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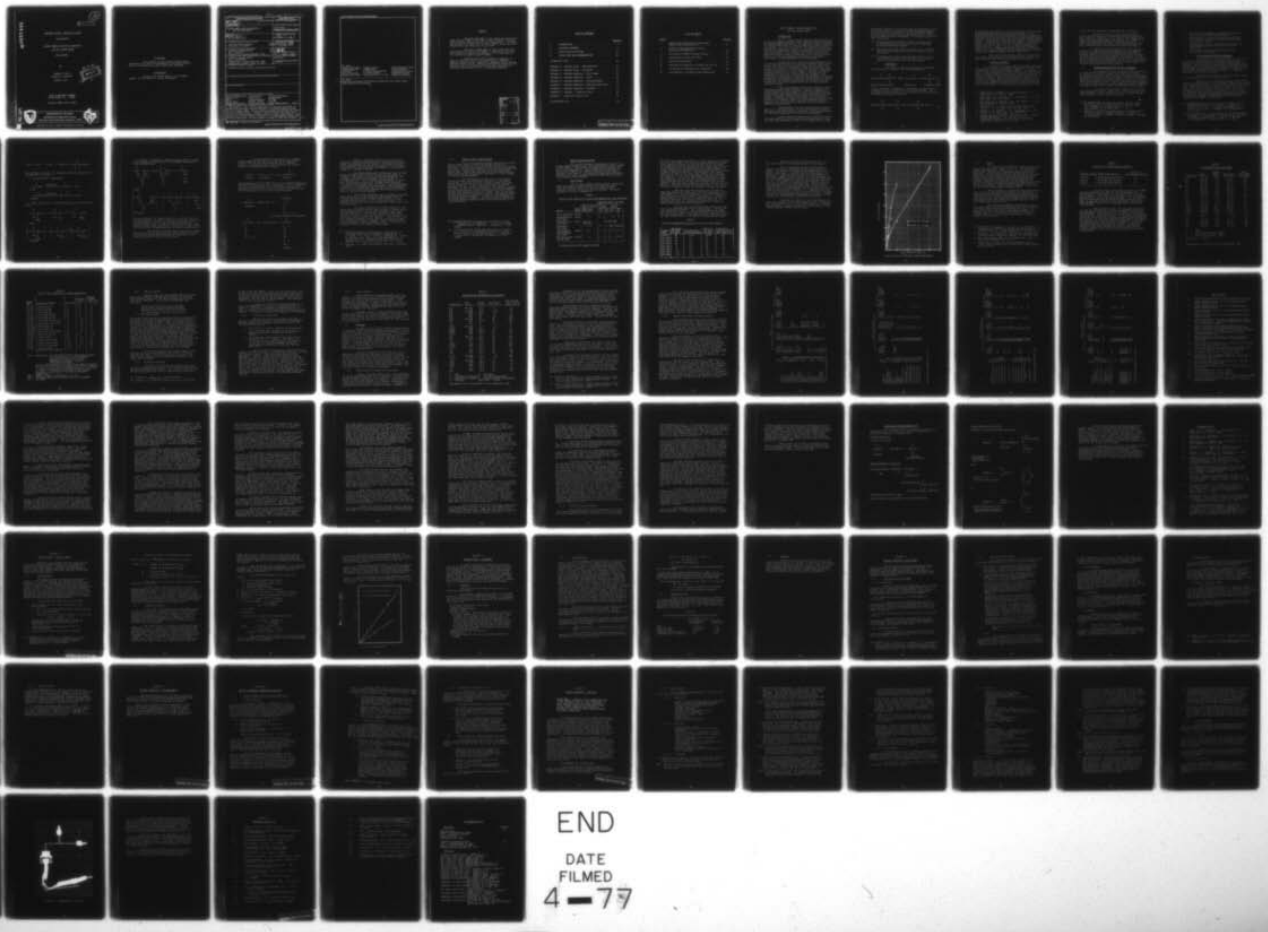

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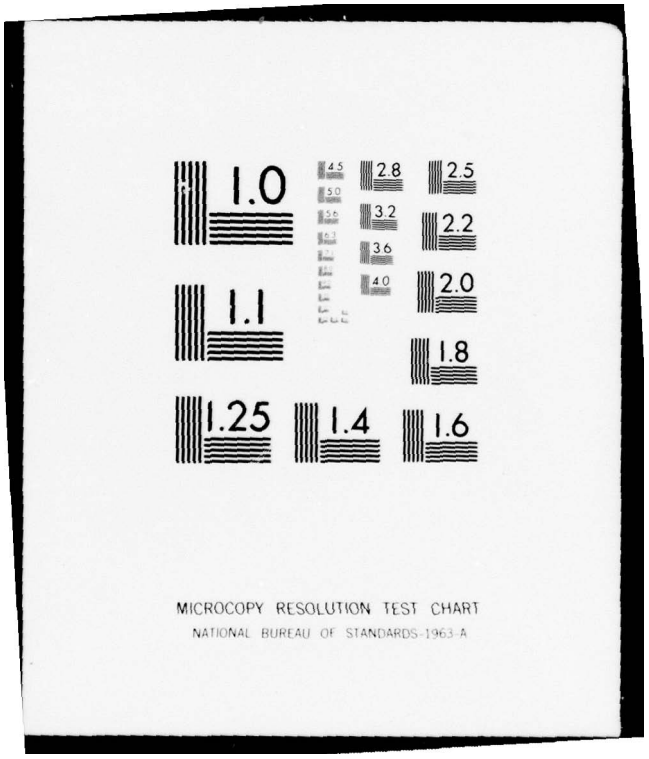
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EDGEWOOD ARSENAL CONTRACTOR REPORT

ED-CR-76093

ENZYME IMMOBILIZATION ALTERNATIVES FOR THE ENZYME ALARM

FINAL REPORT

By

Herman W. Levin
Evelyn S. Erenrich

October 1976

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LEEDS & NORTHRUP COMPANY
North Wales, Pa. 19454

Contract DAAA 15-75-C-0053

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1. REPORT NUMBER (18) (19) ED-CR-76093	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) (6) ENZYME IMMOBILIZATION ALTERNATIVES FOR THE ENZYME ALARM.	5. TYPE OF REPORT & PERIOD COVERED: Final Report, October 1974 to August 1976
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) (10) Herman W. Levin, Evelyn S. Erenrich	8. CONTRACT OR GRANT NUMBER(s) (15) Cont. DAAA15-75-C-0053
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Leeds and Northrup Company, North Wales, PA 19454	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS (16) 1W762710AD27-01 (17)
11. CONTROLLING OFFICE NAME AND ADDRESS: Commander, Edgewood Arsenal, Attn: SAREA-TS-R, Aberdeen Proving Ground, Maryland 21010	12. REPORT DATE (11) Oct[illegible]76
	13. NUMBER OF PAGES: 93 (12) 88p.
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): Commander, Edgewood Arsenal, Attn: SAREA-DE-DDA, Aberdeen Proving Ground, Maryland 21010, (Andrew Davis, Project Officer 671-2882)	15. SECURITY CLASS. (of this report): UNCLASSIFIED
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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Hydrazopolyacrylamide	Ethylenediamine	Tolylene diisocyanate
Bio-Gel P-150	Hexanediamine	Glutaraldehyde
Nylon	Polyethyleneglycol	FHP 2000
Imido esters	Dimethyl sulfate	FHP 3000
Acetylcholinesterase	Dimethyl sulfoxide	Enzyme immobilization (over)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The objective of this research program was to investigate new methods for immobilizing the enzyme, acetylcholinesterase, on selected supports suitable for use in enzyme alarms. Various coupling procedures were developed for glass, nylon and urethane supports. Products of these procedures were evaluated for initial and retained enzyme activities relative to 2-week storage tests at -40°C and 65°C and to 12-hour periods of operation in an electrochemical enzyme alarm at ambient temperature. The best products are shown to result from covalent →

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Dimethyl adipimidate	Cyanogen bromide	Acetylthiocholine iodide
N-Ethylmorpholine	Urethane pads	Drying procedures
Starch gel	s-Triazine hydrocholoride	Cholinesterase activity
Derivitized starch	Activated urethane	Diaminobicyclooctane
Enzyme cross-linking	Diphenylmethane diisocyanate	Dibutyltin diacetate

20. Contd.

coupling of the enzyme to derivatized urethane foam plus further enzyme stabilization by cross-linking.

PREFACE

The work described in this report was authorized under Contract DAAA 15-75-C-0053, titled "Studies on Enzyme Immobilization Alternatives for the Enzyme Alarm". This work was started in October, 1974 and this report covers the total program which was completed in July 1976.

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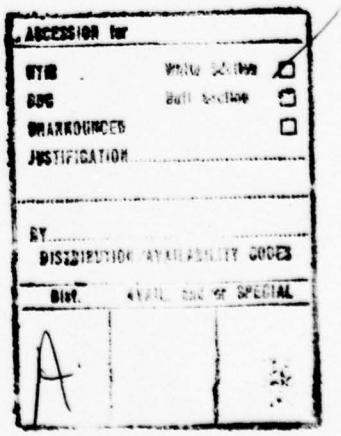

TABLE OF CONTENTS

		Page No.
1.	INTRODUCTION	7
2.	TECHNICAL APPROACH	9
3.	RESULTS AND DISCUSSION	19
4.	CONCLUSIONS AND RECOMMENDATIONS	50
LITERATURE CITED		53
APPENDIX A - Methods Assay — Cholinesterase		57
APPENDIX B - Methods Assay — Isocyanate		61
APPENDIX C - Methods Chemistry — Glass Beads		65
APPENDIX D - Methods Chemistry — Nylon		69
APPENDIX E - Methods Chemistry — Polyacrylamide		73
APPENDIX F - Methods Chemistry — Modified Starch Gel . .		75
APPENDIX G - Methods Chemistry — Urethane		79
APPENDIX H - Methods — Stability Tests		87
APPENDIX I - Materials Source List		91
DISTRIBUTION LIST		93

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LIST OF TABLES

Table		Page No.
1.	Activity and Stability of Glass-Bead-Immobilized Cholinesterase	19
2.	Effect of Concentration of ChE on Activity and Stability	20
3.	Activities of DMS-Activated Products	24
4.	Activation of Nylon with PAPI	25
5.	PAPI-Activated Nylon	26
6.	Activity and Stability of Starch Derivatives . .	28
7.	Activation of Urethane with Isocyanate	32
8.	Test Results of Urethane Foam Preparations . . .	35

ENZYME IMMOBILIZATION ALTERNATIVES FOR THE ENZYME ALARM

1. INTRODUCTION

The U. S. Army's Field Alarm for detection of biological and chemical warfare agents requires a highly sensitive and stable immobilized enzyme. The specific enzyme of interest is cholinesterase which is rapidly inhibited in the presence of such agents. To avoid false alarms, this detector requires an immobilized enzyme which retains a high level of activity from the time of preparation through a reasonable period of operational deployment. Thus, reliability of the field alarm is dependent on maintaining the measured activity of this enzyme under all of the environmental and operating conditions encountered in field deployment of the instrument.

The design of the field alarm electrochemical cell for measuring enzyme activity requires an enzyme support which allows free flow of fluids and maintains positive electrical contact with the inert electrodes. Open-pored urethane foam, because of its porosity, wettability and resiliency, seems most suitable physically as a basic support. The most successful enzyme preparation prior to this report involved physical entrapment in starch gel supported by the urethane. This starch gel is susceptible to melting when subjected to elevated temperatures encountered in field use, which allows the enzyme to wash out with resultant degradation of measured activity. Furthermore, the enzyme tends to leak out of the gel during long term storage.

Previously Edgewood Arsenal has conducted limited research on enzyme immobilization by covalent attachment of cholinesterase to various supports. Chemical bonding of the enzyme should offer advantages of better temperature stability, longer storage and should provide faster electrochemical cell response to enzyme inhibitors than physically immobilized enzymes. Furthermore, chemically bonded enzymes should be more accessible to inhibitor agents than gel-entrapped enzymes because of the diffusion time required for the inhibitor to penetrate the gel matrix.

Unfortunately, the prior enzyme products did not meet all of the requirements for the field alarm instrument. Therefore, continued research, preferably involving new technical approaches to enzyme immobilization, was needed.

Leeds & Northrup Company, which has been involved with development of enzyme immobilization processes for over six years, was awarded a contract on 24 October 1974 to study

alternative processes for coupling the cholinesterase enzyme to various supports in order to achieve an enzyme-support product which can be produced in large quantities, stored for reasonable periods and then provide at least 12 hours of reliable operation after deployment. The specific goals of this project are:

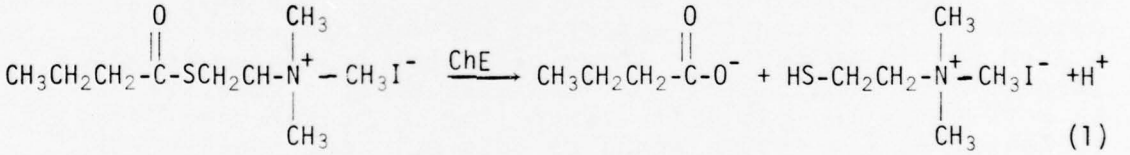
(a) Investigate mass-producible methods of making immobilized enzyme pads that can be rapidly inhibited by anticholinesterase agents.

(b) The enzyme pads shall retain at least 50% of initial activity after undergoing 2 weeks of storage at -40°C and at 65°C.

(c) The enzyme pads shall retain at least 50% of initial activity when operated continuously in the electrochemical enzyme alarm over a 12-hour period.

1.1 Background

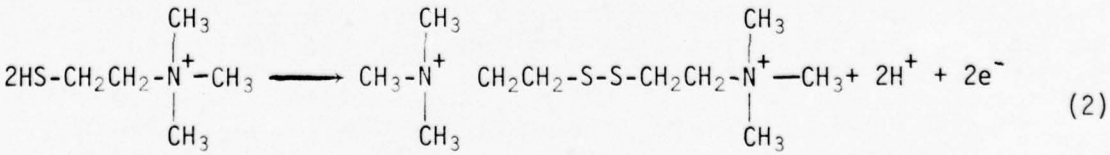
The chemical reaction catalyzed by the enzyme cholinesterase is shown by the following equation:



(1)

Butyrylthiocholine Iodide Butyric Acid Thiocholine Iodide

In this reaction a thioester is cleaved by the enzyme cholinesterase to give a product with a free thiol group. The thiocholine iodide product is electrochemically active and can be readily dimerized as follows:



(2)

Guilbault and colleagues[1-5], first proposed using this system as an enzyme alarm for chemical warfare agents. If the potential between two inert electrodes is measured under the condition of a small but constant current, the electrochemical potential of the anode (where the above oxidation takes place) gives an indication of the presence or absence of thiocholine iodide. If the enzyme is functioning properly, thiocholine iodide is produced, and since it is readily oxidized, the anode potential is small. If the cholinesterase enzyme is inactivated, by a threatening enzyme inhibitor, thiocholine iodide is no longer produced and the anode potential increases toward the oxidation potential of the iodide ion.

Our plan for developing a means of immobilizing the enzyme, cholinesterase, on a support that is compatible with the above type of electrochemical cell follows.

2. TECHNICAL APPROACH

There are four basic means for enzyme immobilization. Falb, of Battelle Columbus Laboratories, defines these as adsorption, physical entrapment, chemical or covalent coupling and intermolecular cross-linking.[6] The latter represents a combined chemical and physical binding in which the enzyme is cross-linked to itself on a porous solid to form an insoluble product. Adsorption of enzymes to surfaces of solids is generally weak and therefore not suitable for the field alarm. Edgewood Arsenal has extensively investigated physical entrapment for immobilization of acetylcholinesterase as demonstrated in the starch gel/urethane pads for the

1. Kramer, D. N., Cannon, P. L., and Guilbault, G. G., Anal. Chem., 34, 842-845 (1962).
2. Guilbault, G. G., Kramer, D. N., and Cannon, P. L., Jr., Anal. Chem. 34, 1437 (1962).
3. Guilbault, G. G., Kramer, D. N., and Cannon, P. L., Jr., Anal. Biochem. 5, 208-216 (1963).
4. Guilbault, G. G., Tyson, B. D., Kramer, D. N., and Cannon, P. C., Jr. Anal. Chem. 35, 582 (1963).
5. Bauman, E. K., Goodson, L. H., Guilbault, G. G., and Kramer, D. N., Anal. Chem., 37, 1368 (1965).
6. Immobilization of Enzymes: An Overview. R. D. Falb in Enzyme Engineering, L. B. Wingard, Jr., Ed., Biotechnol & Bioeng. Symp. No. 3, 177-184 (1972), John Wiley & Sons, Inc., N. Y. 1972.

field alarm and Continuous Aqueous Monitor Instruments[7].

Covalent coupling can be achieved either by direct chemical reaction of an enzyme with active groups on the surface of a support or via a bifunctional reagent which forms a bridge between enzyme and support. The bridge length and chemical properties can be varied at will. This offers the opportunity to modify the microenvironment and, thus, provide product optimization for the field alarm.

The first task we undertook was to establish a baseline for immobilized cholinesterase on glass supports. We then investigated covalent attachment to nylon and to urethane supports, applying reactions known to be applicable to nylon and modifying these methods for urethane. In the early phases we also investigated intermolecular cross-linking to form insoluble enzyme aggregates as well as cross-linking starch, containing entrapped or chemically bound enzyme, in an effort to increase its melting point.

2.1 Enzyme Immobilization on Glass Supports

We started with glass because we had a broad base of experience in covalent coupling of enzymes to glass. Furthermore, successful immobilization of acetylcholinesterase by covalent coupling to glass has been reported by Baum, Ward and Weetall of the Corning Glass Works[8]. Baum et al chemically bonded cholinesterase to silanized fritted glass discs using diazo and diimide processes. We had employed similar processes for coupling many enzymes to glass supports and were therefore confident of achieving a stable cholinesterase-glass product.

This product established a base-line for evaluating subsequent products, using other coupling processes and other supports, as they evolved from this research program. The objectives of this initial task were:

7. An Immobilized Cholinesterase Product for Use in the Rapid Detection of Enzyme Inhibitors in Air or Water. L. H. Goodson, W. B. Jacobs and A. W. Davis, Anal. Biochem. 51 362-367 (1973).

8. Stability, Inhibition and Reactivation of Acetylcholinesterase Covalently Coupled to Glass. G. Baum, F. B. Ward and H. H. Weetall, Biochim. Biophys. Acta 268 411-414 (1972).

1. Validation of procedures for preparing and assaying the cholinesterase enzyme products.
2. Investigation of alternative coupling processes which might be useful for immobilizing the enzyme to activated urethane.
3. Determination of means of packaging the resultant cholinesterase-glass products for use in the field alarm.
4. Investigate stability of immobilized enzyme activity at -40° and 65°C.

2.2 Enzyme Immobilization on Urethane

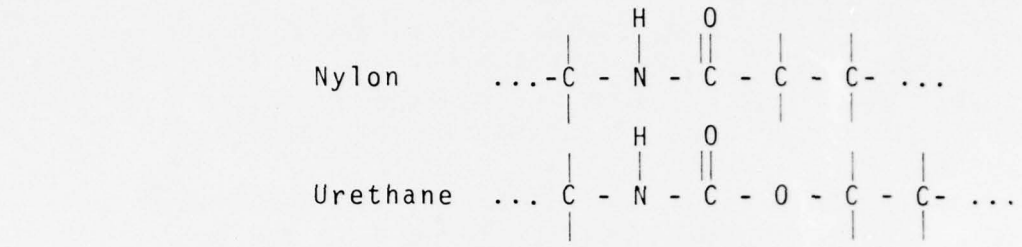
In planning our program for covalent immobilization of acetylcholinesterase on urethane foam, we took advantage of the similarity in structure between nylon and urethane. We investigated three methods for activating nylon without breaking the polymer chain in the hopes that at least one of these could be extended to urethane. In addition, we investigated an innovative approach, based on isocyanate activation of nylon and urethane.

Descriptions of each of the investigated methods are included here for its historical value.

Past efforts in covalently binding acetylcholinesterase to urethane foam involved breaking some of the urethane bonds in order to produce active sites for attaching the enzyme. This weakens the polymer and changes its physical characteristics. This problem has been solved by Hornby, et al and Goldstein, et al for nylon solid support[9,10]. The similarity of structures for nylon and urethane suggested that either the Hornby or Goldstein methods may be applicable to urethane. The similarity between nylon peptide bonds and urethane carbamate ester bonds is shown below.

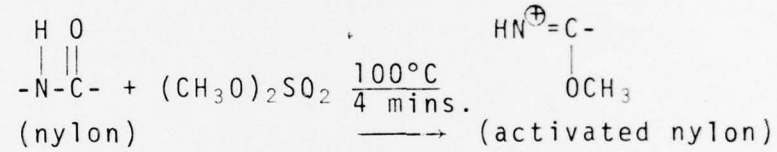
---

9. Preparation of Immobilized Enzymes for Application in Automated Analysis. W. E. Hornby, J. Campbell, D. J. Inman and D. L. Morris in Enzyme Engineering, Vol. 2, Eds. E. K. Pye and L. B. Wingard, Jr., Plenum Press, N. Y., 1974.
10. Derivatized Nylon: A New Support for the Immobilization of Enzymes. L. Goldstein, A. Freeman and M. Sokolovsky in Enzyme Engineering, Vol. 2, Eds. E. K. Pye and L. B. Wingard, Jr. Plenum Press, N. Y., 1974.

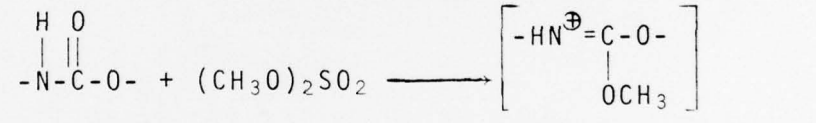


Furthermore, both supports are acid amides; urethane being the half amide half ester of carbonic acid.

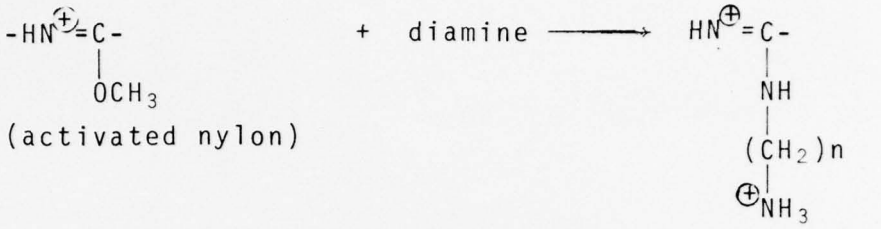
The Hornby approach yields activated nylon because secondary amides can be directly methylated with dimethyl sulfate as follows:



We proposed activating urethane in a similar way yielding:

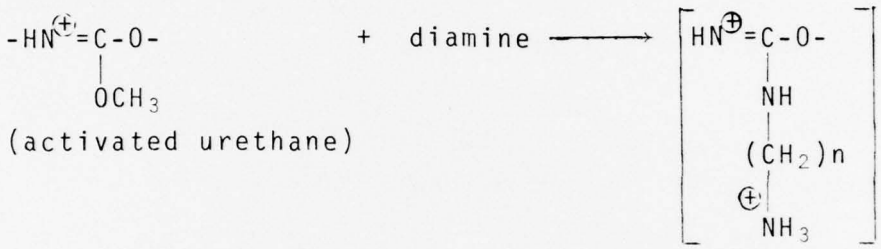


We showed the reaction was usable with nylon but did not try it with urethane because the isocyanate activation looked more promising by that time. These reactive centers can then be used for introduction of primary amino groups by reaction with diamino compounds, such as lysine, or with polyamino compounds, such as polyethylene imine (Hornby method). Comparison of these reactions for nylon and urethane is as follows:



(activated nylon)

(nylon with amino active binding site)



(activated urethane)

(urethane with amino active binding site)

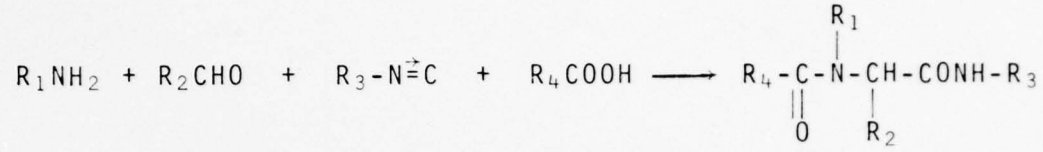
After this step, any standard bifunctional reagent (such as glutaraldehyde), which binds enzymes to primary amines, may be used to immobilize the acetylcholinesterase.

By judiciously selecting the diamine and/or bifunctional reagent, this approach also offered possibilities of modifying the micro-environments and varying the bond length for attaching the enzyme through a long chain to displace it from the support surface.

Goldstein, et al, has proposed an alternate means for activating the amide bonds of nylon[10]. This method may also be applicable to urethane but we did not try it during this contract. In this method, the amide bond is first hydrolyzed and then reformed as an activated amide bond. Goldstein calls this the 4CC Reaction for four component condensation of amine, carboxylaldehyde and isocyanide as shown below.

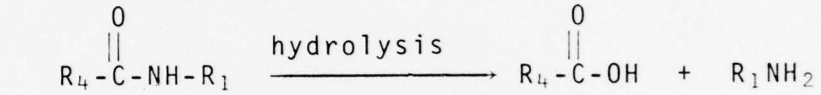
---

10. Derivatized Nylon: A New Support for the Immobilization of Enzymes. L. Goldstein, A. Freeman and M. Sokolovsky in Enzyme Engineering, Vol. 2, Eds. E. K. Pye and L. B. Wingard, Jr. Plenum Press, N. Y., 1974.

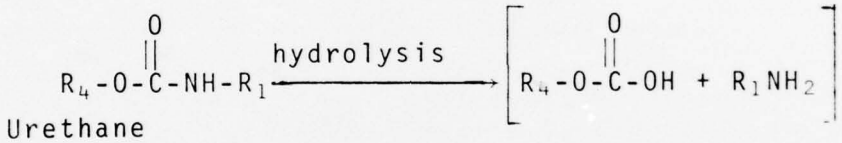


The reactions for nylon and comparable reactions proposed for urethane are as follows:

1. The amide bond is hydrolyzed:

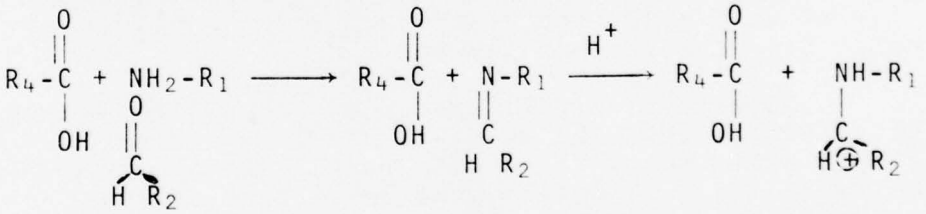


Nylon

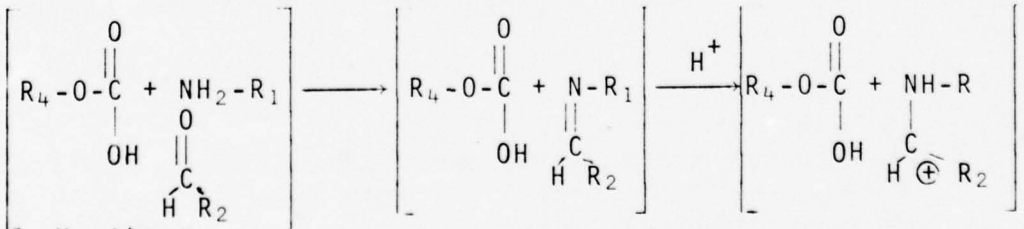


Urethane

2. Then immonium ion is formed producing an activated amino group:

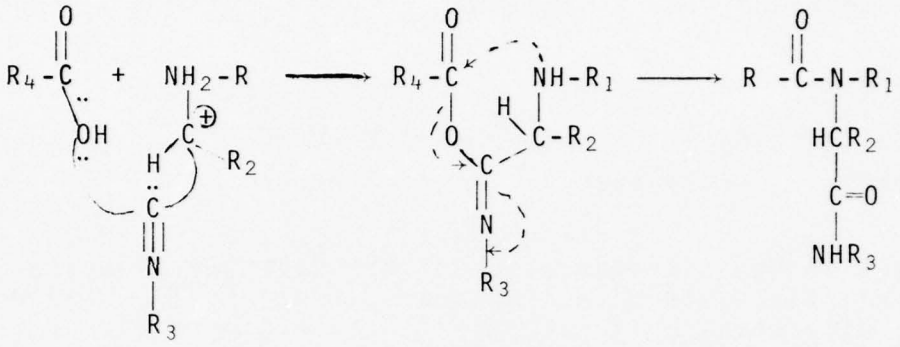


Nylon

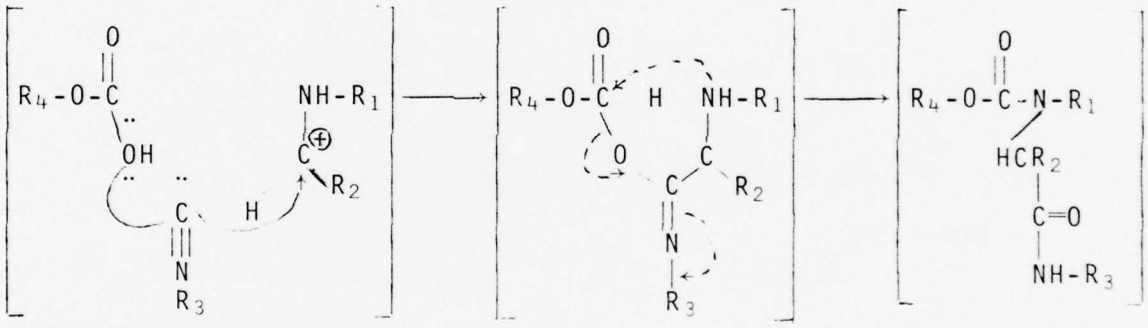


Urethane

3. Finally, α-addition of immonium and carboxyl to isocyanide followed by intermolecular rearrangement produces an N-substituted amide:



Nylon

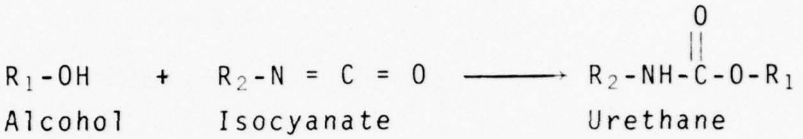


Urethane

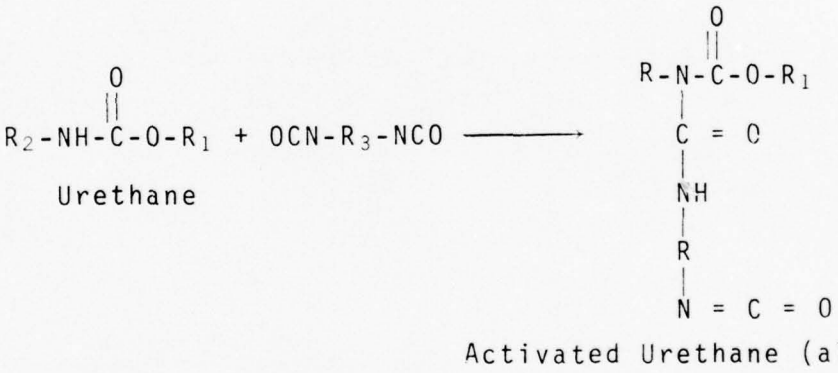
The active group (R_3) used by Goldstein is derived from 1,6 diisocyanahexane. As shown in the final reaction, one of the isocyano groups is used to reclose the nylon bond and the second isocyano group is used to immobilize the enzyme. The enzyme provides either the amino or the carboxyl group needed for the 4CC reaction depending upon the other reagents used.

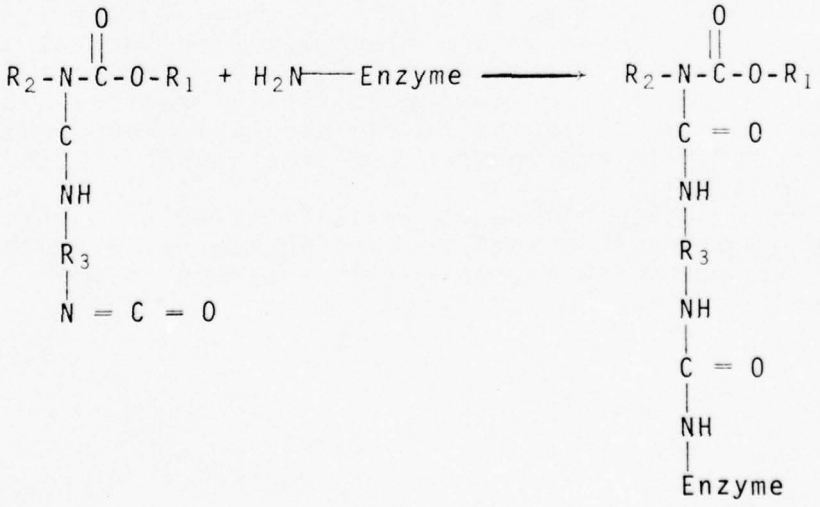
This method of enzyme immobilization also permits modifying the micro-environment and varying the chain length of the group through which the enzyme is attached to the polymer by choice of the R_2 and R_3 groups.

A third approach for activation of urethane, without breaking the chain, is based on the same type of reaction that is used to produce the basic urethane polymer. A polyalcohol and a polyisocyanate are reacted to form the urethane bond as follows:



The hydrogens on the nitrogen atoms of the urethane groups are capable of reacting with more isocyanates to form allophanates. By use of a diisocyanate it was possible to activate the urethane directly with one of the isocyanate groups and then attach the enzyme to the second isocyanate group.





Again, a judicious choice of R_3 permits modification of the micro-environment and variation of the chain length for attachment of the enzyme. Further modification can be achieved by reacting the isocyanate with a diamine or a polyamine and then attaching the enzyme by a difunctional reagent as in Hornby's method.

We investigated several additional types of bonding as alternate means of immobilizing cholinesterase on urethane. The first of these involved intermolecular cross linking of the enzyme by a process similar to that of D. Thomas et al[11]. In this method glutaraldehyde is used to form intermolecular cross-links between enzyme molecules. This cross-linked enzyme forms, in effect, a polyenzyme which is insoluble. A urethane pad coated with a very concentrated solution of the enzyme and then treated with glutaraldehyde forms a cross-linked enzyme membrane which covers the entire interior surface of the foam pad.

The second alternative was an attempt to increase the melting point of the starch gel immobilized enzyme by treating the starch with a bifunctional reagent, such as cyanogen bromide, which will cross link the starch. The resultant starch should be less susceptible to wash out in the Field Alarm unit.

In another approach we tried to chemically link the enzyme to the starch or at least produce a tighter enzyme-starch structure which would reduce leakage of the enzyme from the gel matrix. For example, the book "Immobilized Enzymes" lists several way for activating the OH groups of cellulose, starch, agarose and Sephadex[12]. One approach used an S-triazine compound, such as cyanuric chloride, followed by a dihydroxy or diamino compound. Another approach used sodium periodate to form dialdehyde starch. This could react directly with the amino groups of the enzyme.

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11. Films bearing Reticulated Enzymes: Applications to Biological Models and to Membrane Biotechnology, D. Thomas, M. C. Tran, G. Gellf, D. Domurado, B. Paillot, R. Jacobsen and G. Broun in Enzyme Engineering, L. B. Windard, Jr., Ed. Biotechnol & Bioeng. Symp. No. 3, 299-309 (1972), John Wiley & Sons, Inc., N. Y. 1972.

12. Zaborsky, O., Immobilized Enzymes, CRC Press, Cleveland. 1973.

2.3 Enzyme Product Optimization

All of the covalent coupling processes discussed earlier offer means of optimizing enzyme activity or improving its sensitivity by varying the length of the attachment arms. The microenvironment for the particular enzyme may be similarly modified by judicious choice of substituted spacer arms. These types of molecular surface modifications have been reported by Katchalski and Levin[13,14] and in papers on affinity chromatography.

Our multifaceted attack early in the program proved valuable in bringing the contract to a successful conclusion. Although our prime candidate method eventually proved successful, it took time to work out the optimum procedure for the initial activation of the urethane. During that time, as part of the investigation of alternate methods, we were learning which spacer arms and which cross-linking methods were compatible with this enzyme. When the urethane activation phase was completed, we had at hand methods proven to work with other supports. In addition, the knowledge we had gained about the chemical reactivity and the stability of the enzyme greatly speeded up the final optimization of the selected process.

13. A Water-Insoluble Polyanionic Derivative of Trypsin. I. Preparation and Properties. Y. Levin, M. Pecht, L. Goldstein and E. Katchalski. Biochemistry 3 1905 (1964).

14. A Water-Insoluble Polyanionic Derivative of Trypsin. II. Effect of the Polyeslectrolyte carrier on the Kinetic behavior of the Bound Trypsin. L. Goldstein, Y. Levin and E. Katchalski, Biochemistry 3, 1913 (1964).

3. RESULTS AND DISCUSSION

This section presents evaluations of each of the enzyme-support products which were developed during the course of the program. The methods for producing the products are given in the appendixes. These methods and discussions of the various products are ordered according to the support materials investigated — starting with glass beads, extending procedures to nylon, polyacrylamide and modified starch gels, and concluding with urethane.

3.1 Glass Beads

The six basic immobilization methods, which were found applicable to cholinesterase, are shown in table 1 along with typical results of initial assays and their retained activities after hot and cold storage. The assay procedure is given in appendix A.

Table 1

ACTIVITY AND STABILITY OF GLASS-BEAD-IMMOBILIZED CHOLINESTERASE

Method	Sample number	Specific activity (IUB Units/ml) of packed bed of preparation	Remaining specific activity +65°C for 2 weeks		Remaining specific activity -40°C for 2 weeks	
			Beads dry	Beads wet	Beads dry	Beads wet
Azo on glass beads	256-5A	17	<1 *	<1 *	<1	17
Glutaraldehyde on glass beads	256-58	7.1	<1	<1	9.4	12
Carbodiimide on glass beads	254-12	Low, not quantified	Not evaluated			
Hydrazide on glass beads		7.4	0	0	Not evaluated	
Glutaraldehyde cross-linking on glass beads	254-45	18	<1	<1	9.1	23
DMA cross-linking on glass beads	254-31	7.9	<1	<1	<1	10.3

* Azo beads stored at 65°C without evacuation.

The first four methods covalently link the enzyme to the beads. The last two methods cross link the enzyme molecules to each other forming an immobilized polyenzyme film on the beads. Our highest activity derivatives on glass beads (and incidently the highest activity obtained by any of the methods we tried) are the azo coupled and the glutaraldehyde cross-linked products (256-5A and 254-45). Unfortunately, the thermal stability of none of the glass bead products was acceptable. The stability of the glass bead product stored wet at -40° was excellent. In fact the freezing process actually seemed to further activate the enzyme. With the exception of the glutaraldehyde coupled and the glutaraldehyde cross-linked products, the glass bead preparation did not fare well at -40° when dry. We used both wet and dry materials in this series of tests in hope of finding an acceptable storage condition.

When the glass bead products were tested for simulated operational stability in the flow apparatus at 50°C (See appendix A), the glutaraldehyde cross-linked material was found to be extremely stable. See table 2. A sample of product 254-45 was submitted to Edgewood Arsenal for test in an Enzyme Alarm but its activity level was too high. It required ten times the standard concentration of agent to inhibit the enzyme for alarm condition.

We then investigated decreasing the enzyme loading in the glutaraldehyde cross-linked product. The results are summarized in table 2. We varied the amount of enzyme per milliter of beads and used two different concentrations of cross-linking agent. The cyanaborohydride was also varied but kept proportional to the amount of glutaraldehyde added.

Table 2

EFFECT OF CONCENTRATION OF ChE on ACTIVITY AND STABILITY

Product code	mg enzyme offered/ ml beads	Concentration glutaraldehyde	Activity IUB units/ ml beads	% Activity retained after 50°C flow test
254-45	40	1.1%	18-25	99
277-10A	40	1.1	37	92
277-10B	10	1.1	13	31
277-10C	10	0.5	30	37
277-10D	5	1.1	14	0
277-10E	5	0.5	19	10
277-10F	2.5	1.1	6	7
277-10G	2.5	0.5	10.5	42

Product 277-10A is the same as 254-45. The test results verify the reproducibility of the method.

Table 2 shows that we were able to effect a decrease in activity by decreasing the concentration of enzyme. The relation of enzyme concentration and activity is shown in the figure. The data also show that there is a marked effect when the concentration of glutaraldehyde is decreased. Immobilizations carried out in the presence of 0.5% glutaraldehyde exhibit greater activity than those done at 1.1%. With respect to stability, we found that application of less than 40 mg. enzyme/ml beads results in an unexpected, marked loss of flow stability. Immobilization in the presence of 0.5% glutaraldehyde seems to give better stability than 1% glutaraldehyde, but in neither case are the products particularly attractive. It is possible that the cross-linking reaction is effective in stabilizing the enzyme only when a high concentration of enzyme is present. Perhaps insufficient intermolecular aggregation occurred in 277-10B through 10F to effectively stabilize the structure.

Since the net results of our investigation of glass bead products did not show great promise, we addressed our continuing efforts to other support materials. However, the phenomenon of decreased stability with decreased enzyme loading deserves further investigation.

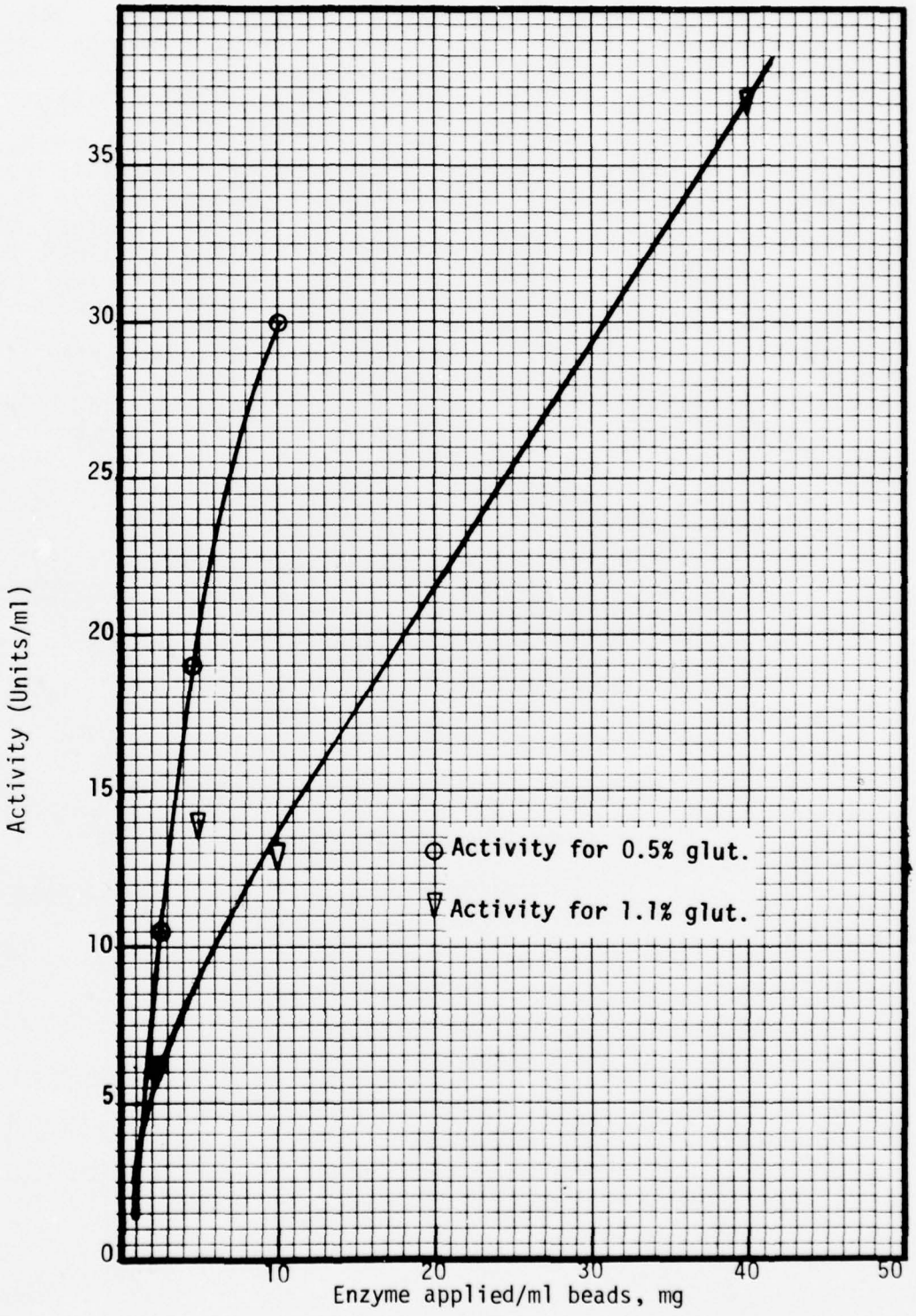

Figure. ACTIVITY AS FUNCTION OF ENZYME CONCENTRATION

3.2 Nylon

As cited in the introduction, nylon has been used in many forms as enzyme support[9,15,16,17]. We used nylon powder (see appendix D) since in that form it is similar to glass beads. We have also immobilized cholinesterase on nylon net. We were struck with the similarities in structure between nylon and urethane and felt that any method which would activate nylon, without breaking the peptide bond, could be modified to activate the similar amide bond in urethane. Based on our knowledge of nylon chemical reactivity, we also knew that isocyanate activation of nylon could be easily accomplished. These procedures applied to nylon then served to guide the urethane activation work.

Initial attempts to immobilize the cholinesterase used nylon which was hydrazide-cleaved, non-hydrolytical cleaved with N,N-dimethyl propane diamine, or hydrolytically cleaved with acid followed by dimethyladipimidate-activation (See appendix D). Both products had lower activity than the glass bead immobilized enzyme and neither did well in the high temperature stability test.

The two methods depend on breaking the chain of the nylon polymer strands and therefore are not as desirable as methods which do not require breaking the chain. They were used to gain experience in the general methods for working with nylon. Activating the nylon with dimethyl sulfate (DMS)[9] or with isocyanate gave superior results as shown in tables 3 and 4.

9. Hornby, W. E., Campbell, J., Inman, D. J. and Morris, D. L. Preparation of Immobilized Enzymes for Application in Automated Analysis. Enzyme Engineering 2, 401-408 (1974).

15. Horvath, C. and Solomon, B. Open Tubular Heterogeneous Enzyme Reactors, Biotechnol. Bioeng., 14, 885-914 (1972).

16. Eng. Tech. Digest, Natl. Sci. Foundation Res. Appld. to Nat'l. Needs Program 3,24 ('74).

17. Faulstich, H., Schafer, A. and Weckauf-Bloching, M. α-and β-Galactosidases Bound to Nylon Nets, FEBS Letters, 48, 226 (1-74).

Table 3

ACTIVITIES OF DMS-ACTIVATED PRODUCTS

Product code	Product description	Activity IUB units/g support
254-73	Nylon-DMS-HxDA-GlutxChE	5.0
256-59	Nylon-DMS-PEI-GlutxChE	1.2
254-75	Nylon-DMS-HxDA-DMAxCHE	2.2

The activities of the dimethyl sulfate activated nylon are comparable to that for the non-hydrolytically cleaved nylon, but are about 70% less than that of our best glass products. This may be attributable to the considerably greater surface area of porous glass beads. (In the past, we have observed significantly higher enzyme loading on porous beads as opposed to smooth or textured beads.) None of the three products did well in the two week stability tests at -40° and 65°C.

The preliminary work with isocyanate activation of nylon was encouraging. At first we used PAPI, which is a polyisocyanate, since it was not as volatile as the monomer diisocyanates. The results of our investigation of activation conditions are summarized in table 4. To achieve reproducible results we had to be sure there was adequate agitation during the reaction. As with most chemical reactions, an increase in temperature and/or time increased the degree of activation. The catalysts (diaminobicyclooctane (DABCO) or dibutyltin diacetate (DBT)) also affected the results. The catalyst effects on activation of nylon will be discussed in the urethane section since both activation studies bear strongly on each other.

Table 4

ACTIVATION OF NYLON WITH PAPI

Preparation	Code run no.	Sample weight (mg)	Activation time (hrs.)	μ Moles NCO groups gm support
P/O	245-36A	55.3	22 hrs.	837
P/O	245-36B	53.3	24 hrs.	1257
P/S	245-43C	58.1	30 min.	387
P/S	245-43D	57.6	60 min.	128
P/S	245-43E	61.5	90 min.	0
P/S	245-43F	56.5	19 hrs.	507
P/S	245-43A	55.8	30 min.	527
P/O	245-43B	58.1	30 min.	145
P/O/S	245-45A	25.3	60 min.	273
P/O/S	245-45B	252	90 min.	294
P/O/S	245-45C	250.8	120 min.	77
P/O/S	245-45D	250.1	21 hrs.	153
P_r/S	245-47	50.8	3.5 hrs.	419
P/S	245-48A	50.9	30 min.	402
P/S	245-48B	50.1	60 min.	385
P/S	245-48C	50.5	90 min.	287

P - PAPI

O - Diaminobicyclooctane (DABCO)

S - Dibutyltin diacetate (DBT)

subscript r - reflux

See page 40 for explanation of the preparation code.

Using some of the activated nylons, we investigated the conditions for bonding the enzyme. Our first attempt was at pH 9.5 in borate buffer (see table 5). Although the bound activity was low, we were greatly encouraged. We then studied the effect of pH on the bonding reaction. The enzyme activities were low at all the pH's tried but it showed that the approach was indeed feasible. These preparations were not subjected to the stability tests since their initial activities were too low. The best results were achieved between pH 7 and 8.

Very little more was done with activation of nylon by isocyanate because the urethane activation was run concurrently and showed great promise, and urethane is the preferred material for the Enzyme Alarm. Further comments about immobilization of cholinesterase on nylon and discussion of these results are incorporated in the section on urethane.

Table 5

PAPI-ACTIVATED NYLON

Preparation	Code run no.	Special notes on activation time (hours)	Foam activation μM (NCO) groups per mg.	Specific activity units AcHE per gm pad	Immobilization pH
P_r/S	245-51A	3.5 hrs.	184	0.46	pH 7
	245-51B	3.5 hrs.	184	0.48	pH 7.5
	245-51C	3.5 hrs.	184	0.50	pH 8
	245-51D	3.5 hrs.	184	0.30	pH 8.5
	245-47	2.5 hrs.	419	0.099	pH 9.5

3.3 Polyacrylamide

The Mid-West Research Institute reported earlier that active and stable immobilized cholinesterase was obtained using the support polyacrylamide derivatized with hydrazine[18]. Therefore, we attempted to duplicate and extend this work. For methods used, see appendix E.

18. Goodson, L. and Jacobs, W., Studies on Use of Immobilized Enzymes in Chemical Agent Detection Kits, Final Comprehensive Report. Edgewood Arsenal Contractor Report ED-CR-74005 (Feb. 1974).

The measured enzyme activity of the hydrazide polyacrylamide (product #254-70) was only 0.5 IUB units/ml support. This activity was lower than that of the glass and nylon products we had prepared up to that time. We then learned from the Project Officer that Mid-West Research Institute was not able to reproduce the previously reported success. Based on these two factors, we abandoned further work on polyacrylamide.

3.4 Modified Starch Gel on Urethane

In our original proposal we suggested that an alternative to covalent attachment of cholinesterase to urethane foam was modification of the starch gel product to produce a more stable enzyme pad. We investigated methods that would both stabilize the starch and stabilize the enzyme in the starch gel. Before starting the starch modification we had to learn the standard Edgewood method for starch gel entrapment of the enzyme on urethane pads. This served as a baseline, not only for the starch modification experiments, but also for the covalent attachment experiments.

The procedures for the chemical reactions discussed below are described in appendix F.

We tried means of modifying the -OH groups in starch so we could then either directly couple the enzyme or further derivatize the starch. The latter allowed us to branch out in several directions. The enzyme could be coupled to the derivatized starch, that is, cross-link the enzyme to the starch, or cross-link the starch itself with the enzyme trapped within. It also enabled us to investigate the effect of spacer arms on the starch-enzyme complex. We also investigated first coating the urethane with activated starch and then incorporated the enzyme or first reacted the enzyme with the activated starch and then coated the pad.

The activity and stability data for the various starch derivatives are shown in table 6. The cyanogen-bromide modified starch was not soluble even at boiling water temperature, and therefore, all the pads in the series (256-92, -95A, -98A, B, C) were coated with a slurry rather than a solution. The s-Triazine activated starch, pre-reacted with enzyme, also would not dissolve and had to be coated on the urethane pads as a slurry.

Table 6

ACTIVITY AND STABILITY OF STARCH DERIVATIVES

Product Code	Product Description	mg Enzyme[2]	Activity (units/g pad)	Residual activity (after flow thru test)
256-88A	Starch Coat/ChE	40	28	n.t.
256-88B	Starch Coat/ChE	80	49	40%
256-92	Starch-CNBr-ChE	10	0	n.t.
256-95A	Starch-CNBr-EDA-ChE	10	17	4%
256-98A	Starch-CNBr-EDA-ChE	10	1.3	n.t.
256-98B	Starch-CNBr-EDA-ChE-Glut[3]	10	0.31	n.t.
256-98C	Starch-CNBr-EDA-ChE-Glut	10	0.82	n.t.
272-5A(1)	Starch-$NaIO_4$-ChE	20	8.3	35%
272-5A(2)	Starch-$NaIO_4$-ChE	10	7.0	0
272-6B(1)	Starch-$NaIO_4$-EDA-GlutXChE	80	0.8	n.t.
272-19A	Starch-sTT-coat/ChE	40	41	2.5%
272-19B	Starch-sTT-ChE	40	0.1	n.t.
272-8A	Starch Coat/ChE-XPAPI	13.3	1.7	66%
272-8B	Starch Coat/ChE-XGlut	13.3	5.6	68%
272-9A	Starch Coat/ChE-XDMA	13.3	10	35%
272-9B	Starch Coat/ChE (Control for 8A, 8B and 9A)	13.3	18	n.t.

Note [1]: Abbreviations: ChE—acetylcholinesterase; CNBr—cyanogen bromides; EDA—ethylenediamine; Glut—glutaraldehyde; $NaIO_4$—sodium periodate; sTT—s-Triazine; PAPI®—polymeric isocyanate (Upjohn Co.); DMA—dimethyl adipimidate.

Starch or starch derivatives are assumed to be coated on a urethane pad at the end of the reaction sequence, unless otherwise indicated. If coating is carried out as an intermediate step, it is indicated as "-coat-" and followed by a description of the subsequent process(es). "Starch coat/ChE" indicates coating by the Edgewood method.

Note [2]: Weight of enzyme is normalized to 4" x 4" pad.

Note [3]: Starch-CNBr-EDA-ChE was dried before soak in glutaraldehyde.

n.t. = not tested.

3.4.1 CNBr Activation

Table 6 shows that the Edgewood physical entrapment method (products #256-88A and B) results in markedly higher activity than with the CNBr modified starch (except for product #256-95A). Two possible explanations seem plausible:

1. The lack of solubility of the activated starch, even in boiling buffer, may have prevented satisfactory coating on the pad.
2. The initial CNBr activation step may have been unsuccessful.

The lengthy time required for washing of the activated starch before the ligand can be coupled makes the latter the more plausible; the imidocarbonate linkage in activated starch is not especially stable in water. Also, the lack of activity of the CNBr-ChE preparation lends credence to this hypothesis, especially since chymotrypsin, a serine protease like ChE, was reported to be successfully immobilized by this procedure[19]. On the other hand, the respectable activity of the CNBr-EDA-ChE pad implies that active imidocarbonate groups were present in order to allow the diamine to be coupled. However, the amine might have been physically immobilized or entrapped rather than chemically coupled. This seems plausible when one considers the dramatic loss of activity of these pads when they were either soaked in glutaraldehyde or subjected to flow. Furthermore, when the CNBr-EDA starch was suspended in a solution of FLURAM (Roche Diagnostics), the fluorescence characteristics of free amino groups were not observed.

We tried to stabilize the enzyme linked to CNBr modified starch by glutaraldehyde cross-linking (products #256-98B and -98C). We did not draw any conclusions from these results because of the uncertainties involved in the prior CNBr activation.

3.4.2 Periodate Oxidation

Activation of starch by periodate oxidation resulted in respectable activities for all cases except the PEI derivatized product (#272-6B(2)). The low activity of the PEI derivative may have been caused by the physical properties

19. Porath, J., Nature, 215, 1491-1492 (1967).
20. Hornby, W. E., Biochim. Biophys. Acta, 387, 307 (1975).

of the starch-PEI adduct. Since it is a viscous gel, it is difficult to coat; furthermore, since the gel could not be easily concentrated, it was necessary to work with dilute solutions. The lability of PEI at alkaline pH may also be responsible for its lack of effectiveness. (The coupling reaction was carried out at pH 8.)

In terms of stability, product #272-6B(1), in which ethylenediamine is coupled to dialdehyde-starch as a spacer, is superior to the other periodate products; in fact, it proved to be the most promising of the three starch products. A sample was submitted to Edgewood for evaluation.

3.4.3 s-Triazine Trichloride

The lack of activity of product #272-19B, in which s-TT modified starch was reacted with cholinesterase and then coated onto urethane, may be due to one of the following possibilities:

1. The initial s-TT-starch reaction was unsuccessful.
2. The s-TT-starch was incapable of reacting with enzyme amino groups, perhaps due to steric factors.
3. The enzyme was inactivated in the course of its reaction with s-TT. Possibly, the serine -OH at the active site displaced one of the reactive -Cl on the s-TT ligand; although, one would expect enzyme amino groups to be more reactive than -OH's.

At first glance, the unusually high activity of product #272-19A (in which s-TT-starch was coated on a pad and then the pad soaked in ChE) would speak against these hypotheses. However, closer scrutiny reveals that were hypothesis 1 correct, enzyme may still have been adsorbed (but not coupled) to the pad during the soaking step. This would account for the high activity. However, during flow, the enzyme should be expected to wash off, which, indeed, is what is observed. A mere 2.5% of initial activity is retained after flow. In the case of product #272-19B, the lack of active s-TT groups would have, of course, prevented any coupling of enzyme to the starch. Any adsorbed enzyme would have been rinsed away during the salt wash which preceded coating.

3.4.4 Cross-linking

Pads prepared by the Edgewood method and then soaked in a solution of a cross-linking reagent show lower activity than those prepared without such a soak. Nevertheless, the activity of the cross-linked pads is still acceptable and moreover, in several cases the flow stability at 50° looks encouraging. Samples of product #272-8A (PAPI soak) and product #272-8B (glutaraldehyde soak) were submitted to Edgewood for evaluation.

Although a number of these modified starch pads showed great promise for future improvement, we discontinued further work because the covalent coupling to urethane showed even greater promise. We felt the project funds could be best utilized by concentrating on the latter for the remainder of this project.

3.5 Urethane

Our goal for the contract was the preparation of a covalently immobilized cholinesterase which would be usable in the field alarm and have the degree of temperature and flow stability required to give reliable operation. The urethane foam used with the starch gel immobilized enzyme had the mechanical properties needed for proper operation of the detector cell. We reasoned, therefore, that immobilizing the enzyme on urethane foam would require no change in the alarm design. To our knowledge, we are the first to achieve a means of covalently attaching an active enzyme to urethane foam without first weakening the structure by hydrolyzing some of the carbonate bonds.

Our plan to accomplish this goal was first to demonstrate that the urethane could be activated without destroying its mechanical properties and then investigate standard methods for immobilizing the enzyme. Because of the similarity of structure, we chose those methods which had been shown to activate nylon without breaking the chain. We also developed a method using isocyanates which, because the reaction was similar to that used to make the urethane, would not likely change its mechanical properties.

3.5.1 Activation of Urethane with Isocyanate

Table 7 lists the results of our first attempts at activating urethane foam with isocyanate. The experimental design was almost identical to that used for isocyanate activation of nylon (See Section 3.2, table 4). Aside from the longer reaction time required for urethane, the degree of activation is very similar in both polymers. The method for assaying the activation of urethane is given in appendix B.

Table 7

ACTIVATION OF URETHANE WITH ISOCYANATE

Preparation	Code run no.	Sample wt.(mg)	Activation time (hrs.)	Foam activation μM (NCO) groups per mg
P/O	245-34A	49.2	3	0
P/O	-34B	37.1	3.5	140
P/O	245-35A	42.8	3.75	0
P/O	-35B	40.4	4	30
P/O	-35C	42	4.5	52
P/O	-35D	35	15	243
P/O	245-36A	32.7	24	142
P/O	-36C	34.4	24	138
P/S	-36D	49	4.5	61
P/O/S	245-38A	31.7	3	97
P/O/S	-38B	34.3	3.5	47
P/O/S	-38C	35.2	4	131
P/O/S	-38D	35.3	22	157
P/O/S	-38E	36.3	22	14
P/O	245-41A	30.1	65	200
P/O	-41B	28.8	65	132
P/O	-41C	31.7	65	170
P/O/S	-41D	28.4	65	264
P/O/S	-41E	31.8	65	251
P/O/S	-41F	24.7	65	411
P/S	-41G	28.5	65	140
P/S	-41H	35.8	65	140
P/S	-41I	27.1	65	55
Pr_1/S	245-48A	32.8	3.5	609
Pr_0/S	-48B	30.8	3.5	877
F3/S	245-63A	30.5	21	92
F3/S	-63B	34.4	21	0
F2/S	-63C	35.4	21	20
F2/S	-63D	39.4	21	71
T/S	-63E	29.1	21	69
D/S	-63F	32.8	21	116

P - PAPI
O - diaminobicyclooctane
S - Dibutyltin diacetate
F_2 - FHP 2000
F_3 - FHP 3000
T - Tolylene diisocyanate
D - Diphenylmethane diisocyanate
Subscript r - reflux

See page 40 for an explanation of the preparation code.

Although not all of the preparations shown yielded consistent results, we found subsequently that proper agitation permitted us to activate urethane with good reproducibility. Furthermore, at methylene chloride reflux temperature (40°C) (#245-48A and -48B), the number of isocyanate groups per gram of foam increased significantly; whereas chlorobenzene reflux (132°C) did not produce significantly greater activation.

The catalyst used has an interesting effect. Diaminobicyclooctane seems to be more effective than dibutyltin diacetate (DBT) in activating nylon or urethane. However, the DABCO affects the physical properties of the polymers more than does the DBT. The DABCO treated polymer displays a decided yellow cast on its surface and, in the case of the urethane foam, the polymer loses some resiliency. The DBT does not have this effect.

Following the success with PAPI, we evaluated four additional isocyanates for their ability to activate urethane foam. Two were standard monomers in the urethane industry (tolylene-2, 4-diisocyanate [TDI] and diphenylmethane diisocyanate [DPMD]) and two are from Dewey Almy Chemical Division of W. R. Grace and Company (Hypol FHP 2000 [F2] and Hypol FHP 3000 [F3]), the latter are prepolymers similar to PAPI but are advertised as hydrophillic isocyanates. A hydrophillic reagent should offer advantages for both coupling of the enzyme and for subsequent use in the field alarm.

Preparations #245-63A to F in table 7 show that all of these isocyanates produced the same order of magnitude of bound isocyanate groups per gram of urethane. The same concentration by weight of the reagents were used for the comparison. The data for PAPI (#245-36A - C) in the table serves as a base line.

In addition to the methods for activating urethane, first described in the First Quarterly Report[21], we also worked on an alternative method, column activation[22], which will be discussed later in this section. As far as enzyme is concerned, the method by which the urethane is activated has little effect on subsequent immobilizations. Therefore, at various times we have used the standard (room temperature) reflux (methylene chloride B. P. 39°C) or column activated pads for the enzyme binding studies depending on which were most readily available at the time.

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21. Levin, H. and Erenrich, E., Enzyme Immobilization Alternatives for the Enzyme Alarm, First Quarterly Report. Contractor Report ED-CR-75014 (1975).

22. Levin, H. and Erenrich, E., Enzyme Immobilization Alternatives for the Enzyme Alarm, Second Quarterly Report. Contractor Report ED-CR-76005 (1975).

For a detailed description of the various enzyme immobilization techniques developed for these studies, see appendix G. Briefly, we used variations on three basic methods: Direct method, the enzyme is allowed to react directly with the isocyanate groups on the activated pad; Indirect method, a spacer arm (a di-or poly-amine or a glycol) is first reacted with the activated pad and the enzyme is bound to the spacer; and, Cross-link, the enzyme is physically adsorbed to the previously untreated foam followed by treatment with a difunctional agent such as glutaraldehyde or a polyisocyanate. As shown below, our best results were obtained when two or all three of these methods were combined.

3.5.2 Immobilized Enzyme Urethane Products

We had demonstrated that nylon could be activated by isocyanate and verified that enzyme could then be directly bound to give an active product. However, the specific activity was low relative to our other immobilized enzymes. Some possible reasons for this low activity include: inappropriate coupling pH, a competitive reaction between the water in the enzyme solution and the amine groups, or a reaction of the enzyme itself with the isocyanate.

The selection of pH for the immobilization involved a number of trade offs. Isocyanate groups react more rapidly with free amino groups than with hydroxyl groups. This argues in favor of a high pH at which the amino groups are unprotonated. On the other hand, isocyanate groups are rapidly hydrolyzed at high pH and furthermore, the enzyme is destroyed. Since the measured activity for the activated nylon at pH 8.5 was low, we decided to use pH 7.5 and 6.5 for the first attempts at direct immobilization of cholinesterase activity (product #245-68A and B). Nor did we get any measurable bound enzyme at pH 8.5 (product #245-71A) or even more alkaline pH 9.0 (product #245-71B).

See table 8 for listing of all experimental preparations for ChE immobilized on urethane foam. The two left hand columns in this tabulation identify the preparation; column 1 gives a shorthand summary of the reaction sequence, column 2 lists our code number. The notes, column 3, are listed on pages 39 and 40 following the table. Column 4 lists the degree of activation of the urethane in μM of isocyanate groups per mg of foam and column 5 is the specific activity of the immobilized enzyme product in international units per gram of foam. The results of the stability tests are listed in columns 6 through 8.

Table 8

TEST RESULTS OF URETHANE FOAM PREPARATIONS

Preparation	Code run no.	Special notes on activation time (hrs.)	Foam activation μM (NCO) groups per mg.	Specific activity units AChE per gm pad	Stability tests: 16 hr. flow at 50°C % activity retained	Stability tests: 2 wks. storage at 65°C % activity retained	Stability tests: Enzyme alarm 8 hr. at 23°C % activity retained
P_c/O/S-E_b	245-68A	Note 1,2	590-1600	0			
P_c/O/S-E_b	-68B	Note 1,3	590-1600	0			
P_c/O/S-E_b	245-71A	Note 4	30-210	0			
P_c/O/S-E_s	-71B	Note 5	30-210	0			
P_c/O/S-E_{sb}	245-73	Note 6,7	253-273	0			
P_c/O/S-E_s	245-77	Note 6,8	253-273	0			
P /O/S-PE-E XG	245-80	Note 9	30-180	0.20-0.38			
F3 /O/S-PE-E XG	245-82	Note 9	0-106	0.0-0.13			
E_d-XP/O/S	245-85A		N.A.	2.52			
P_{r_2}/O/S-E	-85B	Note 10	650-1000	0.26			
$F3_{r_3}$/O/S-E_d	245-86A	Note 10,11	254-609	0.1			
Pr_2/O/S-PE-EXG	-86B	Note 10,12	650-1000	0			
$F3_{r_3}$/O/S-PE-EXG	-86C	Note 11	254-609	0			
E_d-XP/O/S	245-89A		N.A.	0.65-1.04			
E_d-XF3/O/S	-89B		N.A.	2.06-2.35	53		
E_d-XT/O/S	245-91A		N.A.	1.65-1.92			
E_d-XT/O	-91B		N.A.	2.68-3.29	44-56		
E_d-XT	-91C		N.A.	2.14-2.93			
P /O/S-E -XT	-91D	Note 10	650-1000	1.41-1.74			
*$F3_{r_3}$/O/S-E -XT	-91E	Note 11	254-609	0.03-0.06			
T_{r_4}/O/S-E	245-92		155	0			
E_d-XP/O	245-93A	0.5	N.A.	1.92-1.93			
E_d-XP/O	-93B	1 hr.	N.A.	1.80-1.95			
E_d-XP/O	-93C	2 hr.	N.A.	1.43-1.54			
E_d-XP/O	-93D	3 hr.	N.A.	2.04-2.28	20		
E_d-XP/O	-93E	4 hr.	N.A.	2.05-2.22	12		
T_{r_4}/O/S-A-E_bXG	245-94	Note 9	155	0			
T_{r_4}/O/S-A-G-E_b	245-95A	Note 9	155	0			
T_{r_4}/O/S-A-G-E_bXG	-95B	Note 9	155	0			
E_wXG	245-97	Note 13	N.A.	0.63-1.28			

*Sample submitted to Edgewood Arsenal for testing.

Table 8 (continued)

TEST RESULTS OF URETHANE FOAM PREPARATIONS

Preparation	Code run no.	Special notes on activation time (hrs.)	Foam activation μM (NCO) groups per mg.	Specific activity units AChE per gm pad	16 hr. flow at 50°C % activity retained	Stability tests: 2 wks. storage at 65°C % activity retained	Stability tests: Enzyme alarm 8 hr. at 23°C % activity retained
E_wXF3	277-1		N.A.	0.84-3.32			
*P_{rs}/O/S-A-E_bXG-XT	277-3A			0.42-0.53	24		
*P_{rs}/O/S-A-E XG-E -XT	-3B			6.47-7.42	65		
*P_{rs}/O/S-A-E -XT	-3C			0.87-1.01	27		
*E_d-XP-A-E -XT	277-4		N.A.	1.28-1.99	36		
*E_d-XT	277-5		N.A.	0.72-0.89	50		
P_c/O	277-98	5060-337	N.A.				
O_c-P_c/O_c	277-100	150-1976	N.A.				
*P/O-A-E/G-E -T/O	295-10		3200	2.65	49	19	95
E_d-XF3/O	295-16		N.A.	1.6	25		
E_d-XP/O	295-18B		N.A.	2.0	15		
*E_d-XT/O	-18C		N.A.	1.8	39	33	8
P/O-PE6-E/G-E_d-XD/O-E_d	295-25		1700	1.8	11		
	-28		1700	0.5	40		
*P/O-PE6-E/G-XD/O	295-30		1700	1.2	33		
P/O-PE6-E/G-XD/O-E_d-XD/O	295-31		2600	<0.1	1		
	-32		2600	2.6	23		63
P/O-PE6-E/G-XD/O-E_d-XD/O	295-33		2600	2.6	10	69	67
P/O-PE18-E_d-XD/O-E_d-XD/O	295-36		1600	1.6	31		
*	-37		1600	2.8	47	61	54
P/O-PE18-E_d-XT/O-E_d-XT/O	295-40		1600	1.0	30		
*	-42		1600	2.4	42	91	75
F3-PE18-E_d-XD/O-E_d-XD/O	295-43		1000	1.2	39		
*	-44		1000	1.6	38	69	44
F3-PE17-E_d-XT/O-E_d-XT/O	295-45A		1000	2.0	20		
*	-45B		1000	2.5	36	48	39

*Sample submitted to Edgewood Arsenal for testing.

Table 8 (continued)

TEST RESULTS OF URETHANE FOAM PREPARATIONS

Preparation	Code run no.	Special notes on activation time (hrs.)	Foam activation μM (NCO) groups per mg.	Specific activity units AChE per gm pad	16 hr. flow at 50°C % activity retained	Stability tests: 2 wks. storage at 65°C % activity retained	Stability tests: Enzyme alarm 8 hr. at 23° % activity retained
P/O-PE18-E_d-XD/O-E_d-XD/O	295-48						
*	-49		1000	0.8	38	87	50
D-PE18-E_d-XD/O-E_d-XD/O	295-55		800	1.0	70		
*	-57	Note 19	800	1.7	65	76	51
D-PE18-E_d- XT/O-E_d-XT/O	295-59A		800	1.1	45		
*	-59B		800	2.25	62	>95	80
P/O-PE18-E_d-XT/O-E_d-XT/O	295-62A		1000	1.1	55		
*	-62B		1000	0.9	78	>95	80
P/O-PE12-E_d-XT/O-E_d-XT/O	295-70	Note 14	1000	1.3	38		
			1000	1.55	45		
			1200	1.4	39		
P/O-PE12-E_d-XT/O-E_d-XT/O	-71	Note 14	1000	1.25	>95	69	71
			1000	1.25	65	78	93
			1200	1.45	>95	60	61
P/O-PG-E_d-XT/O-E_d-XT/O	295-74		1100	0.8	63		
	-76		1100	1.2	75	58	64
P/O-PG-E_d-XD/O-E_d-XD/O	295-77A		1100	1.3	61		
*	-77B		1100	2.8	43	21	56
P/O-PG-E_d-XT/O-E_d-XT/O	295-78	Note 22	1000	0.1	>1		
	-80		1000	0.2			
P/O-PG-E_d-XD/O-E_d-XD/O	295-84A	Note 22	1000	0.3			
	-84B		1000	0.9	44	>10	60
P-PE18-E_w/PE18-XT/O-E_d-XT/O-E_d-XT/O	295-86		800	0.2	50		72
	-88		800	1.0	70		73
	-89		800	0.9	>95	>95	88,63
P/O-PE18-E_w/G-XT/O	295-95		600	0.1			

*Sample submitted to Edgewood Arsenal for testing

Table 8 (continued)

TEST RESULTS OF URETHANE FOAM PREPARATIONS

Preparation	Code run no.	Special notes on activation time (hrs.)	Foam activation μM (NCO) groups per mg.	Specific activity units AChE per gm pad	Stability tests: 16 hr. flow at 50°C % activity retained	Stability tests: 2 wks. storage at 65°C % activity retained	Stability tests: Enzyme alarm 8 hr. at 23°C % activity retained
P-PE18-E_w/PE18-XT/0-	295-96A		800	0.3	>95		
E_w/PE18-XT/0	-96B		800	0.9	67	67	82
P/0-PE18-G-E_d-XT/0-E_d-XT/0	295-100A	Note 20	600	2.4	91	65	>95
	-100B		600	2.3	83	87	87
0-P_c/0-$PE18_c$-E_c-XT_c/0	306-8			.46-1.38	67		
0-P_c/0-$PE18_c$-E_c-XT_c/0	306-12A	Note 2		.23-1.7			
P_c	306-15		1650-2100				
P_c-$PE18_c$-E_c-XT_c	306-18			.3-1.4			
P_c-$PE18_c$-E_c/PE18-P_c-E_c/ PE18-XT_c	306-20			<.2			
P_c-$PE18_c$-E_c-XT_c-$PE18_c$-E_c/ PE18/XT/0	306-23			.1-4.3			
*P/0-PE18-G-E_d-XT/0-E_d-XT/0	317-5A	Note 15	1600	5.4	76	>95	83
			1600	3.3	73	79	85
			1600	3.4	65	94	59
*	317-5B		1600	2.9	93	>95	55
			1600	2.7	>95		
			1600	2.4	>95		
*P/0-PE18-G-E_d-XT/0	317-7	Note 16	1600	3.0	60		>95
P/0-A-G-E_d-XT/0	317-10	Note 16	500	0.8	37	37	>95
P/0-PE6-G-E_d-XT/0	317-14	Note 16	1000	3.6	56	66	66
*P/0-A-G-E_d-XT/0	317-15	Note 17	500	1.2	58		>95
*P/0-PE6-G-E_d-XT/0	317-17	Note 16,19	1000	5.7	77		>95
P/0-PG-G-E_d-XT/0	317-22	Note 18	700	1.6			
	-22		700	2.8	>95		85
*P/0-PG4-G-E_d-XT/0	317-23	Note 18	1600	2.1	>95		>95
	-23		1600	1.5			

*Sample submitted to Edgewood Arsenal for testing

TABLE 8 NOTES

1. High NCO activation due to insufficient solvent wash.
2. Enzyme dialyzed against pH 7.5 0.1M Borate buffer before immobilization.
3. Enzyme dialyzed against pH 6.5 0.1M Borate buffer before immobilization.
4. Enzyme dissolved in pH 8.5 0.1M Borate buffer before immobilization.
5. Enzyme dissolved in pH 9.0 0.1M Borate buffer before immobilization.
6. Column broke after 2-1/2 hours (usual column activation time 4 hours). Activation completed in a beaker for an additional 2 hours.
7. Enzyme dissolved in 0.1M Borate buffer pH 8.5 - 2 parts of enzyme buffer solution diluted with 1 part DMSO before being contacted with the activated urethane.
8. Enzyme suspended in pure DMSO before contacting activated urethane pad.
9. $NaBH_3CN$ (200 µl/4 ml) added to reduce and stabilize amine-aldehyde addition product. Used substrate protection.
10. After reflux the urethane was not washed with methylene chloride but was allowed to dry.
11. Some of the dye from the indicating drying agent may have been extracted into the reaction mixture.
12. PEI aqueous used with no dilution with DMF.
13. Special test for air drying stability - 17 hrs. at room temp. - 50% retained activity; 17 hrs. at 35°C - 45% retained activity.
14. Test of the effect of urethane porosity. Used 45, 60 and 80 ppi urethane foam.
15. Test of glutaraldehyde wash time - used 10, 30 and 60 seconds.
16. Glutaraldehyde wash time 10 seconds.
17. Glutaraldehyde wash time 60 seconds.
18. Test of glutaraldehyde wash time - used 10 and 60 seconds.
19. Enzyme for coating used at 4 mg/ml which is twice the usual concentration.

20. Glutaraldehyde rinse for 15 minutes. The foam structure was almost totally destroyed.

21. See Nylon Table for 306-12B.

22. Contact time for bonding PEG to activated foam increased to 16 hours.

Reaction Sequence Code

P	PAPI
F3	FHP 3000
F2	FHP 2000
T	Tolylene diisocyanate
D	Diphenylmethane diisocyanate
O	Diaminobicyclooctane
S	Dibutyltin diacetate
E	Acetylcholinesterase
PE	PEI 30% aqueous solution with DMF
PE6	PEI 600 avg. molecular weight in solvent
PE12	PEI 1200 avg. molecular weight
PE18	PEI 1800 avg. molecular weight
G	glutaraldehyde
X	cross-link
A	Ethylenediamine
PG	Polyethylene glycol 1540 average molecular weight
PG4	Polyethylene glycol 400 average molecular weight

Subscripts

r_1	reflux
r_2	reflux
r_3	reflux
c	column
d	dry - enzyme slurred in water applied to foam and allowed to dry
b	buffer - enzyme dissolved in buffer
w	water - enzyme dissolved in water
s	DMSO - dimethyl sulfoxide
/	Separate substances in same solution during any one step in the process
-	Separates steps - no wash or solvent wash

We tried adding a very concentrated aqueous slurry of enzyme, letting it dry, and then react with the activated surface of the pads before washing off excess enzyme (products #245-85B and #245-86). This yielded small, but not usable, amount of bound activity.

There was no immediate explanation for these results since we could introduce about 3 to 4 times as many active isocyanate groups per gram of urethane foam as we could per gram of amino functional glass beads or nylon. We obtained very high enzyme activity on glass beads and moderate activity on nylon.

We thought perhaps the isocyanate activated pads were not adequately wet by the buffer solution or the water was reacting with the isocyanate groups before the enzyme could react. We tried dissolving the enzyme in pH 8.5 buffer made up in water-dimethy sulfoxide 2:1. When this did not produce an active pad (product #245-73), we tried suspending the enzyme in pure dimethyl sulfoxide. Again there was no detectable activity on the urethane pads (product #245-77). The enzyme suspended in the dimethyl sulfoxide retained full activity. Pure dimethyl sulfoxide adversely affects the physical properties of the urethane foam; the foam becomes less rigid and after compression literally takes hours to regain its shape.

Dimethyl formamide was also tried. The activity of the enzyme suspended in this solvent is not adversely affected but the urethane pads are completely destroyed.

Following the apparent failure of direct binding of enzyme to isocyanate groups on urethane foam, we decided to try the indirect approach. Polyethyleneimine (PEI) or ethylene diamine (EDA) could react with the isocyanate groups on urethane in a non-aqueous solvent. The resulting free amino groups, we reasoned, would produce a more hydrophilic surface and, in addition, would allow us to use a common amino specific reagent, such as aqueous glutaraldehyde, to immobilize the enzyme. Our work with glass beads and nylon, activated by reagents other than isocyanate, had shown that the enzyme could be immobilized using glutaraldehyde.

Products #245-80 and 245-82 using PEI gave very low activity pads but products #245-94, 245-95A and 245-95B, using EDA, gave no measurable activity when glutaraldehyde was the cross-linking agent.

At this time we knew we could obtain very active preparations on glass beads and nylon powder by cross-linking with glutaraldehyde (as contrasted to the two-pot glutaraldehyde immobilization procedure). We reasoned that difunctional or polyfunctional isocyanates should also be very effective cross-linking agents. Indeed, we obtained a reasonably active immobilized enzyme product (#245-85A) using these reagents. The procedure was slightly different from the glutaraldehyde method in that the pad was first coated with enzyme which was allowed to dry. Then the isocyanate (in a non-aqueous solvent) was added. This prevented the destruction of the isocyanate by water.

We tested three different isocyanates – PAPI, FHP-3000 (F3) and tolylene diisocyanate (TDI) – and found them equally effective cross-linking agents (products #245-89 A and B and #245-91 A, B and C). When TDI was used, a mixed catalyst of DABCO and DBT was less effective than DABCO alone or no catalyst. The latter two systems appeared to be comparable. Although DABCO appeared to have some positive effect, the difference could not be considered significant with so few samples (products #245-91A, B and C).

We also ran a set of experiments using PAPI and DABCO to determine the reaction time needed (products #245-93 A, B, C, D and E). We found that well over 90% of the activity was fixed in 30 minutes.

For comparison, we tried glutaraldehyde cross linking by the same method that worked well with glass beads (product #245-97). The activity was about half that obtained using isocyanates. This was improved by letting the enzyme dry on the pad first, as is done with the isocyanates, and then treating with glutaraldehyde. This product (#245-97) was used to study the effect of letting the urethane immobilized enzyme pad air dry. In contrast to the glass bead case or the starch impregnated urethane case, the isocyanate activated urethane pad immobilized enzyme lost 50% of its activity on air drying overnight at either room temperature or at 35°C.

We also tried one immobilization using F3 with an aqueous enzyme solution, following the one pot glutaraldehyde procedure. Because we had to work quickly to impregnate the urethane (before initiation of the foaming reaction), we did not get uniform coating (product #277-1). However, the center of the urethane pad had a significantly higher activity than any other preparation we had made to that time with isocyanates.

Up to this point in our work, the highest levels of activity were achieved by cross-linking methods, i.e., by methods which did not strongly bind the enzyme to the urethane foam. Furthermore, methods, which were assumed to produce a multilayer of enzyme, gave a more active preparation. A multilayer of enzyme can be produced in two ways. One is to mix the cross-linking reagent with the enzyme prior to impregnating the urethane pad (product #245-97) or with F3 (product #277-1). The other method is to apply the enzyme to the pad as a thick slurry and let it dry (compare product #245-85B with #245-68A and B). If this is followed by a cross linking agent, the activity is further increased (compare #245-85B with #245-91D).

By combining several of these methods we obtained a very active preparation (product #277-3B). The foam was activated by reflux. This was followed by reaction with a spacer, ethylenediamine. Then the enzyme was bound and cross-linked to this surface by glutaraldehyde mixed in with the enzyme solution. As pointed out above, this would be expected to produce a relatively thick layer of bound enzyme. The reactions, up to this point, are similar to several other preparations which yielded little, if any, activity (compare #277-3B with #245-80 and #245-95B). To complete preparation of #277-3B, another layer of enzyme was applied as a thick slurry, allowed to dry and then cross-linked with TDI to stabilize it.

Preparation #277-3B was also very stable in the flow test. It retained 65% of its activity after 16 hours at 50°C. Several other samples retained 50% or more of their activity in this flow test but not enough samples had been tested to reach any firm conclusions about the effectiveness of cross-linking agents. The only direct comparisons we had were among products #245-89B, -91C, -93D and E. PAPI did not seem to stabilize the product as well as cross-linking with F3 or TDI.

Indeed, the results on binding of the enzyme to urethane foam were quite baffling. Methods, which worked well with glass beads or nylon, produced totally inactive urethane products. Finally, we postulated that enzyme tightly bound to a flexible foam could be inactivated by mechanical stresses. Dr. M. Weibel of the University of Pennsylvania suggested that when enzymes are bound to an elastic material, the activity can be decreased by stretching the polymer. When the polymer is allowed to relax, the activity increases again but there is hysteresis in the system; that is, with each subsequent stress cycle more and more of the enzyme is permanently inactivated.

Most of Weibel's work has been done with nylon fibers which are much less flexible than urethane. Therefore, it seemed likely that some inactivation occurs each time urethane is flexed, compressed or stretched.

Despite our limited data, this hypothesis seemed to explain most of the anomalies. It accounted for the greater activity and stability of the directly coupled enzyme on glass beads and nylon powder as compared to that coupled directly to urethane foam. The starch entrapped enzyme retained its activity since it was not coupled to the urethane surface at all. A preparation in which the enzyme is adsorbed on the foam and then cross-linked should be more active than one which is first chemically bound to the surface. This was observed.

This hypothesis could also explain the greater stability of the TDI cross-linked preparations compared with the PAPI cross-linked system in the flowing tests. TDI is a much smaller molecule than the PAPI and is therefore more rigid. For a cross-linked enzyme, subjected to the stresses postulated here, the smaller stiffer molecule would tend to stabilize the structure more than the larger, more flexible molecule. This is in contrast to the characteristics of a desirable spacer arm, that is, a spacer arm should be a long flexible molecule.

It may be that past attempts at immobilizing the enzyme on urethane foam, as with our earlier attempts, coupled it too tightly to the surface. A long flexible spacer arm would tend to relieve mechanical stresses on the enzyme — thus, the requirements for a good spacer arm are just the reverse of those for a good cross-linking agent. In fact, in our most active preparation (#277-3B), the first layer of enzyme might be considered to be the spacer. Note that preparation #245-94, with only one layer, but otherwise identical to #277-3B, was totally inactive. If we assume as a very rough approximation that this first layer was 4 to 10 enzyme molecules thick, it acts as a long spacer arm.

The above hypothesis was tested by adding spacer arm between the support and the enzyme. We had intended to add spacer arms to enhance exposure of the enzyme to the air flow in order to improve sensitivity. The spacer arms, thus, may be shown to have more than one desirable function.

We decided to use either polyethylene imine (PEI) or polyethylene glycol (PEG) as spacer arms. Both are available in a variety of chain lengths. For PEI, we selected average molecular weights of 600, 1200 and 1800. These correspond to, respectively, 14, 28, and 42 ethylene imine

units per molecule or maximum coil lengths of about 70, 140, and 210Å (angstrom units), respectively. We used PEG of 1540 molecular weight which has a 35 degree of polymerization and is therefore approximately 170Å long. If we assume the enzyme to be about 35Å diameter, we see that the shortest spacer arm is about two enzyme diameters long and the longest about six enzyme diameters. Because the spacer arms are random coils and not rigid rods, these distances are the maximum distance obtainable between the enzyme and the point of attachment of the spacer arm to the polymer surface. In its usual configuration the spacer arm would act like a spring between the enzyme and the polymer.

The criteria for a cross-linking agent for stabilizing the enzymes are almost exactly opposite from those for a spacer arm; a short rigid molecule, which will prevent the enzyme from unfolding, is wanted. It has to be just long enough to allow for the movement of the enzyme as it changes configuration during the reaction but not long enough to allow the enzyme to change its configuration permanently. We chose to investigate glutaraldehyde, tolyene diisocyanate (TDI) and diphenylmethanediisocyanate (DPMDi), respectively about 8, 12, and 18Å long, as cross-linking agents. The phenyl groups in the isocyanates make them fairly rigid molecules.

The stability data for all our urethane foam preparations are summarized in table 8, columns 6 through 8. Column 6 shows the percentage of activity remaining after the 16 hours flow test at 50°C. (See appendix H.) Column 7 lists the percentage of the activity remaining after 2 weeks storage at 65°C. Column 8 presents the results of a flow test at room temperature in a modified Enzyme Alarm (DDE #8) which was provided from Contract DAAA15-75-C-0134. No data is shown for the 2 week stability at -40° since all preparations were stable under those conditions.

Where there is more than one code number under a reaction sequence, in table 8, it means that a sample was tested part way through the sequence. Generally when a reaction sequence called for two or more layers of enzyme to be bound to the pads, samples were tested after each enzyme layer was cross-linked. The first lines of data show that not all of these intermediate samples were subject to the complete series of stability tests.

Analysis of the data indicate that PEI 1800 and PEI 1200 are equally effective spacer arms (compare #295-62B with #295-71). The PEI 1800 gave pads with a slightly higher

enzyme activity than does the PEI 600 (compare #295-25 through #295-33 with #295-36 through #295-40). Pads number 295-76, -77B, -80 and -84B indicate that the PEG does not bond as much enzyme activity as any of the PEI's.

In tests to determine which cross-linking agent gave the more stable product, we found that Tolyene diisocyanate was more effective than the diphenylmethanediisocyanate (for example, compare #295-37 with #295-42). The comparison shows that the cross-linking agents had little effect on the amount of bound enzyme but the TDI cross-linked material was more stable at 65°C and in use in the field alarm. Using PEI 600 spacer, we had tried cross-linking with glutaraldehyde and found that either the bound activity was low (#295-28), unstable (#295-25) or both (#295-31).

We also rechecked our earlier conclusion that PAPI was the agent of choice for activating the urethane. Using PEI 1800 spacer arms and both TDI and DPMD cross-linking agent, we compared PAPI, FHP 3000 and DPMD for their ability to bond enzyme activity and its subsequent stability. In comparing #295-37 with #295-44 and #295-57, which all use the less effective cross-linking agent DPMD, one finds little difference among the activating agents. In the test which used TDI as the cross-linking agent, (#205-42, #295-45B, #295-59B and #295-62B), we found that PAPI and DPMD gave products which were approximately equal and were more stable than the product using FHP 3000. We decided to continue to use PAPI to activate the urethane, because it was less volatile, and therefore safer to use than the DPMD.

The effect of support pore size was also studied by using 45, 60, and 80 ppi urethane foam. These results are summarized as code numbers 295-75 and 295-71. The numbers in parenthesis represent the three different porosities of urethane foam used. Sample #295-70 are the intermediary samples. We thought that since the 80 ppi material had a greater internal surface area than the 45 ppi foam, we would get a higher enzyme activity. No significant differences in either amount of bound enzyme nor resultant stabilities could be detected as a function of pore size. Therefore, there was no reason to change from the 45 ppi foam which had been used for all the other preparations.

As a result of the column activation method work, we investigated the feasibility of not using DABCO catalyst in activating the urethane. (Note the test data on runs #295-57 and #295-59B in which DPMD was used, and runs #295-89 and #295-96A in which PAPI was used as activator). It is

difficult to tell from the data, but based on activation runs using catalyst, there is a reduction in the number of isocyanate groups introduced. This reduction may be as high as 20%. Despite the reduction, the amount of enzyme bound is not significantly decreased. We can infer from these results that with or without catalyst the activation produces enough isocyanate groups to saturate the surface of the urethane foam with enzyme.

We tried several other parameter variations such as: increase PEG contact time (#295-80 and #295-84B); three enzyme coats (#295-89) and mixing PEI 1800 with the enzyme prior to coating (#295-89 and #295-96B).

At this point in the investigation we had a number of preparations which passed the stability tests (#295-42, -57, -59B, -62B, -71 (45, 60 and 80 ppi). Samples of these and several others were delivered to the Project Officer for evaluation at Edgewood Arsenal.

These pads had somewhat lower initial activity, as compared to the starch pads. Therefore, we attempted to increase the activities by a factor of 2 to 4. It had been observed that the reaction between glutaraldehyde and the PEI spacer arm is extremely rapid. Activating the spacer with glutaraldehyde for 15 minutes almost totally destroys the enzyme pad structure but gives a product with reasonably good activity and excellent stability (#295-100A and -100B). (The effect of glutaraldehyde in the foam structure may explain the anomalous results obtained earlier when we tried immobilizing the enzyme by mixing it with glutaraldehyde prior to application to the pads.) Runs #317-5A and -5B were then made using very short contact time between the glutaraldehyde and the foam with attached PEI 1800 spacer arms. It can be seen from runs #318-5A and -5B that the 10 second glutaraldehyde wash gave a more active, very stable, product than did the 30 or 60 second washes. Moreover, it became apparent that adding a second layer of enzyme actually decreased the activity of the pad although it did slightly improve the stability. Using this technique (10 second treatment with glutaraldehyde followed by rapid water washing) produced several single enzyme layer, highly active pads with excellent stability (#317-7, -14, -17).

3.5.3 Column Activation Method

Once we had some success with methods for activating urethane foam and binding the enzyme on it, we realized that these reactions could be carried out in a column packed

with urethane foam discs. If precut foam discs are packed in a glass tube (whose diameter is such that the discs fit snugly), the various reagents and wash solutions can be pumped through the column to produce immobilized enzyme pads without having to handle the foam between operations. This approach holds promise for mass producing enzyme pads, but, unfortunately, we did not have the time to optimize it.

In our initial attempts we used a short column which held 9 pads and the solution was pumped into the bottom of the column to minimize formation of air bubbles. A wide difference in the degree of activation of the pads at the top and bottom of the columns were observed. (See runs #245-68A through #245-82). Toward the end of this series of runs, we started determining the degree of activation of every pad. Prior to that, we assayed only the two at the top and bottom and one in the middle. We then found that the four pads in the middle were reasonably close in activity but the pads at each end were widely different.

We then obtained a longer column which held 27 pads, thus providing a larger middle section. We still observed a large difference between the top and bottom pads (#277-98) but more than half of the middle pads were reasonably close in activity. We had been adding the catalyst to the isocyanate and we reasoned that the DABCO must have a high affinity for the urethane and, therefore, was preferentially absorbed by the pads near the inlet of the column. We then made another run (#277-100) in which we presoaked the pads in a catalyst solution before packing the column. We still obtained the same wide variance between top and bottom pads but it was reversed! The pads near the inlet were poorly activated (150 μM/mg) compared to the pads at the top (1976 μM/mg).

We made two runs (#306-8 and #306-12) in which we performed all the steps to make the immobilized enzyme product right in the column. These results were encouraging because all pads showed active immobilized enzyme but the pads at the bottom were less active than the pads at the top. A pad from run #306-8 was subjected to the 50°C flow test and was found reasonably stable. In run #306-12, nylon clothed discs, interspaced between the urethane pads, did not show the same degree of variation.

We reasoned that if presoaking with catalyst could make such a dramatic change, then our problems must be due to catalyst. An activation run with no catalyst present

(#306-15) showed that we not only got uniformity of activity (± 20%) between the top and bottom but also greater activation was achieved. This was confirmed by standard activation runs in which no catalyst was used. Three columns were then run (#306-18, -20, -23) in which the total process to an immobilized enzyme product was carried out. All columns yielded active products. However, we got a large variance between top and bottom pads again, but the pads in the center were within a range of 20%.

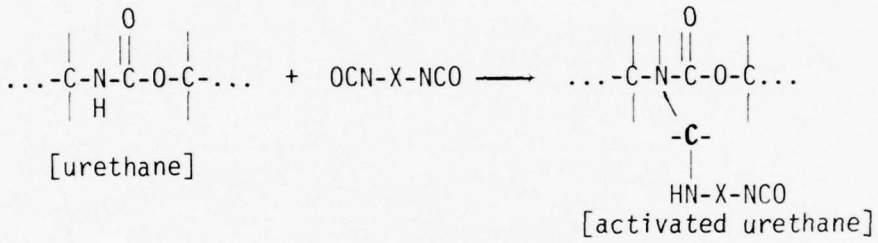
These results are extremely encouraging since the most active pads were nearly as active as the best pads from the standard process. We feel that this column process can be optimized to yield uniform active pads.

4. CONCLUSIONS AND RECOMMENDATIONS

The following chemical equations illustrate our understanding of the reactions involved in immobilizing acetylcholinesterase on urethane foam.

Urethane Activation

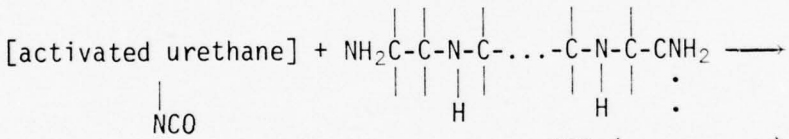
X= PAPI or FHP 3000



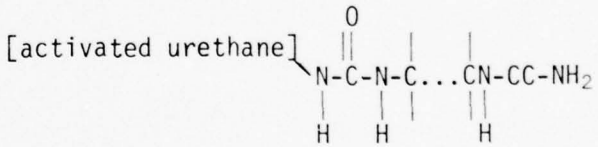
[urethane]

[activated urethane]

Chemical Attachment of Spacer Arm



PEI (spacer arm)



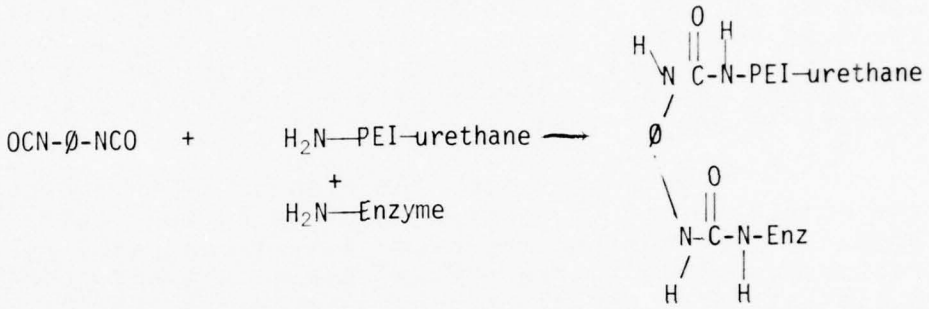
[activated urethane + spacer arm]

The secondary amine may also react.

Enzyme is then physically adsorbed to the PEI modified urethane.

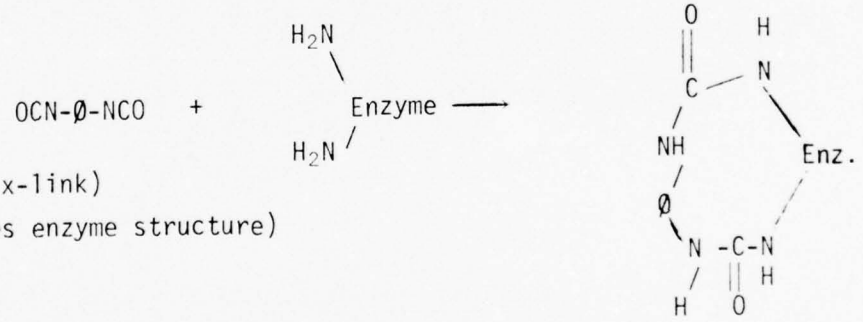
Covalent Bonding and Cross Linking

OCN-Ø-NCO = any low molecular weight diisocyanate



(Covalent bonding to
solid support
through spacer arms)

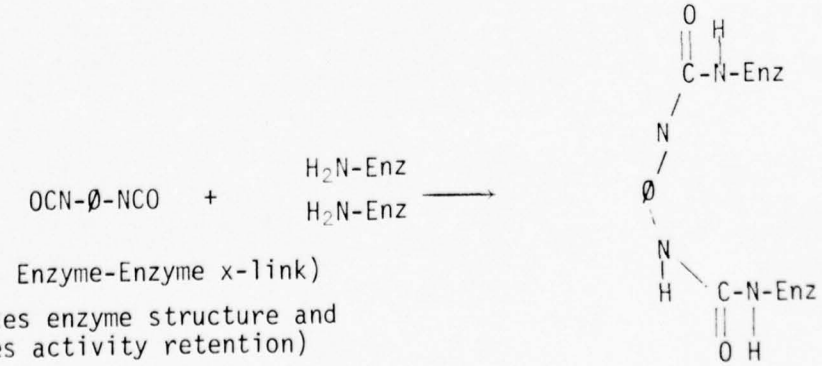
Also,



(internal x-link)

(stabilizes enzyme structure)

and



(external Enzyme-Enzyme x-link)

(stabilizes enzyme structure and
increases activity retention)

Although we achieved satisfactory immobilized enzyme products, we realize that further optimization is needed. We don't know whether more or less activation of the foam or whether a different spacer arm will give a pad better suited to the alarm instrument requirements. Furthermore, no attempt was made to optimize the sensitivity or speed of response. Both of these factors may be improved by the proper selection of reagents and reaction conditions. The column method is very promising but we only scratched the surface in the limited time available.

In conclusion, the results of this program satisfy the objectives of the contract but we believe there is potential for further improvement of these pads. It is recommended that future effort involve closely coordinated optimization of the electrochemistry of the Enzyme Alarm and of the enzyme immobilization processes to achieve maximum improvement in sensitivity, speed of response and stability of the enzyme.

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APPENDIX A

METHODS ASSAY – CHOLINESTERASE

Before we could proceed with the immobilization of the cholinesterase (ChE) we had to have a method for determining enzyme activity. Initially, we worked out an assay for the free enzyme in solution, and then adapted it first to glass bead immobilized enzyme and then to the other immobilized enzyme products.

A.1 Soluble Assay

The soluble assay is based on the procedure of Ellman[23]. This method involves the reaction of a thiol, produced by the ChE – catalyzed hydrolysis of the thio analog of butyrylcholine, with a chromogen, bisdithionitrobenzoate (DTNB). A deep yellow color is formed, which has an absorption maximum at 412 nm. Although the Ellman assay was originally devised to be run at pH 8, we chose to run at pH 7.4 instead, despite the fact that enzyme activity is higher at pH 8. This decision was made because in going from pH 8 to pH 7.4, the spontaneous autolysis of substrate decreases to a much greater extent than the corresponding enzyme activity.

The procedure for the assay is as follows:

1. Into a clean, dry 1 cm. path length cuvet, place the following:
   a. 20 µl of 0.0-5M butyrylthiocholine iodide (BuSChI)
   b. 100 µl of 0.01M DTNB
   c. 2.00-2.99 ml of Tris buffer (0.1M pH 7.4) such that
      Σ (Tris + enzyme) = 3.0 ml
2. Insert cuvet into spectrophotometer. Determine spontaneous rate of substrate hydrolysis by monitoring ΔA_{412}.
3. Inject appropriate volume (usually 10-50 µl) of enzyme solution.
4. Record at 412 nm until linear rate is assured.

23. Ellman, G. L., Courtney, K. D., Andres, V., Jr. and Featherstone, R. M., A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity, Biochem. Pharmacol. 7, 88-95 (1961).

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Specific activity is calculated as follows:

$$\text{Specific Activity} = \frac{10^6 \times \Delta A' \times V}{\varepsilon \times W} \ \mu\text{moles/min/mg enzymes}$$

where $\Delta A'$ = (change in absorbance/min)$_{\text{sample}}$ - (change in absorbance/min)$_{\text{blank}}$

V = reaction volume

W = mg enzyme injected into reaction

ε = extinction coefficient = 13.6×10^3 mole^{-1}cm^2

This assay was found to be linear with both enzyme concentration and time.

We also investigated the effect of substrate concentration on the assay. The reaction seems to follow Michaelis-Menten kinetics, with substrate inhibition at high concentration of BuSChI. At the substrate concentration used in the assay, 4.8×10^{-4} M, the velocity is actually a sharp function of the substrate concentration. Therefore, the specific activities which are reported are valid only for the particular concentration of BuSChI used in the assay, and would be higher if higher substrate concentrations had been used.

A.2 Immobilized Assay

This assay is an adaptation of the soluble assay. It was first worked out using glass beads and then extended to the other products. Immobilized enzyme, substrate and chromogen are incubated at 25° with shaking. At fixed time intervals the immobilized enzyme is allowed to settle, an aliquot of the reaction supernatant is withdrawn, and its absorbance at 412 nm recorded. Activity can be calculated directly from the absorbance.

We found that if the volume of immobilized enzyme preparation is $\geq$ 10 µl (approx.), then the assay was neither linear with time nor with volume of immobilized enzyme; the observed absorbance leveled off as either of these parameters was increased. There are a number of possible causes for this phenomenon, but we did not have the time to investigate these in depth. Instead, we modified the procedure to achieve a linear assay. From 0.2 to 5 µl of beads could be measured by sucking up the beads into a thin capillary tube. Both the

height and the total volume of the capillary were known to within one percent. The height of the column of the beads (which had been tapped down to ensure uniform packing) was then measured by means of an optical micrometer and converted into volume.

When we employed this technique for our assay, the absorbance at 412 nm was directly proportional to the quantity of beads used, implying that the absorbance should be a reliable measure of activity.

The detailed procedure for the assay is as follows:

1. Into a 25 ml Ehrlenmeyer flask, place
   a. 10 ml of 0.1M Tris buffer, pH 7.4
   b. 1 ml of 0.01M DTNB
   c. Measured volume of beads
2. At t = 0, inject 250 µl of 0.1M BuSChI.
3. Shake at 25° for a fixed time, t, usually 5 minutes.
4. Withdraw 1 ml of reaction supernatant and read A_{412}.
5. Subtract A_{412}(blank) from A_{412}(sample)

   for blank, shake 1 ml Tris buffer
   100 µl DTNB
   and 25 µl BuSChI

   for time, t.
6. Calculation:

   Bead activity (µmoles hydrolyzed/min/ml beads)

$$= \frac{10^6 \times A_{412} - A_{blank} \times V}{\varepsilon \times t \times V_{beads}}$$

where $\varepsilon = 13.6 \times 10^3$ mole^{-1}cm^2

V = assay volume - 11.25 ml

V_{beads} = volume of beads (in ml)

We found that results were not affected when DTNB was not included in the reaction mixture, but was instead added to an aliquot of the supernatant.

The assay could be used unmodified with the nylon powder and the polyacrylamide bead derivatives. In all cases, at least three different volumes of immobilized enzyme were assayed to ensure that specific activity was not a function of sample size.

Urethane pads were assayed by the same procedure, except that a small weighed piece of dry pad was used rather than a measured volume of beads. If the pad was wet before the assay, it was recovered after the test, dried and weighed. (Pad pieces were weighed on the Sierra Auto Balance AM-1.)

To ensure linearity, we tried incubating various weights of pads; for the assay to be meaningful, a plot of activity vs. weight of sample should be linear. This is shown in figure A.

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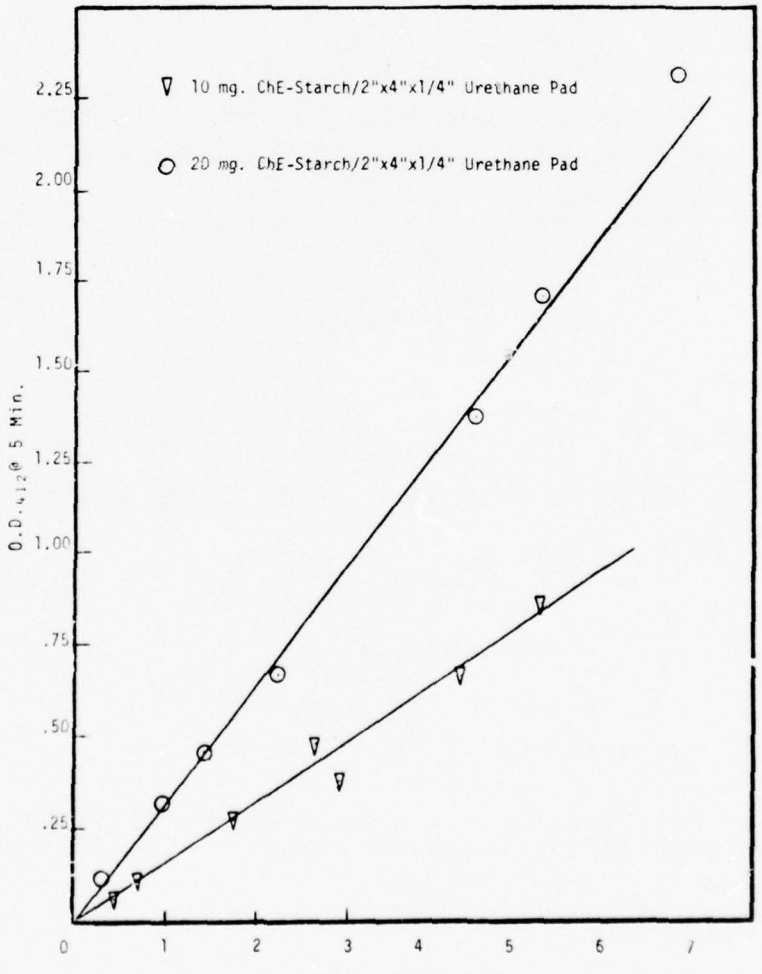

Figure A. Wt. of Starch-ChE Pad (mg)

APPENDIX B

METHODS ASSAY – ISOCYANATE

In order to develop the method for activating urethane and nylon with isocyanates, we first had to have an assay procedure for isocyanate groups. The following assay is a modification of a method recommended by Upjohn Company for isocyanate analysis. It depends on the rapid reaction between amines and isocyanates. If an excess of a secondary amine is used, this excess can be measured by titration and the isocyanate, therefore, determined by differences. Since each amino hydrogen can react with one isocyanate, a secondary amine is used because it has only one reactive hydrogen.

B.1 Materials

Methylene Chloride dried with zeolite or an equivalent drying agent.

Dibutylamine (DBA) stock solution – 1 gram DBA brought to 100 ml with dry Methylene Chloride. This solution must be standardized just prior to use and must be restandardized several times during a day because of the volatility of the solvent used.

Distilled or other high purity water.
Standardized 0.1M HCl.
Acetone - reagent grade.
Hot water bath.
Automatic titrator – we actually used a Leeds & Northrup pH meter together with a Leeds & Northrup strip chart recorder and a constant speed peristaltic pump. The titration was started by placing the pump delivery tube in the titration vessel and simultaneously the recorder chart drive was turned on. The time required to reach the inflection point in the titration curve was determined from the recorder chart. The pumping rate times the time gave the volume of acid required to neutralize the amine.
Magnetic stirrer and stirring bars.
Micropipettes.
Test tubes, beakers, glass rods and other glassware as needed.

B.2 Assay Details

The isocyanate activated polymer, in a test tube, (about 30-50 mg. dry weight) is covered with dry methylene chloride (about 2-3 ml). An accurately measured volume of standardized DBA is added. The volume added should provide excess amine. Usually 1 ml of DBA stock is sufficient. The amine is allowed to react with the isocyanate groups for from 5 to 15 minutes with occasional stirring with a glass rod. Usually 5 minutes is sufficient, but sometimes a longer reaction time is needed to get reproducible results. The excess DBA is removed from the polymer by washing 4 to 5 times with methylene chloride; the supernatant solution and washings are combined in a test tube and brought to dryness in a hot water bath. Ten ml of water is then added to the test tube to dissolve the unreacted amine. Some of the isocyanates produced a residue which did not easily disperse in pure water. In these cases up to 3 ml of acetone (usually 1 ml) were added, the residue dispersed, then the water was added. The pH probe was inserted and the titration started. The pump speed was set to deliver 100 µl/min. in almost all cases although up to 300 µl/min. pump rates have been used when this would not shorten the titration time much below 4 minutes. In our system we needed that much chart paper to get a sufficiently accurate time estimate in order to get a reliable estimate of the volume of acid delivered.

The DBA was standardized by going through the same procedure except that the polymer was omitted. The acid delivery pump rate was the same as used for the polymer isocyanate determination to avoid errors due to changes in the pump delivery rate.

The difference between the volume of acid required to neutralize the amine when no isocyanate was present and when isocyanate was present provided the measure of the number of isocyanate groups in the sample.

B.3 Sample Calculations — Standardization of Dibutylamine

One millimeter of dibutylamine stock solution required 4.61 minutes to reach equivalence point when 0.1M HCl was pumped in at a rate of 100 µl/min.

4.61 min x 100 µl/min x 0.1 µm HCl/µl =

46 µmoles HCl

∴ 46 µmoles dibutylamine/ml

Determination of the number of isocyanate groups per gram of powder:

To 30.1 mg of activated urethane foam was added 1 ml of standardized dibutylamine stock solution. After reaction the residual amine required 4 minutes to reach the equivalence point when 0.1M HCl was pumped in at a rate of 100 µl/min.

46 µM dibutylamine - (4.0 min x 100 µl/min x 0.1 µM HCl/ml)

= 46 - 40 µmoles

= 6 µmoles isocyanate groups/30.1 mg urethane

= 200 µmoles isocyanate groups/gm urethane

B.4 Reproducibility

When we first developed this assay, we checked it by determining the concentration of isocyanate groups in the di-or poly-isocyanates we were using to activate the polymers. The results are given in Table B-1. From these results we estimate a reproducibility of ± 10 to 15%. This is more than adequate for our application.

Table B-1

Isocyanate groups/mg of Urethane Activating Agents

	Calculated or given by manufacturer (µmoles/mg)	Measured (µmoles/mg)
PAPI	7.3-7.5	7.4
Hypol FHP 2000	1.75-2.00	1.34
Hypol FHP 3000	2.00-2.75	2.11
Tolylene 2,4-diisocyanate	11.4	10.31
Diphenylmethane diisocyanate	5.9	5.6

B.5 Comments

This assay system has wide utility since, with minor modification, it can be used to determine active groups other than isocyanate in a polymer. In fact, any active group such as amine, hydrazide, urea or hydroxyl, which can react with isocyanate can be estimated by modifying this method. Many of the methods now used to estimate these groups in an insoluble polymer are much more tedious and laborious.

APPENDIX C

METHODS CHEMISTRY – GLASS BEADS

The basic activation and preliminary derivatization of the glass beads is briefly described here. For more details see the paper by M. Weibel[24]. We used six different immobilization methods with the glass beads; four involved silanized beads and two involved cross-linking the enzyme on untreated beads.

C.1 Activation of Glass Beads

(1) Silanization:

Controlled pore glass (50/80 mesh, pore size 550Å) was dried under vacuum and then refluxed overnight in dry toluene with γ-aminopropyltriethoxysilane. This reflux step was repeated a second time. The beads were subsequently refluxed with ethyl triethoxysilane in dry toluene.

(2) Further derivatization of silanized beads for azo coupling:

Silanized beads were refluxed overnight with p-nitrobenzoyl chloride in dry chloroform in the presence of triethylamine. After the benzoylated beads were dried, they were reduced by reaction with sodium dithionite in boiling water for one hour.

(3) Further derivatization of silanized beads for hydrazide coupling:

Silanized beads were refluxed overnight with terephthaloylchloride in dry chloroform in the presence of triethylamine. The beads were then reacted with hydrazine in dry ethanol for one hour in the cold.

(4) Isocyanate Coupling:

Silanized beads were allowed to react with PAPI by the same procedure described under nylon activation with PAPI (see Appendix D, Section D.1.d.).

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24. Weibel, M. K., Dritschilo, W., Bright, H. J. and Humphrey, A. E. Immobilized Enzymes: A Prototype Apparatus for Oxidase Enzymes in Chemical Analysis Utilizing Covalently Bound Glucose Oxidase. Anal. Biochem. 52 402-414 (1973).

C.2 Covalent Immobilization

The following are general points to keep in mind concerning immobilization on glass or other bead material:

a. For every x ml of support, we generally use from 1.5 - 2x ml of enzyme solution at a concentration of 20 mg/ml. Enzyme should be dialyzed against the coupling buffer prior to immobilization.

b. To allow good surface contact between enzyme and beads, while minimizing physical fragmentation of beads, it is best to shake the beads gently in a shaker bath during immobilization.

c. Presence of a substrate or inhibitor during the immobilization process helps to maintain an active conformation while the enzyme is binding to the support. We have found addition of acetylthiocholine iodide (approx. 2-3 mg/ml beads) to an immobilization reaction to be effective.

d. Enzyme uptake from solution is monitored by withdrawing aliquots of the reaction mixture during immobilization and measuring the A_{280} as well as the remaining activity of the sample.

e. After immobilization is complete, the supernatant is recovered. The enzyme preparation is then washed 10 times with 1M NaCl to desorb the enzyme which has been adsorbed by electrostatic interactions. The beads are then washed with storage buffer, usually 0.1M potassium phosphate containing 0.1% benzoic acid as a preservative.

f. We can obtain an estimate of the efficiency of coupling by comparing the A_{280} of the reaction supernatant and rinsings with that of the original enzyme sample applied to the beads.

C.3 Procedures for Covalent Immobilization on Glass Beads

a. Azo

Silanized beads which have been derivatized for Azo coupling were reacted in an ice bath for 1-1-1/2 hours with sodium nitrite and HCl in order to form an active azo group. After the activated beads were washed, a solution of cholinesterase was added. Reaction was allowed to continue

at room temperature for at least two hours. After that time, it may be left in the cold overnight if desired. Overnight reaction generally results in a slight, though not significant, increase in the units of enzyme taken up by the beads.

b. Glutaraldehyde

Silanized beads were reacted for two hours with 10% glutaraldehyde in phosphate buffer, pH 7.5 at 0-4°C in the presence of the reducing agent sodium cyanoborohydride. For the first half hour of reaction, suction was applied. After the reaction was complete, the glutaraldehyde solution was removed, the beads rinsed, and a solution of enzyme (previously dialyzed against 0.1M phosphate buffer, pH 7.5) added. Reaction was allowed to continue at 0-4°C with shaking for at least two hours, and overnight if desired. This two step immobilization contrasts with the one pot cross-linking described below.

c. Carbodiimide

Cholinesterase solution, water soluble carbodiimide [1-ethyl, 3-(3-dimethylaminopropyl) carbodiimide - EDC] and silanized beads were combined and shaken at 4°C for two days with periodic monitoring of pH. Acid or base was added to maintain the pH near 5.0. A typical reaction mixture consisted of 20 mg. cholinesterase, 50 mg EDC and 1 ml silanized beads in a total volume of 4 ml water.

d. Hydrazide

Beads which had been derivatized for hydrazide coupling were activated to yield an azide group by reaction with $HCl/NaNO_2$ in ice. After rinsing the beads, enzyme solution was added and the slurry shaken at 4°C for at least two hours.

C.4 Cross-linking on Glass Beads

We believed that cross-linking the enzyme around the support might lend additional stability to the immobilized product. To accomplish this, we worked with two cross-linking reagents, glutaraldehyde and dimethyladipimidate (DMA).

a. Glutaraldehyde

This procedure involved a one-pot reaction among the silanized glass beads, enzyme and glutaraldehyde, as opposed to a two-step process as described above.

The beads were first shaken with a solution of cholinesterase for 15 minutes, during which time much of the enzyme was adsorbed to the bead surface. A sufficient volume of aqueous glutaraldehyde solution was then added to bring the final concentration to 1-2 percent. Sodium cyanoborohydride was also added at a concentration of between 0.1 - 0.2M. The mixture was shaken at 4°C overnight.

b. Dimethyl Adipimidate (DMA)

DMA has been described as an effective bifunctional cross-linking reagent for proteins[25]. Zaborsky[26] noted that chymotrypsin adsorbed on porous silica and then cross-linked with DMA had increased thermal stability. Since both cholinesterase and chymotrypsin are serine esterases, we reasoned that cholinesterase might also be favorably stabilized by means of DMA cross-linking.

To carry out the reaction, a mixture of cholinesterase, DMA (0.02M in phosphate buffer, pH 7.5) and glass beads were shaken at 4°C overnight

25. Wold, F., Bifunctional Reagents, Methods of Enzymology, 11,617 (1967).

26. Zaborsky, O., Stabilization and Immobilization of Enzymes with Imidoesters, Enzyme Engineering 2,115 (1974).

APPENDIX D

METHODS CHEMISTRY – NYLON

A 6% solution of nylon 6/6 in 90% formic acid was prepared by stirring and heating to 60°C. Water was added until a slight turbidity developed in the solution held at 60°C. A small amount of formic acid was added to clear up the turbidity. The solution was then removed from the heat. After cooling, the nylon precipitated out to give a white viscous suspension. The precipitated nylon was filtered and washed with water on a medium pore size sintered glass funnel. The nylon powder cake was air dried and stored at room temperature.

D.1 Activation of Nylon Powder

Three of the following five methods cleave the nylon backbone and the last two methods activate the amide bond without breaking it:

a. Hydrolytic cleavage of nylon[9,27] – Nylon powder was stirred with 3.65N NCl for 40 min. at 40°C and then rinsed with water.

b. Nonhydrolytic cleavage of nylon[9,27] – Nylon powder was refluxed overnight at 70°C with N,N-dimethyl 1,3-propanediamine (neat) and then rinsed with water.

c. Hydrazinolysis[28] – Nylon was reacted with a 6M solution of hydrazine hydrate at 50°C overnight, and then rinsed with water and absolute alcohol.

d. Isocyanate Activation – For about 50 mg. of nylon powder in a test tube add 1 ml of PAPI stock solution and 1 drop of catalyst stock solution. At the end of the reaction time the nylon is filtered and washed 5 times with solvent. The activated nylon is air dried and stored at room temperature.

9. Hornby, W. E., Campbell, J., Inman, D. J. and Morris, D. L. Preparation of Immobilized Enzymes for Application in Automated Analysis. Enzyme Engineering. 2,401-408 (1974)

27. Hornby, W. E., Inman, D. and McDonald, A., The Preparation of Some Immobilized Dehydrogenases and Their Use in Automated Analysis. FEBS Letters, 23,114 (1972)

28. Inman, J. K. and Dintzis, H. M., The Derivatization of Cross-Lined Polyacrylamide Beads, Biochemistry 8, 4074 (1969)

Solution

PAPI stock - 10 gm PAPI made up to 100 ml with methylene chloride (CH_2Cl_2)

Catalyst stock - 0.1 gm DABCO made up to 1 ml with CH_2Cl_2

- 0.1 mg DBT made up to 1 ml with CH_2Cl_2

e. Dimethyl Sulfate Activation – 10 ml of nylon powder was made into a thick slurry with dioxane. The slurry was brought to 100°C with stirring. 10 ml DMS (Aldrich) was added. The powder clumped into large, intractable masses, which could be only partially broken up. After 9 minutes, the powder was rinsed with dioxane and DMF.

D.2 Enzyme Immobilization on Activated Nylon

a. Nonhydrolically cleaved nylon – The reaction scheme was based on the work of Hornby[9]. Nonhydrolically cleaved nylon was taken up in 30% w/v N-ethyl morpholine in ethanol, which contained 0.5% DMA. After shaking at room temperature for two hours, the nylon was rinsed free of DMA, and cholinesterase solution in 0.1M N-ethyl morpholine buffer, pH 8, was added. This was shaken at 4°C overnight.

b. Hydrazide Nylon – The azide group was generated by reaction with $HCl/NaNO_2$. Cholinesterase was then added, and the mixture shaken overnight.

c. Isocyanate Activated Nylon – To immobilize the enzyme, about 50 mg of activated nylon was placed in a small side arm suction flask. About 1.5 ml of enzyme (20 mg) in buffer was added. The flask was placed in a shaking water bath (25°C) and suction was applied for the first half hour of the reaction. The total reaction time was 2.5 hours.

d. Dimethyl Sulfate Activated Nylon – Derivatization of activated nylon with di- or poly-amines. The activated powder was shaken for 2-1/2 hours with a 2M solution of either ethylenediamine (EDA), hexanediamine (HxDA) or polyethylenemine (PEI, 30% aqueous solution, Aldrich) in DMF. The residues were then recovered by centrifugation and washed with distilled water. The EDA and PEI derivatives were very viscous and difficult to filter. The amino-nylon powders were dried at 70° in vacuo overnight.

9. Hornby, W. E., Campbell, J., Inman, D. J. and Morris, D. L. Preparation of Immobilized Enzymes for Application in Automated Analysis. Enzyme Engineering. 2,401-408 (1974)

D.2.1 Immobilization

(a) Glutaraldehyde cross-linking – 30 mg ChE in 3 ml 0.1M phosphate buffer, pH 7.5, were added to 1 ml of HxDA-nylon or PEI-nylon. Acetylthiocholine iodide was added for substrate protection. After shaking for 15 min. at 3°C, glutaraldehyde and sodium cyanoborohydride ($NaBH_3CN$) were added to bring their respective concentrations to 0.83% and 5mM. After shaking at 4° with suction for an additional 1.25 hour, the powder was rinsed with water, 1M NaCl, and buffer.

(b) Dimethyl Adipimidate (DMA) cross-linking – 30 mg ChE in 1.2 ml of 0.1M N-ethylmorpholine buffer, pH 8, was added to 1 ml of HxDA-nylon. An additional 2 ml of buffer, pH 9, was added. After 15 min. shaking, 3 ml of 0.08M DMA in N-ethylmorpholine buffer, pH 9, was added. The reaction was shaken overnight and rinsed as in (a).

APPENDIX E

METHODS CHEMISTRY – POLYACRYLAMIDE

Hydrazide-polyacrylamide, available commercially as Bio-Gel P-150 (Bio Rad Laboratories) was reacted with HNO_2 to form an active azide group. Cholinesterase was then immobilized thereon. The method used was as follows:

300 mg. of dry Bio-Gel P-150 was wetted for 2 hours in 8 ml of 0.1M borate buffer, pH 8.5. These beads were rinsed with 0.6NHCl and suspended in 14 ml of Tris buffer. An azo group was generated by addition of $NaNO_2$ at 4°C. After rinsing the beads with sulfamic acid and borate buffer, they were shaken with 50 mg ChE in 3 ml borate buffer for 2 hours at 4°C.

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APPENDIX F

METHODS CHEMISTRY — MODIFIED STARCH GEL

F.1 Chemical Modification of Starch-OH Groups

F.1.1 Cyanagen bromide

A slurry of 10 g of starch and 20 ml of 5M phosphate buffer (K_2HPO_4 + K_3PO_4) was cooled in an ice bath. An ice cold solution of CNBr (0.3g in 3 ml water) was added drop wise over a 2 minute period. After stirring for another 8 min., the suspension was filtered and the residue washed with ice cold water until the rinsings were at pH 8.7. The starch was then washed with 0.25M sodium bicarbonate and divided into three portions for enzyme coupling.

(a) Approximately 2g starch + 20 mg ChE in 2 ml of 0.25M $NaHCO_3$ (pH 9).

(b) Approximately 2g starch + 40 mg ChE in 4 ml of 0.25M $NaHCO_3$.

(c) Approximately 6g starch + 5 ml ethylene-diamine and 5 ml $NaHCO_3$.

(These were shaken gently at 4° C for 24 hours)

Preparations (a) and (b) were rinsed with: 0.1M borate buffer, pH 8.5 containing 1M NaCl; 0.1M acetate buffer, pH 4.5 containing 1M NaCl; deionized water, and 0.1M Tris buffer, pH 7.4. We then attempted to coat these preparations onto urethane by the Edgewood method, but the starch-enzyme product would not dissolve at 45°C. As a result, a uniform coating could not be achieved.

The thick suspension of preparation (c) was dialyzed overnight against 0.1M phosphate buffer, pH 7.5. This was expected to eliminate excess, unreacted amine while retaining the starch. The starch was then rinsed as (a) and (b) above. Approximately 1g of the starch-CNBr-EDA preparation was suspended in 2.5 ml of Tris buffer. This was then coated on a urethane pad by the Edgewood method (10 mg ChE used for a 4" x 4" x 1/2" pad). Note that the starch-CNBr-EDA did not dissolve, even when in boiling Tris buffer.

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Finally, other samples of the derivatized starch (prep. c) were treated with glutaraldehyde in order to link up appropriately situated -NH_2's in the amino-starch. Three preparations were made as follows:

(d) A starch-enzyme solution was prepared as usual for coating a urethane pad, but before coating, glutaraldehyde was added to bring its final concentation to about 1 percent. This mixture was then coated on the pad.

(e) Pad was coated as usual (i.e. no glutaraldehyde added to mixture). After pad was dried overnight at 35°, it was soaked in a 1.5% glutaraldehyde solution, pH 8, for 2-1/2 hours.

(f) Same as (e), except pad was dried only 30 minutes at room temperature before soaking.

F.1.2 Periodate Oxidation

A procedure was devised, based on the work of Fleche[29], to make aldehyde starch. To a paste of 5g starch in 6.5 ml of 0.05M acetate buffer, pH 3.6, was added a solution of sodium periodate (Sigma S01878) (9.8g $NaIO_4$ in 60 ml buffer). This mixture was stirred for 4 hours. 500 ml of ethylene glycol was added to remove unreacted periodate. The resulting gel was filtered and washed with o.1M borate buffer, pH 8. The gel was divided into two portions for:

(a) Reaction with enzyme — Aldehyde starch was used to coat a urethane pad by the Edgewood method, using 10 or 20 mg ChE for a 4" x 4" x 1/4" pad.

(b) Reaction with ligand:

1. 7.5 ml of ethylenediamine was reacted with the starch for 2 hours. The product was dialyzed overnight to remove unreacted amine, and then concentrated with an Amicon ultrafiltration cell.

2. 10 g of PEI (30% solution in water) and 10 ml of 0.1M buffer, pH 8, were reacted with the starch as in 1. Because of its viscosity, the product was impossible to filter or concentrate after dialysis. Both products were then reacted with ChE and approximately 1% glutaraldehyde for 2 hours (after which time, the solutions had turned orange-yellow in color). Urethane pads were coated with the products.

29. Fleche, G. Die Starke, 20,50 (1968).

F.1.3 S-Triazine Trichloride (s-TT)

A 5% solution of s-TT in dioxane-xylene (1:1) was added to starch which had been soaked in 0.01M NaOH for 15 minutes. The mixture was stirred for 30 minutes and then washed with acetic acid/H_2O dioxane (1:1:2). Part of the modified starch was prepared for storage by washing with acetone and drying in vacuo.

The remaining starch was divided into two batches:

(a) The starch slurry was brought to boiling and coated on a urethane pad. After drying, the pad was soaked in a 1.2 mg/ml ChE solution for 1 hour; the enzyme solution was absorbed by the pad.

(b) The starch was reacted with ChE at pH 5.5 for 1 hour. Light flocculent particles formed which were difficult to filter. Unreacted groups were capped by reaction with NH_4OH buffer (1M NH_4Cl/NH_4OH, pH 8.5) for 30 minutes. The paste was rinsed with NaCl and buffer. It was heated to 45°C for 10 minutes, and although it did not dissolve, was coated on a urethane pad.

F.2 Cross-linking of Enzyme Entrapped in Starch

Four urethane pads were coated by the Edgewood method. After overnight drying, the 1 x 1-1/2 pads were immersed in one of three cross-linking agents and soaked overnight:

1. Soaked in 10 ml of PAPI (10% solution in methylene chloride) and 0.5g DABCO. (It was determined previously that DABCO did not affect ChE activity adversely.) Excess PAPI was washed out with Ch_2Cl_2.

2. Soaked in 1.2% glutaraldehyde with 8mM sodium cyanoborohydride.

3. Soaked in 10 ml of 0.1M phosphate buffer, pH 7.5, containing 4.5 mg dimethyladipimidate·2HCl.

The three pad preparations were washed with 2M KCl and with Tris buffer.

APPENDIX G

METHODS CHEMISTRY – URETHANE

SAFETY NOTE – BECAUSE OF THE EXTREME TOXICITY OF THE CHEMICALS USED IN THESE PROCEDURES, ALL REACTIONS MUST BE RUN IN A WELL VENTILATED HOOD. RUBBER GLOVES AND A RESPIRATOR SHOULD BE WORN WHILE HANDLING THESE MATERIALS. DISPOSAL OF THE WASTE LIQUIDS SHOULD CONFORM TO SAFETY REGULATIONS.

To obtain highly active and stable immobilized cholinesterase on urethane foam requires four basic operations: activation of the urethane, further derivatization of the activated urethane, covalent binding of the enzyme to the derivatized foam and enzyme stabilization by cross-linking. We have obtained active foam pads when step two is omitted, when steps one and two are both omitted, or when step three is omitted; that is, when the enzyme is immobilized by cross-linking only. These preparations, when compared with the best preparations, are either less active, less stable, or both.

This Appendix differs in format from the preceding ones in that all four of the important operations mentioned in the preceding paragraph are adequately described by the two best procedures for activating, chemically modifying and immobilizing enzyme on urethane foam. In the first procedure all reactions are carried out on batches of foam in a flask or other reaction vessel. In the second method the foam pads are packed in a column and the reactions are carried out by pumping the reagents through the packed column. Other operations mentioned in the urethane activation results and discussion section, but not covered by these two procedures, are, with one exception, described in the other Appendices on glass beads or nylon.

G.1 Best Methods for Urethane Pads

As mentioned above, we have obtained active immobilized enzyme by two different routes, batch and column. A detailed procedure for the best immobilized cholinesterase produced by each route is described below.

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G.1.1 Batch Method

This procedure produced the most stable and most active pads we have obtained.

G.1.1.1 Materials

3 neck, 3 liter-round-bottom flasks (ȝ 24/40)
Water condenser (Liebig-300mm, ȝ 24/40)
Stirrer with Teflon blade (ȝ 24/40)
Variable speed stirrer
Stoppers (ȝ 24/40)
Nitrogen Tank and Regulator (dry)
Rubber Gloves (Neoprene)
Respirator (organic vapors)
Vacuum Oven
Fleaker - 500 ml
Drying Tube (for condenser)

G.1.1.2 Chemicals

Z foam 45 ppi - Scott
Methylene Chloride (ACS) dried with Mol. Sieve-4A
PAPI-135 (Polymeric Isocyanate) - Upjohn
Tolylene - 2,4 diisocyanate (5 ml ampoule) - Polysciences, Inc.
DABCO (1,4-Diazabicyclo[2.2.2]octane) - Aldrich Chem. Co.
Cholinesterase (C-7512) - Sigma Chemical Co.
Polyethyleneimine (1800 mol. wt.) - Polysciences, Inc.
Molecular Sieve - 4A (Linde)
Potassium phosphate buffer - 0.1M, pH - 6.5 (0.1% benzoate)

G.1.1.3 Procedure

(1) Preparation of urethane – Cut the urethane foam (45 ppi) into 10 - 2"x2"x1/4" pads, and dry in a vacuum oven (30" Hg.) at 30°C for sixteen hours.

Note: The foam does not have to be washed but it does have to be dry. A drying method other than a vacuum oven may also be used.

(2) Activation of Urethane Foam — Into a clean 3-liter flask add 1.0 liter of methylene chloride (dried), plus 100g of PAPI-135. Add the ten dried urethane pads. Add 1.0 gm of DABCO. Stir at room temperature for four hours. Decant the liquid and wash six times with methylene chloride (250 ml/wash). Washing of the pads is performed in the original 3-liter flask. Drain all liquid.

Note: This reaction can be carried out in any container (test tube, beaker, or flask) of a size appropriate for the amount of urethane used and which offers some protection from moisture in the air.

If for some reason the process must be stopped at this point, the activated pads must be protected against moisture from the air until after the derivatization step (3). We find that we can easily get through the enzyme immobilization step in an eight hour day.

(3) Derivatization of Activated Foam — To the 3-liter flask with pads, add 1.0 liter of methylene chloride (dried). Add 50 grm. of polyethyleneimine (1800). Stir at room temperature for one hour. Decant the liquid and wash six times with methylene chloride. Drain all liquid. Dry the pads, while in the flask, with nitrogen (dry).

(4) Further Derivatization and Attachment of Spacer Arms — Transfer the dry pads to a Fleaker. Add 250 ml of glutaraldehyde (2.5% soln.) to the pads, and shake for ten seconds. Decant rapidly and wash quickly with water (D.I.), ten times. Drain the excess liquid, and dry between paper towels.

Note: For this step, any container can be used which can be stoppered but allows rapid addition and rapid decantation of the reagents. Timing in this step is critical. A contact time of more than 10 seconds causes deterioration of the physical structure of the foam.

(5) Enzyme Coating and Immobilization — To each 2"x2"x1/4" pad, add 4.0 ml of cholinesterase solution (2.0 mgm./ml H_2O). Spread the ChE solution evenly on both sides of the pad, by kneading on a flat glass surface, with a glass rod. Air-dry the pads on a support of glass rods, for two hours. Transfer the pads (and its support) to a 30°C circulating oven, and dry for sixteen hours.

Note: We find that we can easily get through to this stage of the procedure in one day. The drying step can then take place overnight which allows us to complete the

entire preparation before noon of the following day. The drying time can be shortened but the pads must be as dry as possible to avoid side reactions with the water during the cross-linking operation.

(6) Cross-linking – Place the ten dried pads in a clean 3-liter flask and add 1.0 liter of methylene chloride (dried). Add carefully the contents of a 5 ml. ampoule of tolylene diisocyanate (highly toxic). Add 0.050 gm of DABCO. Stir at room temperature for one hour, then decant the liquid. Wash six times with methylene chloride. Dry the pads while still in the flask, with nitrogen (dried).

Note: Handle the tolylene diisocyanate carefully. Do not bypass any of the safety precautions outlined above. The material is highly toxic and has an appreciable vapor pressure.

(7) Final Wash – Transfer the dried pads to a 500 ml Fleaker and wash ten times with 1M NaCl solution. Wash five times with H_2O (D.I.). Wash five times with 0.1M potassium phosphate buffer, pH = 6.5 (0.1% benzoic acid). The enzyme pads can be stored in this buffer in the refrigerator or they can be dried after the water wash and stored dry.

Note: The 1M NaCl solution washes out any enzyme which is not covalently coupled to the urethane but which is held by physically adsorption. The vessel used for this step should be similar to that in step (4).

G.1.2 Column Method

Our application of this procedure showed that the method might be optimized to produce pads as good as those obtained by the batch method. We could not bring this method along as rapidly as the batch method because of the large number of assays which result from the number of pads in each column.

The procedure which follows produced the best pads we obtained from the column method.

G.1.2.1 Materials

Glass column 10 mm ID x 15 cm
Peristaltic pump - variable speed
Silicone rubber pump tubing
Polypropylene connectors (for column and tubing)
Vacuum oven
Vacuum pump
Cold traps
Dry ice
Recirculating blower - 30°C
Beakers – various small sizes (below 150 ml)
Ernlenmyer flasks, small sizes (25, 50, 125, 250)
Rubber stoppers
Plastic film wrap
Tank dry nitrogen and regulator
Rubber gloves (neoprene)
Respirator (organic vapors)

G.1.2.2 Chemicals

Z foam - Scott
Chlorobenzene (ACS) - dried with mol. sieve 4A
PAPI-135 (polymeric isocyanate) - Upjohn
DABCO (1,4-Diazabicyclo[2.2.2]octane - Aldrich Chemical Company
Molecular sieve - 4A (linde)
Polyethyleneimine (1800 mol. wt.) (PEI) Polysciences, Inc.
Acetone (ACS)
Cholinesterase (C-7512) - Sigma Chemical Co.
Tolylene - 2,4 diisocyanate (5 ml ampoule) Polysciences, Inc.
Dibutylamine ACS
Potassium phosphate buffer - 0.1M, pH 6.5 (0.1% benzoate)

G.1.2.3 Procedure

(1) Packing the Columns – Cut 27 disks from a sheet of 1/4" thick Scott Z-foam, 45 ppi, with a #7 cork borer. Stack them into the glass column loosely. The column I.D. should be such that the pads fit snugly against the glass walls but the pads should not be compressed longitudinally in the column. The column is capped with inert connectors, attached via silicone rubber tubing to the pump output and set up vertically for upward flow. The input to the pump is connected via small bore silicone rubber tubing

to the solution reservoir. An Ernlenmyer flask, because of the small neck, is an ideal reservoir. For any step use the minimum size flask which will contain the reagent. A slow flow of Nitrogen gas is introduced through a small bore polyethylene tube. This serves both to stir the solution and to protect the reactive reagents from moisture in the air. To change the solution going through the column of pads, simply transfer both tubes from one flask to another. The effluent from the column is allowed to flow back to the solution reservoir through silicone rubber tubing.

Note: The pads are individually positioned in the column using a blunt end glass rod or a dowel stick.

(2) Activation of Urethane — A solution of 2.7 grams of PAPI (100 mg PAPI/disk) in 100 ml of dry cholorobenzene (C_6H_5Cl) was placed in the reservoir and the pump started. The solution is recirculated through the column for two hours at a flow rate of 1 ml per minute. The reaction is stopped by first directing the column effluent to waste and then pumping C_6H_5Cl through for 10 minutes at 10-15 ml/min followed by 30-40 minutes at a flow of 5 ml/min.

Note: PAPI is toxic, handle with care.

(3) Derivatization of Activated Foam and Attachment of Spacer Arms — A solution of 32 grams of PEI in about 200 ml of dried C_6H_5Cl is then recirculated through the column for 1 hour at a flow rate of 5 ml/min. The excess PEI is then washed out with acetone for 10 minutes at 10 ml/min followed by water (D.I.) at first for 10 minutes at 10 ml/min and then for 30 minutes at 5 ml/min. The washings are run out to waste.

Note: The acetone is the transition solvent between C_6H_5Cl and water, which will be used in the next stage.

(4) Enzyme Adsorption — Recirculate a solution of 10 mg of ChE in about 30 ml of water through the column for one hour at 5 ml/min. The enzyme physically adsorbs to the PEI spacer arm. The column is then disconnected from the recirculation apparatus, the excess enzyme solution drained and it is placed in a vacuum oven at 30" Hg vacuum and 40°C for one hour. Any remaining water is then allowed to evaporate by placing the column in a recirculating oven at 30°C overnight.

(5) Cross-linking and Covalent Binding of Enzyme — The column is reattached to the recirculating pump apparatus and a solution of 5 grams of TDI in 100 ml of dried C_6H_5Cl is recirculated through the column for two hours at 1 ml/min. Excess TDI is washed out with dry C_6H_5Cl pumped through at 10 - 15 ml per minute for 10 minutes. The unreacted isocyanate groups on the pads are then capped off by recirculating 50 ml of a DBA solution (200 µl DBA in 50 ml of dry C_6H_5Cl) for about 30 minutes at 5 ml/min.

(6) Final Wash — The final wash sequence consists of an acetone wash (10 min. at 10-15 ml/min); a water wash (10 min. at 10-15 ml/min); a 1M NaCl wash (5 min. at 10 ml/min followed by 30 minutes at 1 ml/min), and a D.I. water wash (15 min. at 5 ml/min). The pads can then be stored in 0.1M phosphate buffer, pH 6.5 (0.1% benzoate) or they can be stored dry.

G.2 Other Operations

This Section describes the methods and chemicals which are mentioned in the Results and Discussion Section but which have not been included in the procedures which were detailed in Section G.1, above.

G.2.1 Activation

G.2.1.1 Reflux activation. The procedure is the same as described for the Batch Method, Step (2), except that the reaction vessel, equipped with a reflux condenser, is heated gently. To obtain the different reaction temperatures, we have used methylene chloride, choloroform, or chlorobenzene as reaction solvents.

G.2.1.2 Isocyanate activation. To activate urethane foam with isocyanates other than the PAPI, simply use the same procedure but replace the PAPI with an equal weight of the desired isocyanate.

G.2.2 Cross-linking

G.2.2.1 Glutaraldehyde cross-linking. Using foam derivatized with amino groups, the procedure for glutaraldehyde cross-linking is exactly as described for glass beads in Appendix C (C.3.b or C.4.a). The method described in C.4.a, one pot method, was also used with foam which was not previously activated.

G.2.2.2 Isocyanate cross-linking. The basic diisocyanate cross-linking methods are described in paragraphs G.1.1.3(6) and G.1.2.3.(5). As pointed out in Section G.2.1.2, to use a diisocyanate other than TDI in the above methods, simply replace the TDI with an equal weight of another diisocyanate.

G.2.3 Enzyme Immobilization

G.2.3.1 Direct method. In this method the enzyme is covalently bound directly to the isocyanate activated foam, that is, steps G.1.1.3(3) and G.1.1.3(4) for the Batch Method or G.1.2.3(3) for the Column Method are omitted from the overall procedure.

G.2.3.2 Multiple enzyme layers. To get multiple layers of enzyme onto the foam, it is not necessary to go through the entire procedure from the activation step, although we did reactivate at the beginning of this work. For the Batch Method (G.1.1) it is only necessary to go back to paragraph G.1.1.3(5) In the Column Method (G.1.2) it is only necessary to start the second layer with paragraph G.1.2.3(4). In many of the early multilayered preparations we also omitted the exhaustive wash step (G.1.1.3(7) or G.1.2.3.(6)) between the enzyme layers.

APPENDIX H

METHODS – STABILITY TESTS

H.1 Storage Stability Test at -40° or 65°C for 2 Weeks

A 9mm disk is cut from the ChE treated foam pad. It is washed five times with distilled water, excess water removed on a paper towel and then placed in an oven at 30°C for three hours. The test disk is then placed in a 5 ml serum vial which is capped with a septum and aluminum retaining ring. A small hypodermic needle (28 ga) is inserted through the septum and evacuated for 5 minutes against a vacuum of 30" Hg. The needle is removed while the pump is still running. The vial is then placed in the test chamber (either -40° or 65°C) for two weeks. At the end of the test the sample is allowed to come to room temperature and then assayed as described in Appendix A.

The drying step can be omitted for the cold storage test but must be used for the 65°C storage test. Even traces of water can accelerate the inactivation of the enzyme.

H.2 Description of Flow Test Apparatus

A test apparatus was assembled to simulate the temperature and flow conditions of the Enzyme Alarm. This test set up enabled us to evaluate the stability of the various enzyme/support products at the high temperature extreme expected in the field.

The flow cell was fabricated from acrylic plastic. A photograph of this cell is shown in figure H. In use the cell is mounted in a temperature controlled oven and air, pre-heated to 50°, enters at the top of the cell. Its flow is monitored by a flow meter at the oven exit. The substrate-buffer solution is pumped through 2 feet of 1/16" I.D. silicon rubber tubing inside the oven to bring it to 50° $\pm$ 5°C before entering the cell from the right. The two streams meet and the air, now mixed with substrate solution, is pumped through the immobilized enzyme pad at the bottom of the cell by application of vacuum to the exit line. Three thermocouples monitor temperature of the incoming air, the oven enclosure and the exit stream. These temperatures are continuously recorded. The exit stream thermocouple is mounted within about 5 mm of the enzyme sample.

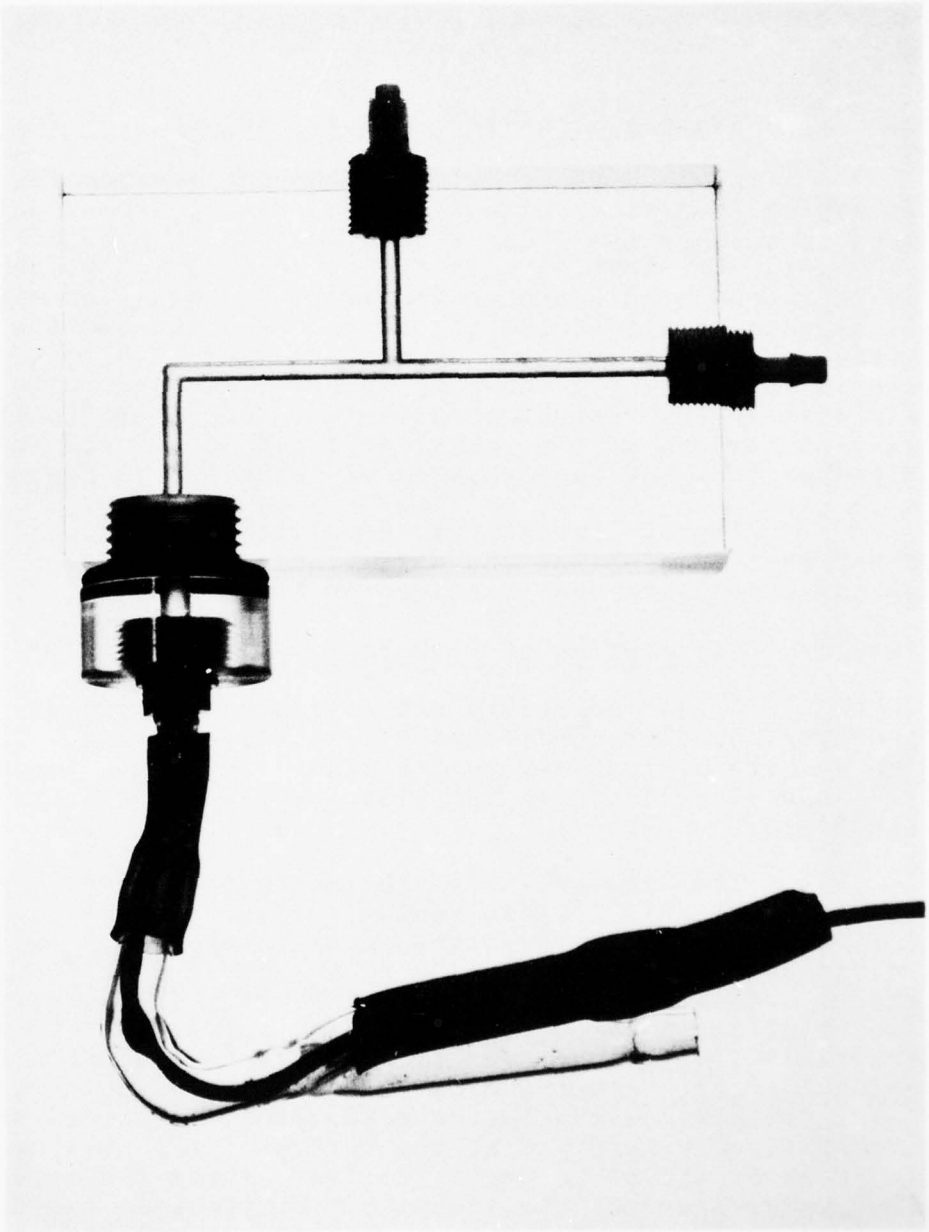

Figure H. Photograph of Flow Cell

Urethane pads coated with enzyme or containing chemically bound enzyme were punched out into 10mm disks to fit into the flow cell. When glass beads or nylon particles were to be tested, a urethane disk was hollowed out and the beads (about 50 µl) packed into the indentation. (80 ppi urethane was used to retain 80/120 mesh beads.) We found that a disk of 100µ nylon net placed at each end of the flow chamber ensured retention of the beads inside the cell.

With liquid flowing, the temperature of the exit stream is between 30° and 40°C. The temperature drop is due to evaporative cooling. With no liquid flow the temperature of the exit air remains at 50°C. Since this system closely duplicates the conditions in the Enzyme Alarm, we assume that this temperature drop (15° to 20°C below ambient) is typical of actual operations.

Although the specification in the contract calls for a 12 hour test, we found it more convenient to run for 16 hours. We required 50% retention of activity to pass the test. At the end of the test, the sample was assayed.

APPENDIX I

MATERIALS SOURCE LIST

1. Acetone - Fischer Scientific Company

2. Butyrylthiocholine iodide: Nutritional Biochemicals or Sigma Grade II.

3. Carbon Tetrachloride - Mallinckrodt Chem. Works

4. Cholinesterase (horse serum): Sigma, Type IV.

5. Chlorobenzene - Fischer - Lot #740748.

6. 1,4 Diazobicyclo[2.2.2]octane 97% – Dabco Aldrich Chem. Co., Inc. - Lot #05094

7. Dibutylamine - Eastman Chemical Company.

8. Dibutyltin Diacetate – DBT – Eastman Chemical Company.

9. Dichloromenthane – Fischer Scientific Company and Matheson, Coleman and Bell.

10. Dimethyl adipimidate, dihydrocholoride – DMA – Pierce Chemical Company.

11. N,N-dimethylformamide – DMSO – Fischer Scientific Company, Lot #78303.

13. 1,4 – Dioxane – Fischer Scientific Company, Lot #734353.

14. Diphenylmethane diisocyanate – DPMDi – Polysciences, Inc., Lot 2L-227.

15. 5,5′ – Dithio-bis (2-nitrobenzoic acid) - Pierce Chemical Company.

16. 1 – Ethyl-3 -[3-dimethylaminopropyl]carbodiimide - Ott Chemical Company.

17. Ethylenediamine – EDA – Matheson, Coleman & Bell.

18. Formic Acid, 90% - Fischer Scientific Company.

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19. Hypol, Foamable Hydrolphilic Polyurethane Polymer, (FHP 3000 #999990402, FHP 2000 #999990403) - Dewey and Almy Division, W. R. Grace & Company.

20. Nylon — Nylon beads: Nylon 65 - Aldrich Chemical Company.

21. PAPI - Upjohn Company - polyisocyanate.

22. Phenol liquified - Fisher Scientific Company Lot #723241.

23. Polyethyleneimine — PEI - Aldrich Chemical Co., Inc.

24. *Sodium cyanoborohydride - Alfa Inorganics, Ventron.*

25. Standard HCl 0.1N - Fischer Scientific Company.

26. Tolylene 2,4 diisocyanate - Polysciences, Inc.

27. Urethane Foam - Scott's Z Foam 45, 60 and 80 PPI - obtained from J. M. McClintock & Company.

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