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Project Summary

Biochemical Analyses for Detection and Assessment of Pollution in the Subsurface Environment

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Selected biochemical analysis techniques were investigated for potential use in detecting and assessing pollution of subsurface environments. Procedures for determining protein, nucleic acids, organic phosphate, lipopolysaccharides, and various coenzymes and enzyme systems were evaluated. These procedures were modified and adapted for application to environmental samples, and sensitivities were determined in terms of numbers of *Escherichia coli* cells which could be detected.

Standard spectrophotometric and fluorimetric methods for protein, DNA, RNA, and organic phosphates lacked sufficient sensitivity for successful application to subsurface environmental samples. Methods for coenzymes and enzymes which employed enzymatic cycling procedures could be made highly sensitive but required use of very sophisticated and difficult microprocedures. Two highly promising procedures were the Limulus amebocyte lysate test, which embodies a built-in amplification, since lipopolysaccharide activates an enzyme which then catalyzes the reaction to be measured, and the bioluminescence or chemiluminescence procedures. These methods are currently applicable to many environmental samples, and it should be possible to significantly increase their sensitivity, reliability, and applicability by further study.

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This Project Summary was developed by EPA's Robert S. Kerr Environmental Research Laboratory, Ada, OK, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Basic to technological advancements in any scientific area are sensitive, accurate, and facile analytical techniques. The past several decades of extensive methods development research have supported an astounding advance in biochemical research and in molecular biology. The present studies selected biochemical analysis techniques resulting from this developmental research to determine their applicability for detection and assessment of pollution in the subsurface environment.

First, the reproducibility of results available from published procedures was established, and modifications necessary to improve on the ease and reproducibility of the assays were made.

Second, the sensitivities of the procedures were assessed for both minimum quantities of biochemicals and minimum numbers of cells of *Escherichia coli* which could be detected under the assay conditions.

Third, procedures which showed particular promise, or a high degree of sensitivity, including enzymatic, cycling, and chemiluminescent assays, were studied in detail. Where possible, the assay conditions were optimized.

Fourth, the most promising assay, firefly luciferase for ATP, was studied extensively. Commercially available reagents and equipment were evaluated. Each component of the reaction mixture was examined for its essentiality and the proper concentration to be used. Less extensive work was done on the Limulus amebocyte lysate test for lipopolysaccharides.

Finally, selected assays were applied to authentic environmental samples.

Standard Biochemical Tests

Standard biochemical tests on protein, DNA and RNA were evaluated:

Protein—Lowry; dye binding with Coomassie blue and bromosulfalein; and o-phthalaldehyde without and with hydrolysis.

DNA—Diphenylamine; diaminobenzoic both spectrophotometric and fluorimetric; and fluorimetric using ethidium bromide or DAI.

RNA—Orcinol and ethidium bromide;

Organic phosphate—Phosphomolybdate and with extraction.

Several independent analyses were repeated often enough to establish both statistical confidence and operator experience. Since the assays measured a particular substance in a culture of *Escherichia coli* cells, the sensitivity of the assay could be expressed as the amount of a given substance required, and as the number of *E. coli* cells required to give the minimum detectable response. Table 1 presents ranges and sensitivities for these assays and makes note of complications encountered.

While each of these assays was satisfactory within its recognized inherent limits and biochemically understandable limitations, none was of sufficient sensitivity to be applicable to the sparse populations of organisms likely to be present in subsurface environmental samples.

Enzyme Assays

In part, the goal of this research was to increase and/or optimize the sensitivity of assays by using normally available laboratory equipment. Since enzymes act

Table 1. *Standard Biochemical Determination Methods and Their Sensitivities*

Substance	*Method or Principle*	*First Author*	*Specific Methods*	*Range**	# E. coli *cells†*	*Complication Remarks*
Protein	*Lowry*	*Lowry*	*Folin phenol, biuret*	*10 - 80 µg*	10^8	*Phenol, tyrosine interfere*
	Dye binding	*McKnight*	*Coomassie blue*	*0.5 - 10 µg*	10^7	*Not all proteins bind dyes to the same extent*
	----	*McGuire*	*Bromosulfalein*	*0.5 - 10 µg*	10^7	
	Fluorescent	*Kutchai*	*Without Hydrolysis*	*1 -25 µg*	2×10^5	*Only amino groups*
	----	*Butcher*	*With Hydrolysis*	*1.5 - 12 ng (Ala)*	----	*6 ng of protein; background is the problem*
	Radioisotopic	*Schutz*	*Labeling amino terminal*	*0.08 - 2.5 µg*	----	*Reproducibility poor*
DNA	*Burton*	*Abraham*	*Diphenylamine*	*5 - 50 µg*	6×10^7	*A Deoxyribose determination*
	DABA	*Setaro*	*Spectrophotometric*	*25 - 750 µg*	10^8	*Not as sensitive, but one can run spectrophotometric or*
	----	*Cattolico*	*Fluorimetric*	*0.1 - 1.6 µg*	10^6	*fluorimetric assay depending on the DNA content of the unknown*
	Fluorescent	*Various*	*Ethidium bromide*	*0.02 - 0.16 pg*	6×10^5	*Also reacts with RNA*
	----	*Kapuscinski*	*DAPI*	*1 - 16 ng*	3×10^4	
RNA	*Orcinol*	*Ceriotti*		*1 - 50 µg*	10^8	*For pentose analysis*
	Ethidium bromide		*Fluorimetric*	5 - 20 ng	10^6	*Also reacts with DNA*
Organic Phosphate	*Molybdoantimontyl-phosphoric acid*	*Going*	*Direct*	*40 - 640 ng*	10^6	
	----		*Extraction*	*0.2 - 6.4 ng*	10^5	*6 fg ATP/*E. coli *cell*

**Range found in this laboratory to yield linear and accurate results.*

†The number of Escherichia coli *cells required to yield the minimum amount of substance that is determined by particular methods.*

catalytically, they produce an amplified effect; that is, several hundred, thousand or so molecules of product for each molecule of enzyme. For example, the fluorimetric limit of detection of NADH is 60 ng and that of alcohol dehydrogenase as a protein is also 60 ng. If the alcohol dehydrogenase is used to reduce NAD+ to NADH which occurs at a rate of 1000 molecules of NADH produced per minute of incubation by each molecule of alcohol dehydrogenase, a 10-minute incubation would yield a 10,000-fold increase in the sensitivity of measuring alcohol dehydrogenase over just a protein determination.

Further increases in sensitivity can be achieved by extended incubations; however, care must be taken because of inactivations and inhibitions. The additional sensitivity given by incubation assays as compared to the normal continuous assays was determined. Table 2 presents some of these results and also lists the minimum number of *E. coli* cells detectable by that enzyme assay.

None of these direct or incubation assays for enzymes was of sufficient sensitivity for the expected subsurface environmental samples.

Coupled Enzymes and Enzymatic Cycling

When the products and reactants of a reaction are not easily determined, sometimes it is possible to utilize one of the products in a succeeding reaction catalyzed by another enzyme.

The following scheme differentiates between these two processes.

Coupled:

$$S_1 + S_2 \xrightarrow{E_{Aux}} P_1 + P_2$$

$$P_1 + S_3 \xrightarrow{E_{Ind}} P_3 + P_4$$

$$S_1 + S_2 + S_3 \longrightarrow P_2 + P_3 + P_4$$

Cycling:

$$S_1 + S_2 \xrightarrow{E_1} P_1 + P_2$$

$$P_1 + S_2 \xrightarrow{E_2} S_1 + P_3$$

$$S_2 + S_3 \longrightarrow P_2 + P_3$$

In coupling, a product of the first reaction is used as a substrate for the second reaction producing two more products. Usually one of these latter products is measured. For enzymatic cycling, the product of the first reaction which is used as a substrate for the second is converted to a substrate for the first. Thus the cyclic conversion $S_1 \rightarrow P_1 \rightarrow S_1$ allows production of much greater amounts of the products.

With the coupled assay for ATP, a linear range of 0.2 to 20 nmol was obtained. The enzymatic cycling procedure for measuring ATP yielded an effective range of 0.1 pmol to 10 nmol. Thus, the increase in sensitivity obtainable with enzymatic cycling procedures is clear. Table 3 summarizes some of the sensitivities obtained using coupled and cycling assays.

Table 2. *Sensitivities of Enzyme Assays as Bioindicators in Terms of* E. coli *Cell Number*

Enzyme E.C. #	*Assay Method*	*Limit of Detection*	*Minimum Cell Concentration (ml^{-1})*
Lactate Dehydrogenase	*Continuous*	*0.1 µg*	
1.1.1.27	*Incubation*	*1.0 ng*	*1 x 10^6*
Alkaline Phosphatase	*Continuous*	*20 ng*	
3.1.3.1	*Incubation (1 h)*	*1.0 ng*	*8.5 x 10^6*
	Incubation (24 h)	*0.09 ng*	
Catalase	*Continuous*	*0.2 µg*	
1.11.1.6	*Incubation*	*1.0 ng*	*1 x 10^7*
Adenylate Kinase	*Continuous*	*3.0 ng*	
2.7.4.3	*Incubation*	*0.1 ng*	*4 x 10^5*

Bioluminescent and Chemiluminescent Assays

Several symposia, conferences, and reports devoted to bioluminescence and chemiluminescence, and the commercial development of reagents and instrumentation, have brought these methods to the forefront. Their sensitivity is greater than most of the methods (except cycling) discussed above. In fact they can be used even after cycling reactions to determine the final product.

The following scheme shows the reactions involved in bioluminescence and chemiluminescence.

Bioluminescence

Firefly

$$LH_2 + Lu + MgATP \rightleftharpoons Lu - LH_2 - AMP + MgPP$$

$$Lu - LH_2 - AMP + O_2 \rightarrow Lu + AMP + CO_2 + OL + Light$$

Bacterial

$$BLu + FMNH_2 + O_2 \rightarrow BLu\text{-}FMNHOOH$$

$$BLu + FMNHOOH + RCHO \rightarrow BLu + RCOOH + FMN + H_2O + Light$$

It is also possible to use NAD(P)H : FMN oxidoreductase

$$NADH + H^+ + FMN \rightarrow NAD^+ + FMNH_2$$

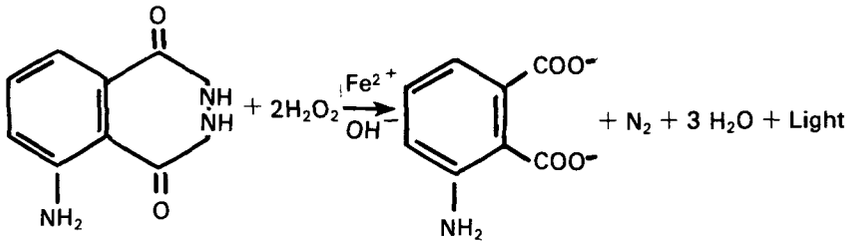
which allows any NADH yielding reaction or couple to be assayed.

Table 3. *Sensitivities of Coupled and Cycling Assays*

Bioindicator	*Assay Method*	*Limit of Detection*	*Minimum Cell Concentration (ml^{-1})*
Pyridine Nucleotides	*Cycling*		*5 x 10^6*
	$NADP^+$	*37.0 pg*	----
	NAD^+	*3 ng*	----
Adenosine Triphosphate	*Coupled Enzyme*	*100 ng*	----
	Cycling	*100 pg*	*5 x 10^5*

Chemiluminescence

Luminol



The properties of most of the commercially available firefly luciferases were compared. Experiments revealed that Tricine buffer yielded a conformation of firefly luciferase which was especially reactive. The various assay conditions were studied and the assay was optimized. Table 4 lists a few of the factors tested and shows the increase in sensitivity. Table 5 shows an example of the assay requirements for one of the commercial preparations. Table 6 shows a comparison of ATP determination by the firefly luciferase (bioluminescence) and enzymatic cycling procedures. As indicated, a sensitivity of 50 fg of ATP was achieved by the bioluminescence method using commercial reagents and instrumentation.

Using bacterial luciferase, as little as 0.25 ng of FMN could be detected. With the luminol assay, it was possible to measure 2 pg of iron porphyrins which corresponded to 10 *E. coli* cells. The bacterial luciferase system in the form of dried bacterial cells was tested as an indicator as described in the Microtox procedure.

Cascaded Reactions

The response of the Limulus amebocyte lysate to lipopolysaccharides from gram-negative bacteria is formation of a gel. Since this reaction series is similar to that in blood coagulation, and the lipopolysaccharide activates an enzyme or factor which acts catalytically, the assay is very sensitive. As little as 100 fg of lipopolysaccharide, and as few as 10 *E. coli* cells, were detected by this method.

Environmental Samples

Many of the assays were applied to spring water samples, soil samples, and core materials. The bioluminescence and cascaded reaction assays were particularly promising, but interfering substances found in the soil and core materials require further research to optimize the application of these biochemical determinations to the difficult environmental solid samples.

Table 4. *Improvements in the Firefly Luciferase Assay*

Factor	*Change*		*Fold Increase*
Reaction vessel	*Glass* →	*Plastic*	*2*
Reaction volume	*0.5 ml* →	*0.2 ml*	*5*
Luciferin	*Commercial* →	*Synthetic*	*2*
Buffer	*Phosphate* →	*Tricine*	*3*
Additives	*None* →	*Complete*	*10*
Enzyme	*Crude* →	*Partially purified*	*4*
Light effect on glass reaction vessel (Pico-Lite)	*Fluoro. light* →	*Dark maintained*	*9 (decr. in bkg.)*
Instrument	*Initial* →	*Microprocessor*	*8*

Table 5. *Requirements for the Firefly Luciferase Assay**

	Light Units (1 ng ATP)
Omissions	
None	*20.6*
- 5 mM $MgSO_4$	*0*
- 0.5 mM EDTA	*12.6*
- 50 µg Luciferin (LH_2)	*5.2*
- 0.5 mM DTT	*13.6*
- 50 µg Bovine serum albumin (BSA)	*20.0*
Additions	
None	*0.1*
$MgSO_4$	*3.7*
LH_2	*0.5*
DTT	*1.1*
BSA	*0.3*
$MgSO_4 + LH_2$	*13.8*
$MgSO_4 + LH_2 + DTT$	*13.6*

**This experiment done with Firelight enzyme.*

Table 6. *Comparison of Enzymatic Cycling and Firefly Luciferase Determinations of ATP*

Parameter	Assay	
	Firefly Luciferase	Enzymatic Cycling
Range*	0.2 pmol - 100 pmol	0.3 pmol - 10 pmol
Sensitivity**	0.1 fmol (50 fg)	0.1 pmol (50 pg)
Cost†	6¢/assay	9.5¢/assay
Productivity	25/hr or 200/8-hr day	96/5-hr or 192/8-hr day
Inhibitors	Metal ions, PO_4^{3-}	None encountered to date
Equipment	Photometer	Fluorometer
Technical competence required	Technicians	Enzymologist
Turnaround time	30 min	5 hr
Specificity	ATP only	ATP, NADH, NADPH

*Useful range of ATP which can routinely be measured.
**Smallest amount of ATP detected by the assay.
†Based on 1979 prices, when the experiment was done.

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The complete report, entitled "Biochemical Analyses for Detection and Assessment of Pollution in the Subsurface Environment," (Order No. PB 83-182 303; Cost: $14.50, subject to change) will be available only from:
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