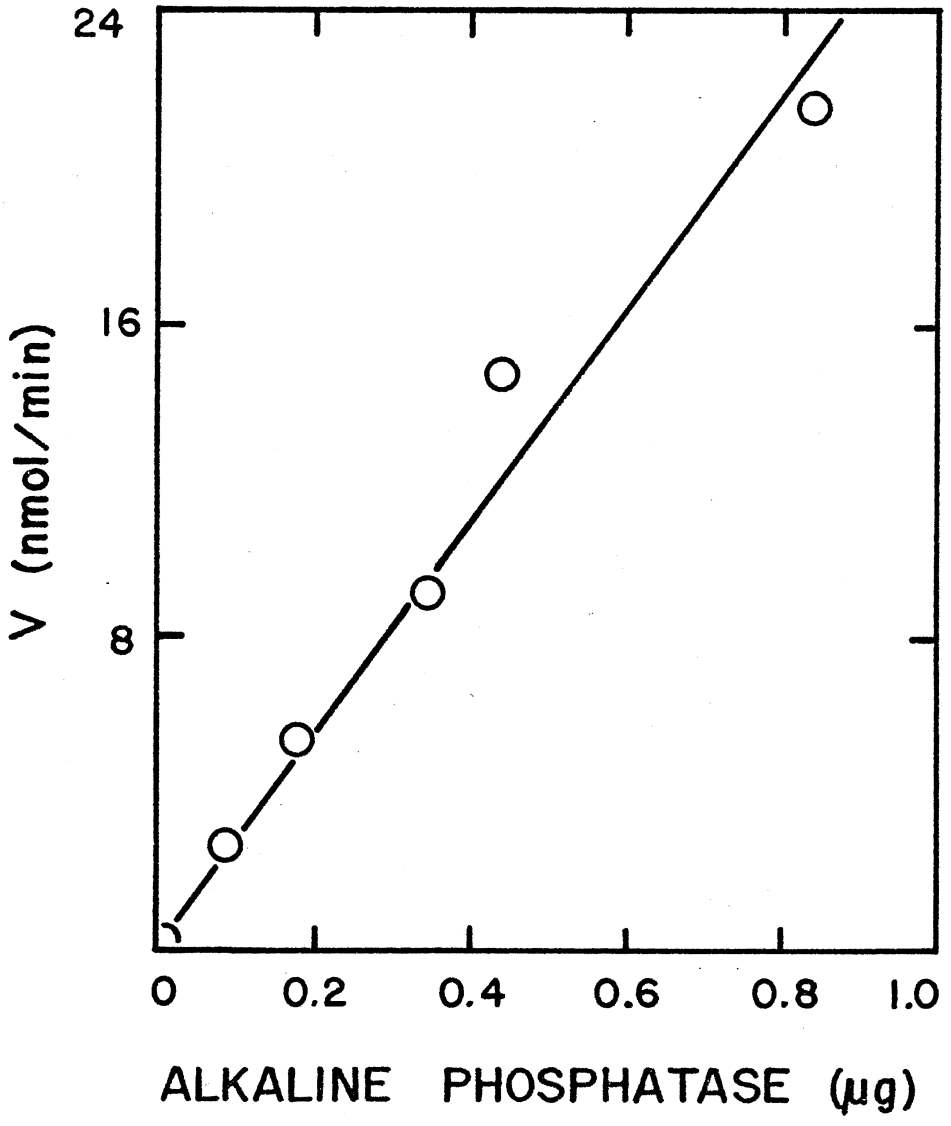
Alkaline phosphatase (E. C. No. 3.1.3.1) catalyzes the hydrolysis of a number of natural and synthetic monophosphate esters (Equation 5). Enzymatic activity may be determined by measurement of either phosphate or alcohol production and the sensitivity of the assay can be increased by increasing the incubation time before product measurement.

Continuous Assay

Alkaline phosphatase activity was determined by measurement of the rate of *p*-nitrophenol formation spectrophotometrically (Figure 7). The limit of detection was 20 ng alkaline phosphatase and the range of the assay was 0.02-2.0 μg alkaline phosphatase.

Figure 7. Continuous Assay of Alkaline Phosphatase.

ρ-Nitrophenol production was measured spectrophotometrically at 410 nm using a chart recorder calibrated for a full-scale deflection of one absorbance unit. The recorder was operated at 5 cm/min and the minimum change detected was 0.005/min. The sample holder was kept at 30° C for the assays. The molar absorptivity coefficient of ρ-nitrophenol is $1.62 \times 10^{4}\ M^{-1}cm^{-1}$.


24
16
8
V (nmol/min)
0
0.2
0.4
0.6
0.8
1.0
ALKALINE PHOSPHATASE (µg)

Incubation Assays

One-Hour Incubation Assay. Alkaline phosphatase samples (0.1 ml) were mixed with 0.9 ml of the *p*-nitrophenyl phosphate for one-hour incubations (Chapter II) and incubated at 37° C. *p*-Nitrophenol was measured spectrophotometrically (Figure 8). The limit of detection was 1 ng and the range of the assay was 1-50 ng alkaline phosphatase. A one-hour incubation increased the sensitivity of the assay 20-fold over the continuous assay.

Long-Time Incubation Assay. Incubations of up to four days were performed to determine if the sensitivity of alkaline phosphatase could be increased by longer incubation times. The *p*-nitrophenyl phosphate reagent specified for long incubations (Chapter II) was used for the determinations. The time-course of *p*-nitrophenol formation was measured for three different enzyme samples (Figure 9A) and the response of the alkaline phosphatase samples after 24-hour and 72-hour incubations were plotted (Figure 9B).

Figure 9A shows results from a 96-hour incubation of three alkaline phosphatase samples. The two larger enzyme amounts (8.8 and 0.88 ng) showed a linear response for three days but a decreased activity between 72 hours and 96 hours. The smallest enzyme amount tested, 0.09 ng, showed a linear response for the 96-hour incubation. Figure 9B shows that a 24-hour incubation gave a linear response over the 100-fold range of alkaline phosphatase tested. Therefore, a long incubation of one or more days can be used to increase the sensitivity of the alkaline phosphatase determination at least 10-fold over a one-hour incubation assay.

Figure 8. One-Hour Incubation Assay of Alkaline Phosphatase.

ρ-Nitrophenol production was measured spectrophotometrically at 410 nm after a one-hour incubation of 0.1 ml sample with 0.9 ml reagent (0.0066 M ρ-nitrophenylphosphate in 0.6 M Tris-HCl pH 8.2) at 37° C. The values have been corrected for blank hydrolysis of substrate.

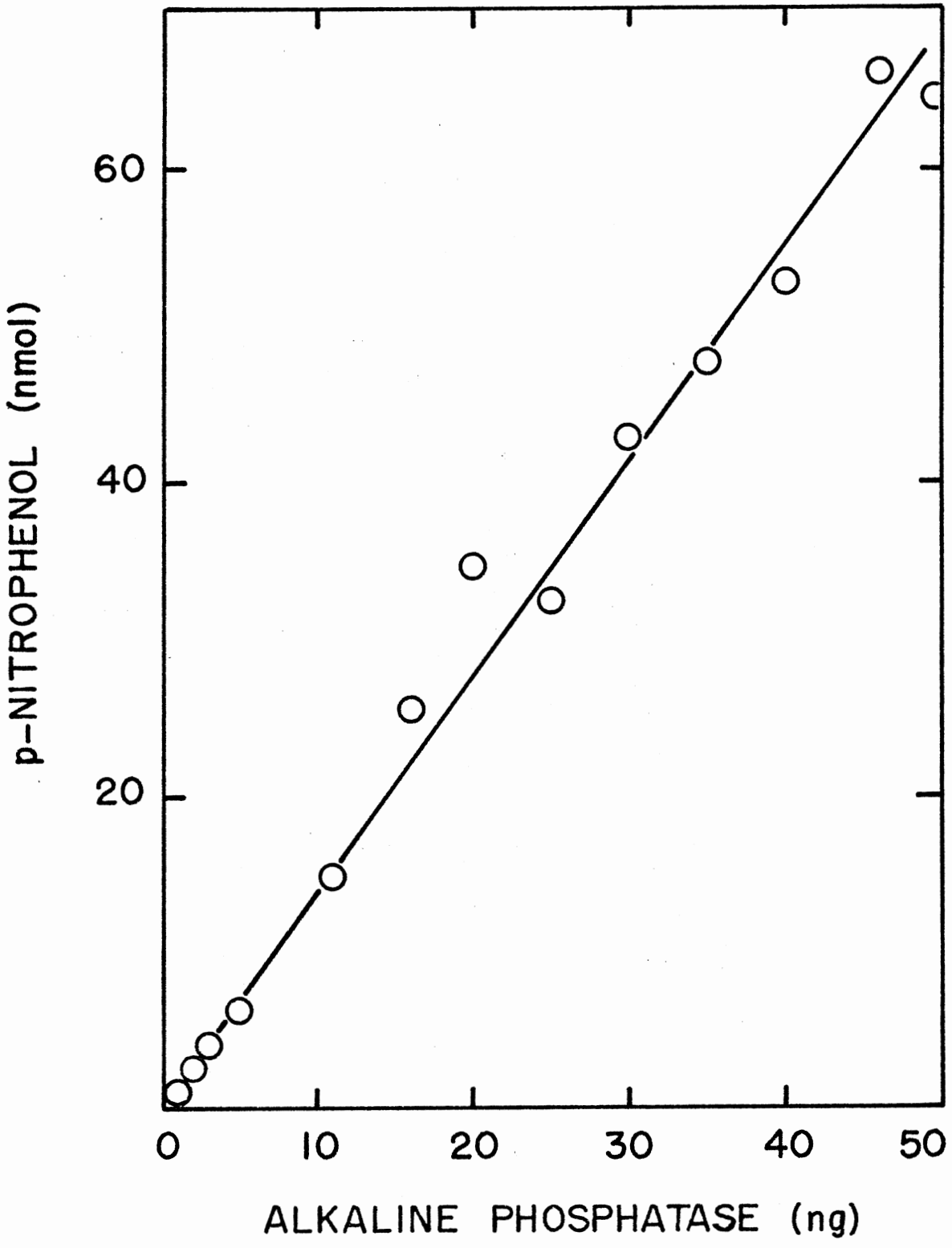
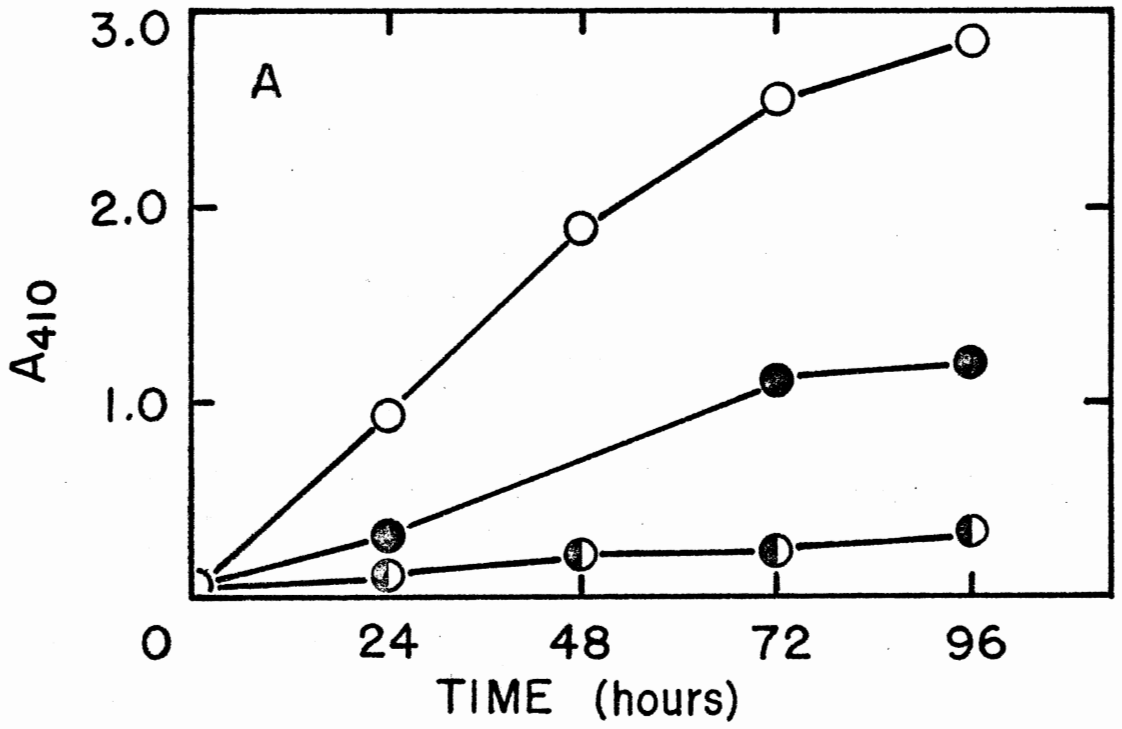

p-NITROPHENOL (nmol)
60
40
20
0
10
20
30
40
50
ALKALINE PHOSPHATASE (ng)

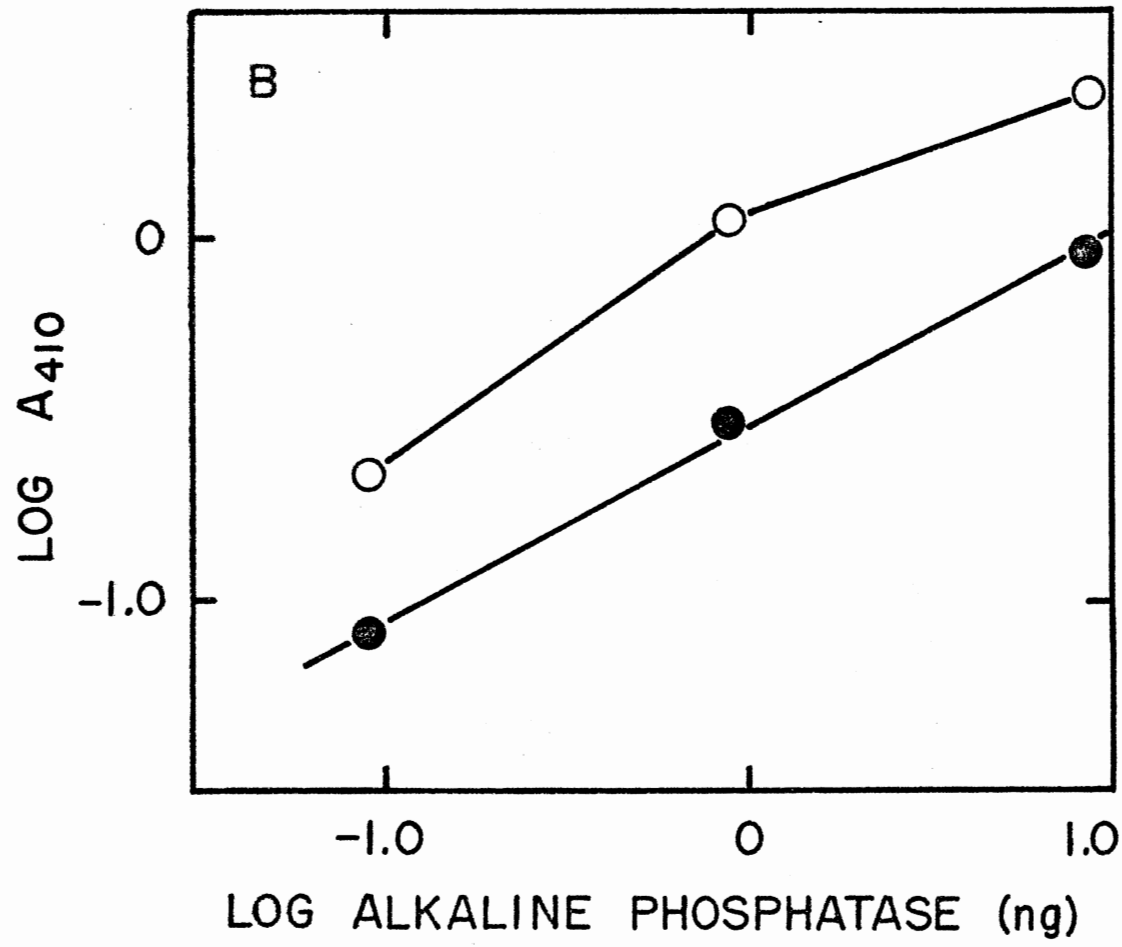
Figure 9. Long-Time Incubation Assay of Alkaline Phosphatase.

Samples (0.5 ml) were mixed with 0.5 ml reagent (0.0135 M *p*-nitrophenyl phosphate in 1.0 M Tris-HCl pH 8.0) in capped plastic vials and incubated at 37° C. Duplicate samples were removed and *p*-nitrophenol production measured spectrophotometrically at 410 nm at 24 hour intervals. Reagent blanks were also read each day so that nonenzymatic *p*-nitrophenol formation could be determined and used to correct the results of the enzyme samples.

A. Time course of product formation by alkaline phosphatase - 8.8 ng (O), 0.88 ng (●), and 0.09 ng (◐), measured at 24 hour intervals for four days.

B. Plot of enzyme amounts after a 24 hour incubation (●) and 72 hour incuabtion (O).


A
3.0
2.0
1.0
A410
0
24
48
72
96
TIME (hours)


B
0
-1.0
LOG A410
-1.0
0
1.0
LOG ALKALINE PHOSPHATASE (ng)

Measurement of Alkaline Phosphatase in E. coli

Alkaline phosphatase was measured in E. coli ($8 \times 10^6 - 8 \times 10^9$/ml) treated with 0.1% toluene using a one-hour incubation assay. Cell samples (0.1 ml) were mixed with 0.9 ml reagent and incubated one hour at 37° C. Cell blanks (cells + buffer) were used so that absorbance caused by the cells could be substracted from the final absorbance readings of the samples. The limit of detection was 8.5×10^6 cells/ml or the activity of one ng of alkaline phosphatase (from Figure 8).

Catalase

Hydrogen peroxide produced by cellular oxidases is scavanged by catalase (E. C. No. 1.1.11.6). Catalase activity is measured by quantitation of hydrogen peroxide, either continuously in a spectrophotometer or after an incubation period.

Continuous Assay

Catalase was measured by spectrophotometric determination at 240 nm of the rate of hydrogen peroxide destruction (Figure 10). A three ml assay volume was used because oxygen bubbles produced by the catalase caused fewer problems in the spectrophotometer in three ml than in one ml. The limit of detection was 0.2 μg and the range tested was 0.2 - 10 μg catalase.

Incubation Assay

A one-hour incubation assay of catalase was used to increase the sensitivity of catalase determination (Figure 11). The amount of hydrogen

Figure 10. Continuous Assay of Catalase.

Catalase samples (2 ml) were mixed with 1.0 ml buffered hydrogen peroxide in a 3 ml cuvette and the decrease in absorbance was measured spectrophotometrically at 240 nm using a chart recorder calibrated at 1 absorbance unit/full scale deflection and running at 5 cm/min. The limit of detection was 0.005/min. Sample temperature was held at 30^{o} C for the assays.

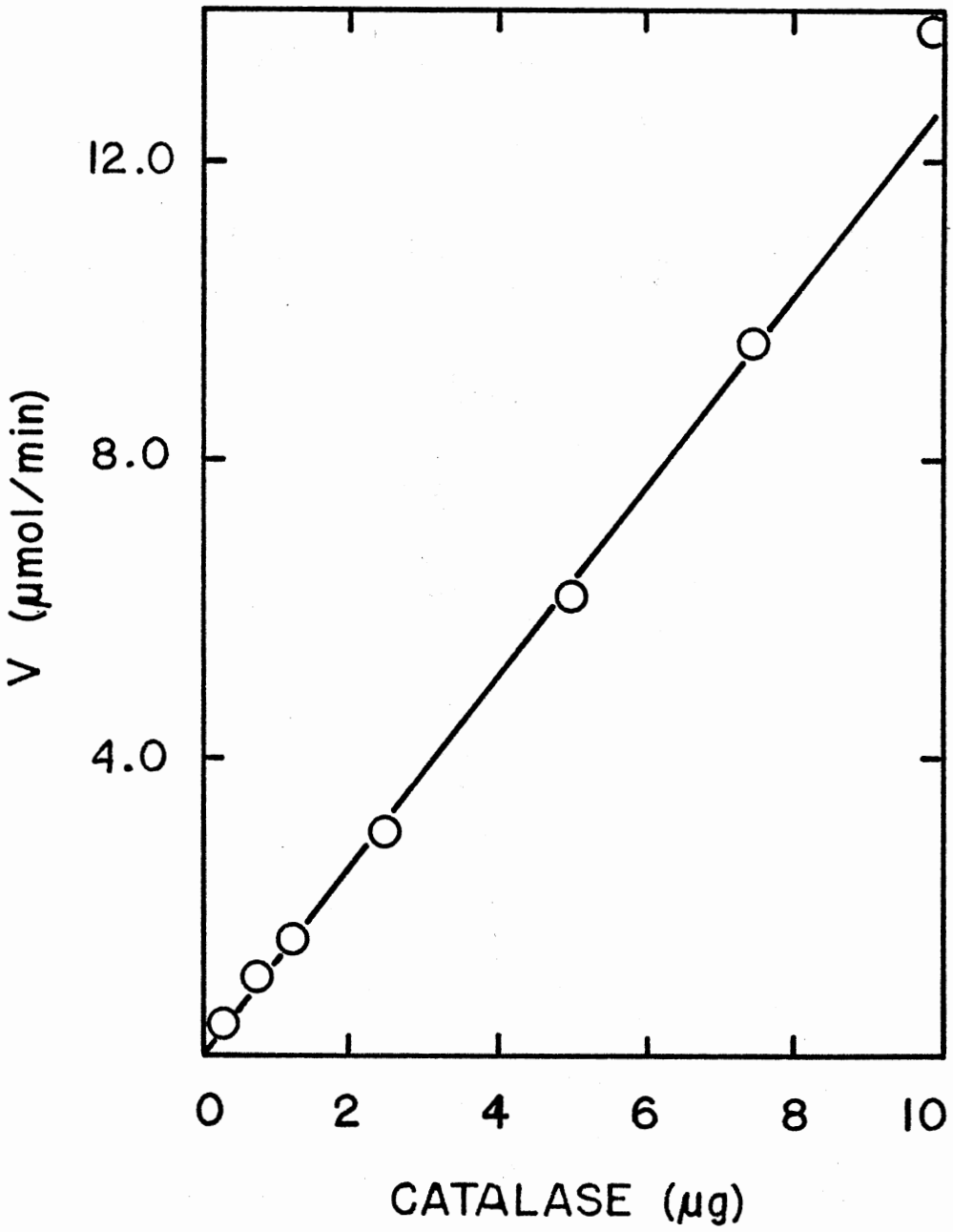
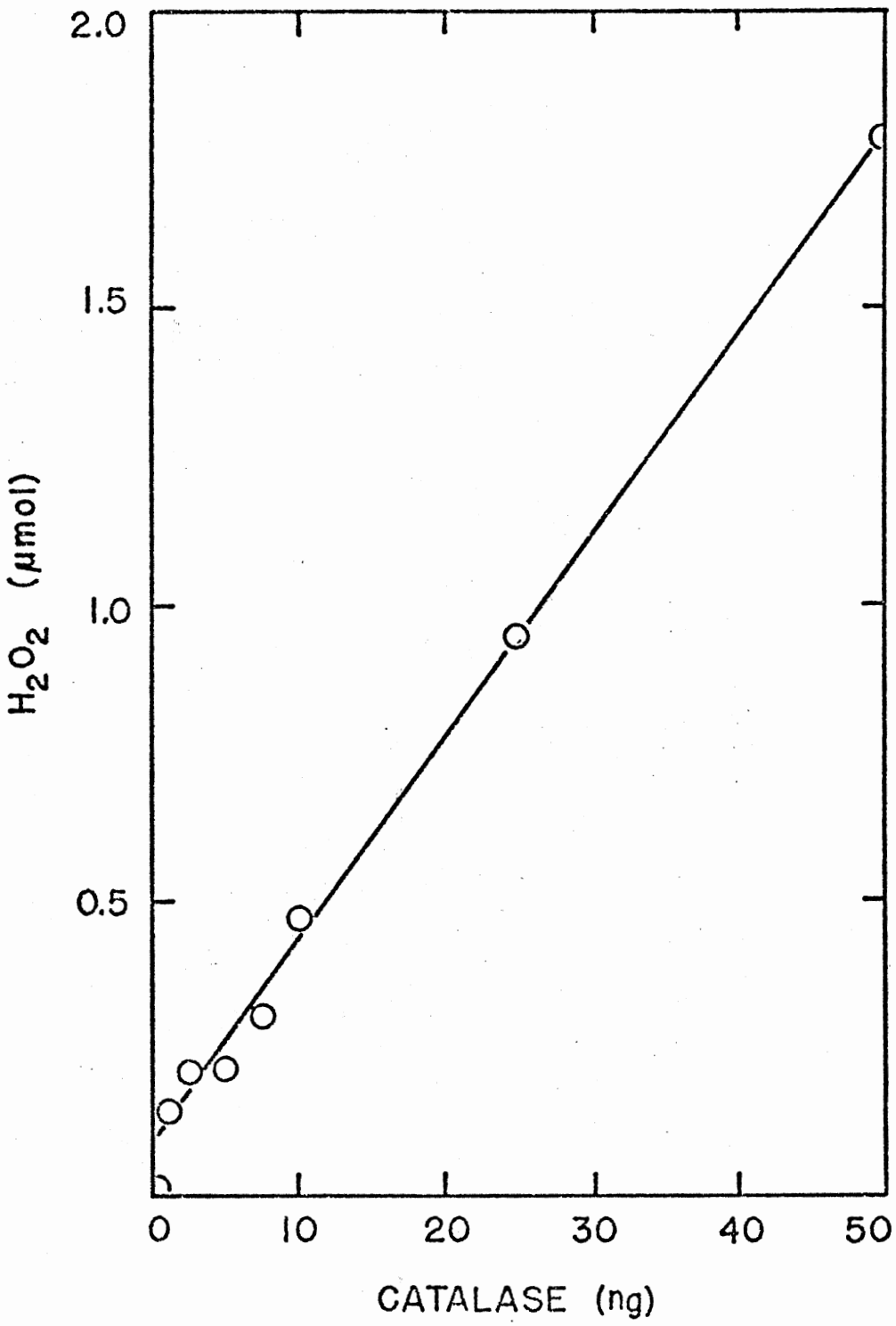

12.0
8.0
4.0
V (µmol/min)
0
2
4
6
8
10
CATALASE (µg)

Figure 11. Incubation Assay of Catalase.

Catalase samples (0.1 ml) were mixed with 0.9 ml buffered hydrogen peroxide and incubated one hour at 37° C. Hydrogen peroxide was measured spectrophotometrically at 240 nm and catalase activity was measured as the difference in absorbance between samples and blanks.


2.0
1.5
1.0
0.5
H_2O_2 (µmol)
0
10
20
30
40
50
CATALASE (ng)

peroxide utilized was determined by the difference in absorbance between hydrogen peroxide blanks and catalase samples. The limit of detection was 1 ng and the range was 1-100 ng catalase.

Measurement of Catalase in E. coli

Catalase was measured in E. coli cells (10^4-10^9/ml) which had been suspended in distilled water. Toluene treatment was not used since toluene absorbs strongly at 240 nm. A one-hour incubation assay was used in which 0.1 ml sample was added to 0.9 ml reagent in capped vials and incubated so that catalase activity could be measured. The limit of detection was 10^7 cells/ml, which had the equivalent of 1 ng catalase/10^6 cells.

Adenylate Kinase

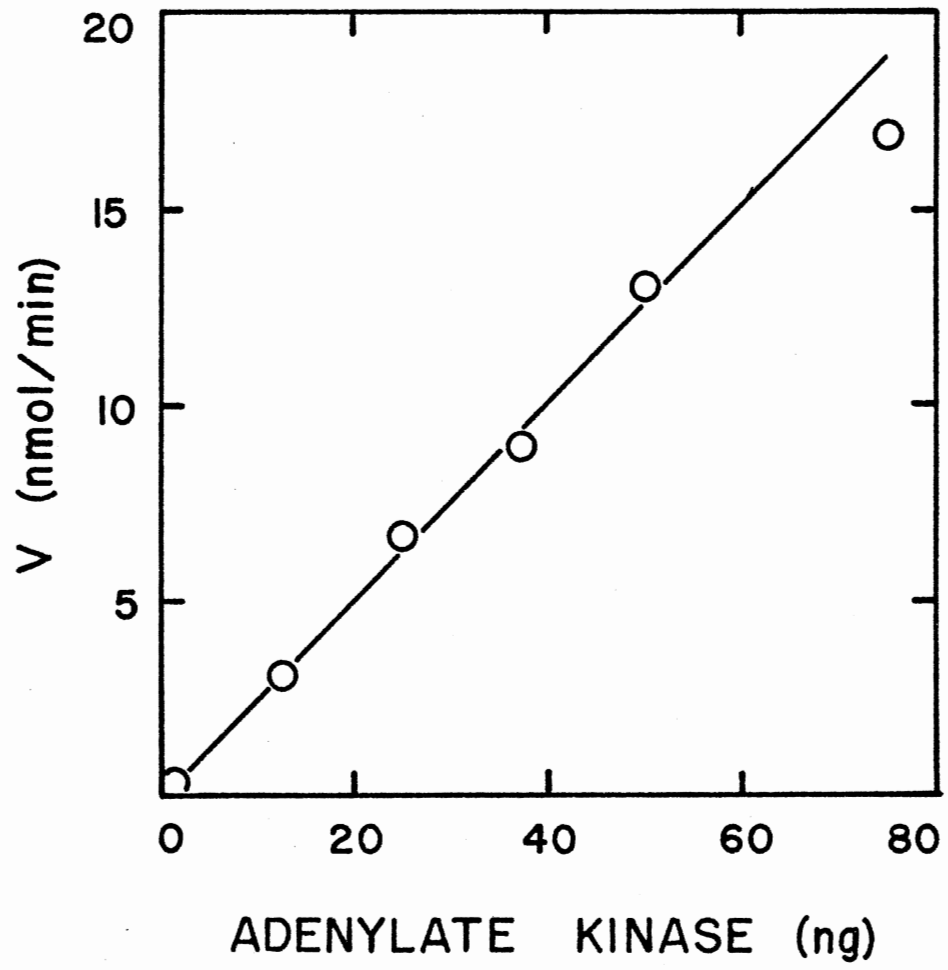
The ratio of adenine nucleotide concentrations in the cell is regulated by adenylate kinase (E. C. No. 2.7.4.3). Enzyme activity was determined by quantitation of ATP produced when ADP was used as substrate by a coupled enzyme assay (Equation 8) and by firefly luciferase. The coupled enzyme assay was used for this study because the firefly luciferase preparations had adenylate kinase activity which interfered with the determination of small amounts of enzyme.

Continuous Assay

Adenylate kinase was measured by following the rate of NADPH formation spectrophotometrically (Figure 12). The limit of detection was 3 ng and the range tested was 3-75 ng adenylate kinase. It was discovered during these assays that ADP may be contaminated with

Figure 12. Continuous Assay of Adenylate Kinase.

Adenylate kinase samples (0.1 ml) were mixed with 0.9 ml reagent in a 1.0 ml cuvette in a spectrophotometer thermostatted to 30° C and the rate of NADPH formation measured at 340 nm using a calibrated chart recorder at 5 cm/min. The limit of detection was 0.005/min.


20
15
10
5
V (nmol/min)
0
20
40
60
80
ADENYLATE KINASE (ng)

significant amounts of ATP. An ADP preparation from Sigma contained 1.3% ATP and an older preparation from Pabst Laboratories had 2.4% ATP. Subsequent assays were performed using an ADP preparation that had been treated with hexokinase and glucose to remove ATP.

Incubation Assay

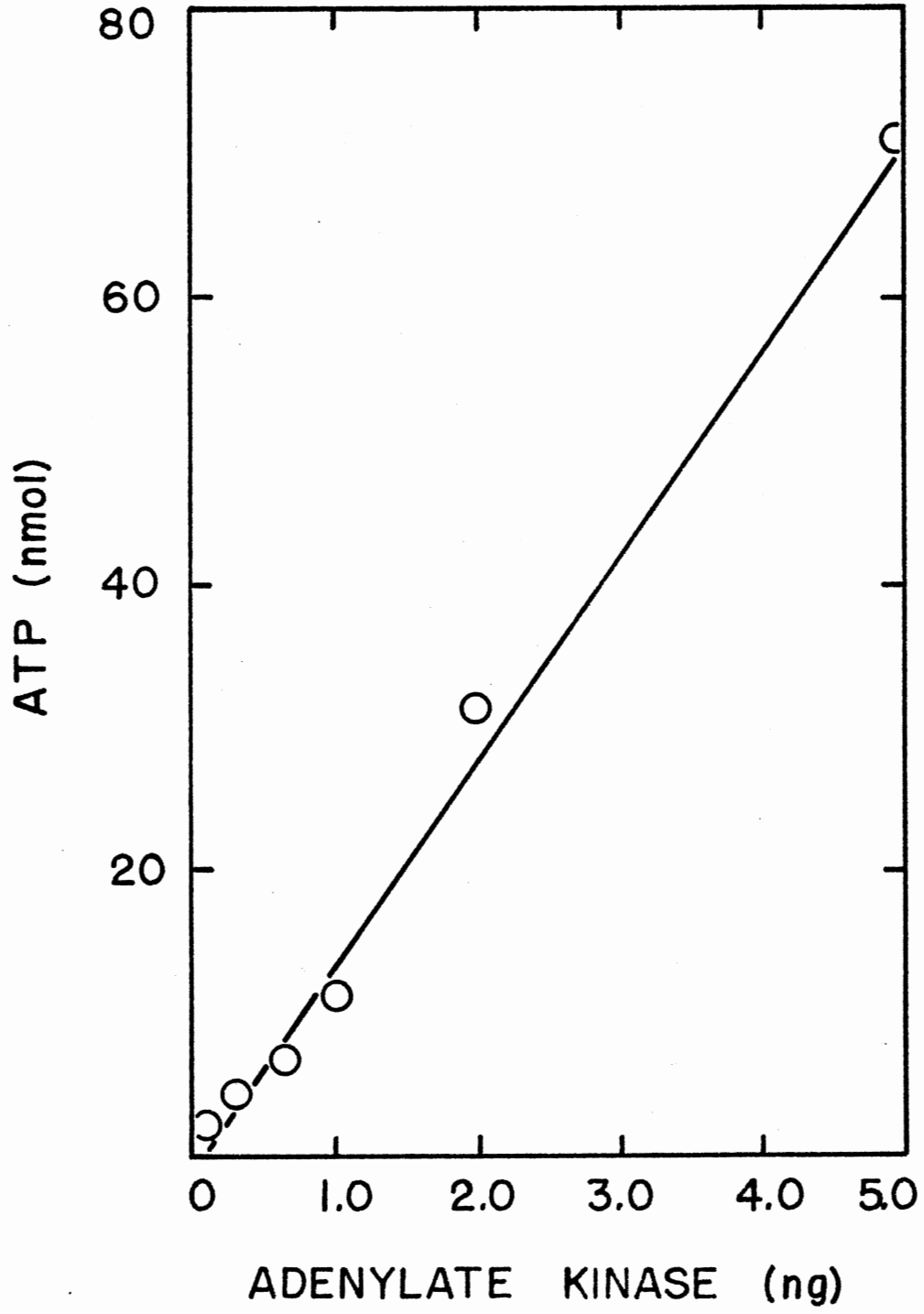
A one-hour incubation assay was used to determine whether sensitivity of the continuous assay of adenylate kinase could be improved. The same reagent was used as in the continuous assay but the incubation was carried out at 37^{o} C instead of 30^{o} C. Adenylate kinase was measured by NADPH formation spectrophotometrically (Figure 13). The limit of detection was 0.1 ng and the range was 0.1-5.0 ng adenylate kinase. This represents a 30-fold increase in sensitivity over the continuous assay.

Measurement of Adenylate Kinase in E. coli

Adenylate kinase was measured in E. coli cells (10^{5}-10^{8}/ml) treated with 0.1% toluene using the one-hour incubation assay. Cell blanks were used to correct for absorbance by cell particles. The limit of detection was 10^{6} cells/ml, which corresponds to 0.3 ng adenylate kinase/10^{5} cells in the assay using the standard curve in Figure 13.

Figure 13. Incubation Assay of Adenylate Kinase.

Adenylate kinase samples were treated as in the continuous assay except they were mixed in plastic-vials and incubated one hour at 37° C. Activity was measured spectrophotometrically at 340 nm and values were reported as ATP production. Reagent blanks were carried through the procedure and subtracted from adenylate kinase samples before being plotted.


80
60
40
20
ATP (nmol)
0
1.0
2.0
3.0
4.0
5.0
ADENYLATE KINASE (ng)

CHAPTER IV

RESPIRATORY COFACTORS

All living organisms obtain energy from their environment (by photosynthesis or substrate oxidation) and use that energy for growth and reproduction. Respiratory cofactors, which function in the transfer and storage of energy in the cell, include pyridine nucleotides (NAD^+/NADH and $NADP^+$/NADPH), adenosine triphosphate, flavin nucleotides (FMN/$FMNH_2$ and FAD/$FADH_2$), and iron porphyrins.

The approach for study of respiratory cofactors was similar to that for determination of enzymatic activities. Available assays were tested for sensitivity and improved where possible. The limits of detection were determined and bacterial cells were assayed, using extraction techniques where appropriate, for each respiratory cofactor.

Pyridine Nucleotides

Pyridine nucleotides are quantitated by the absorbance or fluorescence of the reduced pyridine nucleotides NADH and NADPH or by luminescence produced by bacterial luciferase (Equation 10). The response of $NAD(P)^+$/NAD(P)H can be amplified by enzymatic cycling to improve the sensitivity of detection of pyridine nucleotides.

Direct Measurement of NADH and NADPH

Spectrometry and Fluorometry. NADH was quantitated spectrophotometrically (340 nm) and fluorometrically (excitation 340 nm, emission 455 nm). The spectrophotometric limit of detection was 0.8 nmol and the fluorometric limit of detection was 0.071 nmol NADH in a one ml cuvette using water or buffer as a blank. The responses for NADPH are the same as for NADH (67) and were not tested.

Luminescence Using Bacterial Luciferase. The coupled enzymes NADH: FMN oxidoreductase-bacterial luciferase were used to measure NADH (Figure 14). Two crude enzyme preparations were used in the determinations and had inherent light production which made the method too insensitive for application to bacterial samples.

Enzymatic Cycling of Pyridine Nucleotides

The enzymatic cycling procedure of Lowry and Passonneau (7) was used to amplify the signal produced by pyridine nucleotides in a sample. Since it is possible to couple many enzymatic activities and metabolites to pyridine nucleotides, a number of other bioindicators can be detected through determinations of pyridine nucleotides by enzymatic cycling.

Calculation of Cycling Rate. The amplification obtained by enzymatic cycling is dependent upon the overall cycling rate, which is dependent upon the ratio of the two enzymes, glucose-6-phosphate dehydrogenase and glutamate dehydrogenase. The appropriate amounts of enzymes and the overall cycling rate were calculated by measurement of several parameters of the reaction (Figure 15). The ratio of oxidized/reduced pyridine

Figure 14. NADH Measurement Using Bacterial Luciferase.

The luminescence produced by bacterial luciferase-NADH:FMN oxidoreductase in the presence of NADH was measured by integration of the light emission for five minutes after injection of 0.1 ml NADH sample into 0.4 ml bacterial luciferase reagent containing 9.7 nmol FMN and 10 µg aldehyde in 0.1 M phosphate buffer, pH 6.9. The amount of enzymes in the reagent was not determined.

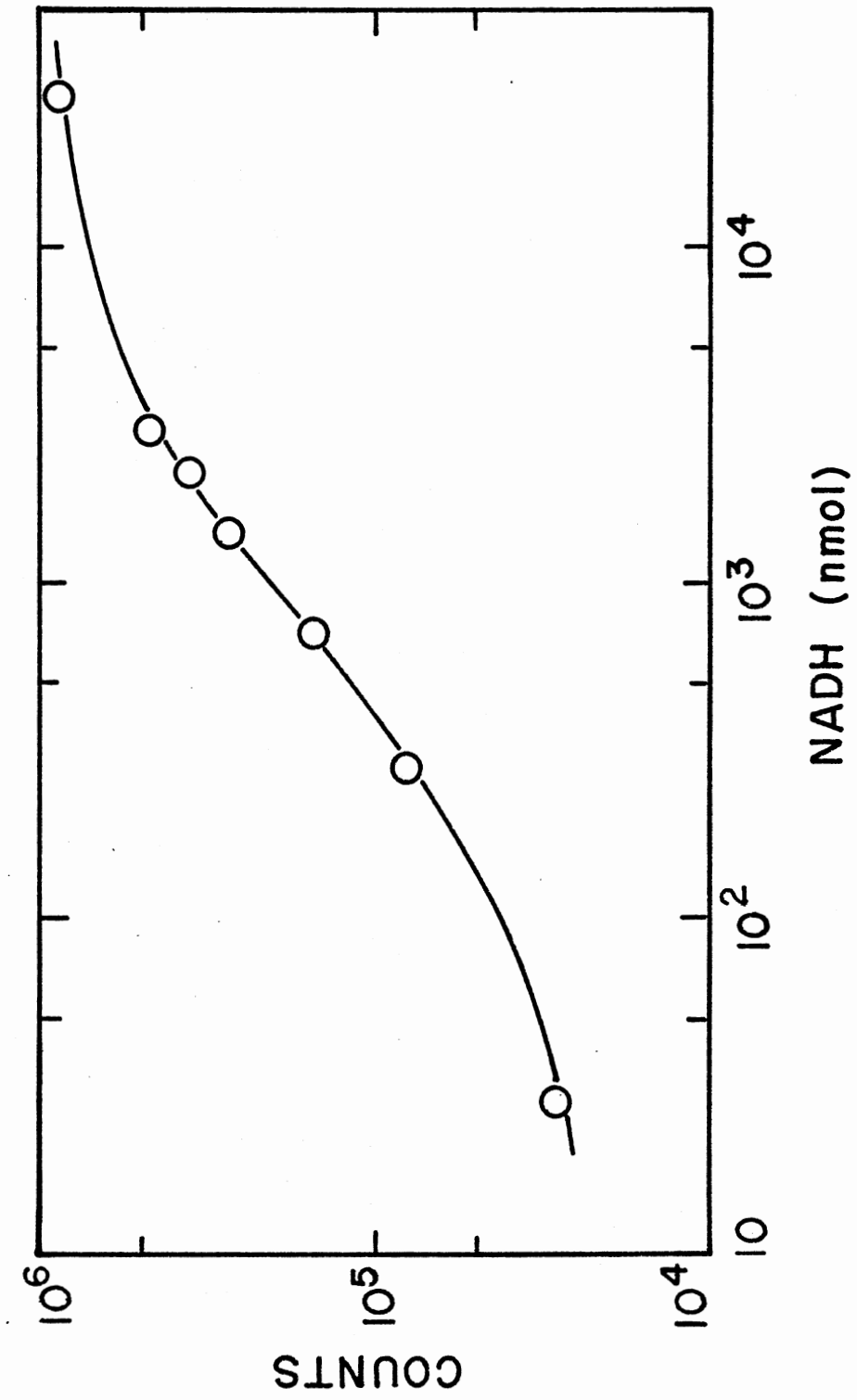
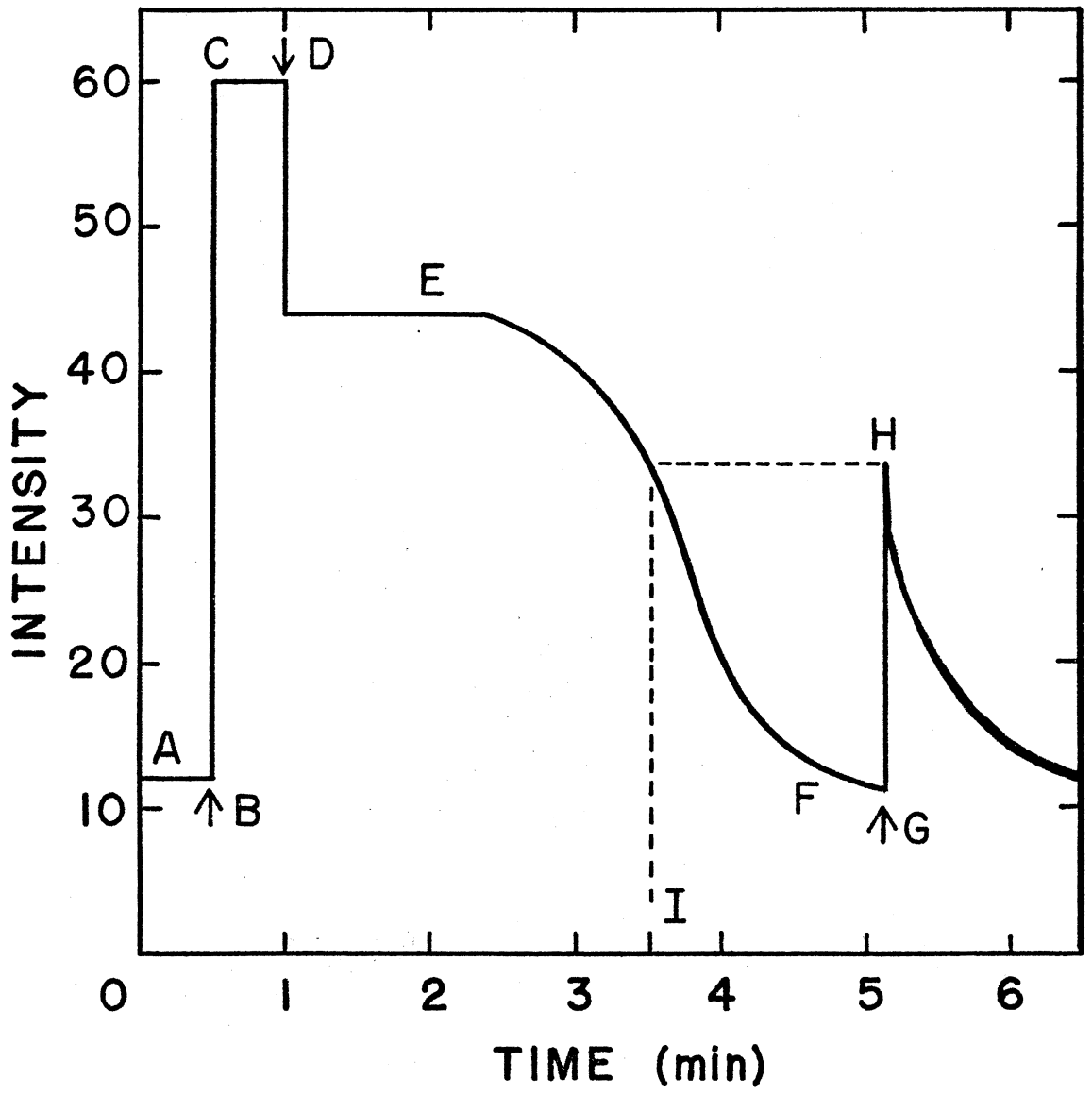

COUNTS
10^6
10^5
10^4
10
10^2
10^3
10^4
NADH (nmol)

Figure 15. Time-Course of Enzymatic Cycling Reactions and Calculation of Cycling Rate.

Cycling reagent (1.0 ml) containing 10 nmol $NADP^+$ and 1 μmol glucose-6-phosphate but no enzymes was placed in a fluorometer (excitation 340 nm, 1 mm slit; emission 455 nm, 2 mm slit) with the sample chamber maintained at 37° C (A). Glucose-6-phosphate dehydrogenase (0.5 U) was added (B), reducing all the $NADP^+$ to NADPH (C). Glutamate dehydrogenase (0.72 U) was added (D) and the cycle started, producing an equilibrium concentration of NADPH (E). After the glucose-6-phosphate was exhausted (F), 0.1 μmol glucose-6-phosphate was added to the reaction mixture (G) to determine the NADPH concentration (H) and time (I) when 90% of the glucose-6-phosphate had been utilized. The time (I) was then used to calculate the overall cycling rate (Equation 12).


C
D
E
H
A
B
F
G
I
60
50
40
30
20
10
0
1
2
3
4
5
6
INTENSITY
TIME (min)

nucleotide is given by the ratio of fluorescence intensities (C-E)/(C-A) from Figure 15 and the overall cycling rate calculated using the amounts of substrates present and the time required for 90% oxidation of glucose-6-phosphate (I) (Equation 12).

$$\text{Cycling Rate } (h^{-1}) = \frac{\text{90\% Glucose-6-P (mol)}}{\text{NADP}^+ \text{ (mol)}} \times \frac{1}{\text{Time for 90\% G-6-P Oxidation (h)}} \quad (12)$$

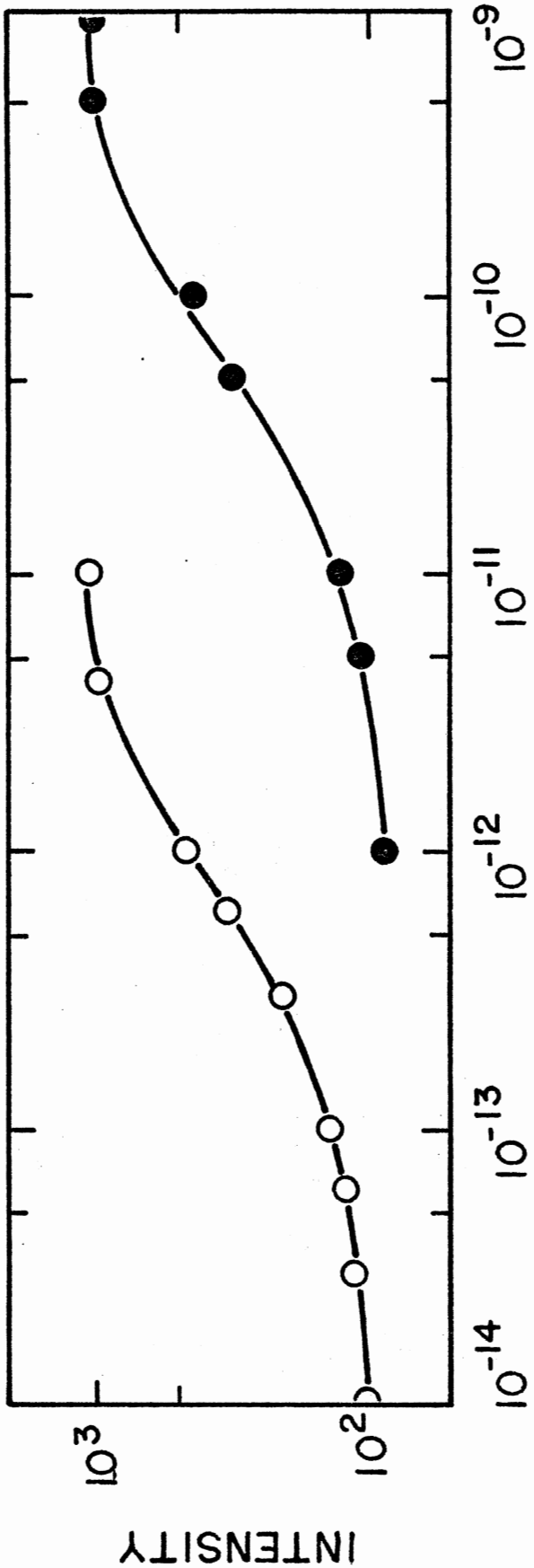
The cycling rates of NAD^+ and $NADP^+$ are considerably different due to the specificities of the enzymes used for cycling. The cycling rate was 3600/h for $NADP^+$ and 800/h for NAD^+. This difference was more pronounced under actual assay conditions. The actual amplification of $NADP^+$ obtained by enzymatic cycling was ~10,000/h, determined by 6-phosphogluconate standards while that for NAD^+ remained at ~800/h. It is not clear what causes this phenomenon but the optimum cycling rate of $NADP^+$ may occur at $NADP^+$ amounts much lower than those used for the calculation of cycling rate, while the NAD^+ cycling rate does not change over the range of concentrations studied.

Measurement of Pyridine Nucleotides

$NADP^+$ and NAD^+ were measured separately by the enzymatic cycling procedure (Figure 16). The incubation time was one hour for both pyridine nucleotides which, because of the different cycling rates, made $NADP^+$ measurement much more sensitive than NAD^+ measurement. The $NADP^+$ range was 5×10^{-14} - 5×10^{-12} mol and the NAD^+ range was 5×10^{-12} - 5×10^{-10} mol with the limit of detection for each being the low value of the range.

Figure 16. Enzymatic Cycling Determination of $NADP^+$ and NAD^+.

$NADP^+$ (O) and NAD^+ (●) were measured using the enzymatic cycling procedure discussed in Chapter II. Samples (50 µl) were incubated one hour with 100 µl cycling reagent at 37° C. Fluorescence of NADPH produced by oxidation of the cycling product 6-phosphogluconate by 6-phosphogluconate dehydrogenase was measured (excitation 340 nm, 1 mm slit; emission 455 nm, 2 mm slit) and plotted against the amount of pyridine nucleotide present in the sample.


INTENSITY
10^3
10^2
10^{-14}
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
PYRIDINE NUCLEOTIDE (mol)

Measurement of Pyridine Nucleotides in E. coli

Pyridine Nucleotides were extracted from E. coli using boiling Tricine buffer and measured by enzymatic cycling. The results were reported as $NADP^+$ since $NADP^+$ standards were used but the extracts actually contained a mixture of $NAD(P)^+$ and NAD(P)H. The extraction was tested using $NADP^+$ standards and a 96% recovery of $NADP^+$ was measured so no correction was made in the values determined for cell samples.

Two cell samples, containing 5×10^8 and 5×10^7 cells, were extracted using 5 ml boiling Tricine and 0.05 ml of the extracts were used in the cycling procedure. The amounts of $NADP^+$ measured in the cell samples were 1.4×10^{-12} mol/50 µl of the 5×10^8 cell extract and 1.7×10^{-13} mol/50 µl of the 5×10^7 cell extract. This may be expressed as 1.4×10^{-10} mol $NADP^+/5 \times 10^8$ cells and 1.7×10^{-11} mol $NADP^+/5 \times 10^7$ cells. The limit of detection was 2.4×10^5 cells carried through extraction and cycling.

Adenosine Triphosphate

Adenosine triphosphate may be measured directly by firefly luciferase or indirectly by a coupled enzyme system using NADPH for spectrophotometric or fluorometric determination. Enzymatic cycling can be used to amplify the response and increase the sensitivity of measurement of ATP.

Coupled Enzyme Assay

A coupled enzyme reaction of hexokinase-glucose-6-phosphate dehydrogenase was used to measure ATP by the fluorescence of NADPH (Equation 8). Tests of the assay using both ATP and glucose-6-phosphate standards showed

that the reaction was complete and that all ATP was reacted in less than five minutes at 37° C. A typical standard curve is shown in Figure 17. The limit of detection was 2×10^{-10} mol and the range was 2×10^{-10} - 2×10^{-8} mol of ATP.

Enzymatic Cycling

The sensitivity of detection of ATP was improved by enzymatic cycling of the NADPH produced by the coupled enzyme assay. Excess $NADP^+$ in the coupled enzyme assay reagent was destroyed by alkali treatment prior to cycling. This approach was selected instead of an ADP/ATP cycle (Figure 3) because the $NADP^+$ cycling procedure had been developed and could be applied to the measurement of another metabolite, ATP.

Destruction of Excess $NADP^+$. For enzymatic cycling to be useful, all unreacted $NADP^+$ must be destroyed so that only the NADPH formed by the coupled enzyme assay of ATP participates in the cycling reactions (Figure 2). This was accomplished by alkali treatment prior to addition of the cycling reagent, since $NADP^+$ is labile at high pH (Table III). The time required for 99.99% destruction of $NADP^+$ was reduced by alkali treatment at 65° C. A one-hour incubation of samples at pH 12.5 at 65° was used, which meant that 5×10^{-13} mol of $NADP^+$ remained of the 1×10^{-9} mol in the reagent after treatment. The undestroyed $NADP^+$ was a major contributor to the blank fluorescence and largely determined the sensitivity of ATP detection for that reason.

ATP Measurement by Enzymatic Cycling. ATP was measured using enzymatic cycling of NADPH after alkali treatment (Figure 18). The limit of detection was 2×10^{-13} mol and the range was 2×10^{-13} to 2×10^{-11} mol

Figure 17. Coupled Enzyme Assay of ATP.

ATP was measured by reaction with hexokinase and glucose-6-phosphate to produce NADPH, which was measured fluorometrically (excitation 340 nm, 1 mm slit; emission 455 nm, 2 mm slit). ATP samples (0.1 ml) were mixed with the coupled enzymes reagent (0.9 ml) and incubated 15 min at 37° C before being measured.

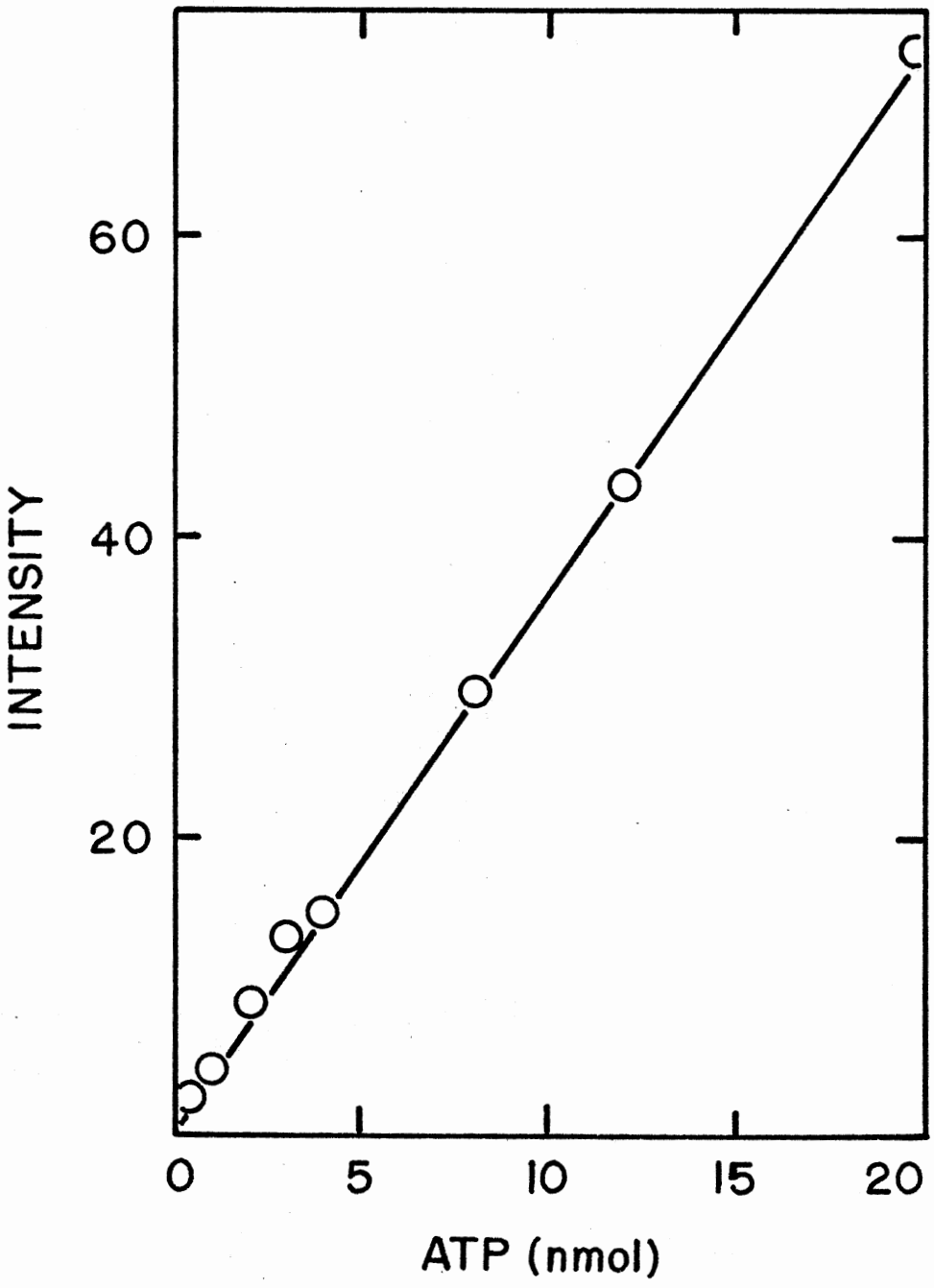

INTENSITY
60
40
20
0
5
10
15
20
ATP (nmol)

TABLE III

$NADP^+$ DESTRUCTION BY ALKALI

Temperature (°C)	Half-Life (min)	Time for 99.9% (min)[a]	Time for 99.99% (min)[a]
38	21.0	209	279.1
65	5.5	54.8	73.0

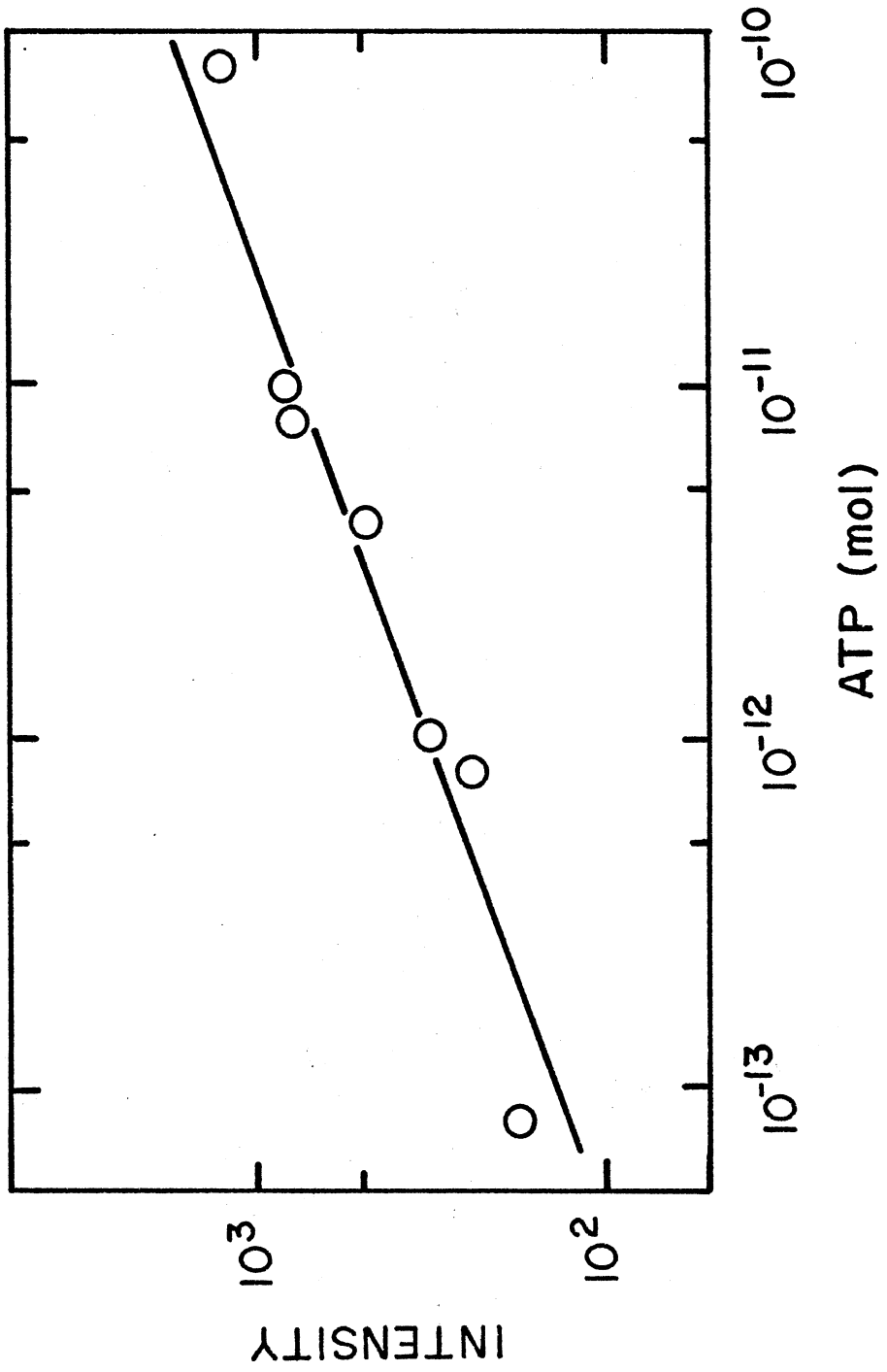
a. The time required to destroy a known fraction of the amount present or the amount left after an incubation time is given by the equation

$$N = N_o e^{-\frac{0.693t}{t_{1/2}}}$$

where N = the amount present
N_o = the starting amount
t = incubation time
$t_{1/2}$ = half-life under the incubation conditions.

Figure 18. Enzymatic Cycling Assay of ATP.

ATP was measured by enzymatic cycling of NADPH produced by the coupled enzyme reactions discussed earlier (Figure 17). Samples (20 µl) were incubated with 10 µl of coupled enzyme reagent 15 min, treated with NaOH and incubated one hour at 65° C, neutralized, and carried through the standard $NADP^+$ cycling procedure.


INTENSITY
10^3
10^2
ATP (mol)
10^{-10}
10^{-11}
10^{-12}
10^{-13}

of ATP. $NADP^+$ standards produced a parallel curve but with a lower blank and so a greater sensitivity. The limit of detection of ATP by enzymatic cycling was improved 1000-fold over the coupled enzyme assay method.

Measurement of ATP in E. coli

ATP was extracted from E. coli using boiling Tricine buffer and measured by the enzymatic cycling procedure discussed earlier in this chapter. The extraction procedure yielded > 90% of the ATP in standard samples so no correction was made for recovery. Since extracts contained ATP and pyridine nucleotides, the response was the sum of ATP and reduced pyridine nucleotides.

Two samples of cells, 5×10^8 and 5×10^7 cells, were extracted using five ml boiling Tricine buffer and 0.02 ml of the extracts were used in the cycling procedure. Excessive concentrations of ATP and pyridine nucleotides were present for the reagent used with the extract from 5×10^8 cells, but there was 5.4×10^{-12} mol of ATP/20 µl extract with 5×10^7 cells. This may be expressed as 1.35×10^{-9} mol ATP and reduced pyridine nucleotides/5×10^7 cells. When the pyridine nucleotides were subtracted, the ATP content was 1.23×10^{-9} mol/5×10^7 cells.

Comparison of Enzymatic Cycling and Luciferase

Since this laboratory has also studied ATP determination with firefly luciferase, comparison of enzymatic cycling and firefly luciferase procedures for the determination of ATP was made (Table IV). The parameters compared were range, sensitivity, cost, productivity (number of samples measured, not counting standards), inhibitors, equipment, turnaround time (time required to get a result and repeat an assay), and

TABLE IV

COMPARISON OF ENZYMATIC CYCLING AND FIREFLY LUCIFERASE DETERMINATIONS OF ATP

Parameter	Luciferase	Cycling
Range[a]	0.2 pmol - 100 pmol	0.3 pmol - 10 pmol
Sensitivity[b]	0.2 fmol (0.1 pg)	0.1 pmol (50 pg)
Cost	6¢/assay	9.5¢/assay
Productivity	25/hour or 200/8 hour day	96/5 hr or 192/8 hour day
Inhibitors	Metal ions, PO_4^{3-}	None encountered to date
Equipment	Photometer	Fluorometer
Turnaround Time	<30 minutes	5 hours
Specificity	ATP only	ATP, NADH, NADPH

a. Useful range of ATP amounts which can be routinely measured.

b. Smallest amount of ATP detected by the assay.

specificity. The firefly luciferase was clearly as good or better in every category except inhibitors, which is of vital importance in the measurement of environmental samples. Enzymatic cycling could be used for those samples that contain inhibitors which prevent determination by firefly luciferase and as such is a useful procedure.

Flavin Mononucleotide

Measurement of flavin mononucleotide (FMN) was undertaken using bacterial luciferase with NADH and flavin reductase acting to generate the true reaction substrate, $FMNH_2$. A crude preparation of bacterial luciferase was used with an excess of NADH present so that light production was proportional to the amount of FMN in the sample. Aging the luciferase preparation to lower its blank luminescence also decreased its response to FMN and so the sensitivity of the assay was limited by the blank luminescence of the reagent. The limit of detection of the assay was 1 ng FMN and the range tested was 1-50 ng FMN (Figure 19).

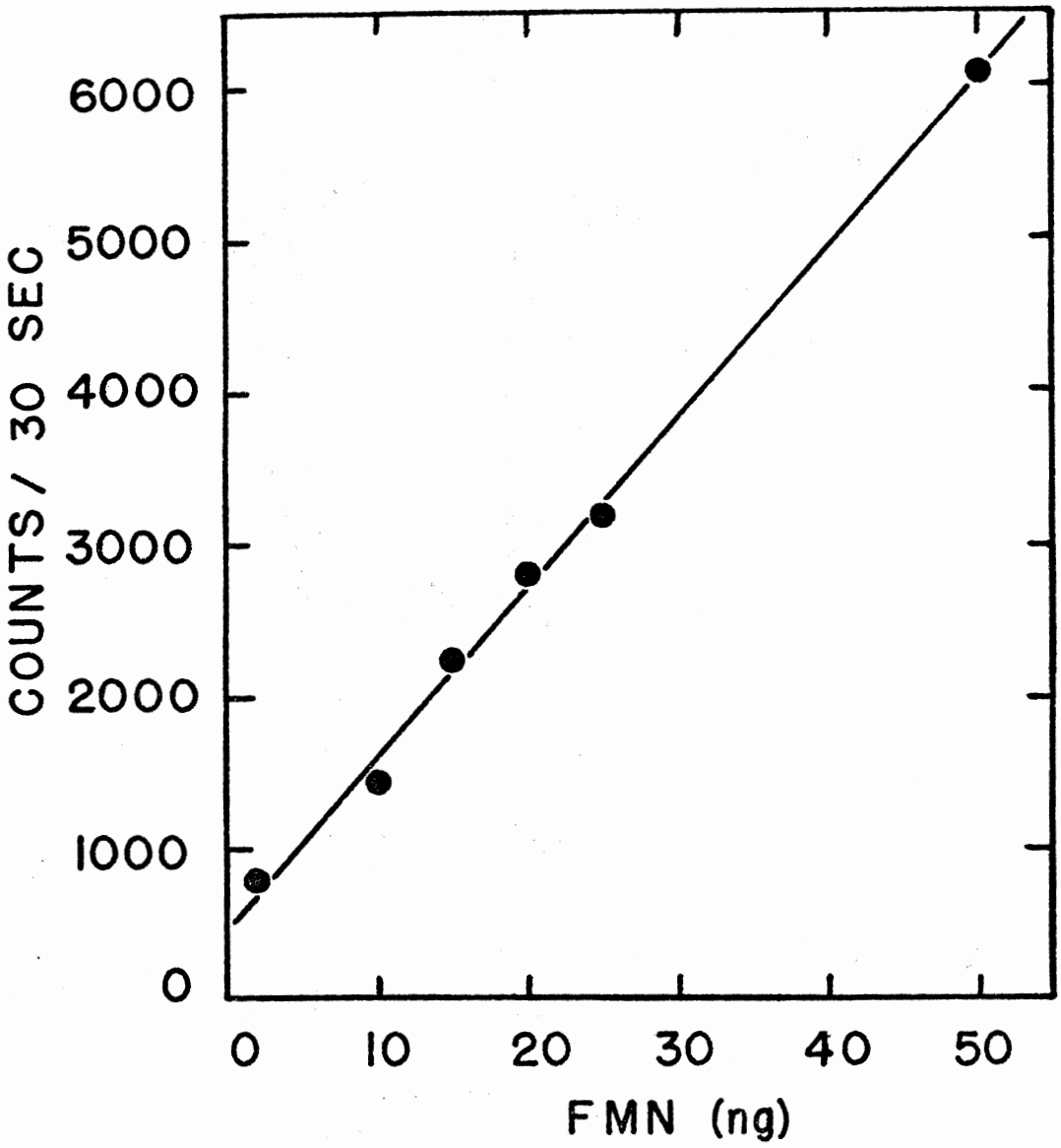
Iron Porphyrins

Iron porphyrins, that is porphyrin ring structures containing quadridentate-chelated iron atoms, act in the transfer of electrons from reduced cofactors (NADH and $FADH_2$) to molecular oxygen in aerobic organisms and as catalytic sites in enzymes such as catalase and horse-radish peroxidase. Proteins containing covalently or non-covalently bound iron porphyrins are sometimes called heme proteins.

Heme-containing proteins can be quantitated by their catalytic effect on the chemiluminescent oxidation of luminol under alkaline conditions. The major application of iron porphyrin determinations has been

Figure 19. FMN Measurement Using Bacterial Luciferase.

The luminescent reaction of bacterial luciferase was used to quantitate FMN as well as NADH. FMN samples (0.2 ml) were mixed with 0.3 ml bacterial luciferase reagent containing 0.2 mg luciferase-flavin reductase, 20 µg NADH, and 1.5 µg dodecyl aldehyde in 0.4 M Tris-HCl buffer, pH 6.4. Samples were counted to 30 sec in the SAI Model 3000 Photometer using a 10 second delay and sensitivity of 400.


6000
5000
4000
3000
2000
1000
0
COUNTS / 30 SEC
0
10
20
30
40
50
FMN (ng)

quantitation of biomass or cell concentrations in environmental samples (57).

Light Emission During Luminol Oxidation

The intensity and duration of luminol chemiluminescence depends on the concentrations of reactants - luminol, hydrogen peroxide, catalyst, and hydroxyl ions (pH). Studies of reaction conditions have established optimum reactant concentrations (57, 59), and these were used as a basis for the application of the procedure to quantitation of iron porphyrins and biomass in this laboratory.

Luminescence measurements were made using the Model 3000 ATP photometer (SAI Company), which has the capability of collecting data in two modes, integration and peak height (Figure 20). Both modes were used during the study, with an accessory injector (0.2 ml) being used for most of the peak height measurements to improve reproducibility. Aluminum disks with holes of several sizes were used in some determinations to attenuate the light emission and extend the range of the determinations.

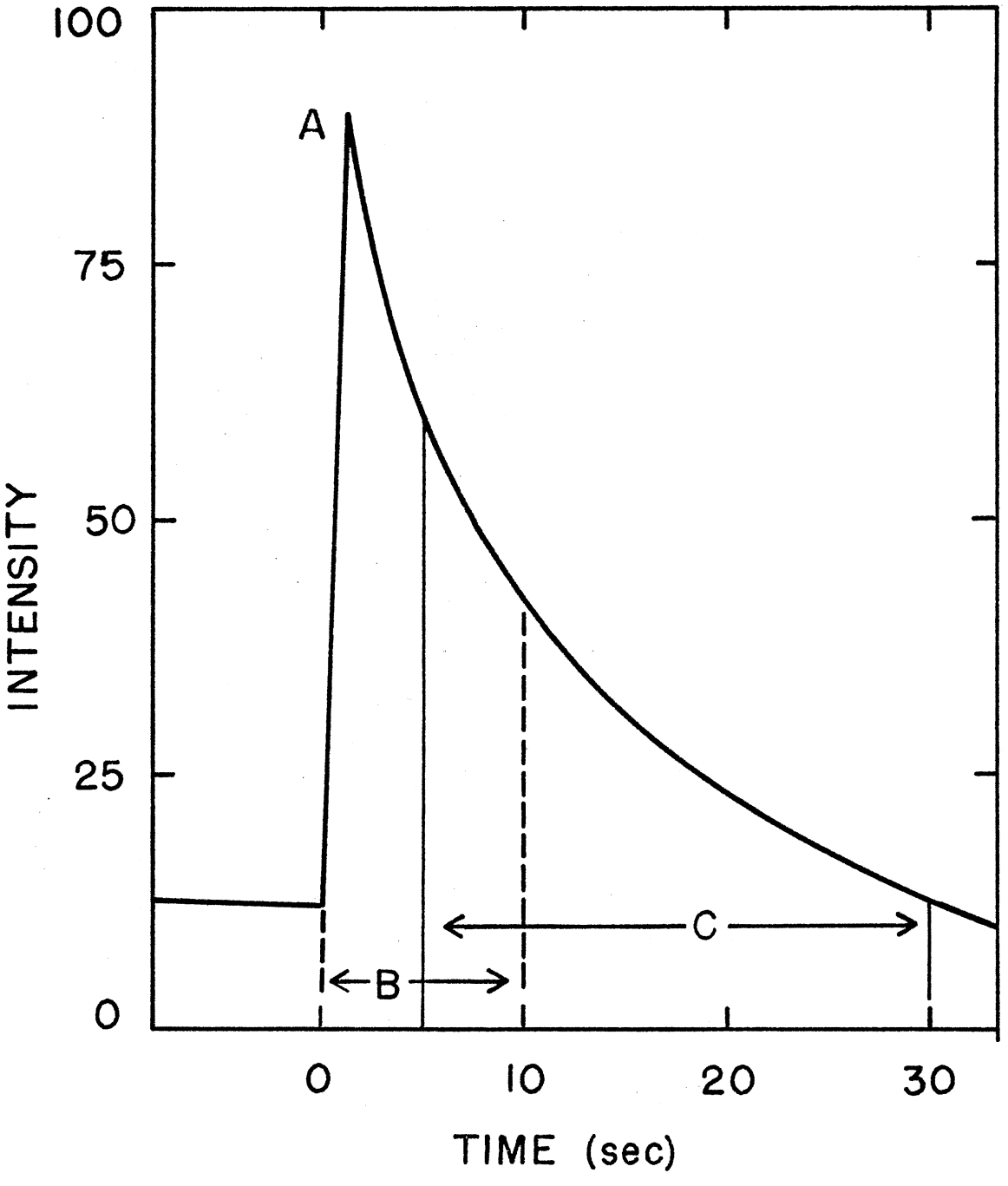
Measurement of Hemoglobin

Human hemoglobin contains four iron porphyrin groups per molecule and so can be quantitated by luminol oxidation. The limit of detection and range of hemoglobin which could be measured were determined by luminescence attenuation using peak height mode and sample injection.

Luminescence Attenuation. The useful range of measurement of luminescent reactions is the difference between the blank and the light intensity which can damage the photomultiplier tube of the instrument.

Figure 20. Light Emission of Luminol.

The luminescence of luminol oxidation was measured by three methods. (A) The height of the initial light emmission after injection of sample into luminol reagent was measured using a chart recorder. (B) The initial peak was integrated from 0.5 sec to 10.5 sec after sample injection into luminol reagent. (C) The light emission curve was integrated for 30 sec after a 5 sec delay which was required when the injector accessory was not used and the sample was pipetted into luminol reagent.


100
75
50
25
0
INTENSITY
A
B
C
0
10
20
30
TIME (sec)

The Model 3000 has a protection device which turns off the voltage when the light intensity is too high and may overload the photomultiplier tube. Luminol is oxidized by oxygen at a slow rate and so has a measurable blank. Aluminum disks having several different-sized holes were used to reduce the amount of light reaching the photomultiplier tube and increase the range of hemoglobin concentrations that can be measured. Counts made with disks in place were corrected for the attenuation by dividing the response by the fraction of light allowed to pass by each disk so that measurements made using several disks could be evaluated together (Table V).

Hemoglobin Standard Curve. A large range of hemoglobin (1 pg - 10 ng) was measured by the luminol reaction using the aluminum disks (Figure 21). Although all samples had responses higher than a distilled water-luminol blank (71,500 counts) a linear response was seen only at higher hemoglobin amounts (200 pg - 10 ng) using the 0.035 in. disk. The failure of sample responses using different disks to describe a straight line in Figure 21 is probable due to geometric factors of the attenuation which have not been eliminated or accounted for in the correction factors used. The limit of detection for hemoglobin was 2 pg and the range was 2 pg - 10 ng.

Measurement of E. coli

The limit of detection which had been reported for bacteria by the luminol reaction was 10^4/ml (57). Peak height and integration modes were examined to see if the sensitivity of the assay could be improved.

The luminescence of a 0.5 ml aliquot of luminol reagent was measured by peak height on a chart recorder and by a 30 sec integration count.

TABLE V

ATTENUATION OF LUMINESCENCE BY ALUMINUM DISKS

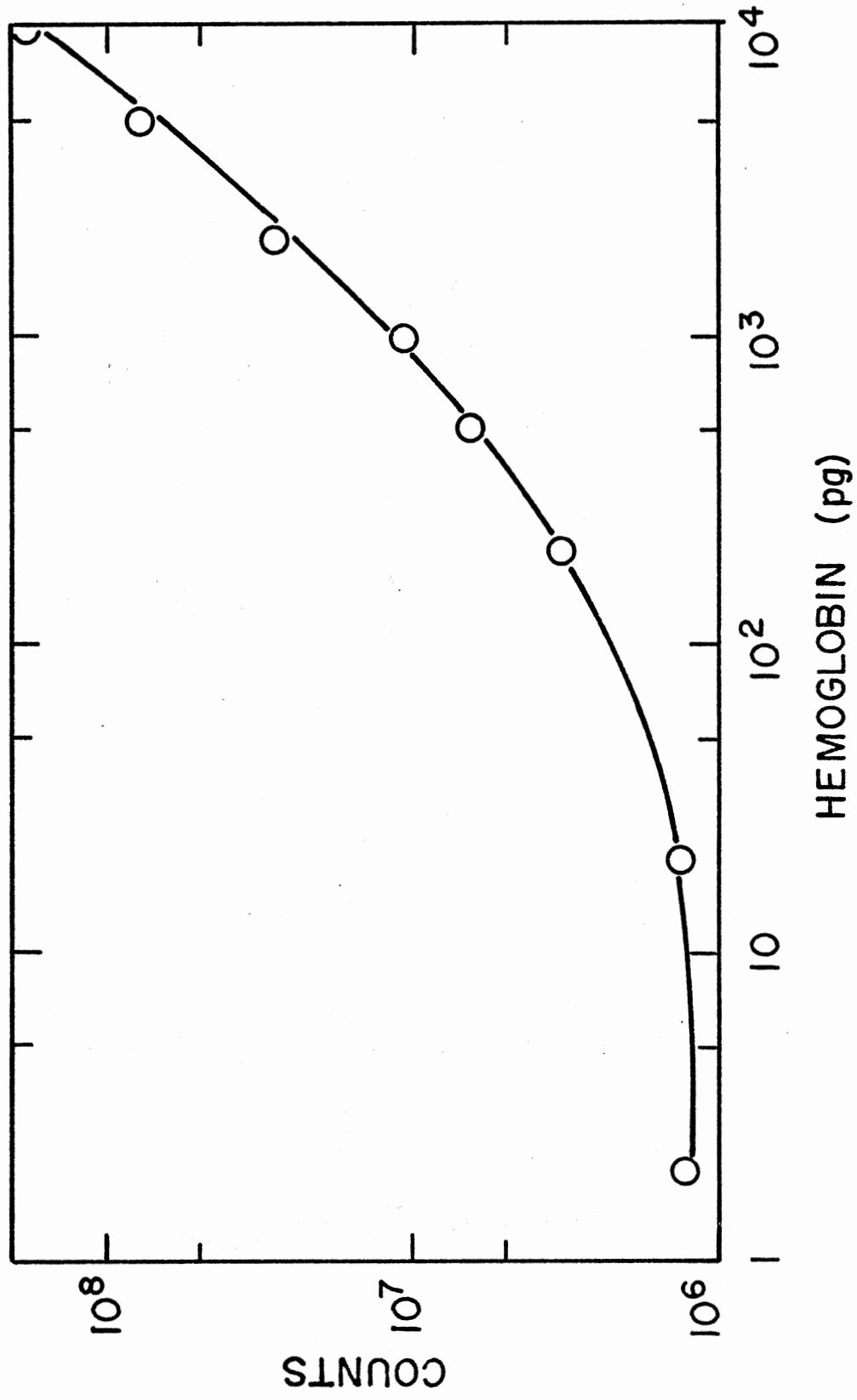
Hole Size (cm)	Area (cm^2)	Fraction of Area	Counts[a]	Fraction of Counts
2.445[b]	18.7767	1	413821	1
0.221	1.4287	0.07609	106943	0.25843
0.111	0.3585	0.01909	32344	0.07816
0.567	0.0937	0.00499	9276	0.02242
0.028	0.0227	0.00120	1963	0.00474
0.015	0.0062	0.00033	218	0.00053
0.007	0.0013	0.00007	0	-

a. A 50 ng ATP standard assayed using the method outlined in Chapter 2 was used as a standard light source.

b. Size of the sample chamber with no disk in place.

Figure 21. Luminescence Determination of Hemoglobin.

Luminol chemiluminescence was used to quantitate hemoglobin. Samples (0.2 ml) were injected into 0.5 ml luminol reagent (0.25 mM luminol, 10 mM EDTA, and 1% H_2O_2 in 50 mM phosphate buffer, pH 11.6) and the light emission measured for 10 sec after a 0.5 sec delay in the SAI Model 3000 photometer with the instrument sensitivity set at 700. The luminescence was attenuated by aluminum disks to extend the range of hemoglobin concentration measured.


COUNTS
10^8
10^7
10^6
1
10
10^2
10^3
10^4
HEMOGLOBIN (pg)

E. coli samples (0.5 ml) were pipetted into the reagent and counted in the photometer using a 5 sec delay for integration mode while peak height was determined from the chart recorder 2 sec after sample addition. The counts or peak height of the reagent alone were subtracted from the total response and net counts were plotted (Figure 22). Under these conditions, the integration mode was more sensitive with a limit of detection of 500 cells/assay or 1000 cells/ml sample. Peak height measurements had a limit of detection of 1000 cells/assay or 2000 cells/ml sample and the range for both modes extended to 10^5 cells/ml.

Measurement of Iron Porphyrins in Spring Water

Water from eight springs in eastern Oklahoma and western Arkansas was collected in sterile pyrogen-free bottles and was tested for iron porphyrins by the luminol assay using hemoglobin as a standard. Samples were measured both before and after treatment with 0.5% hydrogen peroxide so that the response due to intact cells could be determined. Treatment of samples with 0.5% hydrogen peroxide destroys extracellular porphyrins and oxidizes reduced metal ions that may give a response without damaging viable or intact cells (57). The results are shown in Table VI.

Three conclusions can be drawn from the data in Table VI. Firstly, environmental samples did contain measurable amounts of iron porphyrins. Secondly, hydrogen peroxide treatment did substantially reduce the luminescence response of the samples. Lastly, there was no clear correlation between cell numbers measured by nutrient agar plate counts and amounts of iron porphyrins present in the samples.

Figure 22. Luminescence Determination of E. coli.

Chemiluminescence response of E. coli was measured using nutrient agar plate counts to determine cell numbers used. Cell samples (0.5 ml) were mixed with 0.5 ml luminol reagent (0.25 mM luminol, 6.3 mM EDTA and 0.1% H_2O_2 in 0.75 N NaOH) and the light emission counted 30 sec in a SAI Model 3000 photometer using a 5 sec delay and a sensitivity of 500 (O). Peak height measurements were made two seconds after sample addition using a chart recorder (●). The reagent was counted 30 sec and its chart response determined before sample was added and these values subtracted from the measurements made after samples were added.

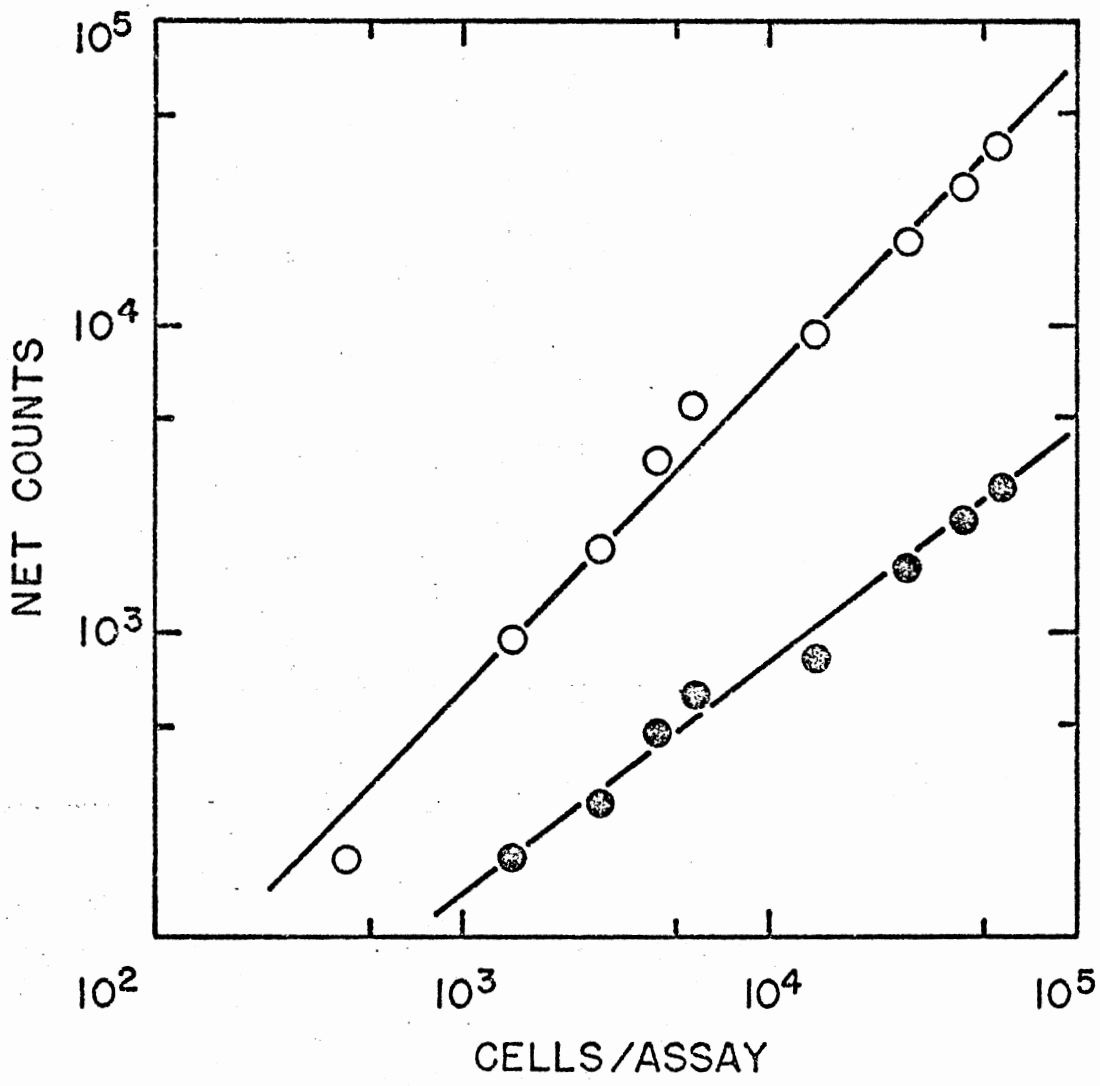

10^5
10^4
10^3
NET COUNTS
10^2
10^3
10^4
10^5
CELLS/ASSAY

TABLE VI

ANALYSIS OF SPRING WATER FOR IRON PORPHYRINS

Sample	Plate counts (ml^{-1})	Iron Porphyrin (pg/ml)[a]	
		$- H_2O_2$[b]	$+ H_2O_2$[c]
Whaley's Old Highway	59	76	9
Behind Speedy's	29	100	20
Billy Brown	219	135	14
Groto Eureka	4	58	17
Charlie Tanihill	2	940	240
Park Spring	5	195	25
Harding Eureka	114	86	2
Murrel House	473	2	2

a. Results expressed in picograms hemoglobin (Figure 21).

b. No H_2O_2 pretreatment of samples.

c. Sample pretreatment with 0.5% H_2O_2.

CHAPTER V

MEASUREMENT OF LACTATE DEHYDROGENASE BY ENZYMATIC CYCLING OF NAD^+

As stated earlier, the limit of detection for an enzymatic activity is the limit of detection for a change in the amount of substrate or product being measured. An increase in sensitivity of product measurement also increases the sensitivity of enzyme measurement. This concept was tested by using enzymatic cycling to measure NAD^+ produced by lactate dehydrogenase (Equation 2) and so improve the sensitivity of detection of the enzyme.

Development of NAD^+ Cycling

Destruction of Excess NADH

All unreacted NADH must be destroyed prior to enzymatic cycling so that the final response will be proportional to the enzymatic activity. This was accomplished by treatment of samples with hydrochloric acid, which destroys NADH without affecting NAD^+, the product of the lactate dehydrogenase reaction. The half-life of NADH at pH 2 at 37^{o} C is 0.07 min so the time required for 99.99% destruction of NADH under assay conditions, using the equation in Table III, is 0.93 min. The reagent used contained one nanomole NADH, and a ten minute incubation at pH 2 was used, which was sufficient to decrease the amount of NADH present

to >1 x 10^{-14} mol without changing the amount of NAD^+ produced by the reaction.

Measurement of Lactate Dehydrogenase

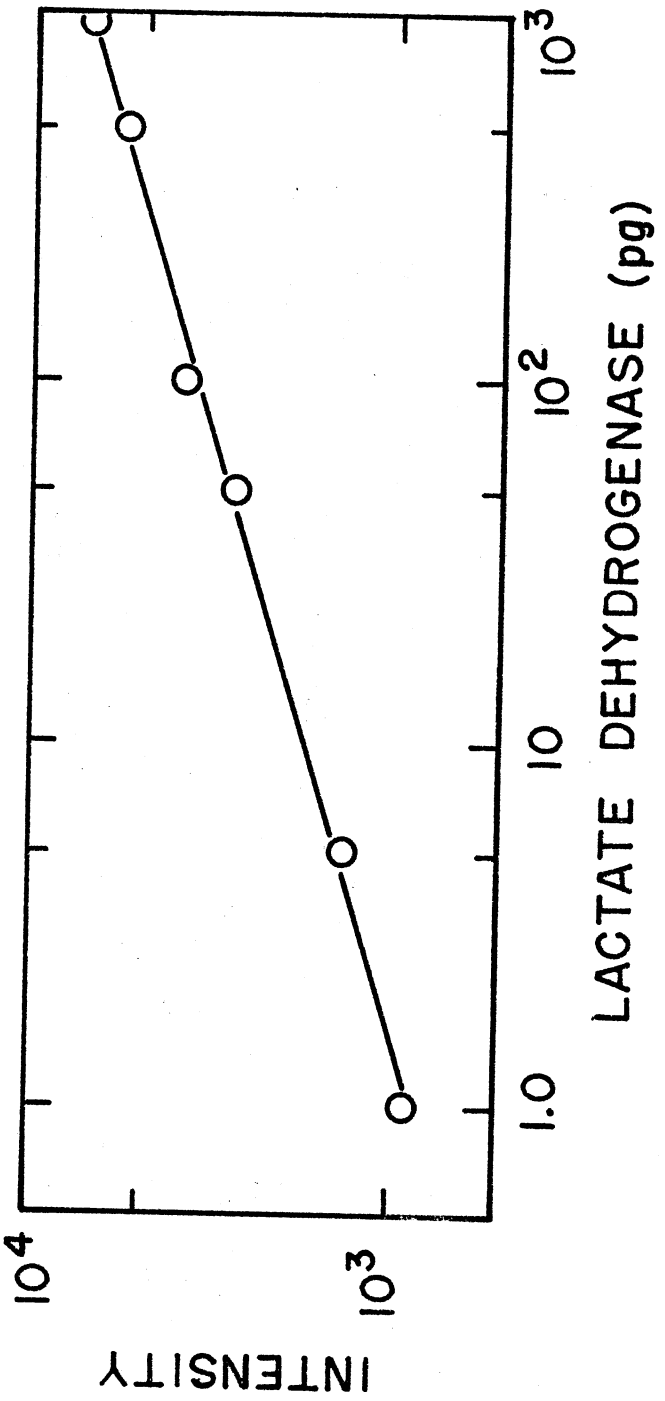
Lactate dehydrogenase samples (1 pg - 1 ng) were incubated with substrates pyruvate and NADH for one hour at 37° C. Unreacted NADH was destroyed by acidification of the samples by hydrochloric acid and incubation for 15 min. The samples were then neutralized with NaOH and the NAD^+ was amplified by the enzymatic cycling procedure discussed in Chapter 2. The results are shown in Figure 22. The limit of detection was 1 pg and the range of the assay was 1 pg - 1 ng lactate dehydrogenase. Enzymatic cycling of NAD^+ improved the sensitivity of lactate dehydrogenase detection 1000-fold over the one hour incubation assay.

Measurement of Lactate Dehydrogenase in E. coli

Samples containing E. coli cells (10^3-10^6/sample) were treated with 0.1% toluene and then lactate dehydrogenase measured by a one-hour incubation with the pyruvate-NADH reagent followed by NADH destruction and enzymatic cycling of NAD^+. Using the lactate dehydrogenase standard curve in Figure 23, the cell samples read as follows: 10^4 cells - 5.4 pg; 10^5 cells - 56 pg; 10^6 cells - 390 pg. The sample containing 10^3 cells did not have a value above the blanks which were run with the samples. The concentration of the 10^4 cells/assay was 5 x 10^5 cells/ml. This represented only a two-fold increase in sensitivity of lactate dehydrogenase measurement in E. coli over a one-hour incubation assay using the dye INT.

Figure 23. NAD^+ Cycling Measurement of Lactate Dehydrogenase.

Picogram amounts of lactate dehydrogenase were measured by enzymatic cycling of NAD^+ produced by a one-hour incubation of 20 µl samples with 10 µl of LDH reagent (10 nmol NADH and 30 nmol pyruvate in 0.09 M phosphate buffer, pH 7.4) at 37° C. Unreacted NADH was destroyed by treatment with 10 µl 1 N HCl and the NAD^+ was measured by the enzymatic cycling procedure already discussed.


INTENSITY
10^4
10^3
1.0
10
10^2
10^3
LACTATE DEHYDROGENASE (pg)

CHAPTER VI

DISCUSSION

Selection of Bioindicators

General Considerations

The bioindicators used in this study were selected because they met one or more of the requirements stated in Chapter I. All indicators were involved in cellular metabolism, cellular energy transfer or storage, and were measurable in nanogram quantities by published analytical techniques. The number of bacteria required to give detectable amounts of a bioindicator was determined for each assay and was an important consideration of the suitability of each bioindicator for use in ground water quality monitoring.

Enzymes

Representatives from three classes of enzymes were studied: lactate dehydrogenase and catalase (oxidoreductases), adenylate kinase (transferase), and alkaline phosphatase (hydrolase). The amounts and variations in concentrations of the enzymes in various cell types have not been studied. The persistence of catalase, alkaline phosphatase, and nonspecific dehydrogenase has been well characterized in soil but not water (68).

Respiratory Cofactors

Of the respiratory cofactors selected for study, ATP, FMN, and iron porphyrins had been used for biomass quantitation (41, 69, 70). The pyridine nucleotides were selected because assay techniques were available which could be used for sensitive measurement of the bioindicator. FMN measurements using commercial preparations of bacterial luciferase did not have the sensitivity considered necessary for bioindicator determinations in environmental samples.

Development of Assays

To be useful for routine monitoring of biological activity in environmental samples, an assay must be sensitive enough to measure small amounts of bioindicator but be usable over a large range of bioindicator concentrations, rapid, utilize commercially available equipment, and require average laboratory skills. Two types of assays were developed to measure bioindicators and the suitablilty for determinations of biological activity were tested: direct determinations (continuous assays and coupled assays), and amplification of responses (incubations and enzymatic cycling).

Direct Determinations

The most rapid assays involved measuring a bioindicator directly or by coupling to the production of some species which could be measured directly. The sensitivity of the assays depended upon the analytical method.

Continuous Enzyme Assays. Continuous assays were used to measure

all four enzymes. The rapidity with which the continuous assays could be performed (5 min/sample) was countered by a lack of sensitivity (0.1 μg lactate dehydrogenase, 3 ng adenylate kinase). However, they could be used to screen samples for the selected enzyme activities to establish the range at which to apply more sensitive determinations.

Coupled Assays. Several bioindicators were measured by quantitation of some other species which was equal to or proportional to the amount of bioindicator present. Coupled assays are important because: 1) usually several different bioindicators can be measured by the same analytical technique (i.e. luminescence measurements), and 2) bioindicators which would be difficult to measure directly can be quantitated.

ATP utilization was coupled enzymatically to NADPH production which could be measured spectrophotometrically or fluorometrically. Four bioindicators were coupled to light-producing reactions: ATP (firefly luciferase), NADH and FMN (bacterial luciferase), and iron porphyrins (luminol). Thus the same instrmentation and inherent sensitivity of luminescent measurements could be utilized.

Amplification of Bioindicator Responses to Improve Sensitivity of Detection

The results of several of the direct determinations (continuous assays and pyridine nucleotide measurements) showed that greater sensitivity of detection was required if the bioindicators were to be used to measure biological activity. Therefore studies of two amplification techniques were undertaken to improve the sensitivity of bioindicator determination.

Incubation of Enzymes. Since enzymes function catalytically, they act on many molecules of substrate to produce many molecules of product each second for each enzyme molecule. Under the appropriate conditions the amount of product formed or substrate reacted at the end of an incubation of substrate with enzyme is proportional to the amount of enzyme present, so the effect of the enzyme is amplified at a rate dependent upon the turnover rate of the enzyme and the time of incubation.

One-hour incubation assays were developed for all the enzymes so that data could be collected in a reasonable length of time. All enzymes could be measured with greater sensitivity using a one-hour incubation than using continuous assays (20-fold to 100-fold improvement).

The possibility of greater sensitivity using a longer incubation time was tested using alkaline phosphatase as a model. Reaction mixtures were incubated for up to 96 hours before total product formation was measured spectrophotometrically. Although nonenzymatic product formation had to be compensated for, the sensitivity of the assay was increased at least 10-fold using a 24-hour incubation over the one-hour incubation assay. Long incubations have been widely used for determinations of both phosphatase (71) and dehydrogenase (72) in soil.

Enzymatic Cycling. Another amplification technique which utilizes the principle of accumulation of an enzymatic reaction product to increase the sensitivity of measurement is enzymatic cycling, which was discussed in Chapter I. The enzymatic cycling of pyridine nucleotides was used to measure pyridine nucleotides, ATP, and an enzymatic activity, lactate dehydrogenase. Since many bioindicators could be coupled to pyridine nucleotides or ATP, enzymatic cycling has the potential of being a

technique that could be used to amplify and detect a number of bioindicators.

The enzymatic cycling of $NADP^+$ and NAD^+ increased the sensitivity of detection 10,000-fold for $NADP^+$ and 1000-fold for NAD^+ over the standard fluorometric determination. ATP measurement coupled to NADPH cycling increased the sensitivity 1000-fold over coupled assay ATP measurement.

The enzymatic cycling determination of ATP was neither as sensitive nor as rapid as the firefly luciferase determination of ATP (Table IV) and so would not be the method of choice for most applications of ATP measurement. However, firefly luciferase is highly sensitive to interferences that are present in soil (73) and the enzymatic cycling might be used effectively for samples where interferences prevent use of the luciferase determination.

Measurement of Lactate Dehydrogenase Using Enzymatic Cycling. The two amplification procedures, incubation of enzymatic activities and enzymatic cycling of pyridine nucleotides, were combined to improve the sensitivity of determination of lactate dehydrogenase. Enzyme samples were incubated one hour as before, but then the reaction product, NAD^+, was cycled one hour to improve the sensitivity of its measurement. A 1000-fold improvement in lactate dehydrogenase detection resulted.

Evaluation of Bioindicators and Assays

Each bioindicator except FMN was measured in E. coli samples to determine the minimum number of bacteria required for detection. These data were then used to evaluate the suitability of the bioindicator

and/or assay methods for measurement of biological activity in environmental samples such as ground water.

Sensitivities of Assays

The sensitivities of the assays obtained in this study were compared with published values (Table VII). The most significant improvements were lactate dehydrogenase measurement using enzymatic cycling (2000-fold) and E. coli quantitation using luminol chemiluminescnece (7-fold). The ATP assay sensitivity using enzymatic cycling was considerably less than the firefly luciferase assay sensitivity, but environmental sample conditions may require an alternative method for ATP measurement which enzymatic cycling provides. The sensitive assay cited (27) is a manometric technique which is not applicable to many routine measurements, but could be used on samples which have less catalase activity than can be measured spectrophotometrically. The other assays have sensitivities comparable to those examined in the literature.

Measurements of Bioindicators in E. coli

The microbial population in the subsurface environment is heterogeneous and subject to changes in numbers and types of organisms present depending on temperature, water, and soil type (1). Since it would be impossible to duplicate the composition of organisms in ground water, a model bacterium, E. coli, was used for bioindicator determinations.

A summary of the sensitivities of the assays developed for this study and the amounts determined in E. coli samples and numbers of E. coli required for measurement are shown in Table VIII. All assays were able to measure one ng or less of bioindicator and to detect $<10^6$ cells.

TABLE VII

COMPARISON OF BIOINDICATOR SENSITIVITIES

Bioindicator	Limit of Detection		Reference
	This Study	Literature	
Enzymes			
LDH	1.0 pg	2.0 ng	74
Alk. Phosphatase	0.1 ng	5.0 ng	75
Catalase	1.0 ng	20.0 pg	27
Adenylate Kinase	0.1 ng	0.1 ng	76
Respiratory Cofactors			
Pyridine Nucleotides	37.0 pg	15.0 pg	62
ATP	100.0 pg	0.2 pg	42
FMN	1.0 ng	0.4 ng	50
Iron Porphyrins			
Hemoglobin	2.0 pg	6.0 pg	77
E. coli	500 cells	3400 cells	69

TABLE VIII

SUMMARY OF RESULTS OF ASSAYS FOR BIOINDICATORS

Bioindicator	Assay Method	Limit of Detection	Minimum Cell Concentration (ml^{-1})
Lactate Dehydrogenase	Continuous	0.1 μg	
	Incubation	1.0 ng	1×10^6
	Cycling	1.0 pg	5×10^5
Alkaline Phosphatase	Continuous	20 ng	
	Incubation(1 h)	1.0 ng	8.5×10^6
	Incubation(24 h)	0.09 ng	
Catalase	Continuous	0.2 μg	
	Incubation	1.0 ng	1×10^7
Adenylate Kinase	Continuous	3.0 ng	
	Incubation	0.1 ng	4×10^5
Pyridine Nucleotides	Cycling		5×10^6
	$NADP^+$	37.0 pg	
	NAD^+	3 ng	
Adenosing Triphosphate	Coupled Enzyme	100 ng	
	Cycling	100 pg	5×10^5
Flavin Mononucleotide	Bacterial Luciferase	1.0 ng	
Iron Porphyrins	Luminol		
	Hb	2.0 pg	
	Pk. Ht.		2×10^3
	Integration		1×10^3

Suitability of Bioindicators and Assays

The number of organisms reported for ground water varies from $<10^4$/liter (78) to $>10^8$/liter (79). Membrane filtration can concentrate microbes in water samples 100-fold or more (80) so a suitable assay should be able to detect biological activity from 10^3 cells/ml to 10^7 cells/ml. Examination of Table VIII shows that, of the assays tested, only the luminol determination of iron porphyrins is capable of detecting the smaller amounts of bacteria that might be present in ground water. The amplification provided by enzymatic cycling improved the sensitivity of detection of ATP and lactate dehydrogenase enough to make them useful for all but the smallest amounts of bacteria. All the bioindicators selected and assays developed would be suitable for biological activity determinations in water samples in which the microbes could be concentrated to 10^6/ml.

Conclusions

The purpose of this study was to develop sensitive assays for selected enzymes and respiratory cofactors and to test the feasibility of using enzymatic cycling of pyridine nucleotides to increase the sensitivity of bioindicator determination. The following conclusions can be drawn from the data presented:

1) Sensitive assays for the enzymes lactate dehydrogenase, alkaline phosphatase, catalase, and adenylate kinase, and the respiratory cofactors pyridine nucleotides, ATP, FMN, and iron porphyrins were developed which could be used to measure nanogram quantities or less of these bioindicators.

2) All bioindicators except FMN were detectable in E. coli cells - lactate dehydrogenase (10^4), alkaline phosphatase (8.5×10^5), catalase (10^6), adenylate kinase (4×10^4), pyridine nucleotides (2.4×10^5), ATP (10^4), iron porphyrins (5×10^2).

3) Several assays were sensitive enough to be used without further modification - iron porphyrins, ATP, and lactate dehydrogenase.

4) Enzymatic cycling is an effective way of improving the sensitivity of bioindicator measurement. Cycling improved $NADP^+$ measurement 10,000-fold, ATP measurement 1000-fold, and lactate dehydrogenase measurement 1000-fold. Since many other bioindicators could be coupled to ATP or $NADP^+$, enzymatic cycling may have the most utility of any method tested as a technique for sensitive determinations of bioindicators.

CHAPTER VII

SUMMARY

The purpose of this study was to develop sensitive assays for selected bioindicators which could be used to monitor the quality of ground water supplies. Two classes of bioindicators were chosen for detailed study: 1) enzymes and 2) respiratory cofactors. The enzymes were lactate dehydrogenase, alkaline phosphatase, catalase, and adenylate kinase; the respiratory cofactors were the pyridine nucleotides, ATP, FMN, and iron porphyrins.

One-hour incubation assays were more sensitive than continuous assays for all enzymes (a 20-fold improvement for alkaline phosphatase (20 ng to 1 ng) to a 200-fold improvement for catalase (200 ng to 1 ng)). The enzymatic activities were measured in toluene-treated E. coli using the one-hour incubation assays and the limit of detection was 10^6- 10^7 cells/ml for the four enzymes.

The respiratory cofactors were measured by several methods and the sensitivity of each method determined. Pyridine nucleotides were quantitated spectrophotometrically, fluorometrically, and in a coupled reaction with bacterial luciferase. Enzymatic cycling was used to amplify the fluorescence response and decreased the limit of detection to 0.05 pmol of $NADP^+$ and 5 pmol of NAD^+. Pyridine nucleotides were extracted from E. coli using boiling Tricine buffer and measured using enzymatic cycling. The limit of detection was 2.4×10^5 cells/assay.

Adenosine triphosphate was quantitated by a coupled enzyme assay which produced NADPH that was measured fluorometrically or by enzymatic cycling. Cycling improved the sensitivity from 200 pmol to 0.2 pmol. ATP was extracted from E. coli using boiling Tricine buffer and measured using enzymatic cycling. The limit of detection was 10^4 cells/assay. The results of the ATP studies were compared to studies using firefly luciferase to measure ATP.

FMN was measured by a luminescence assay using a crude bacterial luciferase preparation with FMN reduction carried out enzymatically using NADH as electron donor. The limit of detection was 1 ng FMN.

Iron porphyrins were measured by their catalytic action on the rate of chemiluminescent oxidation of luminol. Hemoglobin samples were quantitated over a range of 2 pg - 10 ng using aluminum disks to attenuate the intensity of the light emission. E. coli were quantitated by the peak height and integration methods of luminescence measurement with a limit of detection of 2×10^3/ml and 1×10^3/ml, respectively.

A sensitive assay of lactate dehydrogenase was developed by combining a one-hour incubation with enzymatic cycling of the reaction product, NAD^+. The limit of detection was improved 1000-fold (1 ng to 1 pg) and the number of E. coli cells which could be detected was decreased 10-fold (10^5 to 10^4/assay).

It is concluded that the ATP and lactate dehydrogenase assays developed using enzymatic cycling and iron porphyrins could be used to detect changes in their respective bioindicators in environmental samples such as ground water which have as little as 10^4 cells/liter. Measurements of pyridine nucleotides, catalase, adenylate kinase, and alkaline phosphatase could be used effectively for samples with higher cell concentrations.

A SELECTED BIBLIOGRAPHY

1. McNabb, J. F. and Dunlap, W. J. (1975) Ground Water, 13, 33-44.

2. Watson, J. D. (1976) Molecular Biology of the Gene, 3rd Ed., W. A. Benjamin, Menlo Park, CA., 739 pp.

3. Dermer, O. C., Curtis, V. S. and Leach, F. R. (1980) Biochemical Indicators of Subsurface Pollution, Ann Arbor Science Publishers, Inc., in press.

4. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. (1957) Manometric Techniques, Burger Publishing Co., Minneapolis, MN., 338 pp.

5. Werner, M., ed. (1976) Microtechniques for the Clinical Laboratory, John Wiley, New York, 442 pp.

6. Bergmeyer, H. U., ed. (1963) Methods of Enzymatic Analysis, Academic Press, New York, 1316 pp.

7. Lowry, O. H. and Passonneau, J. V. (1972) A Flexible System of Enzymatic Analysis, Academic Press, New York, 291 pp.

8. Rotman, B. (1961) Proc. Nat. Acad. Sci., U.S.A., 47, 1981-1991.

9. Schultz, R. M. and Wassarman, P. M. (1977) Anal. Biochem., 77, 25-32.

10. Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys., 155, 213-220.

11. Lacks, S. (1962) J. Mol. Biol., 5, 119-131.

12. Boer, G. J. (1975) Anal. Biochem., 65, 225-231.

13. Lowry, O. H., Passonneau, J. V., Schulz, D. W. and Rock, M. K. (1961) J. Biol. Chem., 236, 2746-2755.

14. Skujins, J. J. (1967) in Soil Biochemistry, A. D. McLaren and G. H. Peterson, eds., pp. 371-414. Marcel Dekker, New York.

15. Burns, R. G., ed. (1978) Soil Enzymes, Academic Press, London, 380 pp.

16. Preul, H. C. (1972) Water Res., 6, 1141-1154.

17. Kiss, S. and Boaru, M. (1965) Symp. Methods Soil Biol. Bucharest, 137-143.

18. Lenhard, G., Noruse, L. D. and Schwatrz, H. M. (1965) in Advances in Water Pollution Research, Vol. 2, J. K. Baars, ed., pp. 105-127. Permagon Press, New York.

19. Hirte, W. (1963) Zentr. Bakteriol. Parasitenk Infekioskr. Hyg., 116, 478-484.

20. Peterson, N. V. (1967) Mikrolbiologiya, 36, 518-525.

21. Wieser, W. and Zech, M. (1976) Marine Biol., 36, 113-122.

22. Schaefer, R. (1963) Ann. Inst. Pasteur, 105, 326-331.

23. Curl, H. J. and Sandberg, J. (1961) J. Marine Res., 19, 123-138.

24. Glathe, H. and Thalmann, A. (1970) Zentr. Bakteriol. Parasitenk Infektionskr. Hyg., 124, 1-23.

25. Aebi, H. (1974) in Methods of Enzymatic Analysis, Vol. 2, H. U. Bergmeyer, ed., pp. 673-684. Academic Press, New York.

26. Chance, B. and Meahly, A. C. (1955) in Methods in Enzymol., Vol. 2, S. P. Colowick and N. O. Kaplan, eds., pp. 764-775. Academic Press, New York.

27. Weetall, H. H., Weliky, N. and Vango, S. P. (1965) Nature, 206, 1019-1027.

28. Kunze, C. (1972) Experienta, 28, 1897-1398.

29. Johnson, J. L. and Temple, K. L. (1964) Soil Sci. Soc. Amer. Proc., 28, 207-209.

30. Baroccio, A. (1957) Soils and Fertilizers, 21, 2117.

31. Kunze, C. (1972) Landwirt. Forsch. Sonderh., 27, 91-94.

32. Koppen, R. (1955) Water Pollut. Abstr., 28, 2634.

33. Halstead, R. L. (1964) Can. J. Soil Sci., 44, 137-144.

34. Gilmartin, M. (1967) Limnol. Oceanogr., 12, 325-328.

35. Reichardt, W., Overbeck, J. and Steubing, L. (1967) Nature, 216, 1345-1347.

36. Speir, T. W. and Ross, D. J. (1975) New Zeal. J. Sci., 18, 231-237.

37. Berman, T. (1969) Nature, 224, 1231-1236.

38. Ramirez-Martinez, J. R. and McLaren, A. D. (1966) Enzymologia, 30, 243-253.

39. Atkinson, D. E. (1977) Cellular Energy Metabolism and its Regulation, Academic Press, New York, 293 pp.

40. Holm-Hansen, O. and Booth, C. R. (1966) Limnol. Oceanogr., 11, 510-519.

41. Holm-Hansen, O. (1973) in Estuarine Microbial Ecology, L. H. Stevenson and R. R. Colwell, eds., pp. 31-63. Univ. of S. Carolina Press, Columbia.

42. Howard, J. L., Webster, J. J. and Leach, F. R. (1979) Fed. Proc., 38, 670

43. Kornberg, A. (1955) in Methods in Enzymol., Vol. 2, S. P. Colowick and N. O. Kaplan, eds, pp. 497. Academic Press, New York.

44. McElroy, W. D. (1947) Proc. Nat. Acad. Sci., U.S.A., 33, 342-345.

45. Seliger, H. H. and McElroy, W. D. (1960) Arch. Biochem. Biophys., 88, 136-141.

46. DeLuca, M. (1976) Adv. Enzymol. Relat. Areas Mol. Biol., 44, 37-68.

47. Webster, J. J., Chang, J. C., Howard, J. L. and Leach, F. R. (1980) J. Appl. Biochem., 2 (in press).

48. Hafez, Y. S. M. and Allred, J. B. (1977) J. Carbohydrates Nucleotides, 4, 341-348.

49. Bulleid, N. C. (1978) Limnol. Oceanogr., 23, 174-178.

50. Stanley, P. E. (1971) Anal. Biochem., 39, 441-453.

51. Baumstark, A. L., Cline, T. W. and Hastings, J. W. (1979) Arch. Biochem. Biophys., 193, 449-455.

52. Brolin, S. E. and Hjertin, S. (1977) Mol. Cell Biochem., 17, 61-73.

53. Seitz, W. R. and Neary, M. P. (1977) in Methods of Biochemical Analysis, Vol. 23, D. Glick, ed., pp. 161-188. John Wiley, New York.

54. Chappelle, E. W. (1975) in ATP Methodology Seminar, G. A. Borun, ed., pp. 62-103. SAI Technology Co., San Diego.

55. Seliger, H. H. (1973) in Chemiluminescence and Bioluminescence, M. J. Cormier, D. M. Hercules and J. Lee, eds., p. 471. Plenum Press, New York.

56. Hastings, J. W., Baldwin, T. O. and Nicoli, M. Z. (1978) in Methods in Enzymol., Vol. 57, M. DeLuca, ed., pp. 135-152. Academic Press, New York.

57. Thomas, R. R., Picciolo, G. L., Chappelle, E. W., Jeffers, E. L. and Taylor, R. E. (1977) in Second Bi-Annual ATP Methodology Symposium, G. A. Borun, ed., pp. 569-600. SAI Technology Co., San Diego.

58. Ewetz, L. and Thore, A. (1978) Appl. Environ. Microbiol., 36, 790-793.

59. Lee, J. and Seliger, H. H. (1972) Photochem. Photobiol., 15, 227-237.

60. Miller, C. A. and Vogelhut, P. O. (1978) Appl. Environ. Microbiol., 35, 813-816.

61. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. and Britten, R. J. (1955) Studies of Biosynthesis in Escherichia Coli, Carnegie Institution of Washington, Washington, D. C., 521 pp.

62. Chi, M. M-Y., Lowry, C. V. and Lowry, O. H. (1978) Anal. Biochem., 89, 119-129.

63. Slein, M. W. (1963) in Methods of Enzymatic Analysis, H. U. Bergmeyer, ed., pp. 117-123. Academic Press, New York.

64. Malamy, M. H. and Horecker, B. L. (1964) Biochemistry, 3, 1893-1897.

65. Torriani, A. (1968) in Methods in Enzymol., Vol. 12B, L. Grossman and K. Moldave, eds., pp. 212-218. Academic Press, New York.

66. Nygaard, A. P. (1963) in The Enzymes, 2nd Edition, P. D. Boyer, H. Lardy and K. Myrback, eds., pp. 557-565. Academic Press, New York.

67. Lowry, O. H., Roberts, N. R. and Kapphahn, J. I. (1957) J. Biol. Chem., 224, 1047-1064.

68. Skujins, J. J. (1976) CRC Crit. Rev. Microbiol., 383-421.

69. Oleniacz, W. S., Pisano, M. A., Rosenfeld, M. H. and Elgart, R. L. (1968) Environ. Sci. Technol., 2, 1030-1033.

70. Okrend, H., Thomas, R. R., Deming, J. W., Chappelle, E. W. and Picciolo, G. L. (1977) in Second Bi-Annual ATP Methodology Symposium, G. A. Borun, ed., pp. 525-546. SAI Technology Co., San Diego.

71. Kiss, S., Dragan-Bularda, M. and Radulescu, D. (1975) Adv. Agron., 27, 25-40.

72. Casida, L. E., Jr., Klein, D. A. and Santoro, T. (1964) Soil Sci., 98, 371-375.

73. Eiland, F. (1979) Soil Biol. Biochem., 2, 31-35.

74. Howell, B. F., McCune, S. and Schaffer, R. (1977) Clin. Chem., 23, 2231-2237.

75. Doellgast. G. J. (1977) Anal. Biochem., 82, 278-288.

76. Brolin, S. E., Borglund, E. and Agren, A. (1979) J. Biochem. Biophys. Methods, 1, 163-169.

77. Neufeld, H. A., Conlin, C. J. and Towner, R. D. (1965) Anal. Biochem., 12, 303-309.

78. Kuznetsova, Z. I. (1962) in Geologic Activity of Microorganisms, S. I. Kuznetsov, ed., pp. 94-98. New York Consultants Bureau.

79. Davis, J. B. (1967) Petroleum Microbiology, Elsevier Publishing Co., New York, 243 pp.

80. Jeffers, E. L., Thomas, R. R., Taylor, R. E., Picciolo, G. L., Chappelle, E. W. and Deming, J. W. (1977) in Second Bi-Annual ATP Methodology Symposium, G. A. Borun, ed., pp. 601-610. SAI Technology Co., San Diego.

VITA[2]

Jeffrey Lane Howard

Candidate for the Degree of

Doctor of Philosophy

Thesis: DEVELOPMENT OF SENSITIVE ASSAYS FOR BIOINDICATORS IN GROUND WATER

Major Field: Biochemistry

Biographical:

Personal Data: Born in Ardmore, Oklahoma, October 24, 1953, the son of Mr. and Mrs. Melvin J. Howard.

Education: Graduated from Woodward High School, Woodward, Oklahoma, in May, 1971; received Bachelor of Science degree in Chemistry from Northwestern Oklahoma State University in May, 1975; completed requirements for the Doctor of Philosophy degree in Biochemistry at Oklahoma State University in May, 1980.

Professional Experience: Undergraduate chemistry teaching assistant, September, 1972, to May, 1974, Department of Chemistry, Northwestern Oklahoma State University; research technician, May to September, 1975, and research assistant, September, 1975, to May, 1980, Department of Biochemistry, Oklahoma State University.

Honorary Societies: Phi Lambda Upsilon, Blue Key, Sigma Xi.