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Rapid Detection System for Organophosphates and Carbamate Insecticides in Water

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RAPID DETECTION SYSTEM FOR ORGANOPHOSPHATES AND CARBAMATE INSECTICIDES IN WATER

By

Midwest Research Institute
425 Volker Boulevard
Kansas City, Missouri 64110

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Project Officer

Dr. Thomas B. Hoover
Southeast Water Laboratory - EPA
College Station Road
Athens, Georgia 30601

Prepared for

OFFICE OF RESEARCH AND MONITORING
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ABSTRACT

An apparatus for the detection and monitoring of water supplies for hazardous spills of organophosphate and carbamate insecticides has now been designed and fabricated. The new unit is called the Cholinesterase Antagonist Monitor, CAM-1, because it produces an alarm in 3 min. when toxic or subtoxic levels of cholinesterase antagonists are present in water. Response of this apparatus to subtoxic levels of azodrin, sevin, dimetilan, malathion, parathion and DDVP has already been demonstrated. CAM-1 uses immobilized cholinesterase for the collection of cholinesterase inhibitors from the water supplies. The activity of the immobilized cholinesterase is determined automatically in an electrochemical cell by passing a substrate solution over the enzyme at regular time periods. A minicomputer is used to automate the detection process and to signal an alarm when there is a rapid loss of enzyme activity--a situation which occurs in the presence of organophosphate and carbamate insecticides in the water sampled.

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SECTION I

CONCLUSIONS

On the basis of the present investigation of the use of immobilized enzymes for the detection of toxic organophosphate and carbamate insecticides in water the following conclusions have been made:

1. An automatic system for monitoring water supplies for the presence of organophosphate and carbamate insecticides has been designed and fabricated. The new system has been designated as the Cholinesterase Antagonist Monitor, CAM-1, since it responds rapidly to low levels of cholinesterase antagonists in water supplies.

2. The complete CAM-1 detection system and several of its component parts, including specifically the enzyme cell for water monitoring, the automatic enzyme pad changer, and certain circuit design features, are novel and patentable.

3. CAM-1 responds to toxic and subtoxic levels of organophosphate and carbamate insecticides (i.e., based on rat and animal toxicity data). In one response test with city tap water to which 0.2 ppm of DDVP (dimethyl-2,2-dichlorovinyl phosphate) had been added, CAM-1 provided both visible and audible alarms repeatedly. On this basis it is concluded that CAM-1 has adequate sensitivity to prevent accidental poisoning of human and other animal species by cholinesterase inhibitors in water supplies.

4. CAM-1 responds to toxic and subtoxic levels of other organophosphate and carbamate insecticides including the following which have been tested: azodrin, sevin, paraoxon, dimetilan, malathion and parathion.

5. The response data presented in this report suggest that there is a rough correlation between the rat toxicities of the various insecticides and the levels of these insecticides which can be detected by CAM-1. This observation is not surprising since the inhibition of cholinesterase is the cause of the rat toxicity and also the basis of the detection of the inhibitors. Differences in the affinity of specific insecticides for cholinesterase explains why their toxicities are different and why the levels detectable by CAM-1 were observed to be different.

6. The sensitivity of CAM-1 to low levels of insecticides in water is the result of the affinity of these insecticides for the reactive sites on the surfaces of the immobilized enzyme used in the electrochemical enzyme cell. Thus the enzyme is functioning as a selective concentrator of the materials to be detected.

7. CAM-1 is the first instrument which has successfully used an immobilized enzyme product for the automatic monitoring of water supplies for the presence of enzyme inhibitors.

8. CAM-1 operates satisfactorily with enzyme pads made of open-pore urethane foam, starch gel, and cholinesterase complexed with aluminum hydroxide gel. A single pad of this type performed satisfactorily for 56 hr with water flow rates of 1,200 ml/min during the water sampling portion of the detection cycle.

9. An immoblized enzyme product made by covalent bonding of cholinesterase to cheesecloth has been used successfully in CAM-1 but packing of the wet cheesecloth tended to restrict the flow of water through the enzyme product.

10. The present CAM-1 operates on a 3-min detection cycle in which water is sampled for 2 min and the pad activity is determined during the third minute. The sensitivity of the system can be increased by manually increasing the water sampling period using the controls provided.

11. CAM-1 will continue to function even after a single enzyme pad becomes unsatisfactory due to loss in activity since electrical sensing and automatic pad change mechanisms have been provided to replace a nearly exhausted enzyme pad with a new one.

12. CAM-1 will operate for 48 hr without servicing. Continuous unattended operation for even longer periods could be achieved through the addition of a mechanism to convert the dry substrate ingredients into fresh substrate solution.

13. Continuous sampling of a water supply can be achieved by operating two CAM-1 units out-of-phase so that one unit samples water while the second unit tests the enzyme activity of its pad. A sync cable to keep the two detectors operating out-of-phase has been provided. The use of two CAM-1 units simultaneously provides redundancy and, if an alarm is signalled, it can be confirmed in 90 sec.

14. It was necessary to incorporate a minicomputer in CAM-1 to program the detection cycle, to compare the differences in cell voltage for successive cycles, to compare the voltage differences between cycles with the preset alarm threshold, to signal an alarm when the rate of enzyme inhibition exceeds the sensitivity setting, to change enzyme pads before they fail and to permit unattended operation.

15. The CAM-1 unit which has been developed on this program can be useful to operators of water treatment facilities to warn them of hazardous spills of insecticides so that they can take appropriate action.

16. CAM-1's ability to detect insecticides in water is based upon a biochemical reaction known to be involved in animal toxicity.

SECTION II

RECOMMENDATIONS

1. The response profile of CAM-1 should be further evaluated by an exposure of the detector to a wide variety of potential water pollutants. This study should be concerned not only with the detection of compounds but also with establishing the thresholds at which CAM-1 will detect them.

2. Studies on the effects of various water pollutants and environmental parameters on the system and its components should be extended. For example, the effects of salt water, and of various organic and inorganic water pollutants, should be studied in relation to the performance of the system.

3. Studies should be conducted in which the objectives are (1) to determine the reliability of the various components, and (2) to improve reliability for components where failures or malfunctions occur. They would evaluate enzyme pads under long periods of use under a variety of conditions. Testing of this type is needed to provide the assurance of trouble-free operation under anticipated use conditions.

4. A rugged version of CAM-1 should be fabricated for stream monitoring. It should withstand rain, dust, and salt spray, and operate reliably with a minimum of servicing under extremes of environmental conditions.

5. A research program should be initiated to determine if the capabilities of CAM-1 could be expanded by using different enzymes and different substrates in it.

6. The present study adequately demonstrates the possibility of fabricating a toxic hazards detector which will respond to most (if not all) of the toxic substances which are likely to occur at toxic levels in the environment. As the first step in the fabrication of such a toxic hazards detector system, a search for enzymes which are inhibited by specific types of toxic materials should be initiated. The enzymes selected from such a study could be immobilized and incorporated into a multi-enzyme detection system. Although the present CAM-1 uses an electrochemical system for monitoring the activity of the immobilized enzyme after exposure to water suspected of containing the inhibitors, there would be no need to restrict an enzyme-substrate search to systems which could be used in the present electrochemical cell.

7. The unit which has been developed should be evaluated to determine its potential for routine use in the Water Monitoring Network.

SECTION III

INTRODUCTION

Spills of hazardous materials into rivers, streams or lakes create the danger that toxic levels of chemicals will be pumped into the distribution network of municipal water supplies. As one example, a train wreck might well lead to the spill of a tank car of a toxic chemical such as an insecticide into a stream above the water intake of a city water plant. Such hazardous spills must be detected in time to close off the intake valves of these water supplies until the toxic material has flowed past the inlet.

The possibility of hazardous material spills is increasing because of the switch from the chlorinated hydrocarbon insecticides to the organophosphate insecticides which possess greater acute toxicities. However, most environmentalists believe that organophosphates are safer than the chlorinated hydrocarbons because they decompose in soil and water, and because they do not concentrate in food chains or accumulate in the body fat of man or animals. Under certain environmental conditions, particularly low temperature, neutral or slightly acid waters or the absence of water, some of these persist for prolonged periods of time. For example, Thoman and Nicholson[1] (1963) reported that organophosphate wastes containing parathion discharged into a river in the southeastern part of the United States caused an extensive fish kill for 25 miles and affected fish in a reservoir 100 miles downstream.

An important review of the recovery, separation and identification of organic pesticides from natural and potable waters has been published by Faust and Suffet.[2] The following information on the persistance of organophosphates in water has been adapted from the work of Mühlmann and Schrader[3] (Table 1).

TABLE 1

HALF-LIFE OF SOME ORGANOPHOSPHATE INSECTICIDES IN WATER AT pH 1-5

Temperature (°C)	Paraoxon	Parathion	Dipterex	DDVP	Methyl Parathion
		Days			
10	1,200	3,000	2,400	240	760
20	320	690	526	61.5	175
30	93	180	140	17.3	45
50	9.6	15	10.7	1.66	4.0
70	1.2	1.65	1.13	0.164	0.47
		Hours			
70					
pH 3	23.0	21.0	33.0	3.4	11.2
pH 5	24.4	19.5	15.3	2.8	10.7
pH 7	11.5	7.8	0.7	0.45	6.9
pH 9	2.1	2.7	0.1	--	1.5

Both water temperature and pH are important factors in the stability of the organic phosphate insecticides in water. Some of these compounds persist at hazardous levels in water supplies for months, or even years, if the water is cool and the pH is on the neutral or acid side. In waters of high alkalinity, the persistence of the organophosphates is much reduced; but they may, at low temperatures, have half-lives measured in days and weeks instead of months and years. Since some organophosphates persist for long periods of time under conditions common in streams, lakes and ponds, it is imperative that we have the capability of detecting hazardous spills of these materials into water supplies prior to their ingestion by fish, wildlife and humans.

A number of analytical methods for the detection and identification of these compounds have been reported. Because these compounds are toxic at such low levels, it is usually necessary to pass large volumes of water through a column of activated charcoal and to elute with an organic solvent to get a large enough sample to permit gas chromatography, mass spectrometry, enzyme analysis, etc.

For example, Davis and Malaney[4] collected the acetylcholinesterase inhibitors from water supplies using a charcoal column; chloroform was then used to extract the organophosphates from the column before measuring the enzyme inhibition capacity of the chloroform extracts.

Guilbault, et al.,[5] reported an electrochemical assay procedure for organophosphate insecticides based on their inhibition of cholinesterase and its conversion of a substrate to a hydrolysis product which depolarized platinum electrodes to which a constant current was applied. Guilbault, et al.,[6] also showed that cholinesterases from different insect species were inhibited to different degrees by different organophosphates, and that both sensitivity and selectivity could be used to achieve trace analyses of pesticides by enzymatic methods.

In his discussion of equipment for monitoring pollutants in water, Porterfield[7] enumerated some of the common water pollutants for which there was no monitoring equipment available. He implied that the lack of standards for organophosphates in water was tied to a lack of suitable monitoring equipment. Some investigators have felt that perhaps the important thing for them to know was whether there were <u>any</u> toxic substances in the water supplies rather than just the concentration of one or more specific insecticides. To accomplish this goal, Cairns, et al.,[8] set up equipment for continuous monitoring of the activity of aquatic life; in one system he monitored the heart rate and breathing rate of bluegill sunfish for the detection of sublethal concentrations of zinc ions and other toxicants. In spite of their success, a nonliving toxic hazard monitoring system could have many advantages.

In trying to develop a system which could be used to monitor water supplies for the presence of toxic substances, a statement in the report of the National Technical Advisory Committee[9] should be kept in mind: "The most important mechanism of toxic action is thought to be the poisoning of enzyme systems." Although this statement may be an oversimplification of toxic phenomena, enzymes offer a great potential for the detection of toxic substances. It was for this reason we proposed, to the Federal Water Quality Office, that (1) they sponsor a search for a group of enzymes which could be used in the detection of toxic substances in water supplies, and (2) a water monitoring apparatus based upon the use of immobilized cholinesterase as the sensor should be constructed for the purpose of detecting those toxic substances which inhibit cholinesterase, e.g., organophosphate insecticides, some carbamate insecticides, and some heavy metals.

Previous investigation in these laboratories by Bauman, et al.,[10] had shown that cholinesterase immobilized by starch gel on the surface of open-pore urethane foam could be used in an electrochemical cell for the continuous monitoring of air for the presence of cholinesterase inhibitors. In this system the air being sampled and a solution of substrate for the enzyme were pumped simultaneously and continuously through the immobilized enzyme product; activity of the enzyme was then monitored continuously by passing an electric current through platinum electrodes in contact with the immobilized enzyme product and observing changes in the electrode potentials.

Initial experiments, on the current project, concentrated efforts in determining whether the air monitoring system could be adapted for the detection of toxic substances in water, and were conducted with small bench type electrochemical cells. We were unable to monitor the residual enzyme activity in the presence of large volumes of water, and for this reason developed a detection cycle in which the enzyme inhibitors were collected on the immobilized enzymes in the first part of the cycle, and then the activity of the enzyme was determined during the second part of the cycle. After demonstrating the feasibility of the two-cycle system, we collected information about the electrochemical cell design, the proper buffer concentration, pH, and flow rate of the substrate solution, and other information necessary for the fabrication of an integrated detection and monitoring unit which would operate with a minimum of attention.

This report describes the various individual studies which resulted in the development of the Cholinesterase Antagonist Monitor, CAM-1: specifically, the immobilization of the cholinesterase on open-pore polyurethane foam, the design of two electrochemical cells, the selection of the buffer, substrate concentration, flow rate, applied electric current, and a detection cycle; the electrical and mechanical work included design and fabrication of logic and memory circuits, a digital clock, automatic and manual controls, selection of alarm logic, size, shape, and materials of construction of the case, placement of components, etc.

After completion of the assembly, it was necessary to conduct studies on the performance of the detector in both the absence and also in the presence of enzyme inhibitors. After shakedown runs and completion of minor modifications, the CAM-1 was operated continuously for 56 hr to show that its components were functioning in a reliable manner. Following this, the unit was taken to the Edison Water Quality Laboratory, where its response to 1 ppm of DDVP was demonstrated to the sponsor.

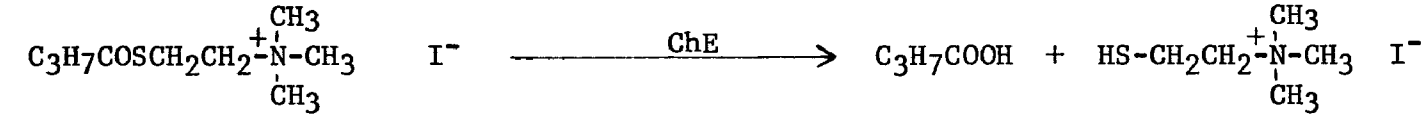
In the following sections of this report the proposed detection principle and the experiments which led to its incorporation into a functioning water monitoring apparatus identified as CAM-1 are described. A variety of immobilized enzyme products were fabricated and several electrochemical cell configurations were investigated in our attempts to perfect an electrochemical enzyme cell suitable for use in water monitoring. A breadboard apparatus was constructed which possessed the components which were necessary for water monitoring. This apparatus permitted consideration of both continuous water sampling and intermittent water sampling and experimentation with various enzyme cell configurations. Also it permitted a selection of operating parameters for the electrochemical cell and a determination of the effectiveness of different detector cycles for the intermittent-type water sampling system. The need for unattended operation resulted in the design and fabrication of an automatic enzyme pad changer-electrochemical cell assembly and a programmer which would control the various detector functions. Data gathered with this breadboard apparatus facilitated the fabrication of the computerized water monitoring apparatus known as CAM-1. Information is presented showing how CAM-1 is operated and how it responds to a group of organophosphate and carbamate insecticides. These experiments and related information are provided in this report.

SECTION IV

THE DETECTION PRINCIPLE

The detection principle used in the monitoring of water for the presence of toxic substance in water can be divided into the following parts: (1) the collection of the enzyme inhibitors on immobilized cholinesterase, (2) the chemical reaction of immobilized cholinesterase with its substrate, and (3) the electrochemical monitoring of substrate hydrolysis products. The mechanisms by which enzyme inhibitors block the activities of enzymes have been extensively investigated. In most, if not all, of the known examples of enzyme inhibition the inhibitor becomes attached (either reversibly or nonreversibly) to the active sites of the enzyme and blocks the substrate from these sites. For this reason immobilized cholinesterase is able to capture pure or mixed organophosphates from very dilute aqueous solutions; measurement of residual enzyme activity gives an indication of the presence of enzyme inhibitors even when very low levels of inhibitors are sampled. We think that noncompetitive inhibitors may also be collected by immobilized enzymes but perhaps to a lesser degree particularly from dilute solutions.

The chemical reaction which we have chosen for determining the activity of the immobilized enzyme is shown by the following equation:



Butyrylthiocholine Iodide (BuSChI) Thiocholine Iodide

In this reaction a thioester is cleaved by the enzyme, cholinesterase (ChE), to give a product with a free thiol group, thiocholine iodide. As will be explained more fully in the description of the electrochemical cell, the formation of thiol from the passage of the substrate over the immobilized enzyme product is evidence for the absence of enzyme inhibitors.

The design of an electrochemical cell adapted for water monitoring of enzyme inhibitors is shown in Figure 1. An immobilized enzyme pad is located between two perforated platinum electrodes. A constant current is supplied by a battery (e.g., 9V) or other source in series with a resistance (e.g., 4.7 meg) so that a current of about 2 μA flows through the circuit. A high impedance voltmeter or electrometer is used to monitor the voltage. During the water pumping part of the cycle only water is passed through the enzyme pad, but during the substrate pumping cycle both air and substrate solution are pumped through the cell. The air flow is needed to push most of the

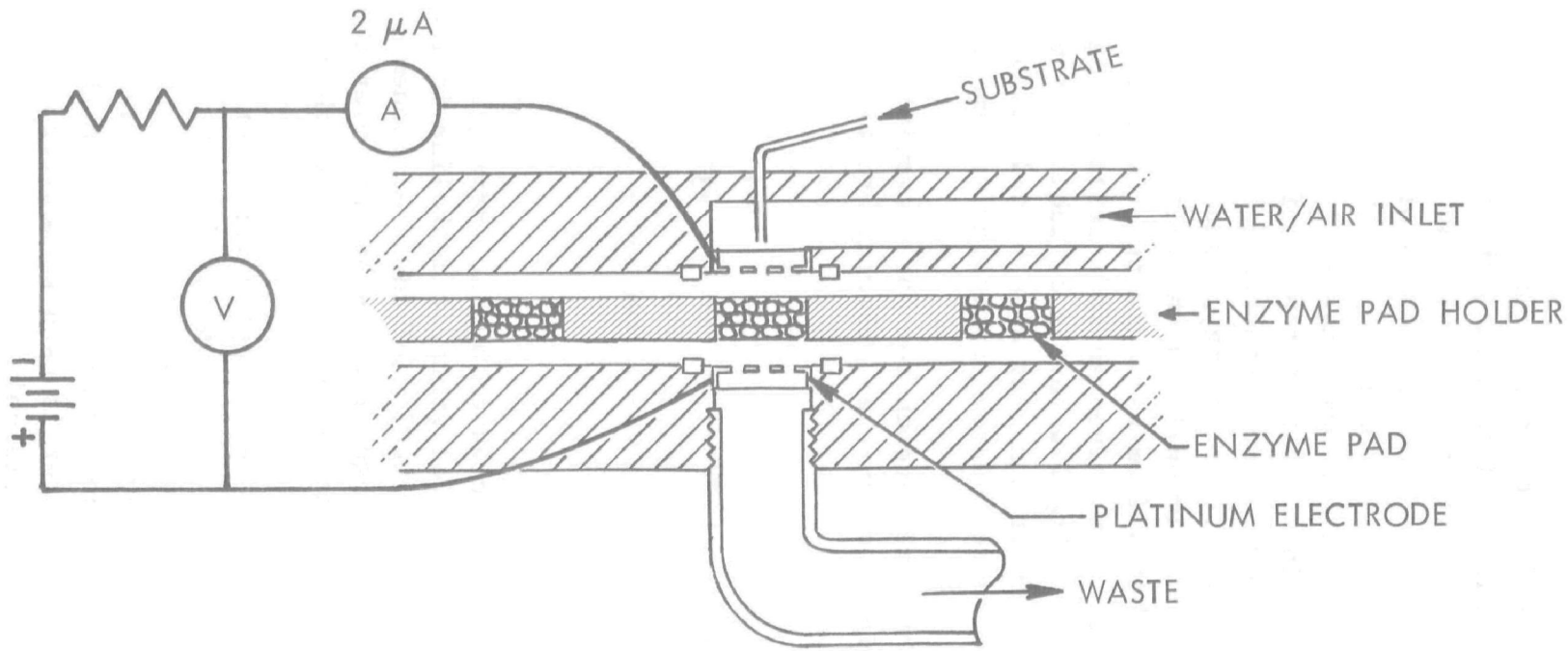


Figure 1 - Cross Section of an Electrochemical Cell Developed for Water Monitoring Showing the Platinum Electrodes Above and Below the Enzyme Pad to Which a Constant Current is Applied.

water from the cell and reduce the dilution of the substrate solution; also the air helps to control the amount of liquid in the enzyme pad during the voltage measurements.

During the water pumping part of the cycle, water is pumped through the immobilized enzyme pad, at which time enzyme inhibitors, if present, are collected on the active sites of the enzyme. During the enzyme-activity-testing part of the cycle, a solution of BuSChI is pumped over the immobilized enzyme product; if enzyme activity remains, then a small part of the substrate is cleaved to give a thiol which then comes in contact with the positive platinum electrode (anode). The lower electrochemical voltage produced between the electrodes when thiol is present (around 200 mV) is due partly to a depolarization of the anode and partly to the electrochemical oxidation of the thiol to the corresponding disulfide. On the other hand, if the immobilized enzyme pad was inactivated by the inhibitors it collected, then there would be no thiol formed, and a higher voltage (around 400 mV) would be observed between the platinum electrodes. Thus by passing a substrate and a current through an electrochemical enzyme cell it is possible to monitor the activity of the enzyme--and also the presence or absence of enzyme inhibitors in the water pumped through the enzyme pad.

We have found no other electrodes which work as well as platinum, and there is a conditioning period for newly prepared electrodes, during which time a coating is formed on the anode; as a result of this invisible coating on the anode, it is different from the cathode, and the two electrodes can function as a battery. The chemical nature of the coating on the anode of a working cell is not known; however, it is formed when current is applied to the cell when thiol groups are present.

Although we have usually used an applied current of 2 μA, either higher or lower currents can also be used. In such cases different voltages will be noted at the electrodes when either substrate or hydrolyzed substrate is present; also, the speed of response to chemical enzyme inhibitors will be different. For example, if lower currents are used, then there is too little electrooxidation of free thiol at the anode, and response to inhibitors is much slower. At higher currents the spread of voltage between hydrolyzed and unhydrolyzed substrate is less. Only a part of the substrate solution passing over the immobilized enzyme is cleaved, and the moles of electrons applied to the cell do not balance the moles of substrate present. The system is quite satisfactory for monitoring of the enzyme activity, although some theoretical aspects of the principles involved are not absolutely clear.

SECTION V

IMMOBILIZED ENZYME STUDIES

The heart of the detection system is the immobilized enzyme product, which removes and concentrates the enzyme inhibitors from the water sampled, and which forms an integral part of the electrochemical cell detection system. For our present investigation we used horse serum cholinesterase (acylcholine acyl-hydrolase, E.C. No. 3.1.1.8 obtained from the Sigma Chemical Company) since we already knew that it was readily inhibited by the organophosphate insecticides. Analyses of the commercial cholinesterase used for the immobilization experiments are shown in Table 2; the procedure for the assay of the soluble enzyme was essentially the same as that reported by Ellman, et al.,[11] except that BuSChI was substituted for acetylthiocholine iodide. It is described below:

Assay Procedure

Apparatus: A Beckman DB-G recording double-beam grating spectrophotometer was used for the assay. We operated the strip chart recorder at 1 in/min and measured the change in absorbancy at 410 mμ as a function of time.

Buffer solution: 0.08 M tris buffer was prepared by dissolving 96.8 g of tris-(hydroxymethyl)-aminomethane in 10 liters of distilled water and adjusting the pH to 7.4 with approximately 33 ml of concentrated HCl.

Indicator solution: 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB), 396 mg, was dissolved in 10 ml of tris buffer by adding sufficient sodium bicarbonate to give a clear solution. This solution was diluted to a volume of 100 ml with buffer and stored in the refrigerator until used.

Enzyme solution: A weighed sample of horse serum cholinesterase was placed in a volumetric flask and diluted with tris buffer to give a solution containing approximately 1 mg/ml. The activity of this solution increases slightly during storage at 4°C for periods up to 48 hr. Vigorous shaking of this solution was avoided.

Procedure: Measured quantities of the following solutions were placed in the cuvettes in the order shown:

0.02 ml of BuSChI solution,
0.1 ml of DTNB solution,
2.0 to 2.98 ml of tris buffer (enzyme + buffer = 3.00 ml),
0.020 to 1.0 ml of enzyme solution depending upon the activity of the enzyme present.

TABLE 2

ACTIVITY OF HORSE SERUM CHOLINESTERASE[a] USED IN IMMOBILIZATION STUDIES

Amount (λ[b])	Activity[c] (μM/min/mg)	
	3-18-71	10-6-71
20	2.96	3.53
	2.77	3.26
	2.91	3.10
40	2.83	3.09
	2.86	3.15
	2.84	3.09
60	2.85	3.11
	2.87	3.18
	3.09	3.12
Mean ($\bar{X}$)	2.887	3.18
Standard Deviation (SD)	±0.093	±0.140
Relative Standard Deviation $\left(\frac{SD}{\bar{X}}\right)$	0.032	0.045

(a) Sigma Chemical Company, Type IVA, Lot 120C-2240.

(b) Quantity of enzyme solution (1 mg/ml) added to the cuvette; see text for analytical procedure.

(c) The assays were conducted by Ellman's procedure using butyrylthiocholine iodide (BuSChI) as substrate.

The blank contained the same volume of solutions but the enzyme solution was replaced with buffer.

Immediately (i.e., within 15 sec) after the addition of the enzyme, the sample was mixed rapidly in the cuvette with a square plastic plunger. Changes in absorbance units per minute (ΔA) were calculated from the percent transmission recorded on the strip chart.

Calculations: The activity of the acylcholine acyl-hydrolase was expressed as micromoles of BuSChI hydrolyzed per minute by 1 mg of the original enzyme preparation:

$$\text{Activity} = \frac{\Delta A \times V}{13.6 \times w} \quad \mu M/min/mg$$

where

ΔA = change in absorbance/min
V = volume of liquid in cuvette (ml)
w = weight of enzyme product (mg) placed in cuvette.

Preparations

A number of different immobilized enzyme products were prepared for trial in the electrochemical cell during our enzyme studies. These products can be divided into two general classes: (1) those in which the enzyme was covalently bound to the support, and (2) those in which the enzyme was complexed with aluminum hydroxide gel, and then entrapped on a polyurethane foam support with starch gel. Both types of products are described in the following paragraphs.

Covalently Bound Immobilized Enzyme Products

A number of methods for the attachment of enzymes to insoluble supports through the use of covalent bonds have been reported in a review by Goldman, et al.[12] Of principal interest to the present investigation, however, are the reports on the attachment of enzymes to insoluble materials with the help of the 2-chloro-s-triazines.[13-15] The chemical reactions involved in the covalent binding of the enzyme to ion exchange cotton is shown in Figure 2. In the first step, diethylaminoethyl cellulose (DEAE-cellulose) is combined with a dichloro-s-triazine dyestuff (Procion Brilliant Blue M-3GS Trademark of ICI and distributed in the USA by Colab) to form a deep blue addition compound. The last chlorine group is reacted with N-methyl morpholine in an effort to facilitate the reaction with the amino groups on the enzyme molecule. The covalently bound immobilized enzyme products made by this

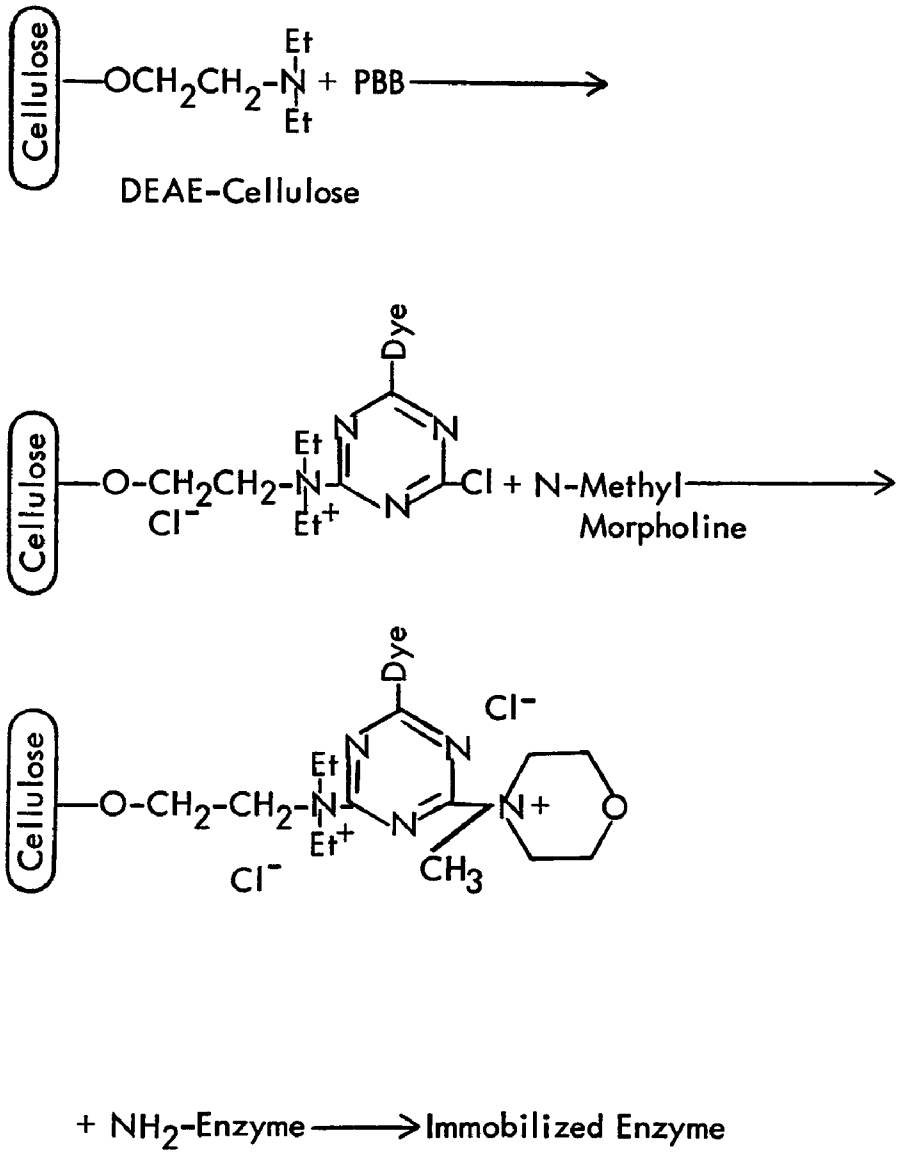


Figure 2 - Reaction Sequences Used for the Preparation of the Immobilized Enzyme Products. PBB is Procion Brilliant Blue and NH_2-enzyme is horse serum cholinesterase.

general procedure are shown in Table 3. Examination of the column identified as "Supporting Matrix" will reveal that the first product was made from absorbent cotton to which the DEAE groups had been attached. The second and third products were made in a similar fashion by attaching DEAE groups to woven cheesecloth. The remaining five products were made by attaching commercially available fibrous DEAE-collulose (Whatman's DE-23) to the surface of open-pore urethane foam with an adhesive and then coupling the enzyme to the outer surface with PBB using the same basic chemical reaction shown in Figure 2.

The covalently bound enzyme products made with the ion exchange cellulose and PBB were evaluated in the electrochemical cells. Attaching the enzyme to absorbent cotton or to cheesecloth gave enzyme pads which presented a good deal of resistance to the flow of water, and tended to pull away from the upper electrode so that changes in electrical contact of the enzyme materials with the electrode tended to produce noise in our system. In an effort to obtain better water flow through the cell, improved contact of the pads with the electrodes and improved uniformity of enzyme activity per pad, we prepared a number of the products in which 45 pores/in. open-pore polyurethane foam (Scott Paper Company) served as the support for the ion exchange cellulose and also the enzyme. Although these products appeared promising, we did not have time to optimize their development nor to conduct exhaustive stability and performance tests with them. Instead, we chose the starch gel immobilized enzyme products described below for use in CAM-1.

Starch Gel Entrapped Immobilized Enzyme Products

In connection with other studies conducted at MRI we developed an enzyme product in which aluminum hydroxide was first used to complex the enzyme, after which the complex was entrapped in starch gel on the surface of open-pore polyurethane foam.[16] The procedure for preparation of enzyme pads for use in the operation of CAM-1 is given in the following paragraphs.

Aluminum chlorohydroxide solution was made by carefully dissolving aluminum chloride (8.7 g) in 100 ml of distilled water and gradually adding aqueous ammonium hydroxide until the pH was 7.0. The resulting gel was spun down in a centrifuge and washed with six portions of water totaling 600 ml. The residue was resuspended in 100 ml of water and the pH was adjusted to 7.01. After standing 1 month the precipitate had dissolved completely, leaving an opalescent solution, pH 5.0. The dissolved material was primarily aluminum chlorohydroxide. The enzyme pads were made in this manner: horse serum cholinesterase (40 mg) was dissolved in 6 ml of tris buffer (0.08 M, pH 7.4) and to this was added with stirring 4.0 ml of the above solution of aluminum chlorohydroxide (note: at this point the aluminum hydroxide gel precipitates and adsorbs the enzyme from the solution).[16] In a separate container 2 g of partially hydrolyzed potato starch (recommended for use in starch gel electrophoresis) was suspended in 10 ml of cold tris buffer pH 7.4 and added to 30 ml of boiling buffer. Heating was continued until the

TABLE 3

COVALENTLY BOUND IMMOBILIZED ENZYME PRODUCTS

Enzyme Preparation[a]	Matrix Preparation: Supporting Matrix	Matrix Preparation: Ion Exchange Capacity (meq/g)	PBB Attachment: Coupling Agents	PBB Attachment: PBB (mg)/ Support (g)	Enzyme Immobilization: Weight PBB-Support Matrix (g)	Enzyme Immobilization: Horse Serum Cholinesterase: Source Lot No.	Enzyme Immobilization: Horse Serum Cholinesterase: (mg/ml)	Product Activity (μM/min/g)[b]
3-15-71	DEAE-Cotton	0.91	PBB (NMM)[c]	2,000/10	10	Sigma 120C-2240	200.12/200	1.77
7-12-71	DEAE-Cheesecloth	0.906	PBB (NMM)	1,500/6	6	Sigma 120C-2240	120.10/120	0.310
9-7-71	DEAE-Cheesecloth	1.59	PBB (NMM)	5,200/27.7	23.7	Sigma 120C-2240	318.30/320	0.179
9-28-71	DE-23-Varnish-Foam[d,e]	1.0	PBB (NMM)	250/4	4	Sigma 120C-2240	20.15/40	0.847
10-11-71	DE-23-Varnish-Foam[e]	1.0	PBB (NMM)	250/4	4	Sigma 120C-2240	20.04/40	0.402
10-21-71	DE-23-Varnish-Foam[e]	1.0	PBB (NMM)	250/4	4	Sigma 120C-2240	10.02/20	0.142
11-22-71A	DE-23-Varnish-Foam[f]	1.0	PBB (NMM)	250/4	4	Sigma 120C-2240	19.95/40	0.205
11-22-71B	DE-23-Sylgard 184-Foam[g]	1.0	PBB (NMM)	250/4	4	Sigma 120C-2240	19.92/40	0.091

(a) The reaction sequences for the preparation of these products are given in Figure 2.

(b) Enzyme activity was determined in a flowing system by pumping BuSChI solution at constant rate over a known weight of product and then measuring the color produced with DTNB by the Ellman method.

(c) NMM is N-methyl morpholine; a 1% aqueous solution was used in these tests.

(d) DEAE-cellulose (Whatman DE-23) with an ion exchange capacity of 1.0 meq/g has been attached to urethane foam circles (6 in. dia x 1/4 in. thick) using binders to form a laminated support matrix for subsequent attachment of PBB and cholinesterase.

(e) These three products used Sear's Interior Polyurethane Varnish (linseed oil modified urethane varnish) as binder for the DE-23. The quantity of the varnish, the method of application and different drying procedures were the variables.

(f) The binder was Penny's Polyurethane Varnish (safflower alkyd toluene diisocyanate).

(g) The binder was Sylgard 184 silicone potting and encapsulating resin catalyzed with 10% of Sylgard 184 curing agent (Dow Corning Corporation).

solution cleared; then the mixture was stirred with a magnetic stirrer to prevent the formation of a scum or lumps in the starch while the mixture was cooling to 45°C. At this temperature the suspension of horse serum cholinesterase complexed with aluminum hydroxide gel was added all at once and carefully mixed with the starch solution. A 10-ml aliquot was withdrawn with a pipette and deposited on a 4 in. x 6 in. x 1/4 in. sheet of 45 pore/in, open-cell urethane foam lying on a piece of warm plate glass. The starch-enzyme mixture was distributed through the urethane foam as uniformly as possible with the aid of a plastic rolling pin filled with 47°C water. In this same manner three other 10-ml aliquots of the warm starch-enzyme slurry were added to other urethane foam sheets as before.

The coated sheets were placed on edge in a wooden rack and allowed to gel for 1 hr at room temperature before drying overnight at 115°F. The dried sheets were then cut into 3/8 in. dia enzyme pads with a motor-driven stainless-steel cutter. The pads were stored in glass bottles with calcium sulfate desiccant in a refrigerator until assayed or used. Additional information about four gel entrapped enzyme products made by this procedure is given in Table 4.

A modification of the procedure of Ellman, et al.,[11] was used for determination of the cholinesterase activity of these enzyme pads. In this case, we solubilized the enzyme from the urethane foam pads by mascerating them in 3 ml of tris buffer, pH 7.4 with 10 units of α-amylase (hog pancreas) and then incubating the mixture for 1 hr at 34°C. The resulting solution of cholinesterase was then assayed by the procedure described for the commercial cholinesterase products (see page 13). α-Amylase does not hydrolyze BuSChI.

The enzyme products made by this procedure were stored over a desiccant in the refrigerator until used. Similar enzyme pads made on another project and stored in this way for 5 years were shown to perform well in CAM-1. In an accelerated aging test, water at 47°C was pumped through the enzyme pad for 2 hr; we found that 48.6% of the initial enzyme activity remained after this extraction. At lower temperatures the enzyme product is more resistant to extraction and/or inactivation.

An Electrochemical Cell Using Immobilized Enzymes

For the design of an integrated electrochemical cell-automatic enzyme pad changer assembly (for use in monitoring of water for the presence of enzyme inhibitors), the new cell must be compatible with the new enzyme products. The first step was to fabricate an electrochemical cell with which experimental immobilized enzyme products could be studied. For this cell, Figure 3, perforated platinum sheet electrodes, through which water and substrate solution could be pumped sequentially or simultaneously, was chosen. We then

TABLE 4

IMMOBILIZED ENZYME PRODUCTS IN WHICH ALUMINUM HYDROXIDE AND STARCH GELS ARE USED TO HOLD CHOLINESTERASE ON POLYURETHANE FOAM[a]

Pad Preparation	Cholinesterase[b] Source	(mg)	Starch[c] (g)	Aluminum Chlorohydroxide Dry Wt (mg)	Enzyme Activity (μM/min/g)
11-10-71	120C-2240	80.0	4.0	300[d]	0.959
11-12-71	120C-2240	80.8	4.0	300[d]	0.764
1-19-72	120C-2240	79.1	4.0	300[e]	1.201
1-31-72	120C-2240	79.9	4.0	300[e]	1.065

(a) Quantities shown are for a batch consisting of four sheets, 4 x 6 x 1/4 in. and yielding 250-300 enzyme pads.
(b) Sigma Chemical Company, Type IV, cholinesterase from horse serum.
(c) Connaught starch-hydrolyzed for electrophoresis, Connaught Medical Research Laboratories, University of Toronto, Canada.
(d) Initial pH of the solution was 3.5.
(e) Initial pH of the solution was 5.2.

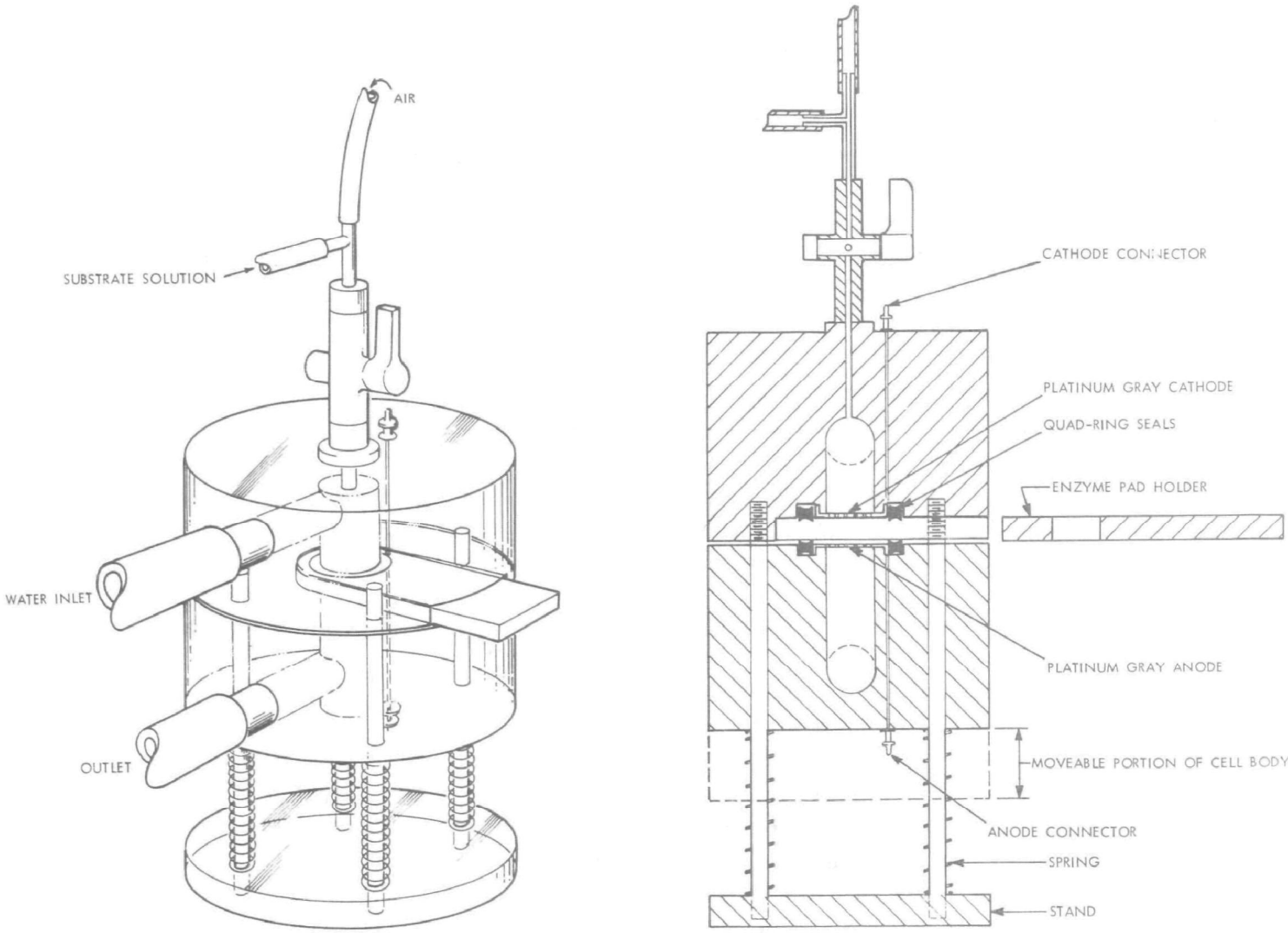


Figure 3 - Experimental Electrochemical Cell for Investigating Immobilized Enzyme Products for Use in Water Monitoring.

tested four types of enzyme products which we described on the basis of the physical support for the enzyme: (1) balls of cotton, (2) woven cotton, (3) fibers laminated on urethane foam, and (4) starch gel on urethane foam. Table 5 gives a comparison of the performance of these same enzyme products but lists them on the basis of their actual preparation. The enzyme was covalently bonded to ion exchange cellulose in the first three of the types and held by physical entrapment in the fourth type shown in the table. As noted earlier, the selection of the urethane foam coated with starch gel and aluminum hydroxide gel entrapped cholinesterase, for routine use in the CAM-1, was based mainly on the advantages shown in Table 5. Information about individual enzyme preparations is presented in Tables 3 and 4.

For the experimental electrochemical cell (Figure 1) we chose dimensions for the water passageways, the perforated platinum electrodes and the enzyme pad so that water flow rates of up to 1,200 ml/min could be achieved with pressures of less than 1 lb/in^2. In this system the enzyme pad was arbitrarily set at 3/8 in. in dia and 1/4 in. thick; probably other sizes would have worked equally as well, but they were not tried.

The electrodes in this cell were made of perforated platinum sheet which had been platinized by electroplating with a 3% solution of chloroplatinic acid which contained 0.1% of lead acetate.

For this platinizing step approximately 2.5 volts was applied across the two electrodes located 1 cm apart and the polarity was reversed every 30 sec until an even black coating of platinum black was obtained (approximately 5 min). The black electrodes were washed in nitric acid and distilled water and then heated to redness in a Bunsen burner to convert the coating to platinum gray, which retains much of its surface area but which is much more resistant to abrasion.

TABLE 5

COMPARISON OF FOUR TYPES OF IMMOBILIZED ENZYME PRODUCTS PREPARED FOR USE IN THE ELECTROCHEMICAL CELL

No.	Type of Enzyme Support	Retention of Enzyme Activity During Use	Ease of Preparing Enzyme Pads With Equal Activity	Contact With Both Electrodes of the Cell	Resistance to Water Flow	Relative Cost of Preparation
1	Ion exchange cotton (long fibers)	Excellent	Difficult	Poor	High	High
2	Ion exchange cheesecloth	Excellent	Less difficult	Fair	Medium	High
3	Short ion exchange cotton fibers laminated on open-pore urethane foam	Good	Less difficult	Excellent	Low	High
4	Urethane foam coated with starch gel + $Al(OH)_3$	Good	Easy	Excellent	Low	Low

SECTION VI

OPERATING PARAMETERS FOR THE ELECTROCHEMICAL ENZYME SENSOR

An explanation has been given of the basic principles involved in the operation of an electrochemical cell for the detection of low levels of organophosphates in water supplies (see Section IV). In order to make this electrochemical enzyme cell both sensitive and reliable for the detection of enzyme inhibitors, it was necessary to control those variables which affected the response of the cell: (1) the buffer solution, (2) the substrate, (3) the applied current, (4) the rate of water sampling, (5) time of water sampling and the like. Some of the variables turned out to be interdependent; others were not.

Tris buffer was selected for use in our system because it was compatible with the platinum electrodes, the enzyme, and the substrate. The selection of the buffer concentration at 0.08 M was arbitrary, and could have been changed to 0.10 or 0.15 M with little effect on the response of the unit to enzyme inhibitors. However, the buffering capacity of the 0.08 M substrate was adequate for our system. The pH of 7.4 for the buffer was a compromise between a mildly acidic pH where the substrate is very stable and pH 8.6 where the cholinesterase is especially active in hydrolyzing the substrate, BuSChI. At pH 7.4 the enzyme is quite active and the spontaneous hydrolysis of the substrate is slow enough at room temperature so that it usually is not a problem.

The selection of substrate concentration and substrate flow rate should be considered together, since the quantity of substrate reaching the electrodes per unit time is the important quantity affecting the base line voltage and sensitivity of the detection system. Greatest sensitivity to low levels of enzyme inhibitors is obtained when an excess of the substrate is avoided; obviously enough substrate solution is needed to produce a lower electrode voltage when the enzyme is active. At the same time, more inhibitor will be required to cause an increase in cell voltage if there is a large excess of substrate. Although several substrate concentrations were investigated 2.5×10^{-4} M BuSChI in 0.08 M tris buffer, pH 7.4 is about the optimum for a pumping rate of about 1 ml/min. The substrate used in these experiments, BuSChI, had a mp of 172°-174°C and was supplied by Pierce Chemical Company or Eastman Organic Chemicals.

As mentioned earlier, an applied current of about 2 μA has been used. This current appears to give a good spread of cell voltages between those encountered in the absence of enzyme inhibitors and those encountered in the presence of inhibitors. The use of higher cell currents could probably be tolerated if the concentration of the substrate solution were increased.

In selecting a combination of applied current of 2.0 μA, a buffer concentration of 0.08 M, a pH of 7.4, a substrate concentration of 2.5×10^{-4} M, a substrate flow rate of 1.0 ml/min and an enzyme pad with approximately 0.02 to 0.04 μM/min/g activity, we were attempting to optimize the various cell operating parameters so as to provide good sensitivity, reliability, and fast voltage responses when the enzyme was inhibited.

SECTION VII

DETECTION CYCLES FOR WATER MONITORING

Originally it seemed possible to mix substrate solution continuously with the water being sampled and obtain a continuous record of the activity of the immobilized enzyme product, but experiments soon showed that this approach was not feasible because of the large quantities of buffer and substrates which would be required.

A second approach to continuous aqueous monitoring is shown in Figure 4. Here two enzyme pads are used alternately for collection of enzyme inhibitors and for measurement of residual enzyme activity. Although the automatic system shown was not built, some studies, which we believe approximated the results to be expected with such a system, were conducted. In these experiments two enzyme pads, A and B, were used; water was passed through pad A for 2 min, while the enzyme activity of pad B was being monitored in an electrochemical cell. Pads A and B were then manually switched for many 2-min cycles, while simulating the two-pad detection system. The voltages observed with pad A were about 50 mV different from the voltages obtained with pad B; as a result of this, we concluded that it would be advantageous to compare each pad only with itself. This conclusion was based upon an inability to make or keep pairs of enzyme pads which had exactly the same amount of enzyme activity or even provided the same electrical pathways between the electrodes. Although a logic circuit could be devised for use with the two-pad system which would permit a comparison of the previous and present voltages of pad A and similarly for pad B, we elected, in the interest of simplicity, to adopt an intermittent monitoring system with only one enzyme pad in use at a time.

Following the decision to build an intermittent sampling type of aqueous monitoring apparatus, the breadboard apparatus shown in Figure 5 was constructed. In this particular system the Haydon electrical timer was adjustable so that we could vary the duration of any particular part of the cycle. The substrate pump was a Holter peristaltic pump with adjustable flow rate control; the electrometer used for most of these experiments was the Keithley Model 602B which had an input impedance of up to 10^{14} ohms. Less expensive high impedance voltage measuring instruments could have been used, but an input impedance of 10 megohms or greater is necessary to prevent masking of the signal. The air pump used in some of the later experiments was a simple vibrator diaphragm pump intended for aeration of aquaria. Detailed construction of the electrochemical cell is shown in Figure 3.

In most of the early investigations with the breadboard detection system a 6-min cycle, in which water was sampled from 3-5 min and the activity of the

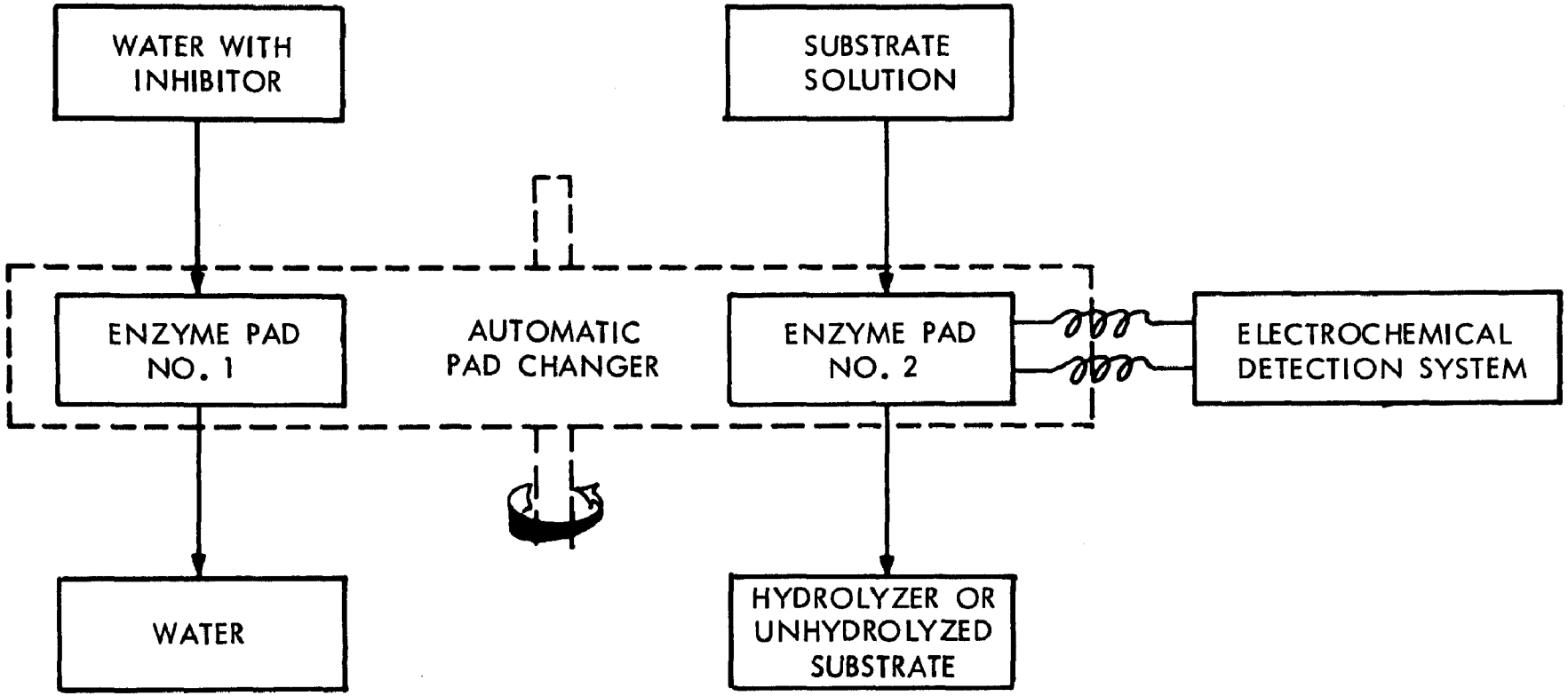


Figure 4 - Proposed 2-Pad Water Monitoring System Showing How Alternate Use of Two Enzyme Pads Might Permit Simultaneous Agent Collection and Readout of Enzyme Inhibitor

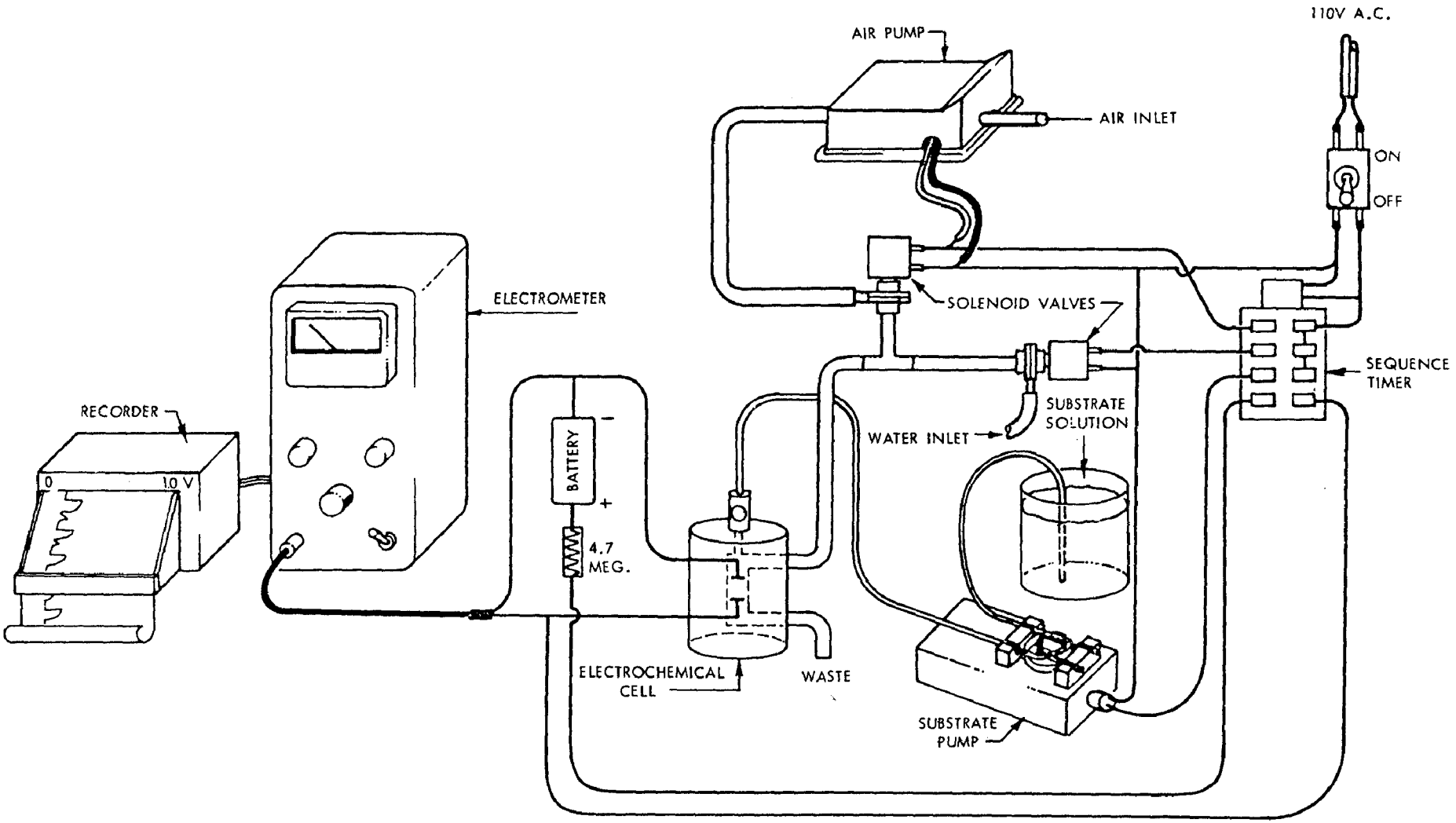


Figure 5 - Breadboard Detector for Toxic Substances in Water

enzyme was monitored during the balance of the cycle, was used. In these studies substrate concentrations of 1.25, 2.50 and 5.0 x 10^{-4} M BuSChI in 0.08 M tris buffer, pH 7.4, and flow rates of 0.5, 0.8, 1.0, 1.5 and 2.0 ml/min, were used. Water flow rates varied from about 450 to 1,200 ml/min; the lower flow rates were mostly encountered while the PBB-cotton and PBB-cheesecloth enzyme products were used, a condition which resulted in partial plugging of the cell and the low flow rates. For pumping the water through the cell an electric fuel pump (diaphragm type), an oscillating pump with Hypalon impeller which was self-priming at a 50-in. depth, a Gelber Model PQ gear pump with a capacity of about 1,200 ml/min, and a Flotec pump made to our requirements with a capacity of approximately 1,200 ml/min (self-priming when wet) were evaluated for use in CAM-1.

One of the most important problems investigated with this breadboard system was a way to obtain a rapid determination of the immobilized enzyme activity after exposure to the water which had been sampled. In the initial experiments, 2-3 min were required for estimation of the residual enzyme activity due to the time required for the electrodes to reach (or approach) an equilibrium voltage after exposure to the water. However, by turning off the current during all of the water sampling portion of the cycle and also during the first part of the substrate pumping cycle, it was possible to shorten the electrode equilibration time. Further shortening of this time was accomplished by blowing the excess water out of the cell with air immediately after turning the water off; increasing of the substrate pumping rate to 1.0 ml/min also speeded the equilibration time. These changes made it possible to obtain uniform voltages from cycle to cycle with 1 min for the electrode equilibration. The detection cycle developed with this breadboard apparatus and used in the CAM-1 is shown in Figure 6. The sponsor selected the 2-min water sampling part of the cycle so that the entire cycle would be complete within 3 min. As shown in subsequent experiments this 2-min water sampling period was adequate for obtaining response of the CAM-1 to both subtoxic and toxic levels of enzyme inhibitors in water supplies.

Figure 7 shows the voltage tracing obtained with the electrochemical enzyme cell operating on the 3-min cycle shown in Figure 6. During the water-pumping part of the cycle, the applied current is turned off and the voltage falls rapidly at first and then levels off somewhat. When the water is turned off and the air is blown out of the cell, there is often a spike in the curve which is of no consequence so far as the operation of the monitoring apparatus is concerned. After the substrate pump has been pumping the substrate solution at the rate of 1.0 ml/min for 20 sec, the current is applied to the cell, and there is a sharp rise in voltage which levels off (and in some cases falls a little from the maximum) at a voltage which is indicative of the activity of the enzyme product in the electrochemical cell. Even though the voltage may not reach a true equilibrium in 1 min, experience has shown

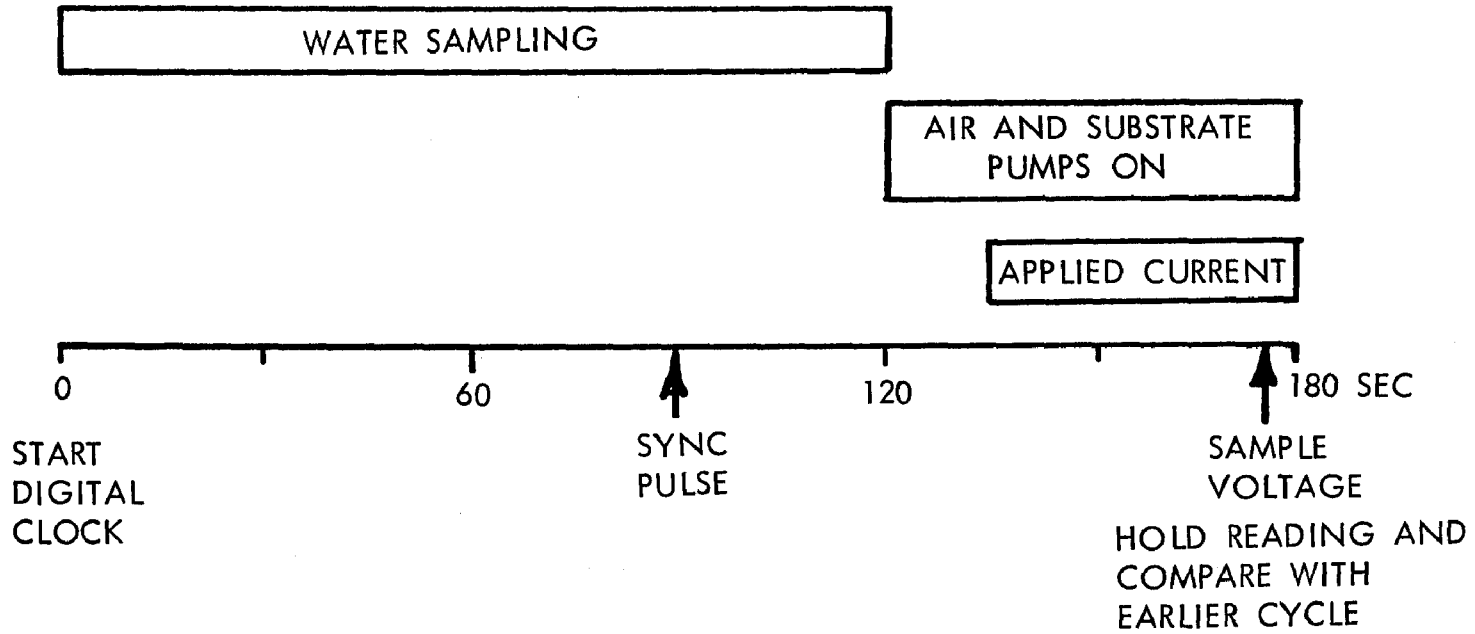


Figure 6 - CAM-1 3-Min Operating Cycle

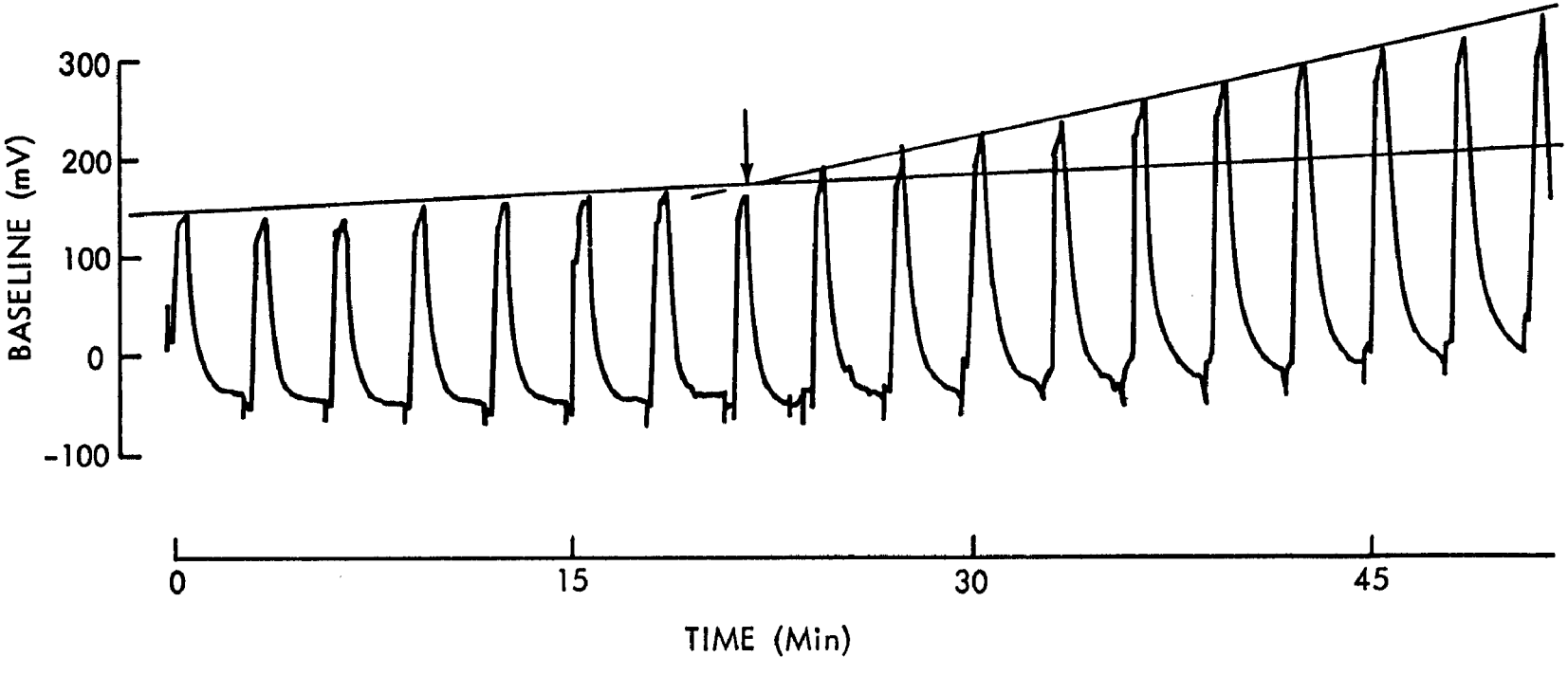


Figure 7 - Response of the Electrochemical Cell Operating on the 3-Min Cycle to Water Containing 0.2 ppm DDVP

that the voltage obtained 55 sec after starting the flow of the substrate and 35 sec after applying the cell current is reproducible, and is satisfactory for monitoring of the enzyme activity. In the figure the difference in peak heights is exaggerated in order to show the kind of noise which can be expected in a system of this type. The straight line over the top of the voltage peaks obtained during the first seven cycles shows a gradual slope upward with time. This slope is due partly to the slow loss of enzyme activity which occurs with the use of the enzyme pads and which does not interfere with the response of the detector so long as enough enzyme activity remains to cause a voltage rise when more of the enzyme is inhibited. As will be noted later, we have provided for automatic enzyme pad rejection when the quantity of residual enzyme activity is marginal to that required for reliable detector performance.

Figure 7 also shows the kind of change in the voltage tracings which can be expected when an enzyme inhibitor is present in the water sampled. The arrow located at the end of the first seven cycles represents the point at which the water supply was changed to a solution containing 0.2 ppm of DDVP. As may be seen from the right-hand portion of the curve, the cumulative alarm signal (i.e., the increase in cell voltage over and above the expected voltage shown by the extrapolated straight line) was 182 mV or 18.2 mV/cycle. If an alarm threshold of 12 mV increase were used as the measure of the presence or absence of significant quantities of inhibitors then it is clear that (1) there would have been an alarm within 3 min after introduction of the DDVP, (2) a single enzyme pad would have given 10 alarms, and (3) there would have been no false alarms during the six preceding cycles of operation. Although not shown on the curve, challenge of the electrochemical enzyme system with higher concentrations of DDVP results in larger (perhaps 100 mV) changes in baseline voltages between cycles.

SECTION VIII

DESIGN AND FABRICATION OF THE CHOLINESTERASE ANTAGONIST MONITOR (CAM-1)

Before designing or constructing the water monitoring system based upon the use of immobilized cholinesterase as the sensor, it was necessary to consider the intended use of the detector, the manipulations and skills to be required of the operators, the period of unattended operation desired, the sensitivity, the selectivity, the permissible size, weight and cost, the need for maintenance, and other parameters affecting the design. From the beginning it was planned that 110 V AC power would be available; that the initial model would be operated indoors so that there would be no need to provide insulation and case heaters; that unattended operation might be as long as a week, although 48 hr was chosen as the immediate goal; and that the weight could be kept below 50 lb and the volume below 2 cu ft because a smaller instrument would be much easier for the operator to use.

If the instrument were to function unattended for long periods of time, every operation should be made as nearly automatic as possible. Of particular importance was a provision for an automatic enzyme pad changer which would change enzyme pads before all of the activity was used up, so that an automatic detection cycle which should signal an alarm when toxic hazards were present in the water sampled could go into operation. The enzyme itself would probably give the unit selectivity, since materials which inhibit this enzyme are likely to be toxic. Speed of response to inhibitors and sensitivity to low levels of inhibitors are dependent variables; it was obvious that longer sampling times would give the greater sensitivity, but as was shown after the unit was fabricated, a 2-min water sampling period is adequate for the detection of subtoxic levels of organophosphates.

The Cholinesterase Antagonist Monitor (CAM-1) which we have designed and fabricated for the rapid detection of organophosphates in water supplies is shown in Figure 8. Basically this apparatus is made up of the same kinds of components as the breadboard detector shown in Figure 5, except that an alarm has been added and operation of the various components has been automated through the addition of logic circuits, interlocks, controls and readout devices. Additional information about the design and construction of CAM-1 and its components is given in the following paragraphs.

Insofar as possible commercially available parts for the fabrication of CAM-1 have been used. However it was necessary to design and fabricate the automatic enzyme pad changer-electrochemical cell assembly shown in Figure 9. The cross-section of the electrochemical cell, Figure 1, shows the shape and configuration of the perforated platinum electrodes which were made from 0.008-in. platinum sheet so that they would have some mechanical strength

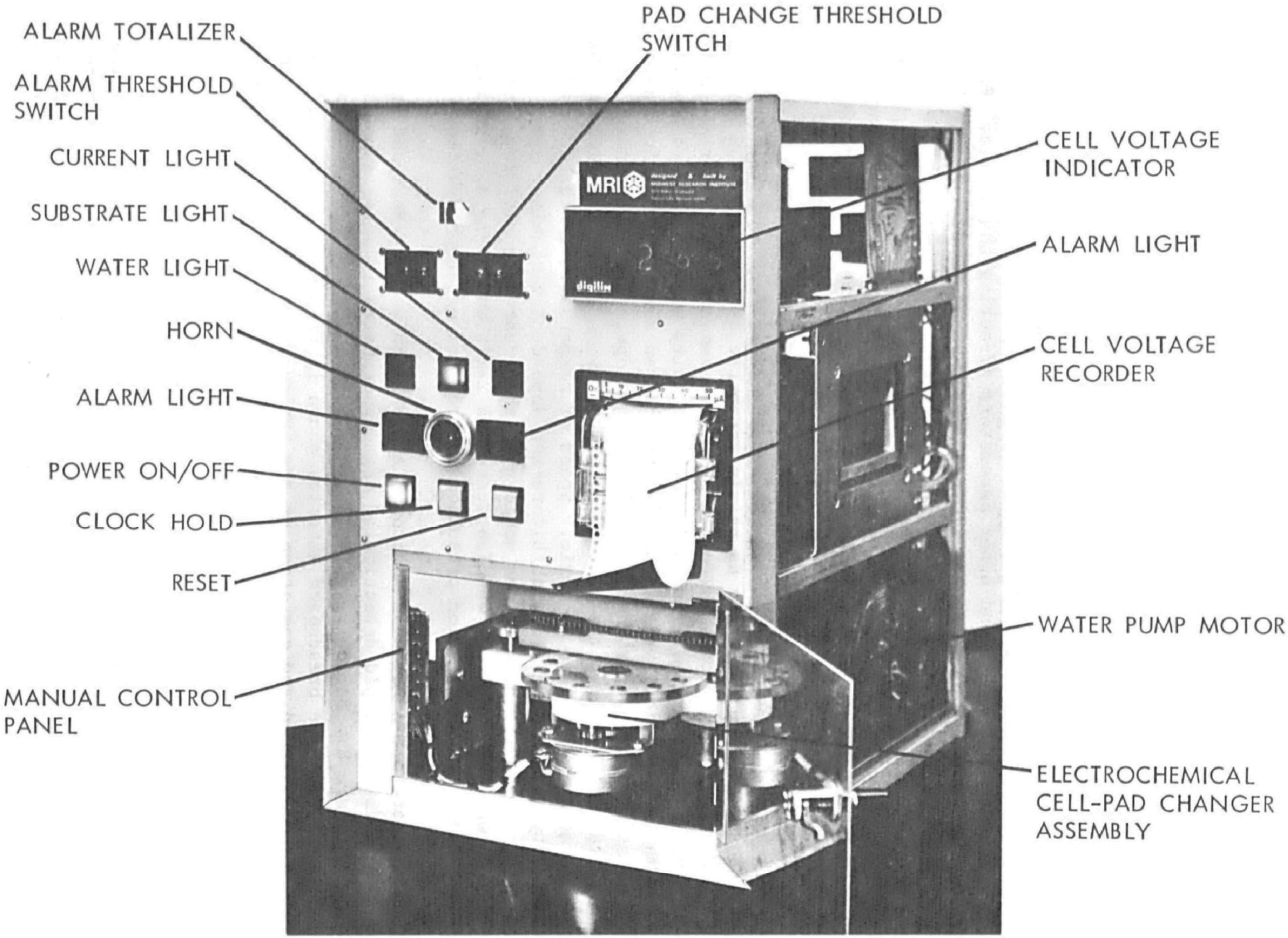


Figure 8 - The Cholinesterase Antagonist Monitor (CAM-1) With Side Panels and Recorder Cover Removed

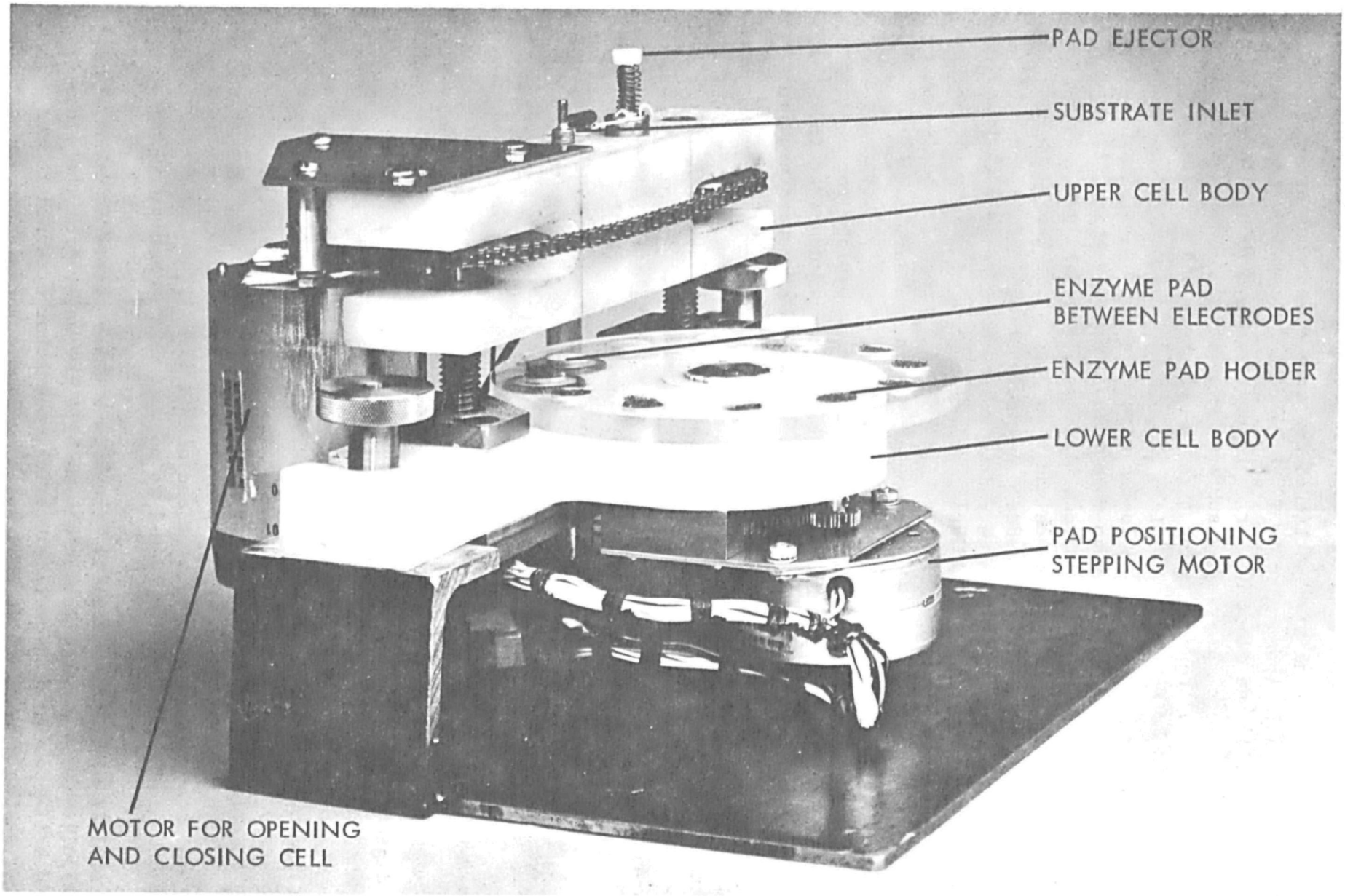


Figure 9 - Automatic Enzyme Pad Changer--Electrochemical Cell Assembly From CAM-1

and retain their shape. The wires attached to these electrodes are platinum and they are welded on. Approximately 34 holes (1 mm in dia) were drilled in the flat surface of the electrode prior to coating it with platinum black and heating to make the platinum gray surface. As may be seen in Figure 9, twin screws driven by a stepping motor through sprockets and a chain are used to open and close the electrochemical cell. When the cell is open, the enzyme pad holder may be removed and loaded with enzyme pads; although there are 12 holes in this plastic holder no more than 11 pads should be inserted at one time. On closing of the electrochemical cell, the pad positioning motor advances the pad holder one space and then ejects the pad which had been placed directly over the electrodes. An operator will, as a consequence, not think that he has good pads in his pad holder when in reality some or all of them may have been exhausted during previous use. Thus every time that a new pad is inserted automatically into the cell, the old pad is pushed out of the enzyme holder and onto the floor of the instrument.

The upper and lower cell bodies are made of delrin, the O-rings for sealing the cell are made of silastic and the substrate inlet is made of stainless steel. Wherever possible corrosion resistant materials have been used in the construction of the pad changer mechanism and also for the case.

Most of the case is made of aluminum; its method of construction is shown in Figure 10(A). The detector is purposely arranged vertically so that the wet components can be localized on the bottom away from the electronic parts--or at least most of them. Figure 10(B) shows the back of CAM-1 with both of the doors open and the accessibility of the various components for servicing and repair, if necessary. The 24 V DC and the 5 V DC power supplies are located on the second level along with the air pump and the recorder (not visible from the back). These two power supplies are fused individually. Figure 10(C) shows the pulse generator used for operating the two stepping motors on the pad changer. Figure 10(D) shows a transformer and three relays; the transformer is used as the source of 6 V AC for operating the digital clock; Relay 1 turns the water on and the air off; Relay 2 controls the substrate flow and Relay 3 is part of the interlock system which keeps the water turned off when the cell is open. Figure 11(A) shows the pad changer assembly installed inside of the CAM-1 case. At the extreme left of this picture are four toggle switches which are used for the manual control of the various components; counting down from the top, Switch 1 opens the cell, Switch 2 controls the water pump, Switch 3 controls the substrate pump and Switch 4 turns the current on and off. All of these switches must be in the down (off) position for CAM-1 to operate in the automatic mode.

When the cell is opened electrically, there is a delay before it opens; this delay was built-in to allow time for air to blow the water from the lines before the cell opened. The small electrical connector attached to the pad changer assembly provides an easy way to disconnect the anode and cathode

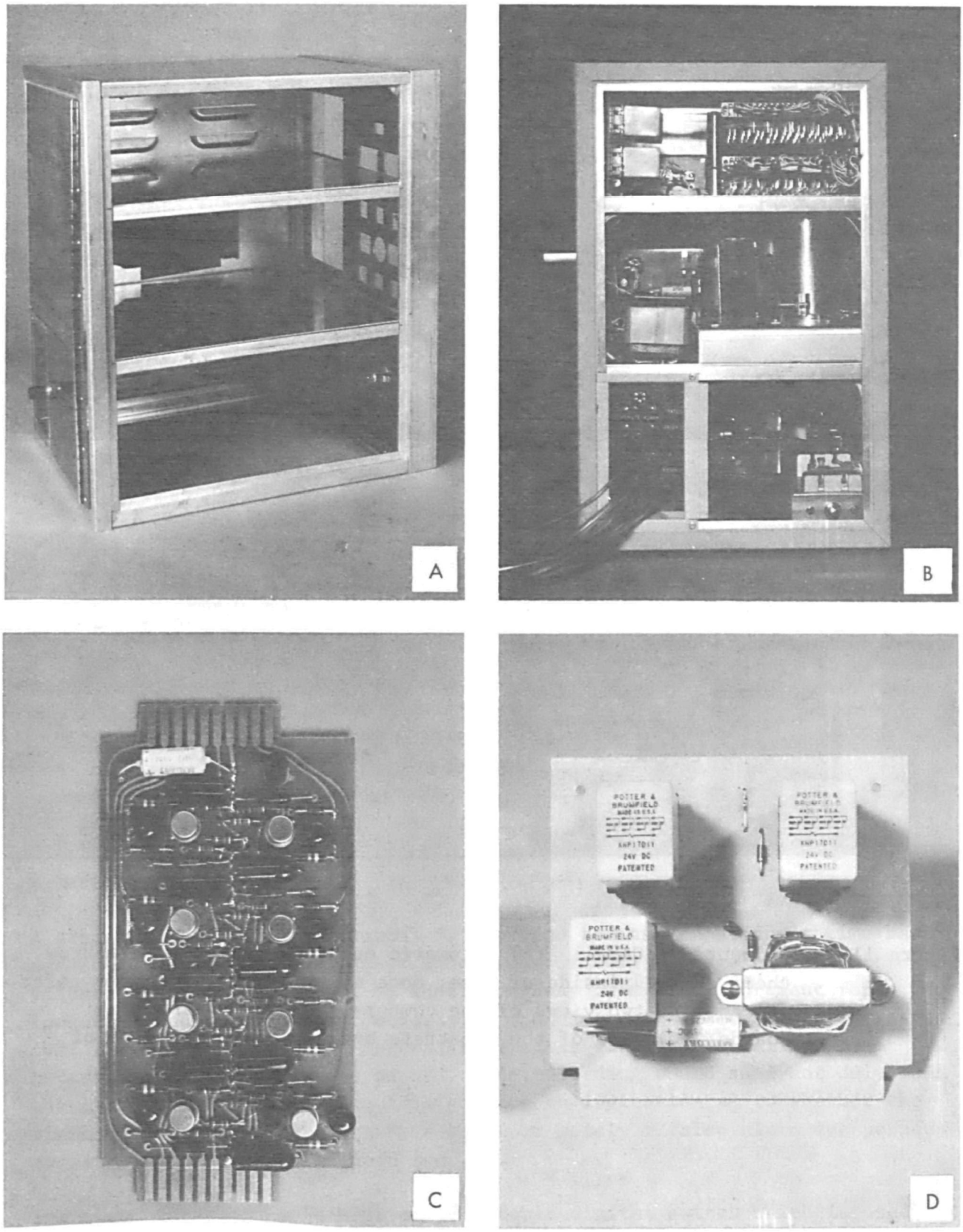


Figure 10 - Components of CAM-1: (A) the case; (B) rear view of CAM-1 showing the relay board and the integrated circuit boards on the upper level, the air pump and the 5V DC power supply on the second level, and the peristaltic pump on the lowest level; (C) pulse generator for operating the stepping motors; (D) relay board.

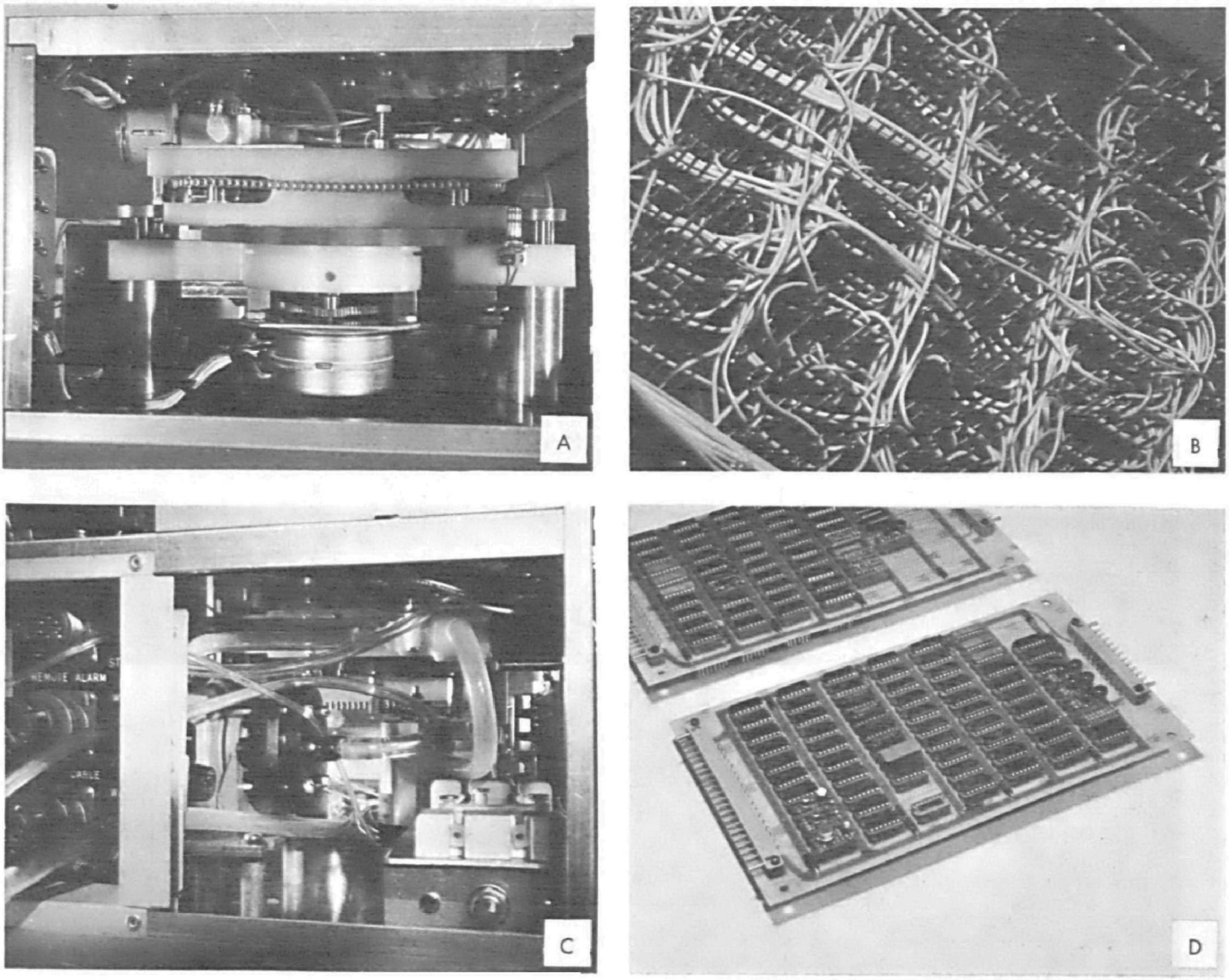


Figure 11 - Components of CAM-1: (A) automatic enzyme pad changer-electrochemical cell inside of case; note manual controls to the left; (B) and (D) two views of the computer and logic circuitry boards; (C) view of the substrate and water pumps inside of case.

when the whole assembly is removed for cleaning or other servicing. The cable attached to the pad changer assembly leads to the recorder housing where the operation amplifier and the constant current power source are located.

Figures 11(B) and 11(D) show two sides of the same circuit boards. The sockets into which the integrated circuits, gates, memory devices, relays, counters, dividers and other solid state logic circuit devices are plugged, are connected by a solderless wirewrap technique developed by Bell Labs. The digital clock for providing all time functions is mounted on one of these boards. Figure 11(C) shows the rear of the lowest level in CAM-1. The peristaltic pump is shown at the lower right, the Flotec pump is shown in the center, and the connector for obtaining a remote readout of cell voltage or an alarm signal is shown in the upper left.

Some of the remaining design features may be seen best by reference to Figure 8. A Digilin digital voltmeter has been mounted on the front of the CAM-1 case for two reasons. The more important is that it is necessary to convert the electrochemical cell voltages into a binary code decimal (BCD) voltage so that it can in turn be processed by the memory and logic circuitry; the digital voltmeter makes this conversion; second, it is useful to be able to read the cell voltage to the nearest millivolt without reading a needle on a meter or looking at a tracing on a recorder. At one point in the detection cycle a voltage-hold function is activated which enables the same cell voltage fed into the memory bank to be held on the meter until either (1) the current is turned on in the next cycle, or (2) the reset button is depressed. In the later case it is possible to obtain exact cell voltage readings during the water pumping part of the detection cycle without reference to the recorder.

A strip chart recorder (Simpson) is provided to make a permanent time-base recording of the cell voltage. Recordings of cell voltage can be particularly valuable to the operator if he should be away from the instrument for a prolonged period of time--particularly if the alarm totalizer should indicate that there had been one or more alarm signals since the totalizer reset button (not shown) had been pushed. The recording would show not only when the alarm occurred, but it would also give an indication as to whether the alarm was the result of a massive spill or merely a false alarm due perhaps to setting the alarm threshold too low.

The alarm threshold switch is an adjustable digital switch which is used to control the sensitivity of the CAM-1 unit. The operator may select alarm thresholds of 0 to 99 mV as the voltage change between successive cycles to which he would like to have the alarm lights flash and the Sonalert horn give its 4-sec whistle. For best results, the alarm threshold should be set high enough so that it does not give false alarms and low

enough so that hazardous material spills are not missed. Our experience indicates that 8-20 mV is usually a good place to set this threshold switch.

Next to the alarm threshold switch is the pad-change threshold switch which is used to activate the automatic-enzyme pad-changer. In order to avoid the possibility that all of the enzyme activity in an enzyme pad has been destroyed and that the pad can no longer respond to enzyme inhibitors, a mechanism by which pads can be replaced automatically before they have lost all of their enzyme activity, has been provided. One way to select a setting for this switch is to place a plain starch-coated urethane foam pad (no enzyme) in the electrochemical cell and operate the CAM-1 for several cycles; following this activity the pad change threshold switch is set 50 mV or more below the voltage obtained with the starch pad in the electrochemical cell. By following this procedure, one can be sure that good pads (i.e., pads which have enough enzyme activity to respond to inhibitors) are always kept in the electrochemical cell.

The detection cycle was shown graphically in Figure 6. The following description of the automatic operation of the detection cycle in CAM-1 shows the timing sequence activated by the minicomputer:

0 sec	Everything off (except for air which comes on when the water is turned off).
1 sec	Turn water pump on and air pump off.
90 sec	Generate sync pulse which can be used to keep the slave detector system out-of-phase and provide total sampling.
120 sec	Turn water off; start air and substrate flow.
140 sec	Apply constant current to electrochemical cell.
175 sec	Enter cell voltage into memory circuit, process this voltage (i.e., compare voltage with voltage from previous cycle); signal an alarm if the voltage increase is in excess of the sensitivity setting; compare cell voltage with amplitude set point and activate automatic pad changer if pad needs changing.
180 sec	Turn off substrate solution and applied current and reset clock to 0.

The three blue lights located above the horn are connected to the water pump, the substrate pump and the applied current relay. These lights come on in sequence, and indicate which part of the detection cycle is then in progress. If these lights fail to cycle, it may be an indication that one of the manual control switches has been left in the up position, or that there is some malfunction.

The three lights below the horn are also push button switches. The first of these switches on the left turns the power on and off. If the detector is stopped in the middle of a cycle and then turned on again, the memory will be erased and the unit will start the first of a new cycle. Depressing the second switch stops the clock at the part of the cycle where the detector happens to be at that instant; pressing this button a second time releases the clock-hold mechanism, and the clock continues with the cycle where it left off. This button has value if the operator should like to increase the duration of any cycle; for example, if there were a desire to increase the water sampling period from 2 min to 6 min, the operator could stop the clock for 4 min during the water pumping cycle and the rest of the cycle would be unaffected. The reset button sets the clock back to zero; it releases the voltage hold on the digital voltmeter but it does not erase the memory of the last voltage entered into the memory bank.

When detector operation is first started with an enzyme pad, there should be no alarm at the end of the first cycle because no voltage is stored in the memory. Even if the voltage exceeds the previous cell voltage reading by an amount equal to or in excess to the alarm threshold, there will be no alarm on the second cycle since CAM-1 was designed to ignore an alarm on the second cycle. On the third and subsequent cycles there will be an alarm every time the alarm threshold is exceeded, except that if the enzyme pad should be replaced automatically, there would be no alarm for the first two cycles whether or not the alarm threshold is exceeded. This provision for no alarm at the end of the second cycle can be changed, but possible false alarms due to the finite time required for new pads to equilibrate can be avoided, and the provision seems wise.

After an alarm signal, the flashing red lights will continue to flash until one of the lights is pushed. The alarms are counted on the alarm totalizer lights which are light-emitting diodes (LED). Since individual segments of this counter could burn out, these lights flash "88" each time the sync signal is generated, i.e., 90 sec after the start of the new cycle to show that all segments are lighting properly.

A block diagram of CAM-1 is presented in Figure 12. This diagram shows in a general way how the various components are interrelated but obviously it cannot show all of the detailed relationships needed to make the unit function. The complete wiring diagrams in a greatly reduced format are included in this report. Figure 13 shows a number of components including: (1) pulse generator for operation of the two stepping motors on the automatic enzyme pad changer, (2) the constant current power supply and high impedance amplifier for the electrochemical enzyme cell (located inside the recorder case), (3) the function control switches, (4) the alarm counter, (5) the relay circuits for operating the various pumps, etc., (located on the relay board shown in Figure 10(D)), etc. Figure 14 shows additional circuit features including: (1) digital clock, (2) counting circuits, (3) analog to digital to binary code decimal converters, programmer, etc. Figure 15 shows still more of the CAM-1 circuitry including memory circuits, flip-flops, inverters, decade counters, binary counters, latches, adders, dividers, comparators and other computer logic components. Together these three figures show the advanced solid state circuitry which enables CAM-1 to operate automatically unattended and to make decisions as to when to change enzyme pads or when to signal an alarm situation. The circuits given on opposite pages of this report may be cut out and pasted together to simplify the tracing of signals.

Table 6 gives a list of the suppliers of components in CAM-1 and these are arranged by position of these components on the three levels in CAM-1.

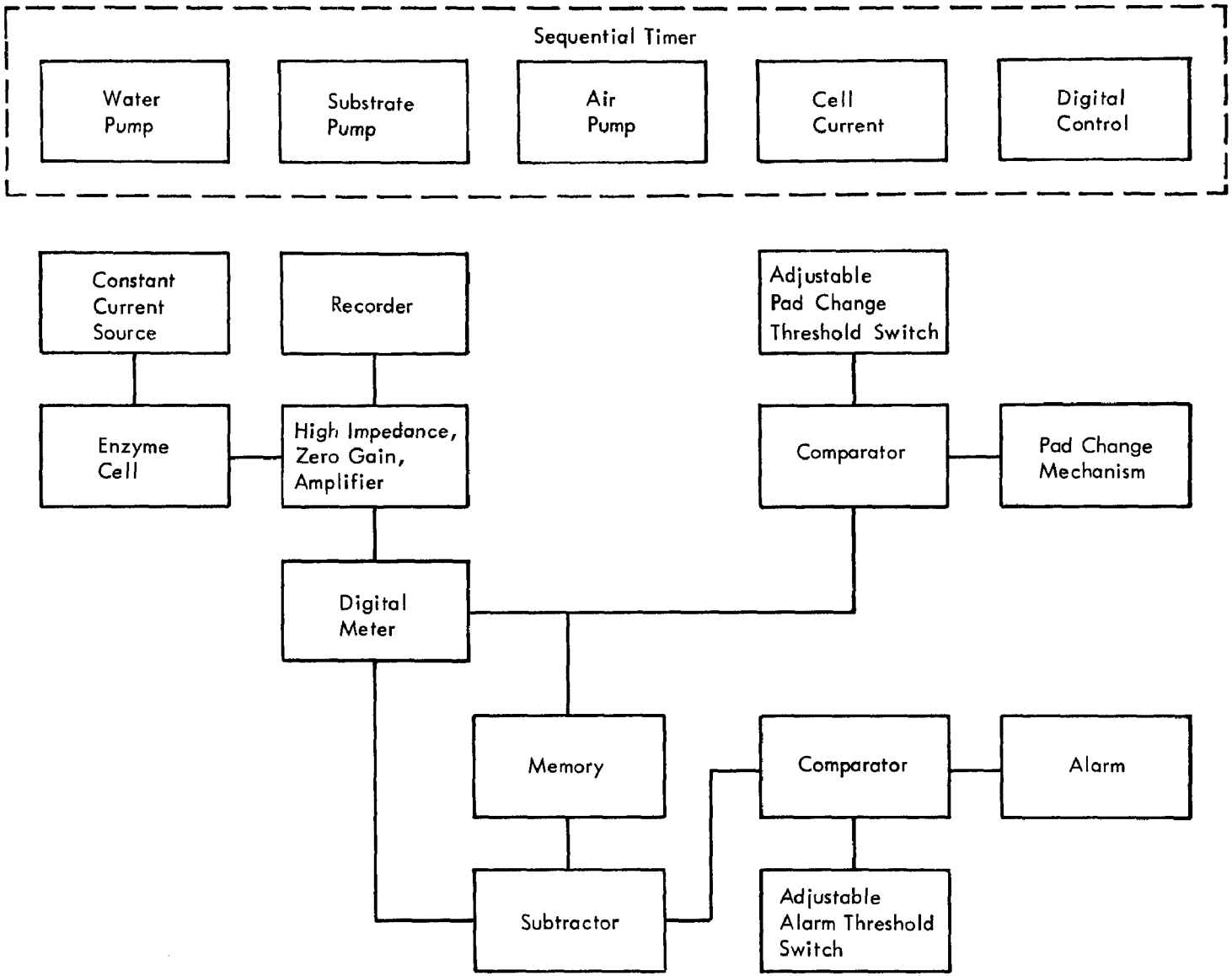


Figure 12 - Simplified Block Diagram of CAM-1 Showing the Principal Relationships of the Logic and Decision Circuits to the Other Components

TABLE 6

SUPPLIERS FOR CAM-1 COMPONENTS

(Lower Level)

Capacitor	Kemet 1 µf 35 V DC
Capacitor	Kemet 100 µf 6 V DC
Capacitor	Mallory 50 µf 25 V DC
Capacitor	Mallory 100 µf 35 V DC
Chain	Pic Design Corporation [a]
Chain Connector	Pic Design Corporation
Connector	Amphenol Mil 3100A - 145 - 2P
Connector	Amphenol Mil 3106 - 145 - 2S
Connector	Amphenol Mil 3102 - 145 - 5P
Connector	Amphenol Mil 3106A - 145 - 5S
Connector	Amphenol Mil 3102A - 145 - 6P
Connector	Amphenol Mil 3106A - 145 - 6S
Connector	Cinch Jones 50 - 20 - A - 30
Connector	Switch Craft TR2A
Fuse Holder	Littel Fuse
Gear	Pic Design Corporation G3-84
Gear	Pic Design Corporation G3-21
Knurled Nut	Pic Design Corporation
Magnet	Hamlin H-31-604
Magnetic Reed Switch	Hamlin MINI-2-115
Motor	A. W. Haydon K82201
Motor	A. W. Haydon K82101
Resistors	IRC - Ohmite
Solenoid	Guardian 3.5 x 9
Solenoid Valve	ASCO 8360-62 [b]
Sprocket	Pic Design Corporation EM-5-36
Sprocket	Pic Design Corporation EM-1-9
Substrate Pump	Extra Corporeal Corporation
Switch	Alco 305D
Switch	Alco 205N
Transistor	Fairchild 2N3569
Transistor	Fairchild 2N4237
Water Pump	Flotec, Inc. FV 158 - 1085 [c]

TABLE 6 (Concluded)

(Second Level)

Air Pump	
Diode	Metaframe Hush I[d]
Diode	Motorola IN5305
Indicator Cover (Amber)	Dialco 300 - 1893
Indicator Cover (Blue)	Dialco 300 - 1874
Indicator Cover (Green)	Dialco 300 - 1872
Indicator Cover (Red)	Dialco 303 - 3471
Indicator	Dialco 533 - 0601
Indicator Switch	Dialco 513 - 0109 - 001
Indicator Switch	Dialco 513 - 0106 - 001
Indicator Switch	Dialco 513 - 1506 - 001
Integrated Circuits	Motorola MC 1741
Lamp (28 V)	Chicago Miniature No. 327
Lamp (6 V)	Chicago Miniature No. 328
Lamp (14 V)	Chicago Miniature No. 330
Power Supply (5 V)	Wanlass Mark III
Power Supply (24 V)	Wanlass
Recorder	Simpson
Relays (24 V)	Potter-Brumfield KHP 17D11
Sonalert	Mallory SC 628
Terminal Strip	Cinch 8 - 140
Trimpot	Bourns 3006P

(Top Level)

BCD Switch	EECO 1776 Series
Digital Panel Meter	Digilin 2330[e]
Integrated Circuits	Motorola
Integrated Circuits	Fairchild
Integrated Circuits	Texas-Instruments

All special items, case, circuit boards, substrate pump, etc., were fabricated in-house. All other parts are commercially available from suppliers such as: Newark Electronics, Allied Electronics, and Manufacturers Representives. All parts not specified are noncritical and substitute items may be used.

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a/ Pic Design Corporation, P.O. Box 335, Benrus Center, Ridgefield, Connecticut, 06877.

b/ ASCO Automatic Switch Company, 50-56 Hanover Road, Florham Park, New Jersey, 07932.

c/ Flotec, Inc., 14510 South Carmenita Road, Norwalk, California, 90650.

d/ Metaframe, Route 17, Maywood, New Jersey, 07607.

e/ Digilin Instruments, 1007 Airway, Glendale, California.

SECTION IX

OPERATION OF THE MONITOR, CAM-1

Water from the Kansas City Municipal Water Supply, raw Missouri River water, and city water in Edison, New Jersey, have all been used with the CAM-1 with no difficulty. It is advisable, however, that the CAM-1 not be connected directly to any pressurized water source since hoses may come off and the electrochemical cell may leak. The water should be run into a beaker or other container in a sink near the CAM-1, and the pump in CAM-1 used to draw the water into the system. The Flotec pump in CAM-1 is self-priming if there is a little water in the pump; if the pump is dry, it is best to prime the pump before operation of the detector. Raw water supplies should be filtered through sand or a similar coarse filter to remove things which might cause plugging of the platinum electrodes or of the enzyme pad.

If two CAM-1 units are used together for the purpose of obtaining continuous monitoring of a water supply, it is necessary to use a sync cable between the two CAM-1 units. When the two units are connected together, one unit becomes the master and the other unit becomes the slave. After the two units have been operated together for 90 sec a sync pulse from the master unit resets the clock in the slave unit to time zero; additional sync pulses are sent from master to slave each 180 sec thereafter. Either CAM-1 unit may be master or slave, depending on which end of the sync cable is plugged into the detector. If the master CAM-1 should change its pad, the second unit would continue to operate without the sync pulse until the master unit was again operating and another sync pulse was sent to the slave. Thus the units would stay out of sync and provide continuous monitoring as planned. The simplified operating instructions given below should be of value to an operator who is unfamiliar with the instrument.

CAM-1 Operating Instructions

To Start:

1. Prepare fresh substrate solution using 40 mg of butyrylthiocholine iodide and 500 ml of 0.08 M tris buffer, pH 7.4 and insert end of small plastic tube.

2. Turn on power (green light switch), and if cell is not open, open it with manual switch.

3. Load pad holder with 1 to 11 enzyme pads.

4. Insert pad holder into the cell with an empty hole between the cell electrodes. Be sure that there is a pad to the left of the empty hole as the pad holder advances clockwise one space before the cell closes.

5. Close the electrochemical cell by moving the top switch to the down position.

6. Prime the substrate lines by moving the substrate switch (third from top) to the upper position for 5 min and then lowering it.

7. The sound of water being pumped indicates that the unit is now operating automatically. If it is not, be sure that the clock-hold light is off, the cell is all the way closed, and all manual controls are in the down position.

8. Adjust alarm threshold to about 12 mV. Greater sensitivity is obtained at 8 mV, whereas lesser sensitivity is obtained at 20 mV.

9. The pad change threshold switch should be set 50 mV below the maximum voltage that is obtained by pumping substrate solution through a plain starch (no enzyme) pad. This setting is usually about 150 mV above the lowest voltage obtainable with a good enzyme pad.

To Stop:

1. Move manual control for the electrochemical cell to the open position (up). (Note: there is a 13.6-sec delay so that water may be blown from the waterlines before the cell will open.)

2. When the cell is all the way open, turn off the power by pressing the green light switch.

3. Remove the plastic disc containing the unused enzyme pads, if any, so that the cell may dry out while not in use.

In an effort to determine if CAM-1 would operate automatically for 48 hr unattended, we conducted a study in which enough substrate solution to last for 2 days without replenishment was made up and enzyme pads were put into the pad holder. CAM-1 was started and observed continuously for 56 hr; a record of the digital voltmeter readings was made for each cycle for the entire period. During this period no one touched CAM-1. The individuals who watched CAM-1 for this period reported that the water and substrate pumps started and ran as expected for every cycle, and that there were no malfunctions of the electronic circuitry. Three false alarms were scattered over the 56-hr period which, judging from the digital voltmeter readings, resulted from abnormally low cell voltage readings and an alarm when the voltage returned to normal voltage on the next cycle. An examination of the voltage tracing showed that no sudden inhibition of the enzyme occurred during this sampling period. If these false alarms should be considered a serious problem, a change in alarm logic could be made to eliminate them;

one such change might be the requirement that the voltage rise for two successive cycles before signalling an alarm. Other alternatives could be worked out if this continues to be a problem.

This 56-hr test did not provide testing of the enzyme pad changer since one enzyme pad gave satisfactory base line voltages for the entire period; however, examination of this enzyme pad after this test revealed that it was near exhaustion, and should have been replaced at about the 56th hr.

The second CAM-1 was also operated during this 56-hr test but some difficulty was encountered due to the numerous alarms produced with it; the difficulty was caused by the use of the sync cable. After disconnection of the sync cable, both of the CAM-1 units performed well. At the conclusion of the test, an error in the wiring of the sync cable connector was corrected. Our experience in operating the two units together with the sync cable is limited, but so far both units have worked well either alone or as slave and master. The first CAM-1 unit was operated about 300 hr before its delivery to the Edison Water Quality Laboratories.

SECTION X

RESPONSE OF CAM-1 TO SEVERAL INSECTICIDES

In the initial studies conducted on the response of the electrochemical enzyme system to an organophosphate, the experimental cell shown in Figure 3, and a solution of tetraethylpyrophosphate (TEPP), were used. Problems with this system developed because of the rapid hydrolysis of the TEPP. In some very crude experiments, three dilutions of Tetron-100 (American Potash and Chemical Company, 40% TEPP and 60% other ethyl phosphates) were prepared in tap water, and stored at room temperature for periods of up to 6 days; these solutions were assayed after various periods of time to determine their ability to inhibit a known quantity of cholinesterase. Although the 1:1,000 solution had some activity after 6 days, the 1:1,000,000 solution had lost more than 25% of its activity after only 24 hr. We decided to pick an organophosphate which was more stable in solution, and which could be used for determining sensitivity of our system. In a study of 2,2-dichlorovinyl dimethyl phosphate (DDVP), we found that a 1:10,000,000 solution of DDVP in distilled water and stored at room temperature retained 63% of its activity after 7 days. This observation led to the conclusion that DDVP would be a better compound to use in testing the CAM-1 than TEPP because of the greater stability of the DDVP in water. Some of the data obtained on the response of an electrochemical cell to DDVP are shown in Figure 7.

Some of the other data collected in a study of response to low concentrations of insecticides in water are presented in Table 7. In Part A of the table some of the results obtained with the experimental electrochemical enzyme cell used with standard laboratory instruments, and operated on a cycle like the one selected for use in CAM-1, are given. Both malathion and parathion can be detected, although the sensitivity to parathion is several times greater. Although time did not permit tests of CAM-1 with these insecticides, we are confident that it will detect them at approximately the same levels as we have reported here.

In Part B of the table the response of CAM-1 to a number of commercially important insecticides, including both organophosphates and also carbamates, is reported. CAM-1 responded to all of them except Ronnel; this failure to respond was probably due to the insolubility of the Ronnel in tap water. Since Ronnel is both insoluble in water and reasonably nontoxic, the failure of CAM-1 to alarm is probably of no consequence. With the compound Sevin, when CAM-1 was set at a threshold sensitivity of 12 mV per cycle, there were five alarm signals prior to the inactivation of the enzyme pad.

The question was raised, "What would happen if the toxic substance were removed after the first or second alarm?" In order to find out, a solution

TABLE 7

DETECTION OF INSECTICIDES IN WATER[a]

Part A - Using an Experimental Enzyme Cell

Insecticide (source)	Chemical Type	LD_{50} (rats) (mg/kg)	Concentration of Inhibitor (ppm)	Number of Alarms
Malathion (Prentiss Drug)[b]	Phosphate	1,640	16.5	(c)
Parathion (Chevron Chemical)[d]	Phosphate	56	3-5	(c)

Part B - Using CAM-1

Ronnel (Dow)[e]	Phosphate	1,720	80[f]	None
Sevin (Union Carbide)[g]	Carbamate	560	50	5
Dimetilan (Geigy)[h]	Carbamate	64	10	3
Azodrin (Shell)[i]	Phosphate	21	20	5
Paraoxon (Cyanamid)[j]	Phosphate	3.5	1	1[k]
DDVP (Shell)[l]	Phosphate	56.0	1	1

(a) All measurements were made with city tap water at 5° ± 3°C.
(b) Dimethyl S-(1,2-dicarbethoxyethyl)phosphorodithioate.
(c) There was no alarm mechanism in this system; responses of 12 mV or more per cycle were obtained for several cycles.
(d) Diethyl 4-nitrophenyl phosphorothioate.
(e) O,O-Dimethyl O-2,4,5-trichlorophenyl phosphate.
(f) The Ronnel was dissolved in warm methanol and added to water at room temperature. Most of the Ronnel came out of solution and plugged up the electrodes and the pad. The concentration in solution was only a small fraction of this value.
(g) N-Methyl-1-naphthyl carbamate.
(h) 2-Dimethylcarbamoyl-3-methylpyrazoyl-5-dimethyl carbamate.
(i) 3-(Dimethoxyphosphinoxy)-N-methyl-cis-crotonamide.
(j) Diethyl p-nitrophenyl phosphate.
(k) The voltage increase for one cycle was 147 mV indicating that much lower levels could be detected.
(l) 2,2-Dichlorovinyl dimethyl phosphate.

of 0.2 ppm of DDVP was prepared, and exposed to CAM-1. After two alarms on successive cycles, the inlet water was switched to tap water. On the third cycle there was an alarm, but on cycles 4, 5, and 6 there was no alarm. The inlet hose was switched back to the jug containing the DDVP and alarms on cycles 7, 8, and 9 were obtained; for all of these 9 cycles, the same enzyme pad was used. The alarm on the third cycle was probably due to the inhibitor's remaining in the waterlines from the previous cycle. This experiment indicates that the alarm can be stopped when the enzyme pad is partially inhibited, and that it can still provide alarms when more inhibitor is encountered. With DDVP and the other enzyme inhibitors investigated so far, the enzyme activity does not return after these inhibited enzyme pads are washed.

The greatest response we have encountered (i.e., the greatest voltage increase between successive detection cycles) occurred when we challenged CAM-1 to 1 ppm of paraoxon and obtained a 147-mV rise in one cycle. It is concluded that CAM-1 will respond to much lower levels of this insecticide, although these have not yet been tried.

There is a rough correlation between the toxicity of the insecticides and the levels which can be detected with the immobilized enzyme system (see Table 7). We believe that the more toxic compounds are collected from the water with greater efficiency due to their greater affinity for the enzyme. At the same time compounds possessing the greatest affinity for enzymes *in vivo* are probably the ones which are going to be more toxic. Thus the similarity of the mechanism of detection in CAM-1 with the mechanism of toxicity of the inhibitors *in vivo* explains why we see the correlation. If the mechanism of action were different in the two systems, and we expect it to be so for some other insecticides, the correlation would probably not exist.

The experiments reported in Table 7 were conducted with tap water at 5° ± 3°C. The response data were collected under temperature conditions much less favorable for the collection of the enzyme inhibitors than 25°C. In some preliminary studies with DDVP, the system was four to five times more sensitive at 25°C than at 5°C. However, the system still possesses enough sensitivity at 5°C to provide adequate protection against accidental poisoning.

The following rough calculation shows that this sensitivity is more than adequate for the protection of individuals using the water supplies: If we assume the LD_{50} of DDVP in man is 6 mg/kg (Heath[17] reports the LD_{50} for rats is 6 mg/kg and the Canada Department of Agriculture[18] reports the LD_{50} for rats is 56-170 mg/kg) and also that a man weighs 70 kg, then we see that a man would need to drink at least 4,200 liters of water containing 0.2 ppm of DDVP in order to receive the LD_{50} dose--an obvious impossibility. CAM-1 need not be operated at this high sensitivity; to lower the sensitivity all that is necessary is to increase the allowable voltage change between successive voltage measurements by adjusting the digital switch on the front of the instrument.

SECTION XI

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SECTION XII

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10 Author(s)
Goodson, Louis H.
Jacobs, William B.

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27 Abstract
An apparatus for the detection and monitoring of water supplies for hazardous spills of organophosphate and carbamate insecticides has now been designed and fabricated. The new unit is called the Cholinesterase Antagonist Monitor, CAM-1, because it produces an alarm in 3 min when toxic or subtoxic levels of cholinesterase antagonists are present in water. Response of this apparatus to subtoxic levels of azodrin, sevin, dimetilan, malathion, parathion and DDVP has already been demonstrated. CAM-1 uses immobilized cholinesterase for the collection of cholinesterase inhibitors from the water supplies. The activity of the immobilized cholinesterase is determined automatically in an electrochemical cell by passing a substrate solution over the enzyme at regular time periods. A minicomputer is used to automate the detection process and to signal an alarm when there is a rapid loss of enzyme activity--a situation which occurs in the presence of organophosphate and carbamate insecticides in the water sampled.

Abstractor Louis H Goodson Institution

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