United States
Environmental Protection
Agency

Hazardous Waste Engineering
Research Laboratory
Cincinnati OH 45268

Research and Development

EPA/600/S2-85/048 June 1985

EPA

Project Summary

Enzyme-Based Detection of Chlorinated Hydrocarbons in Water

Barbara H. Offenhartz and Janet L. Lefko

This study explores a new approach for detecting hazardous levels of high molecular weight chlorinated hydrocarbons based on enzyme-catalyzed reactions. The lactate dehydrogenase (LDH) catalyzed reaction

$$\text{pyruvate} + \text{NADH}^* + \text{H}^+ \xrightarrow[\text{[inhibitor]}]{\text{LDH}} \text{lactate} + \text{NAD}^+$$

is used to detect the presence of chlorinated hydrocarbons, which reduce the rate of the reaction by reversibly inhibiting the enzyme. Detection is straightforward, since the reaction produces a significant pH change. An analysis takes 5 minutes. Inhibition screening experiments show that the LDH method can detect aldrin, toxaphene, DDT, polychlorinated biphenyls (PCBs), and polychlorinated phenols at comparable levels of toxicity at parts per million (ppm) levels. A preliminary study of potential interferences is reported, as well as the results of an extensive literature search for suitable enzymes. In addition to LDH, four other commercially available enzymes were found to be potentially suitable: carbonic anhydrase, hexokinase, phosphorylase b, and an ATPase. LDH was chosen on the basis of a trade-off between sensitivity to inhibition and suitability for commercial method development.

The bench-top method studied in the laboratory appears potentially adaptable to water-quality monitoring systems and pocketable field sensors. The development is patterned on the cholinesterase antagonist monitors (CAMs), developed under the sponsorship of the U.S. Environmental Protection Agency (EPA), which use the cholinesterase enzyme to detect organophosphate and carbamate pesticides. Work has begun on developing immobilized enzyme preparations suitable for use in this type of device.

Since the LDH enzyme is stable in a variety of water/organic solvent mixtures, including methanol, ethanol, and acetone, the method has potential for processing extracts of soils and organic wastes. For water samples, in which the solubility of chlorinated hydrocarbons is often below the ppm level, improvement of detection levels or use of an extraction step will be required to achieve sub-ppm detection levels. In the LDH method reported, the inhibitors were solubilized in a 20 percent methanol solution.

This Project Summary was developed by EPA's Hazardous Waste Engineering Research Laboratory, Cincinnati, OH, 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

Simple and robust field methods for the detection of chlorinated hydrocarbons at hazardous levels are needed to determine the boundaries of an area targeted for decontamination, waste site management, and to rapidly detect spills. Recent advances in the development of analytical methods based on immobilized enzymes have proved useful in the detection

*NAD^+ is the oxidized form of the cofactor nicotinamide adenine dinucleotide.
NADH is the reduced form of NAD^+.

of pesticides. Specifically, enzyme-based CAM water quality monitors, developed under EPA sponsorship, detect hazardous levels of organophosphate and carbamate pesticides by their effect on the rate of a cholinesterase-catalyzed reaction. Design features common to CAM monitors are: an immobilized enzyme preparation, electrometric detection of reaction products, and an automated sampling cycle with automated sample handling.

In the present study, an enzyme-based detection method has been developed for the detection of high molecular weight chlorinated hydrocarbons. Based on an extensive literature review, lactate dehydrogenase was chosen to replace cholinesterase. The reaction

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{[inhibitor]}]{\text{LDH}} \text{lactate} + NAD^+$$

was found to be useful in detecting chlorinated hydrocarbons, which reduce the rate of the reaction. The inhibition of rabbit muscle M_4 isoenzyme by high molecular weight hydrocarbons is reversible and is competitive with respect to pyruvate and the coenzyme NADH. The experimental work described below shows that a wide variety of chlorinated hydrocarbons can be detected at hazardous levels by this reaction. Nonchlorinated hydrocarbon analogs do not interfere. Either pH or electrometric detection of the coenzyme can be used. Enzyme activity is minimally affected by organic solvents, so the method appears adaptable to soil as well as water samples. Since pollutant inhibition is reversible, the LDH enzyme can probably be immobilized on a suitable support medium to enhance the cost-effectiveness of the method. Enzyme immobilization is essential in instrumentation designed for field use, reducing both complexity and cost per test.

Procedure

The objectives of the effort reported were to identify enzymes that could be incorporated in automated water monitoring systems or other field detectors of chlorinated hydrocarbons and to develop an enzymatic detection method that demonstrated potential for adaptation to field use.

Selection criteria were developed to facilitate the literature search and to provide a scheme for ranking the enzymes identified. Sensitivity to inhibition by a broad spectrum of chlorinated hydrocarbons and insensitivity to interferants were of primary importance. Practical considerations dictated that enzymes considered were commercially available and that enzyme characteristics were well-documented. Field method characteristics of reliability, ease of use, and low cost were addressed by requiring electrometric detection of enzymatic reaction products and a reusable immobilized enzyme preparation. Adaptability of inhibition detection schemes to the EPA-developed CAM-1 field monitor was desirable. Lactate dehydrogenase, carbonic anhydrase, hexokinase, and an ATPase have potential for enzymatic detectors. Lactate dehydrogenase, the most promising enzyme identified, was used for field method development.

The inhibition of lactate dehydrogenase by the organochlorine insecticides (dieldrin, aldrin, endrin, chlordane, mirex, kepone, and DDT) has been documented previously. Therefore, laboratory tests on the inhibition of LDH by chlorinated hydrocarbons and potential interferants were carried out to supplement published information. Since the three-dimensional structure and reaction mechanism of LDH are well established, testing focused on compounds most likely to show inhibition.

The spectrophotometric inhibition assay procedure used for the inhibition studies was adapted to a pH assay more suitable for field use. The sensitivities of the two methods are compared. Preliminary results on immobilizing LDH in a polyacrylamide gel are reported.

LDH Inhibition Studies

The results of the experimental inhibition studies are shown in Table 1, which summarizes some 70 inhibition assays on seven representative test compounds. The inhibition results are consistent with the published literature where overlap of data exists, e.g., for aldrin. LDH sensitivity to chlorinated hydrocarbons approximately parallels the relative toxicity of the compounds. The 2,4,5-T inhibition, 19 percent at the highest concentration tested, is marginally significant, and the 2,4,5-T should be retested at higher concentrations.

The "100**" values (Table 1) require an explanation. If an inhibitor that is undetected and undissolved is present at the lower concentrations tested, then the

Table 1. *Inhibition of Lactate Dehydrogenase*

	% Inhibition* at Several Concentrations		
Test Compound	10^{-4} M†	10^{-5} M	10^{-6} M
Cyclodiene Insecticides			
Aldrin	100**	58±8	29±3
Toxaphene	100**	40±2	13±4
Chlorinated Ethane Derivatives			
DDT	100**	22±3	6±5
Polychlorinated Biphenyls			
Aroclor 1242	100**	22±12	0
Polychlorinated Phenols			
Pentachlorophenol	74±8	23±6	0
Chlorophenoxy Derivatives			
2,4,5-Trichlorophenoxy Acetic Acid	19±7	7±7	--
Misc. Chlorinated Hydrocarbons			
Lindane	0	0	--

**Percent inhibition was calculated by assigning 100 percent activity to a control containing no test compound.*

†Conversion factor applicable: 1 x 10^{-5} M = 2-4 mg/l

***Undissolved test compound present.*

Experimental conditions: [NADH] = 0.220 mM; [pyruvate] = 1 00 mM; [test compound] = 0 (control), 1 x 10^{-6} M, 1 x 10^{-5} M or 1 x 10^{-4} M; 0.1 unit of LDH in 0.11 M phosphate-methanol buffer (80:20 v/v%), pH 7.3, 23°C. The addition of enzyme, pre-equilibrated in phosphate-methanol buffer, started the reaction. Enzyme activity was determined by the method of initial reaction rates. The disappearance of NADH was followed spectrophotometrically with a Hitachi Model 100-80 at 340 nm. The 300-340 nm background absorbance was checked for the presence of undissolved reaction components.

data for Table 1 could overestimate inhibition effects projected for field devices. When undissolved inhibitor is present, coprecipitation of enzyme and inhibitor has been observed. This effect mimics inhibition by reducing the active enzyme concentration and has been extensively documented. Undissolved inhibitor, if present in field samples, will react differently with the immobilized enzyme preparations of field devices than with the free enzyme used in the inhibition studies. For this reason, the distinction between coprecipitation and inhibition is of more than academic interest. The possibility of coprecipitation was ruled out by mechanistic studies of the type illustrated in Figure 1. Linear double-reciprocal plots of initial reaction velocity versus substrate concentration are only consistent with reversible inhibition and are inconsistent with the occurrence of coprecipitation.

Interferences

Nonhalogenated aromatic pollutants, excepting phenols and amines, are even less soluble than their chlorinated analogs, so that interference is unlikely. Furthermore, interference is not expected on structural grounds. Phenol was tested and exhibited no significant inhibition at concentrations below 0.1 M.

The organophosphate pesticide Diazinon and the carbamate Sevin inhibited LDH only at concentrations greater than 0.001 M. This indicates that cholinesterase (CAM) and LDH may have complementary detection capabilities toward Category X hazardous substances.* The LDH appears to selectively detect high molecular weight chlorinated hydrocarbons, whereas the CAM method detects organophosphate and carbamate pesticides.

Cyanide tends to inhibit most enzymes. Concentrations as high as 0.01 M were required to exhibit 50 percent inhibition of LDH. The LDH method is hence unusually insensitive to toxic levels of cyanide. On the other hand, heavy metal ions, such as free mercuric ion (Hg^{+2}), showed complete inhibition consistent with the presence of sulfhydryl (-SH) groups on the enzyme. Use of a suitable chelating agent will be necessary to suppress interference in environmental applications when high levels of heavy metal ions are present.

*Category X hazardous substance refers to the 21 organic compounds designated in 40 CFR 117 3 as having a reportable quantity of one pound

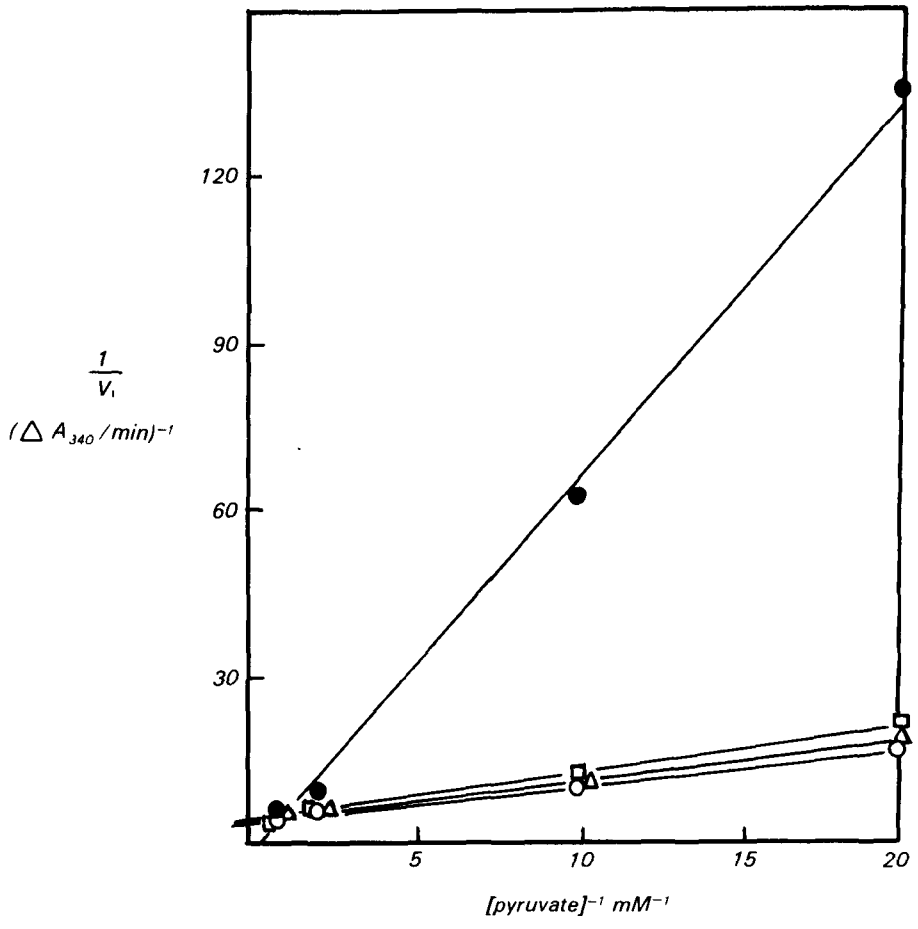

Figure 1. *The effect of Aroclor 1242 on lactate dehydrogenase. Lineweaver-Burk double reciprocal plot of the effect with respect to pyruvate. Experimental conditions. [NADH] = 0.220 mM; [pyruvate] = 0.050 - 1 00 mM; [Aroclor 1242] = (○) 0 mM, (△) 0.005 mM, (□) 0.010 mM, (●) 0.050 mM; 0 1 units LDH in 0.11 M phosphate-methanol buffer (80:20 v/v%) at pH 7.3, 23°C Spectrophotometric determination of the initial rate of disappearance of NADH (v_i) with a Hitachi Model 100-80 at 340 nm*

Detection Methods

In the laboratory work, the rate of the reaction was followed by spectrophotometrically determining the absorbance of the reaction mixture at the 340 nm peak of NADH. However, this detection method is not particularly suitable for field use. Natural waters tend to have background absorption and to foul optical surfaces. Furthermore, optical methods of detection are relatively expensive. Two cheaper alternatives are available, electrometric detection (monitoring of the [NADH]/[NAD^+] couple) and pH detection (the reaction consumes hydrogen ion). Both methods are relatively insensitive to interference, although high concentrations of oxidants (e.g., chlorine) can distort electrometric detection, just as high concentrations of acids and bases can distort pH detection. Only the pH detection method was examined in this study.

Since the reaction consumes hydrogen ion, the pH of the reaction mixture should increase with time. The extent of the pH increase depends upon the buffering capacity of the reaction mixture. When the reaction mixture was titrated with hydroxide, addition of 10^{-4} M OH^- at a pH initially equal to 6 produced a 2-unit pH change rather than the 3-unit change expected in the absence of a buffer. The difference is traceable to the buffering effect of the commercial enzyme preparation. As shown in Figure 2, when the reaction mixture (primarily sodium acetate) is titrated in the absence of enzyme, a far sharper pH change is observed. (A sodium acetate buffer limits pH decrease but has no effect on pH increase.) Never-

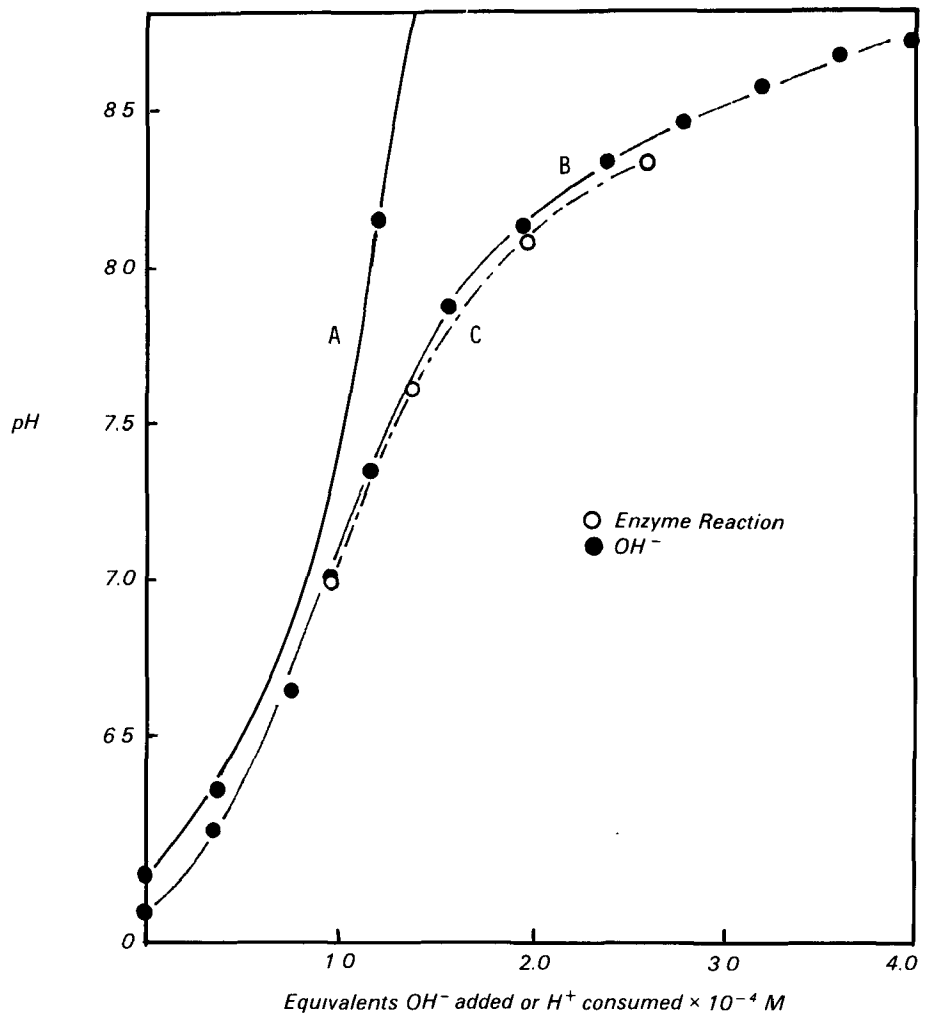

Figure 2. *Titration of the LDH reaction mixture. In titration A the components NADH and pyruvate in buffer are titrated with 0.1 N NaOH In titration B the enzyme in buffer is titrated In titration C the enzyme reaction consumes H^+ (initial pH = 7.0). The change in $[H^+]$ is calculated from the change in [NADH], determined spectrophotometrically at 340 nm; $\triangle[H^+] \equiv \triangle$[NADH] Experimental conditions: [NADH] = 0 220 mM; [pyruvate] = 1 00 mM; 0.4 units LDH in 1 mM acetate buffer-methanol (80 20) v/v%; 23°C.*

theless, as shown in Figure 3, the buffering effect of the enzyme preparation is not so great as to eliminate pH detection as a viable method. In the presence of high concentrations of a chlorinated hydrocarbon, the pH change would be zero; the pH change actually observed, in excess of 1 unit, clearly indicates the absence of inhibitors.

We conclude from these results that a 0.001 M acetate buffer in 20 percent methanol solution supports a viable detection scheme. At this pH and methanol concentration, the enzyme activity is about 25 percent *greater* than the standard LDH assay, and the solution could be adapted readily to acidic water samples. The only potential problems would occur in waters that are more basic than pH 7, such as sea water or brackish water samples. Electrometric detection of [NADH]/[NAD^+] could probably be used in such circumstances.

LDH Immobilization

Immobilization of the LDH enzyme is a necessary step toward developing reliable, cost-effective field methods. Many different types of immobilization techniques have been successfully applied to LDH. Several look quite promising for developing continuous flow applications and field detector designs. However, each immobilization method must be examined with care, since immobilizing an enzyme can also reduce its activity and/or reduce its sensitivity to inhibition. Among the various LDH immobilization techniques, covalent bonding methods, employing a spacer molecule between the enzyme and its support, are best suited to reproducing the free enzyme environment in an immobilized preparation. As of this writing, this technique has not been attempted, although it is clearly recommended for future method development.

Conclusions

An LDH enzyme-based detector has been developed that can detect at least five chlorinated hydrocarbons at concentrations of 10^{-4} to 10^{-6} M in a 20 v/v% methanol/water solution. Based on literature reviews, it is thought that the detector will also be sensitive to other high molecular weight chlorinated hydrocarbons. Phenol test data and analysis of the literature indicate that interference by nonchlorinated analogs will not occur. The simplicity and rapidity of the method indicates promise for field applications. The inhibition of LDH by chlorinated hydrocarbons can be monitored either by pH or electrometric changes in the reaction mixture. This flexibility also enhances the potential of the detection method for field use.

Although the LDH enzyme-based detector has potential for field application, more work is required in several areas including: (1) development of an extraction/concentration procedure (with an enzyme-compatible solvent) to precede the use of the LDH detection method for chlorinated hydrocarbons that have very low water solubility; (2) immobilization of the LDH enzyme on a suitable support to make re-use of the enzyme possible and to simplify the detection procedure; and (3) evaluation of the sensitivity of the detection method to potential interferants in environmental samples.

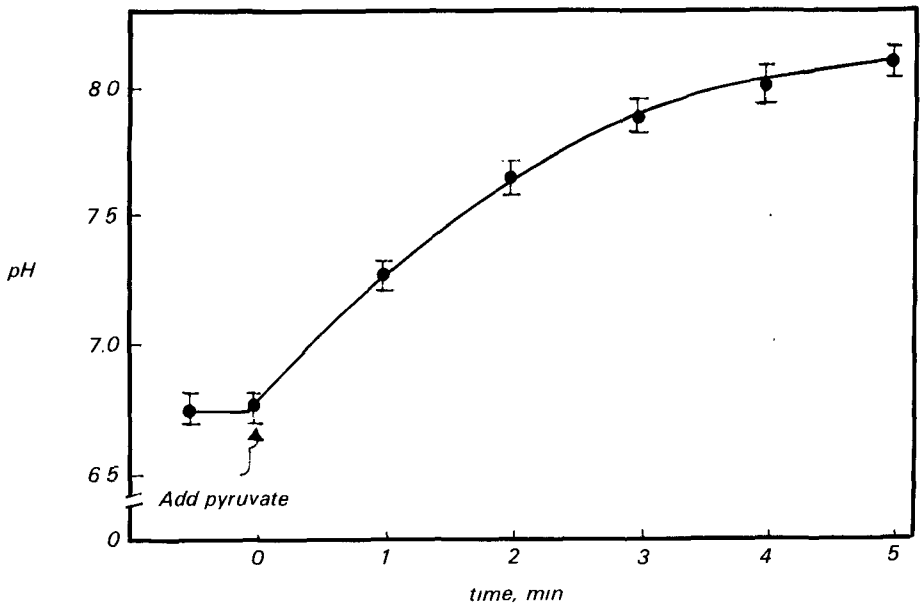

Figure 3. *pH detection of the LDH catalyzed reaction. Experimental conditions [NADH] = 0.220 mM; [pyruvate] = 1.00 mM; 0.4 units LDH in 1 mM acetate buffer-methanol (80:20 v/v%); 23°C. The pH was measured with a Beckman Model 76 pH meter*

Barbara H. Offenhartz and Janet L. Lefko are with B & M Technological Services, Inc., Cambridge, MA 02142.
***Michael D. Royer** is the EPA Project Officer (see below).*
The complete report, entitled "Enzyme-Based Detection of Chlorinated Hydrocarbons in Water," (Order No. PB 85-191 176/AS; Cost: $10.00, subject to change) will be available only from:
National Technical Information Service
5285 Port Royal Road
Springfield, VA 22161
Telephone: 703-487-4650
The EPA Project Officer can be contacted at:
Releases Control Branch
Hazardous Waste Engineering Research Laboratory—Cincinnati
U.S. Environmental Protection Agency
Edison, NJ 08837

☆U.S.Government Printing Office: 1985 — 559-111/10861

United States
Environmental Protection
Agency

Center for Environmental Research
Information
Cincinnati OH 45268
