I. PRODUCTION OF HYDROCARBON ADHERING DERIVATIVES OF WATER SOLUBLE ENZYMES

II. CROSS-LINKING OF ENZYMES BY PHOTOCHEMICAL REAGENTS

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LIST OF SYMBOLS AND ABBREVIATIONS

DDI	A saturated aliphatic 36 carbons diisocyanate
HMDIC	Hexamethylene diisocyanate
FNPA	1-Fluoro-2-nitro-4-azidobenzene
L-Asnase	L-Asparaginase
L-Glnase	L-Glutaminase
L-Asn	L-Asparagine
L-Gln	L-Glutamine
CPA	Carboxypepeptidase A
AME	Arginine methyl ester
Glass-NPA	Glass-nitrophenylazide
Cellulose-NPA	Cellulose-nitrophenylazide
MB-Asnase	Matrix bound asparaginase
IU	International unit(s) of activity

PART ONE

PRODUCTION OF HYDROCARBON ADHERING DERIVATIVES OF WATER SOLUBLE ENZYMES

CHAPTER I

INTRODUCTION

Large bodies of oil layered on water surfaces constitute a major pollution problem challenging our scientific and technological ingenuity.

The ubiquitous microbe is providing man with some ideas and tools for possible solutions to this problem. Some possible problems associated with microbial degradation of oil slicks are:

(1) viability of microorganisms after storage and application

(2) establishment and maintenance of contact with the oil

(3) provision of optimal growth conditions, such as nutrients and temperature

(4) secondary contamination, such as biomass supporting alga bloom and toxin production

We might bypass these potential problems by isolating and using the hydrocarbon oxidizing enzymes from microbial cultures grown on petroleum diets. Since enzymes are the catalysts responsible for the metabolic chemistry of the microbes, relatively small weights of material may be needed to produce rapid oxidation of the hydrocarbons to water soluble products. These water soluble products will be diluted rapidly by the natural water movement.

An early goal of this research program was the development of techniques for producing catalytically active derivatives of water soluble enzymes which would adhere to oil-water interfaces where the oxidative reactions can occur. A chemical modification procedure for accomplishing this goal is the subject of this part of the thesis.

The rationale for our approach to this problem is as follows: Only a small fraction of the total mass of enzyme molecule is intimately involved in the binding and catalysis of substrate molecules. The greater portion of the polypeptide chain material may function primarily by influencing the interactions between the active site and the components of the environment in which the enzyme functions. A worthy goal would seem to be modification or "tailoring" of the enzyme molecule to better adapt it for functioning in a chosen environment. The attachment of lipophilic or hydrocarbon-soluble side chains to the hydrophilic exterior of water-soluble "model" enzymes was our approach to this problem. We chose bifunctional reagents for the possible stabilization of the enzyme molecule by the synthetic intramolecular cross-links which can be introduced in this manner. We chose isocyanate functional groups for the hydrocarbon-soluble reagents because of their reactivity with hydrophilic protein functional groups likely to be on the surface of enzyme molecule, under mild condition of pH and temperature, to produce a substituted urea derivative in the case of amino group reaction.

CHAPTER II

MATERIALS AND EXPERIMENTAL PROCEDURE

Materials:

The reagent of choice for this modification is DDI brand Diisocyanate 1410, a product of General Mills Chemicals, Inc., containing a 36 carbon saturated aliphatic hydrocarbon chain connecting two isocyanate reactive groups. Hexamethylene diisocyanate (1,6-diisocyanatohexane, HMDIC) was obtained from Aldrich Chemicals. Hen egg white lysozyme (Sigma Grade I), bovine pancreatic ribonuclease (Type I-A), wheat germ lipase (Type I) and hog pancreatic lipase (steapsin, crude) were obtained from Sigma Chemical Company. Olive oil (Best, U.S.P., Lot 714498) was obtained from Fisher Scientific Company. Phenylmethylsulfonylfluoride (B Grade) was obtained from Calbiochem. The other chemicals used were Reagent or Certified Spectroanalyzed grade.

Experimental Procedure:

The dry commercial enzyme preparations were dissolved in buffered aqueous solvents, of the chosen reaction pH, to a protein concentration convenient for measurement by absorbance of ultraviolet light (280 nm)

in a 1 cm cuvette (0.3-1.0 mg/ml). Phenylmethylsulphonylfluoride was added (to 0.001 M) to the lipases in order to protect them from inactivation by proteolytic contaminants. The isocyanate reagents were diluted to 0.01 M in the chosen hydrocarbon (usually n-heptane). The volume ratio of organic to aqueous phase routinely used for the reaction mixture was 0.10 ml to 3.0 ml. The ratio of reagent to protein routinely used was 100-200 micromoles per gram protein. In order to minimize the time required for reaction between the diisocyanate in the organic phase with the protein in the aqueous phase, the interface area was increased by brief (about 30 sec) ultrasonic vibration at 0°C to produce a fine emulsion. The emulsion was then stirred at room temperature for 3 hr. The emulsion was subjected to centrifugation at 145,000 x g (average force) for 20 min at 4° or 22°C in order to measure the resulting distribution of protein between the aqueous and emulsion phases. The plastic centrifuge tubes were then punctured at the bottom to allow collection of aqueous fractions and emulsion region for protein and catalytic activity assays. When a film of undissolved material collected at the top of the liquid during centrifugation, this fraction was resuspended in aqueous buffer for measurement.

The rate of reaction between the isocyanate groups and the primary amino groups on the protein (measured by decrease in ninhydrin color yield with reaction time) was found to be much faster than the

rate of adsorption of protein (measured by loss of protein from aqueous phase) in the reaction mixture to the organic interface. In those reactions subjected to rate measurements, the latter process was essentially completed by 3 hr of stirring at room temperature.

REACTION TUBE FOR ENZYME MODIFICATION WITH DIISOCYANATE

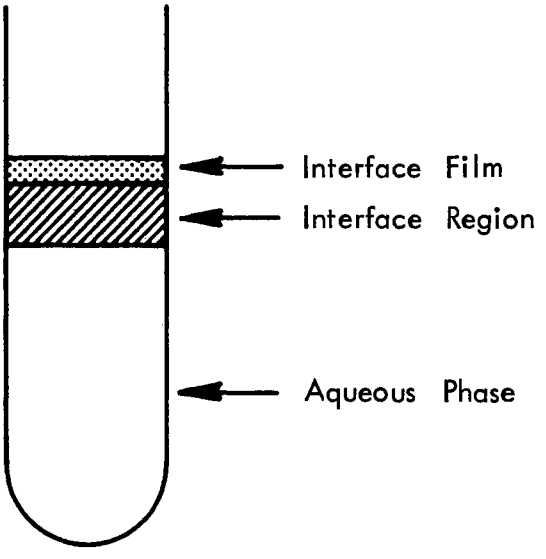

FIGURE 1

CHAPTER III

RESULTS AND DISCUSSION

Representative aqueous phase protein (A_{280}) recovery data from lysozyme treated by this procedure is presented in Table I. While the n-heptane alone caused loss of 44 percent of the lysozyme from the aqueous phase, 64 percent of the hexamethylene diisocyanate-reacted lysozyme and 77 percent of the DDI-reacted lysozyme had been moved from the aqueous phase. Catalytic activity data are not reported for lysozyme since aliphatic hydrocarbon binding to this enzyme causes inhibition (1).

Catalytic activity and protein recovery data from two representative experiments with ribonuclease are presented in Table II. The activities were measured with deproteinized, dialyzed yeast nucleic acids as substrate (2). Experiment B was done with cytidine-2'(3')-monophosphate (10 moles/mole RNase), a competitive inhibitor, mixed with the enzyme in an attempt to improve the catalytic activity yield of the modified enzyme. Removal of protein from the aqueous phase was extensive (51 percent, 63 percent) in the DDI-RNase reaction mixture and slight with heptane-RNase control mixture (4 percent, 13 percent). The catalytic activity recoveries in the modified enzyme

TABLE I

PROTEIN RECOVERIES IN AQUEOUS PHASE FROM LYSOZYME TREATED WITH n-HEPTANE AND ALIPHATIC DIISOCYANATES

Rxn.	n-Heptane	Reagent	Protein Recovery in Aqueous Phase
I	75 λ	-	56%
II	65 λ	10 λ_{DDI}	23%
III	65 λ	10 λ_{HMDIC}	36%

Three milliliter volumes of lysozyme (0.3 mg/ml) in 0.01 M sodium borate, pH 9.0, were treated with (I) n-heptane alone, (II) DDI (0.01 M) in n-heptane and (III) hexamethylene diisocyanate (0.01 M) in n-heptane. Emulsion was formed by ultrasonic vibration and separated by ultracentrifugation. Protein concentration was measured by A_{280} on Beckman DU.

TABLE II

PROTEIN AND ACTIVITY RECOVERIES IN AQUEOUS PHASE FROM RIBONUCLEASE TREATED WITH n-HEPTANE AND ALIPHATIC DIISOCYANATES

Rxn.	n-Heptane	Reagents
I	75 λ	-
II	50 λ	25 λ DDI
III	50 λ	25 λ HMDIC

	A			B		
	I	II	III	I	II	III
Activity Recovery in Rxn. Mixture	92%	53%	54%	97%	57%	62%
Activity Recovery in Aqueous Phase	95%	55%	48%	97%	54%	45%
Protein Recovery in Aqueous Phase	96%	49%	75%	87%	37%	66%

Three milliliter volumes of ribonuclease (0.5 mg/ml) in 0.01 M sodium borate, pH 9.0, were treated with (I) n-heptane alone, (II) DDI (0.01 M) in n-heptane and (III) hexamethylene diisocyanate (0.01 M) in n-heptane in the manner noted in Table I. Activity measurements were made with purified yeast nucleic acids (2). Protein concentrations were measured by A_{280} in Exp. A. In Exp. B, cytidine-2'(3')-monophosphate (10 moles/mole RNase) was included in the rxn. mixture. Protein concentration was measured by Biuret-phenol color reaction.

reaction mixtures were all low (53 percent to 62 percent) compared to the control (92 percent, 97 percent). The presence of the competitive inhibitor protected the reaction mixtures only slightly against catalytic activity losses. Most of the activity of the reaction mixtures was found in the aqueous phase after separating the emulsion. An attempt was made to examine the possibility that the activity losses were due largely to inaccessability of the active site on the hydrocarbon-adsorbed enzyme to the water-soluble substrate rather than to a denaturation or inactivation of the catalytically active site by the chemical modification.

Evidence pertinent to this question was gained by comparing the effect of the DDI treatment on the recovery and distrubition of activity from two functionally similar enzymes which differ in the water-solubility of their substrates (i.e., one with a water-soluble substrate and the other with a hydrocarbon-soluble substrate). Table III contains the activity recoveries of wheat germ lipase (3a) with water soluble triacetin (glycerol triacetate) substrate and hog pancreatic lipase (3b) with olive oil (hydrocarbon soluble) substrate. The DDI reagent showed little effect on the recovery of activity of wheat germ lipase with water-soluble substrate. The interface region exhibited lower effective concentration. The catalytic activity of hog pancreatic lipase preparation, on the other hand, was found to be concentrated (140 percent of initial concentration) in the interface region by the

TABLE III

ACTIVITY RECOVERIES AND DISTRIBUTION
FROM LIPASES TREATED WITH OLIVE
OIL AND DIISOCYANATE

(a) Wheat Germ

	Activity Recoveries**					
	I		II		III	
Sample	(%)	(U/ml)*	(%)	(U/ml)*	(%)	(U/ml)*
Initial Aqueous Solution	100	116				
Reaction Mixture	43	50	37	43	38	44
Aqueous Phase	34	52	34	53	27	42
Interface Region (0.5 ml)	9	41	8	37	6	28
Resuspended Interface Film	-	-	1		4	
Total Recovery in Fractions	43		43		37	

(b) Hog Pancreatic

	Activity Recoveries**					
	I		II		III	
Sample	(%)	(U/ml)*	(%)	(U/ml)*	(%)	(U/ml)*
Initial Aqueous Solution	100	28				
Reaction Mixture	67	19	124	34	77	22
Aqueous Phase	81	23	33	14	14	6
Interface Region (1 ml)			46	39	76	64
Resuspended Interface Film			23		6	
Total Recovery in Fractions	81		102		96	

TABLE III (Continued)

Three-milliliter volumes of lipases (0.5 mg/ml) in 0.01 M potassium phosphate buffer, pH 8.0, plus 0.001 M phenylmethylsulfonyl-fluoride were treated with (I) no additions, (II) 0.1 ml olive oil alone, and (III) DDI in olive oil (0.1 ml), in the manner noted in Table I. Catalytic activity measurements were made in a pH stat (Radiometer), measuring rate of acid release from triacetin by wheat germ lipase (a), and from olive oil emulsion by hog pancreatic lipase (b) (Worthington Biochemical Corporation assay).

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* Concentration of active enzyme.

** Percent of total activity of original aqueous solution.

water insoluble substrate (olive oil) alone (3). A still more extensive concentrating effect in the interface region was seen with DDI derivative (164 percent of the activity concentration found with the unmodified enzyme subjected to the same conditions, 229 percent of the initial concentration).

These preliminary measurements have indicated that the DDI-enhanced transfer of protein to the hydrocarbon interface region results in quite a stable or enduring interaction. Attempts to redissolve the DDI-modified proteins in aqueous buffer were unsuccessful. Equilibrating the olive oil emulsion region containing hog pancreatic lipase with fresh aqueous buffer resulted in significant transfer of the catalytic activity back into the aqueous phase from unreacted protein, whereas little or no catalytic activity could be returned to the aqueous phase from the DDI-reacted protein in the interface region.

These data indicate the feasibility of tailoring enzymes to function as catalysts in chosen environments. The addition of hydrocarbon-soluble side chains to water-soluble enzymes produced a marked enhancement of their tendency to adhere to water-hydrocarbon interfaces. The enzyme which catalyzes the hydrolysis of water-insoluble substrate was hindered only slightly in catalytic function by this chemical modification (4).

Summary

A chemical modification procedure has been developed with egg white lysozyme, bovine pancreatic ribonuclease, wheat germ lipase and hog pancreatic lipase by which 50 to 80 percent of the protein adheres to a water-hydrocarbon interface in a stable emulsion. Up to 85 percent of the catalytic activity against hydrocarbon-soluble substrates was retained in the emulsion phase. This definite enhancement of the tendency of these water-soluble enzymes to adhere to hydrocarbon interfaces was effected by reaction with saturated aliphatic hydrocarbons containing isocyanate groups on each end.

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PART TWO

CROSS-LINKING OF ENZYMES BY PHOTOCHEMICAL REAGENTS

CHAPTER I

INTRODUCTION

The purpose of this introduction is to give a general view of the design, use, and advantages of photo-generated reagents for cross-linking of enzymes as compared with the other methods (1).

Most of the reactions in biological sciences involve the interaction of a small organic molecule, generally called the ligand, with a macromolecule called the receptor. One likes to know how the ligand and receptor interact at the molecular level.

The large size of the receptor makes the spectroscopic techniques less sensitive. The interactions between receptor and ligand are generally reversible and hence the need of having high concentration of ligand decreases the utility of these methods. Moreover these techniques can hardly identify the actual constituents of the binding or active site of the receptor.

Reagents have been prepared which keep the specificity of interaction of the ligand with the receptor and have a chemically reactive group which can tag the active site (2-4). Such reagents have been called "affinity labels" or "active site directed irreversible inhibitors (3)."

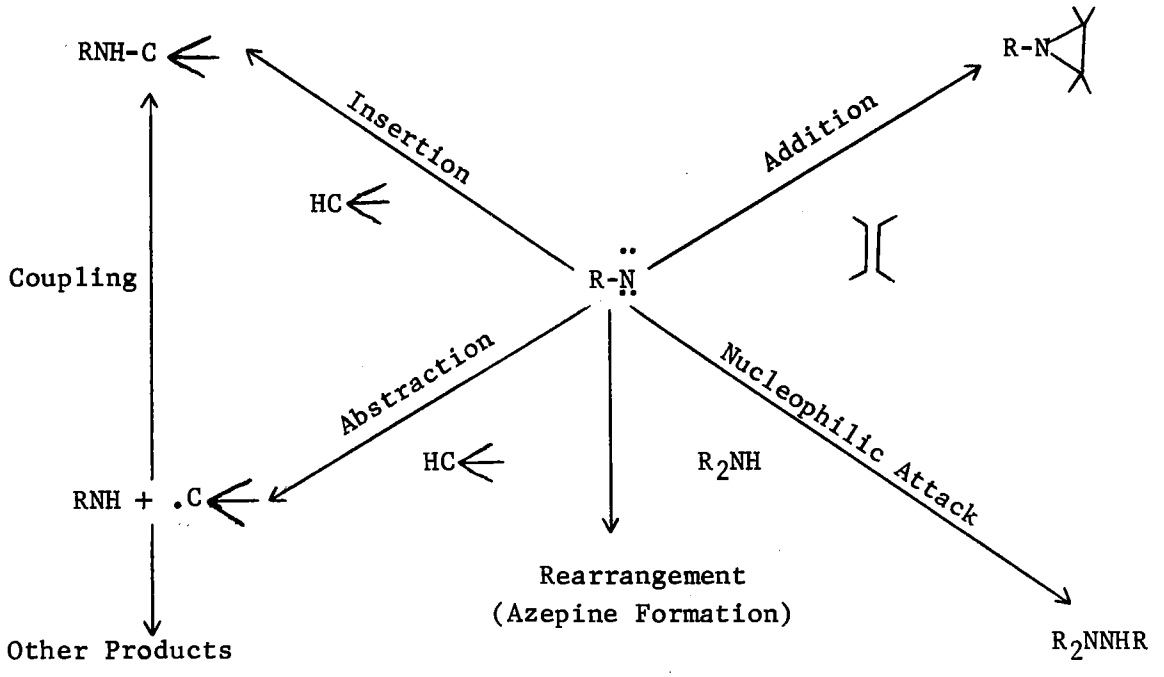
However, most of these reagents have the limitation of requiring nucleophiles for reaction at the active site of enzymes (1-5). It may even be dangerous to use such reagents selective for nucleophiles, since the affinity labeling technique relies on local concentration differences in the reactants. False results may be obtained if the nearest accessible nucleophile is not in the active site. Moreover, any reagent which is to be used in aqueous solution is limited by the fact that water is more reactive than many of the functional groups in amino acid side chains. Furthermore, if the technique of active site labeling is to be generalized, reagents must be designed which are able to insert even in carbon-hydrogen bonds of the constituents at the receptor site. Such common chemical species are only carbenes and nitrenes (6-8).

In general, carbenes arise from diazo compounds and nitrenes from azides by photolysis. Clearly photolytic generation is better than thermolytic as the biological systems retain their structural integrity over only a small range of temperature.

If this method is to be useful the following conditions should be obeyed: (1) the reagent must be stable and specific for the active site of receptor; (2) photolysis must be done at wavelengths clearly distinct from the absorbance bands of the receptor so that it may not be damaged; (3) rearrangement of carbene and nitrene to less active species should be avoided; and (4) the active species (carbene and nitrene) should be nonselective.

Carbenes are less selective than nitrenes (9-12). However, the carbenes undergo rearrangement reactions (13). Aliphatic diazo compounds are only stable when adjacent to a carbonyl or similar electron withdrawing groups, and photolysis of such a system gives a classical situation for the Wolff rearrangement to a ketene. Hence the splendid indiscriminate nature of the carbene is largely lost, ketenes being subject to attack primarily by nucleophiles. It appears that for carbenes there is no precursor which can fulfill all the above mentioned conditions. Nevertheless, earlier experiments with carbenes put this photo-generation idea on a firm ground (14-17).

Alkyl nitrenes suffer even more disadvantages. Generally alkyl azides not only rearrange on photolysis to isocyanates (18) (analogous to diazoketones to ketenes) but also have absorption maxima around 290 nm which may damage the receptor (19). However, aryl nitrenes are much less susceptible to rearrangement (20-22), the major rearrangement path being the formation of substituted azepines (20). Aryl azide half lives vary with substituents (23) and hence reagents with a range of different reactivities can be synthesized. They are chemically stable at 37° and if properly substituted can be photolyzed at wavelength above 350 nm (21,22), well clear from protein absorption. Their main reactions can be summarized as (24):



Aryl nitrenes have been used previously for affinity labeling of antibodies (21) and specific acetylcholine binding sites on membranes (25). We have extended the use for affinity labeling (this thesis slightly touches this aspect) and immobilization of enzymes.

Our goal was to prepare certain derivatives of aryl nitrenes which may prove to be of wide value in the utilization of enzymes for industrial, medical, environmental, and analytical applications, as well as in the basic studies of these functional associations. The initial studies were to prepare certain derivatives of a photochemical bifunctional reagent, 4-fluoro-3-nitrophenylazide (21), (FNPA). A high degree of independence may be expected in the reactions of two functional groups (one thermochemical, the other photochemical) of this

reagent and thermochemical derivatives thereof. The relatively stable nitrene produced upon the absorbance of visible light by nitrophenylazide derivatives will form stable covalent bonds under very mild conditions with a variety of chemicals containing carbon-carbon double bonds and/or carbon-hydrogen bonds (21,22,24). These reagents may be exptected to form stable covalent linkages between insoluble matrix material and: (1) enzymes for immobilization; (2) substrate analogs and or cofactors for enzyme purification by affinity chromatography; and (3) metabolically active whole cell enzyme systems, as well as covalent linkages between (4) enzymes and required cofactors. These intermolecular cross-links have a wide range of applications (26-36).

Immobilization of Enzymes:

In this thesis the term "immobilized enzymes" indicates enzymes which are physically or chemically attached to solid supports (37).

Enzymes are highly efficient catalysts, commonly accelerating chemical reactions one million to one trillion times the speed they would occur in the absence of the enzymes. They accomplish this under mild physiological or life supporting conditions (room temperature, atmospheric pressure, mild acidity or alkalinity, etc.), thus avoiding large energy wastes and operating hazards frequently associated with the use of presently popular industrial catalysts. Enzymes are highly

specific catalysts capable of avoiding large wastes and pollution due to unwanted by-products. They are also capable of catalyzing many reactions which are not possible with ordinary chemical catalysts. They are involved in all the chemical reactions necessary to sustain the living cell and hence control a variety of reactions. In spite of all these advantages, enzymes are not chosen as catalysts for many household or industrial processes.

The main problems are: (1) their high costs of production; (2) difficulties in their recovery from reaction products for eventual recycling; (3) instability toward temperature, pH, storage, etc., and (4) biodegradability. The preparation of synthetic enzymes (38) and improved separation technique of affinity chromatography (28,31) may lower their costs of production. The enzymes can be reused if they are firmly fixed on suitable supports (27). The immobilization may improve their stability toward storage (39-41), temperature (39), pH (39), as well as resistance to microbial attack (27,39) and autolysis (39,42). Melrose (43) compared 50 immobilized enzyme systems with their soluble counterparts. Thirty of the immobilized enzymes were more stable to temperature and storage, eight less stable and 12 showed no difference in stability from the soluble enzymes. This indicates that one will generally expect enhanced stability to result from immobilization, but one cannot be sure it will occur.

The majority of enzymes within the living cell are either in solution with a high concentration of cytoplasmic proteins or contained in solid state assemblages such as mitochondria (26,44). They function in an environment resembling a gel (26). Most laboratory investigations of enzymes are conducted with purified extracts in dilute aqueous solution, under conditions far removed from their natural state. The practical approach to studying enzymes in a simulated natural environment is the immobilization by microencapsulation (45), gel entrapment (46), or attachment to stable artificial matrices (26). Thus, the immobilization technique may lead to practical uses of enzymes and model systems to study them in their natural milieu. In retrospect it is difficult to say whether the interest in enzymes affixed to matrices began as efforts to clarify cell mechanisms or for practical applications (26).

Nelson and Griffin achieved the first immobilization (47). They retained invertase activity after adsorption on animal charcoal. Since then the following major techniques have been used for immobilization (48,49,50).

1. Adsorption: This is simply the adsorption of enzymes on to suitable (hydrophilic) solid supports. As long as certain physical conditions are maintained, such as ionic strength and pH, enzymes remain firmly bound (47,51-55). This is probably due to ionic bonding of the enzyme with the support (26). This method offers the advantage

of extreme simplicity and there is no chemical modification of the enzymes. However, this does suffer the problem of desorption with increased ionic strength, and the properties of matrices necessary to bind the enzyme may not be compatible with the substrate or the product.

2. Entrapment in cross-linked polymeric matrix: Enzymes can be trapped within a gel lattice by carrying out the polymerization reaction leading to gel formation in an aqueous solution containing the enzyme (46,56-59). The pores must be large enough to permit the substrate and the product to enter and leave freely (26). The disadvantages of gel inclusion process are the relative inaccessibility of the enzymes to substrates of higher molecular weight, the diffusion control of substrates and products, and the possible loss of enzymes from loosely cross-linked gels (49). Nevertheless, encouraging results are obtained (46,60).

3. Intermolecular cross-linking: Enzymes can be cross-linked after physical attachment to the matrix material (61-63). Although this method has distinct advantages in some applications (62,63), sometimes it results in burying a large portion of enzyme and making it unavailable to substrate. It may also have the disadvantage of biodegradability. Recently a review of bifunctional reagents for cross-linking of proteins was published (64).

4. Covalent attachment to organic and inorganic carriers: This method involves the covalent binding of the enzyme, via functional groups that are not essential for enzyme activity, to hydrophilic natural (42,54,65-74) or synthetic polymers (31,34,35,75-87) and inorganic substances (27,41,88-101). Coupling has been done successfully either by direct reaction (31,42,71,72,93) of enzyme with the activated matrix or through bifunctional reagents (48,69), one group of which react with the enzyme and the other with matrix material.

Natural polymers which have been used are starch and cellulose. Cellulose has seen the greatest use (72). Activated diethylaminoethyl (54,65,71), iminocarbonate (68), aminoethyl (69,70,73), diazobenzyl (67), chlorotriazine (71,72), titanium complexes (74) and azide (42,72) derivatives of cellulose have been successfully attached to enzymes. Cyanoethylated (66) and dialdehyde (67) activated starch have also been used. Synthetic polymers which are commonly employed are agarose (31,77,84-87), Sepharose (35,79-82), Sephadex (79), polyacrylamide (76), Nylon (34,74), Dacron (83), Enzacryl (35,75) and copolymer of acrylamide-acrylic acid (78). Aminoalkyl activated porous glass (27,74,88-99) and metal oxide (100,101) have been used as inorganic supports. Recently the structure of porous adsorbents (102), their activation by a variety of coupling agents (103) and enzyme immobilization on inorganic carriers (27) have been described.

Generally it is concluded that the inorganic support is better than organic due to: (1) structural stability to pH and solvent; (2) rigidity of size or configuration during usage; (3) variety of configurations readily prepared; and (4) resistance to microbial attack.

Covalent immobilization has the advantage of an attachment which is not being reversed by pH, ionic strength and substrate. Enzymes attached by covalent bonding may be stabilized by being bonded to the matrix in several places. However, this method does alter the chemistry of the enzymes and may change their reactivity (43,67,68,70,104). Supported enzymes are generally less active than the corresponding natural enzymes. In the extreme case the active site may be blocked through the chemical reaction and the enzyme rendered inactive. In other cases, the activity of the enzyme may be altered by the reaction of amino acid side chains which are involved in the operation of enzymes. Hence, the immobilization must be done under very mild conditions most favorable to enzyme integrity. However, the fact that generally immobilized enzymes are more stable to various conditions (temperature, pH, storage, etc.) than the soluble ones and can be used repeatedly (27), offsets the partial loss of activity during immobilization (43).

It was unanimously recommended at a meeting on "Enzyme Engineering" held in Henniker, New Hampshire, August 1971, under the auspices of the Engineering Foundation Conferences, that the term "immobilized enzyme" be used to describe all enzyme preparations by the above outlines procedures (37).

Of the procedures for preparation of immobilized enzymes suggested above, the one currently receiving the most attention by experimentors interested in immobilized enzyme catalysis is the covalent bonding of enzymes to a solid phase (105). However, there is no standard method for the preparation of immobilized enzymes. Special reaction conditions, and in many cases special "tailor made" supports are required to ensure substantial retention of the biological activity of the individual protein to be bound (30).

A large number of reactions are being used for enzyme immobilization (50). Generally α-amino groups, ε-amino groups of lysine, hydroxyphenyl side chains of tyrosine, the guanidyl side chains of arginine, and the immidazole groups of histidine of the enzyme molecule are involved in the reactions (43). Participation of carbonyl groups of enzymes through their activation by carbodiimides has also been reported (93). Accordingly activated matrix materials are prepared which have a variety of reactive groups like isothiocyanate (106), diazonium salts (107), N-hydroxysuccinimide (31,108), iminocarbonate (109), azide (42,72), triazine (71,72), etc., which are subject to nucleophilic

attack by the enzymes. Bifunctional reagents (64), of which one group reacts with the enzymes and the other with solid support, have also been used (48,69). Most of these bifunctional reagents also involve the nucleophilic reactions of the enzymes and activated matrix material. Thus, these methods have the limitation of nucleophilicity. We have tried to immobilize enzymes through photo-generated aryl nitrenes (detail is given in the early part of introduction) to eliminate this requirement.

Excellents reviews are available on immobilization of enzymes. In 1966 Silman and Katchalski (48), discussed the techniques used to prepare reagents and reactive groups on carriers. These authors also discuss the effect of immobilization on the stability of enzymes toward storage and heating, and the effect of the microenvironment of the insoluble matrix on such properties as optimum pH and Km values. The use of immobilized enzymes in column reactors and as part of membranes is discussed. In 1968 Goldstein (110), and Katchalski and Goldstein (111), reviewed preparative procedures, properties and applications of immobilized enzymes. In 1971 "Biochemical Aspects of Reactions on Solid Supports" edited by George E. Stark gives a comprehensive view on the subject (112). In 1972 Lemuel Wingard edited "Enzyme Engineering" which gives papers presented at international symposium, Henniker, August 1971. The 65th annual meeting of American Institute of Chemical Engineers, December 1971, San Francisco, presents status

and future prospectus of enzyme immobilization (113). From July 1972, RANN (Research Applied for National Needs) branch of National Science Foundation have started to publish quarterly "Enzyme Technology Digest" which in addition to a brief review of literature also gives reports of different enzymes currently under investigation for practical applications (114). Most recently a compendium of references are available on immobilized enzymes (115). We have studied the immobilization of L-asparaginase (L-Asnase).

L-Asnase (L-asparagine amidohydrolase, EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine (L-Asn) to L-aspartic acid (L-Asp) and ammonia. This enzyme affords an approach to the chemotherapy of malignant neoplasams by exploiting a metabolic difference between tumor cells and normal cells (116). Normal cells have greater ability to synthesize Asn than tumor cells.

Kidd (117) was the first to note that normal guinea pig serum has antitumor activity, and Broome (118) subsequently showed that the active antitumor substance is L-Asnase which is present in high concentration in the serum of guinea pig. The discovery (119) of E. coli L-Asnase with antitumor activity presented a source of enzyme for further experimentation at both preclinical and clinical levels. Since then international symposia (120,121) have been held and reviews (122, 123,124) written.

L-Asnases are widely distributed in the biological world (124-126). There are two different L-Asnases in E. coli B and are designated EC-1 and EC-2 (127). They differ in their pH activity profiles, substrate affinity and heat stability. Only EC-2 has potent antilymphoma activity and was used by us.

The characteristics which distinguish an Asnase that possesses antilymphoma activity from one that does not, have not been conclusively established. The two principal factors appear to be the rate of enzyme clearance from the host circulation (128) and the substrate affinity (129,130) of the enzyme. Assigning a role in determining antilymphoma effectiveness to clearance time still leaves unanswered what features of the molecule determine clearance time. Recently this subject has been reviewed (124).

EC-2 has been obtained in the crystalline form and physically and chemically characterized (127,131,132). A method has also been described for selective purification by affinity chromatography utilizing the inhibitor D-Asn covalently linked to Sepharose using the CNBr method (133).

Even the most purified enzyme EC-2 has amidase activities toward L-glutamine (L-Gln) and D-Asn, although at a much slower rate than L-Asn (134-135). Examination for L-Gln and D-Asn activity indicated 2-4 percent and 5-10 percent, respectively, of the activity for L-Asn. Each of these substrates appear to compete for the same active site on the

enzyme (134,136). Product inhibition occurs with ammonia at pH 8.5, although not at pH 7.4 or 5.00, but neither hydrolysis nor inhibition is found with L-aspartic acid, D-aspartic acid, L-glutamic acid or D-glutamic acid (134). Glutaminase (L-Glnase) activity is of interest as it has been described to have antineoplastic activity (125,137).

Amino acid analyses have been reported from several laboratories (131,138) following the original report of Whelan and Wriston (139). As indicated by Arens (138), et al., about 50 percent of the molecule is composed of aspartic acid, threonine, alanine, valine and glycine. In comparison to E. coli A, E. coli B contains cystine residues (140). No carbohydrate or phospholipid is present in the enzyme (131).

After some conflicting reports (132,138,139,141) it is established now (142) that L-Asnase is a tetramer having identical subunits and the molecular weight is 133,000 ± 5,000. There are four cystines per tetramer with one intrachain disulphide bond per subunit. There is no intermolecular cross-linking between subunits.

Recent chemical modification of L-Asnase with succinic anhydride and hybridization of native and succinyl-Asnase also constitutes evidence that the enzyme has identical four subunits (143). This paper indicates that some lysyl residues of L-Asnase are involved in inter-subunit association. The cross-linkage between subunits using tetranitromethane has been reported recently (144,145).

The dissociation-reconstitution behavior of L-Asnase has been characterized (132,146). The enzyme can be dissociated into subunits in 7 M urea or 5 M guanidine hydrochloride with no enzymatic activity remaining. This change can be reversed and the enzyme regains nearly its full activity. The physical properties of native and reconstituted enzyme are virtually identical.

Although L-Asnase has a definite place in therapy of leukemia, its use is limited by many side effects (147). A major obstacle to its successful clinical use has been the antigenicity of the enzyme. Production of antibody to the enzyme can result in loss of effectiveness of the enzyme due to shortened half life in the plasma (148). Immobilization may be promising to remove such problems (149).

L-Asnase has been successfully immobilized in recent years by several methods, including microencapsulation with semipermeable (150-153) membrane of free enzyme in emulsion, gel entrapment with polyacrylamide (154) and hydroxyethylmethacrylate (155) (HEMA), adsorption to ion exchange cellulose (156,157), and covalent binding to matrix materials such as dextran and cellulose derivatives (158), dacron vascular prothesis (83), and nylon tubing (159).

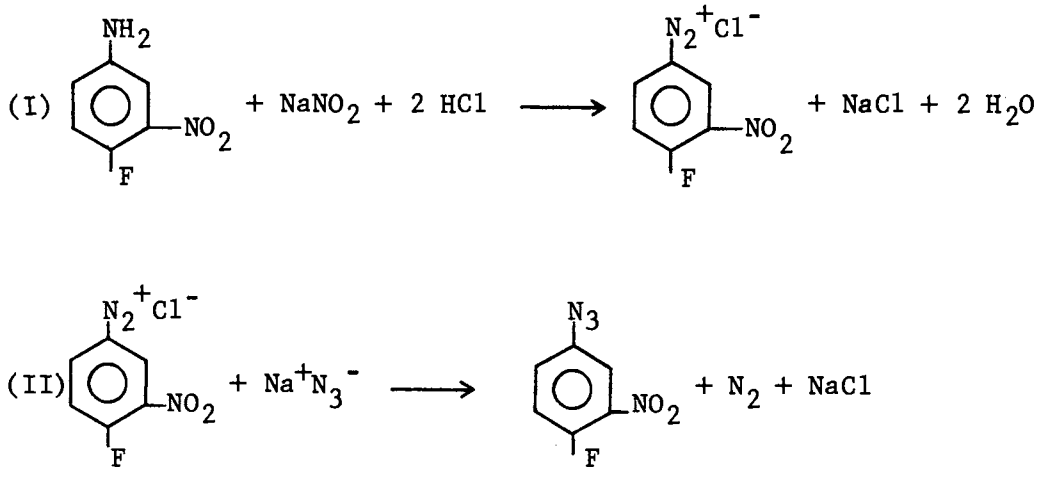
In efforts to improve our control over the exposure of the patient to the catalytic agent, to decrease the antigenicity of the enzyme, and to stabilize the catalytic activity, we have developed reagents and methods for covalent binding of enzymes to chosen matrix materials under very mild conditions. In comparison to our results with succinic

anhydride plus N-hydroxysuccinimide methodology (31) and with glutaraldehyde (technical instructions from R&D Laboratories, Corning Glass Works) as well as with data previously reported (83,150-159), the photochemical reaction technology has provided a distinct improvement in yield of immobilized catalytic activity. L-Asnase immobilized by this method has properties to make it suitable for in vivo study.

CHAPTER II

PREPARATION OF FNPA

FNPA (21), was prepared by adding 5 gm of 4-fluoro-3-nitroaniline (Aldrich Chemical Company Lot 021807) in a mixture of concentrated hydrochloric acid (30 ml) and water (5 ml). The mixture was stirred magnetically at about 40°C for 1 hr and cooled to -10°C (using an external bath of methanol-solid CO_2). An aqueous solution of sodium nitrite (2.8 gm in 3 ml water) was added dropwise over 20 min, and the mixture was stirred for 10 min at -10°C. It was then filtered rapidly into a flask at -10°C. This filtrate was stirred between -20°C and -10°C, and an aqueous solution of sodium azide (2.8 gm in 5 ml H_2O) was added dropwise (using red lighting). If there is frothing at this stage, a few drops of ether may be added. After the addition of sodium azide was finished, the resulting precipitate was collected on a Buchner funnel, washed thoroughly with water, and dried at room temperature in a vacuum dessicator wrapped in aluminum foil (the product is light sensitive). The reactions can be represented by the following equations:



Recrystallization of the crude product from petroleum ether (boiling point 40-60°C) gave straw colored needles in 67 percent yield. The melting point was 52-52.1°C.

Elemental analysis data are given in Table IV. The mass spectra (Table V) showed the molecular ion peak at mass = 182. A number of broad metastable peaks were observed. The NMR spectrum showed a hydroxynitrophenylazide content 3 percent of FNPA. The infrared spectrum gave the characteristic band at 2115 cm^{-1} corresponding to azide. The NO_2 group was indicated by absorption bands at 1365 and 1525 cm^{-1}. Thin-layer chromatography on cellulose silica gel plates using three solvent systems (acetone, chloroform, and 70 percent ethanol) gave only one spot. Attempt to further purify the product by sublimation did not change mass and NMR spectra.

FNPA is insoluble in water but soluble in most organic solvents like acetone, chloroform, ether, ethanol, etc. At alkaline pH it hydrolyses to hydroxynitrophenylazide which is soluble in water. This product showed a molecular ion peak at 180.

TABLE IV

ELEMENTAL ANALYSIS DATA OF FLUORONITROPHENYLAZIDE

Element	Calculated	Found
C	39.6%	39.64%
H	1.65%	1.85%
N	30.8%	31.0%
F	10.5%	10.9%

TABLE V

MASS SPECTRUM[a] OF FLUORONITROPHENYLAZIDE

Major Peaks[b] (m/e)	% of Base Peak	Major Metastable Peaks
182	22.95	130.6 (182-154)
154	20.13	75.6 (154-108)
109	10.95	60.7 (108-81)
108	100	
82	16.07	
81	23.00	
57	56.87	
50	10.63	
33	14.24	
31	23.76	
30	19.14	
28	66.65	

a The mass spectrum was taken by direct inlet system at room temperature. The source temperature was 300°C, probe temperature 50-60°C and electron voltage 70 ev.

b All other peaks were less than 10 percent of the base peak.

CHAPTER III

AFFINITY LABELING TECHNIQUE

Principle: The principle of this technique is essentially the same as when originated by Singer et al.; (160), that is, the reaction of the enzyme with a compound which both resembles a substrate and possesses a group which will form a covalent linkage with amino acid side chains. Displacement of fluorine in FNPA by substrate and/or inhibitor is expected to give the desired compound which may combine with the active site. Then the photolysis of this azide derivative will produce nitrene which may form the covalent bond with amino acid side chains in the active site region.

Results and Discussion: Affinity labeling of bovine carboxypeptidase A (CPA) and trypsin was performed as follows: glycyl-L-tyrosine forms a complex with the active site of CPA (161). The derivative of glycyl-L-tyrosine was prepared by mixing 10 μ moles glycyl-tyrosine in 1 ml, 1 M sodium borate buffer, pH 9, with 40 μ moles of FNPA in 2 ml acetone. This mixture was stirred overnight at 40°C. The product was purified by thin layer chromatography on silica gel plates using diethyl ketone-water (1:1) and/or by sublimation.

This nitroazidophenyl derivative of glycyl-tyrosine (probably linked to the amino group of glycine) was mixed in equimolar quantities with CPA in 0.1 M potassium phosphate buffer, pH 8, having 1 M NaCl concentration. The mixture was photolyzed at 4°C with 100 w bulb through sodium nitrite solution to absorb wavelengths below 400 nm (21). A control experiment was run with glycyltyrosine-nitrophenylazide without photolysis. The reaction mixture was passed through Sephadex G-25 (1 x 25 cm) with the same buffer used in photolysis. The protein fraction was collected and esterase activity measured. The results (illustrated in Figure 2) show that esterase activity of bovine CPA is enhanced. Previously it has been reported (162), that using an active site specific reagent, esterase as well as peptidase activity decreases. Other chemical modification studies reported (163,164), that esterase activity increased while peptidase decreased. No attempt was made to see the chemically modified group in our work.

The experiment with trypsin was done with arginine methyl ester (AME) in a similar way except assay buffer was used in photolysis and chromatography. The results (illustrated in Figure 3) indicate some decrease in apparent esterase activity due to the photoaffinity labeling reagent.

Affinity labeling studies of bovine CPA (162,165), and trypsin (166), using nonphotochemical reagents have been done previously. Our attempt to extend this technique by FNPA derivatives is very preliminary.

Additional experiments must be done before one can present conclusive evidence of the advantages of its use in these enzyme systems. Nevertheless results indicate that this approach may be useful.

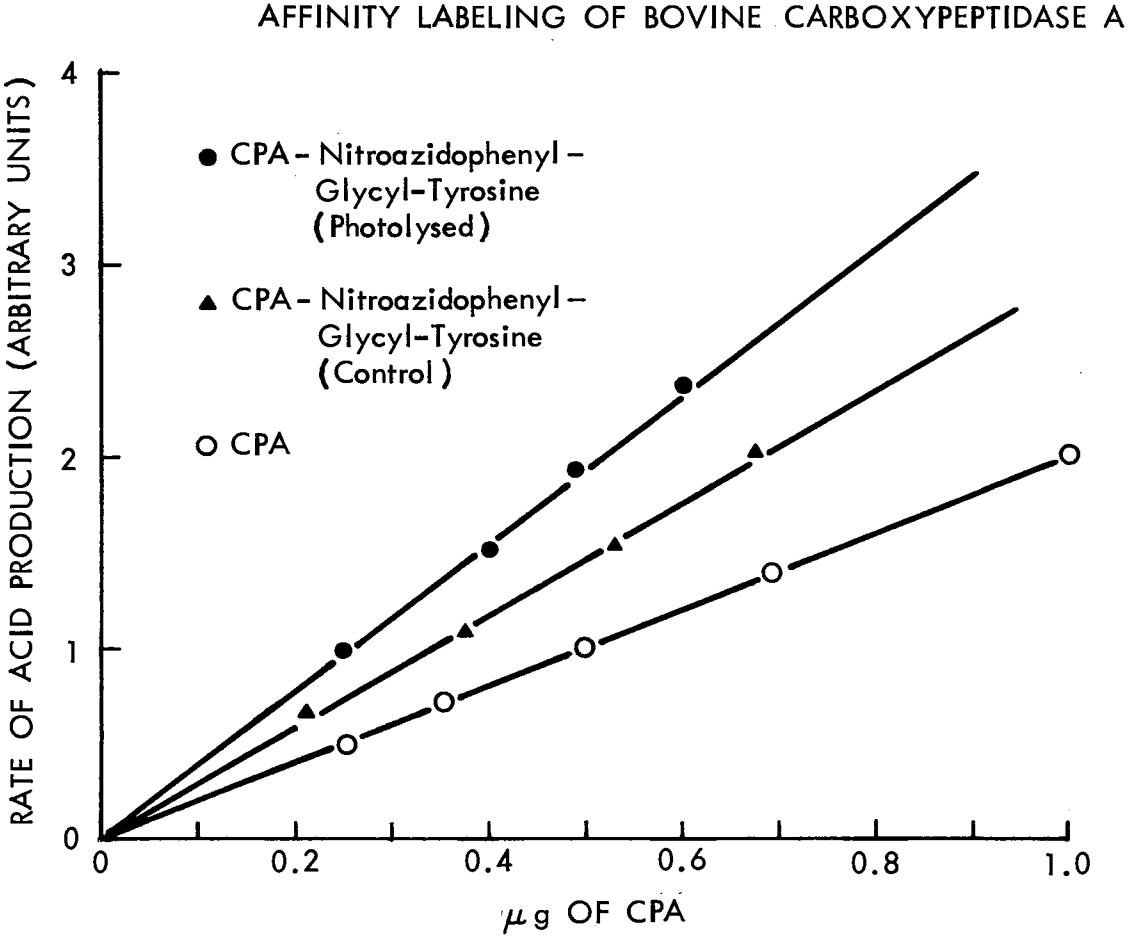

AFFINITY LABELING OF BOVINE CARBOXYPEPTIDASE A
RATE OF ACID PRODUCTION (ARBITRARY UNITS)
0
1
2
3
4
● CPA - Nitroazidophenyl - Glycyl-Tyrosine (Photolysed)
▲ CPA - Nitroazidophenyl - Glycyl-Tyrosine (Control)
O CPA
0
0.2
0.4
0.6
0.8
1.0
μg OF CPA

FIGURE 2

Figure 2 - Assays were performed at 25° with 1 ml of 0.01 M hippuryl dl-β-phenyl lactate in 0.2 M NaCl 0.005 M tris hydrochloride at pH = 7.5. The activity was determined by continuous titration with 0.1 N NaOH of the H^+ released on hydrolysis using pH stat (Radiometer-Copenhagen).

Protein concentration was measured by the absorbance at 278 nm. The molar absorptivity 6.42×10^4 M^{-1} cm^{-1} was used in all cases.

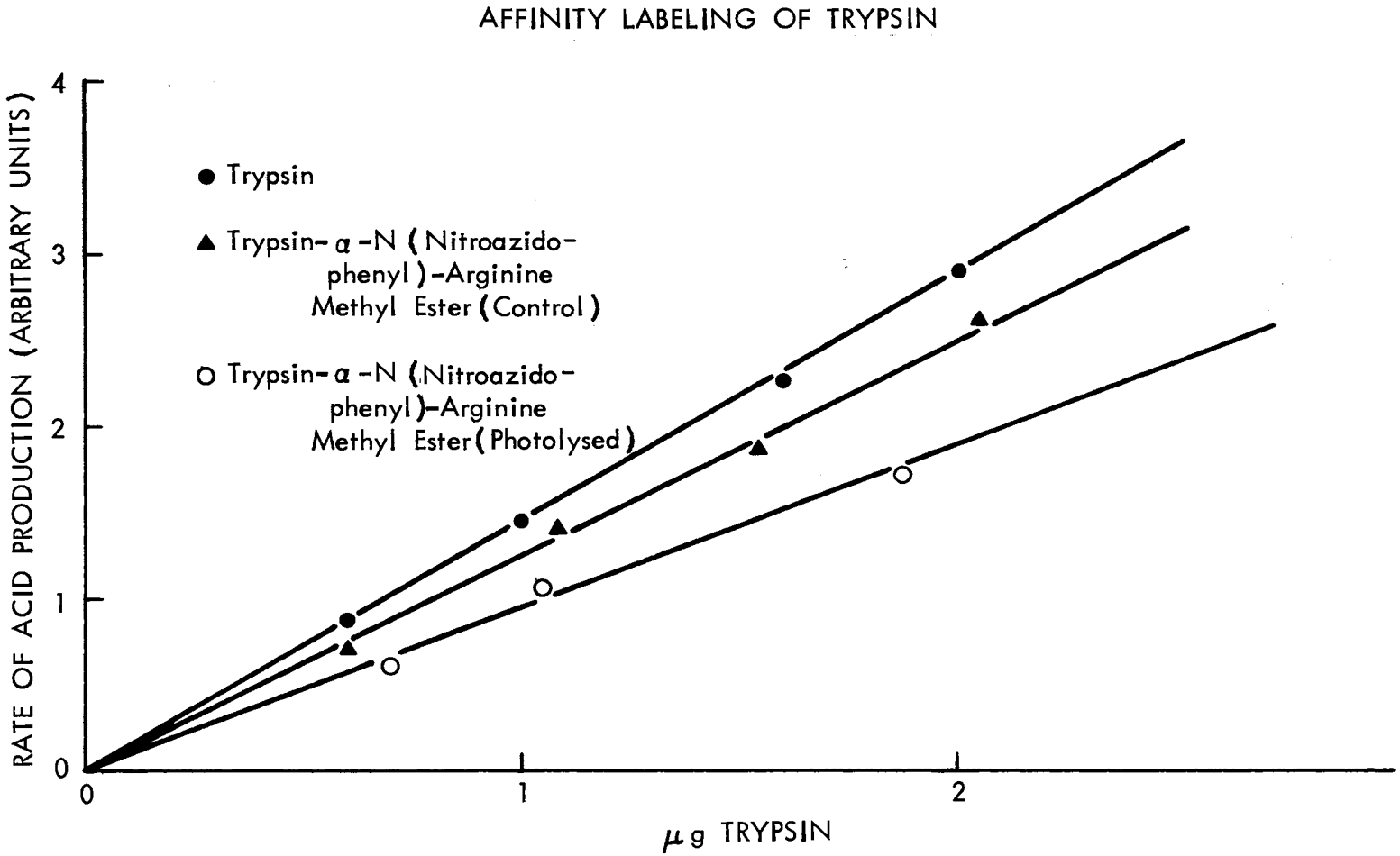

AFFINITY LABELING OF TRYPSIN
RATE OF ACID PRODUCTION (ARBITRARY UNITS)
0
1
2
3
4
● Trypsin
▲ Trypsin-α-N (Nitroazidophenyl)-Arginine Methyl Ester (Control)
○ Trypsin-α-N (Nitroazidophenyl)-Arginine Methyl Ester (Photolysed)
0
1
2
μg TRYPSIN

FIGURE 3

CHAPTER IV

IMMOBILIZATION OF L-ASPARAGINASE

Principle: The matrix material (glass, cellulose, etc.) containing nucleophilic groups are reacted with FNPA to displace fluorine. The resulting activated matrix material is photolysed with the enzyme under very mild conditions for immobilization.

Materials: Glass (96 percent silica glass particles, 550 Å pore diameter, 40 square meters surface area, 40-80 mesh, 0.3 mmoles propyl amino groups per gram of glass, compliments of Corning Glass Works), cellulose (Bio Rad, Cellex-AE, ethylamino groups 0.37 meq/gm), L-Asnase (Squibb and Sons, Lot No.: 335-712/15-5-6, 200 IU/mg, nice gift from Dr. M. K. Patterson of Samuel Roberts Noble Foundation), L-Asn.H_2O (crystalline, Sigma Chemical Company, Lot 81C-0380), L-Gln (Nutritional Biochemical Corporation, Control No.: 6192), Nessler reagent (Sigma Chemical Company, Stock No.: 14-2 and SO-N-20, 76367), glutaraldehyde (solution, Fisher Chemical Company, Biological grade), N-hydroxysuccinimide (Aldrich), dicyclohexylcarbodiimide (Pierce Chemical Company), dioxane (Fisher Scientific Company), succinic

anhydride (Eastman Org. Chem.) and ammonium sulphate (Fisher Scient. Company), alpha chymotrypsin (Worthington Bio. Corp. CD16jF), trypsin (Mann Res. Lab., Cat. No.: 4463) and protease (Sigma, Type VI, from Streptomyces griseus).

Experimental Procedure

Immobilization Methods

Photochemical Method

Dark reaction: All the following steps were done in the dark. One gram of aminoalkyl glass was placed in a colored bottle of about 50 ml capacity. To this was added 10 ml of 1 M sodium borate buffer, pH 9.1, to cover the glass material. FNPA (160 mg) was dissolved in 20 ml absolute ethanol and added to the above bottle. This mixture was stirred for 24 hr at 40°C in a constant temperature water bath shaker. The resulting material was centrifuged and the supernatant fluid (dark straw colored) was removed with a disposable pipet. The solid glass derivative (glass-NPA) was washed extensively with absolute ethanol, 3 M NaCl and water, in succession, to remove all hydroxynitrophenylazide. The pink colored glass product was dried in a dessicator at room temperature under vacuum. This dried activated glass derivative was stored in darkness at room temperature until further use. The azide derivative of amino ethyl cellulose (cellulose-NAP) was prepared in the same manner.

Light reaction: The following steps were done in dark until photolysis. Glass-NPA (100 mg) was placed in a small (approximately 2 ml) glass bottle. To this was added 10 mg L-Asnase and 0.5 ml water to make a thin slurry. A small magnetic stirring bar was used for gentle stirring. The flask was stoppered on the neck by rubber dropper bulb. Photolysis was done at 4°C for 16 hr through 1 M $NaNO_2$ solution of 0.5 cm thickness by a 40 w tungsten focusing microscope illuminator. The photolysed product was washed thoroughly at room temperature with 0.02 M potassium phosphate buffer (pH 8), 3 M NaCl and again with buffer to remove all noncovalently adsorbed L-Asnase. The drying was done in a dessicator at room temperature under vacuum. This same procedure of light reaction was used for cellulose immobilized Asnase preparation.

For comparison the L-Asnase was immobilized by the following methods.

Glutaraldehyde method (Corning Glass Works Instructions): To 200 mg of alkylamine glass was added enough 2.5 percent glutaraldehyde solution (50 percent glutaraldehyde diluted 1 to 20 in 0.1 M sodium phosphate, pH 7) to cover the glass. The entire reaction mixture was placed in a dessicator and attached to an aspirator to remove gas and air bubbles from glass particles and the reaction was continued for 1 hr. The entire product was removed, filtered on Buchner funnel and washed with distilled water. This dried glass derivative (100 mg) was added without storage to 10 mg enzyme in about 0.5 ml

0.1 M sodium phosphate buffer (pH 7) in an ice bath. The reaction was continued for 2 hr. The product was washed (by our method, please see Table VII) thoroughly with sodium phosphate buffer (pH 7), 3 M NaCl and again with buffer. The immobilized Asnase was dried in a dessicator under vacuum.

N-Hydroxysuccinimide method (31): This method was followed as described in the literature (31), and is outlined here. Alkylamine glass beads were succinylated with succinic anhydride in saturated sodium borate. N-Hydroxysuccinimide ester was prepared in dioxane using 0.1 M dicyclohexylcarbodiimide and N-hydroxysuccinimide. This product was washed with dioxane and methanol and reacted for 1 hr with 10 mg/ml L-Asnase in 0.1 M sodium acetate buffer, pH 6.2. The product was washed and dried as described in the above methods.

L-Asnase assay (167): The enzymatic activity of L-Asnase was determined by measuring with Nesslers reagent the rate of ammonia release from L-Asn. Stock 15 mM solution of Asn was prepared in 0.02 M potassium phosphate buffer, pH 8. This solution was kept at 4°C and no hydrolysis of Asn was noted for 2 weeks. In a 25 ml Erlenmeyer flask was added 2.5 ml of 15 mM Asn and the temperature brought to 37°C. To this flask was added the known amount of enzyme (in case of immobilized Asnase, a weighed amount of glass or cellulose product) and the mixture was incubated at 37°C in a constant temperature water bath shaker for the chosen length of time. Generally the

incubation time was 10 min but it varied depending on the activity of immobilized enzyme. After incubation the solution was immediately diluted to 25 ml with deionized water (at room temperature) and 0.5 ml Nessler reagent added. The control was run at the same time under similar conditions with no enzyme. The absorbance was measured at 425 nm (Figure 4).

To calculate international units (IU), a standard curve was determined with $(NH_4)_2\ SO_4$. In a 25 ml Erlenmeyer flask was added 2.5 ml 0.02 M potassium phosphate buffer, pH 8. The known amount of $(NH_4)_2\ SO_4$ was added in this flask. The blank contained only 2.5 ml buffer. The rest of the procedure is the same as used for Asnase assay. However, as expected there was no difference whether we incubate $(NH_4)_2\ SO_4$ at 37°C or not. There was slight difference when two different lots (No.: 14-2, No.: SO-N-20) of Nessler reagent were used.

Effect of drying on wet glass immobilized L-Asnase: The drying effect was determined by weighing known quantities of wet immobilized Asnase in two 25 ml Erlenmeyer flasks. One flask was used for determining the activity of wet immobilized Asnase. The other flask was placed in a dessicator under vacuum at room temperature. This was weighed again to calculate the moisture content. This same flask was used for determining the activity of dry immobilized Asnase.

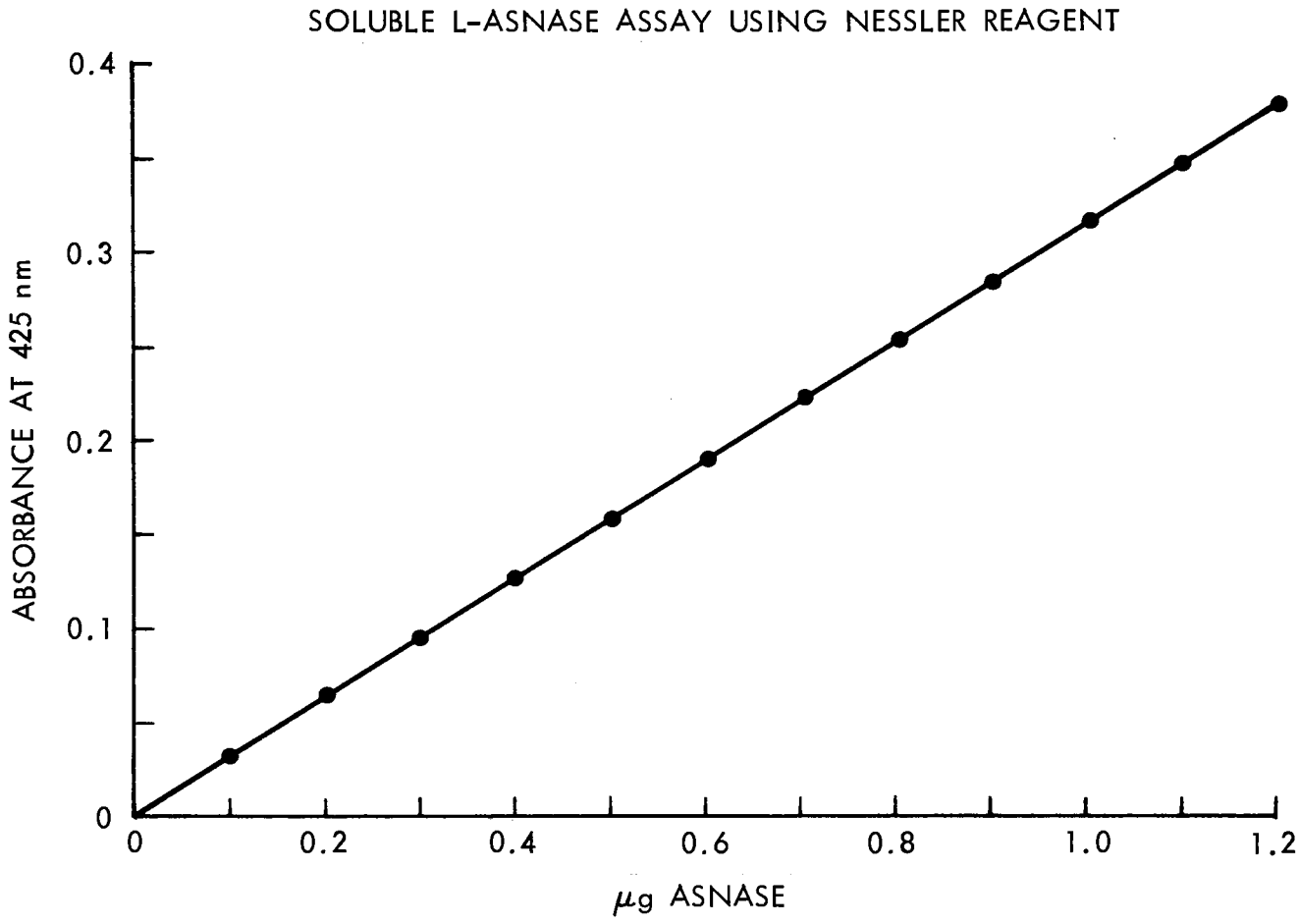

SOLUBLE L-ASNASE ASSAY USING NESSLER REAGENT
ABSORBANCE AT 425 nm
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1.0
1.2
μg ASNASE

FIGURE 4

Effect of temperature on stability of immobilized L-Asnase: The effect of temperature on dry immobilized Asnase was determined by keeping the samples in closed vessels at 4°C and room temperature. The activity was determined with respect to storage time.

Effect of temperature on glass immobilized L-Asnase in buffer: To see the effect of various temperatures on stability of soluble and immobilized Asnase in solution, 1.25 ml, 0.02 M potassium phosphate buffer was added to each of three 25 ml Erlenmeyer flasks. Known quantities of soluble and immobilized Asnase were added in two flasks, the third being used as blank. All three flasks were incubated at the desired temperature (the effect of which is to be determined) for half an hour on a water bath shaker previously adjusted to that temperature. After half an hour the flasks were removed and placed in another water bath shaker at 37°C. Two minutes after placement of these flasks at 37°C, 1.25 ml, 30 mM Asn (previously brought to 37°C) was added to each of the three flasks. The assay was done for 10 min by the usual method.

Influence of temperature on assays: The influence of temperature on the assay of soluble and immobilized enzyme was seen by doing the assays under normal conditions except that, in addition to 37°C, the assays were also done at various other temperatures.

Effect of pH: The pH optimum of soluble and immobilized enzyme was determined by performing the assays under standard conditions at

different buffered pH's. The following 0.05 M buffers were used (168): pH 2-4, sodium acetate-hydrochloric acid buffer; pH 4-6, sodium acetate-acetic acid buffer; pH 6-8, sodium phosphate dibasic-potassium phosphate monobasic buffer; pH 8-11, sodium carbonate-boric acid buffer. There are no acetate, phosphate or borate ion effects on activity of L-asnase (127).

Effect of physiological saline: In order to determine the effect of physiological saline (0.86 percent NaCl solution) at 37°C on stability of immobilized enzyme, 1.25 ml, 0.02 M potassium phosphate buffer (pH 8) having 0.86 percent NaCl concentration was added in two 25 ml Erlynmeyer flasks (four such pairs were prepared for zero and various incubation times). To one flask was added the known amount of enzyme at room temperature and the other flask was treated as blank. Flasks were placed at 37°C in an incubation chamber at 37° and assayed after certain periods of time. For zero time 1.25 ml, 30 mM Asn was added in both flasks (containing enzyme and the blank) and immediately the assay was done by standard procedure.

Effect of proteolytic enzymes: The stability to proteolytic enzyme (trypsin, chymotrypsin, protease) was seen under the following conditions. To three 25 ml Erlynmeyer flasks was added 1.25 ml, 0.02 M potassium phosphate buffer (pH 8) at room temperature. On the basis of activity (in linear range of assay), equal amounts of soluble (5 µg) and immobilized Asnase were added to their respective flasks.

The third was for blank. Five micrograms of proteolytic enzyme was added to all three flasks which were immediately incubated at 37°C for 5 min on a water bath shaker. After 5 min incubation 1.25 ml, 30 mM Asn was added to all flasks and immediately the assay was done for 10 min by normal procedure.

Effect of human plasma: The effect of human plasma on soluble and immobilized Asnase was determined under the following conditions. Human blood was taken and centrifuged. To 2.55 ml of the supernatant was added 1.20 ml, 0.86 percent NaCl solution to obtain the needed volume for subsequent assays. One and a quarter milliliters of this plasma solution was added in three 25 ml Erlenmeyer flasks. On the basis of activity, equal amounts of soluble and immobilized Asnase were added to their respective flasks. The third flask was for blank. These flasks were placed in an incubation chamber for 37°C for 24 hr after which these were assayed. For zero time, three more flasks were prepared as above and immediately assayed. Assays were modified as follows to remove interference with color formation due to the plasma proteins. To each flask was added 1.25 ml, 30 mM Asn and assay incubation was done for 4 min on a water bath shaker at 37°C. The flasks were removed from shaker and 0.5 ml 30 percent trichloroacetic acid was added to all flasks. The entire precipitated material was centrifuged in separate centrifuge tubes. To 1.5 ml supernatant was added NaOH solution to neutralize the trichloracetic

acid. Deionized water was added to make 12.5 ml and then added 0.25 ml Nessler reagent. The absorbance was measured at 425 nm.

L-Glnase activity of L-Asnase: The L-Glnase activity of L-Asnase was determined in the same way as L-Asnase activity but 15 mM L-Gln was used as substrate instead of L-Asn. The incubation time was 45 min in this case, as L-Glnase activity of L-Asnase was found quite low.

All these experiments were done at least twice in triplicates.

Results and Discussion

L-Asnase has been immobilized by several methods in recent years (83,150-159), in attempts to improve its therapeutic value for leukemia treatment. In efforts to improve our control over the exposure of the patient to the catalytic agent, to decrease the antigenicity of the enzyme, and to stabilize the catalytic activity, we have developed reagents and methods for covalent binding of enzymes to chosen matrix materials. This immobilization methodology is very simple to execute and is accomplished under very mild conditions of pH (neutral), temperature (4°C), aqueous medium, etc. These conditions are necessary in order to preserve the steric structure of enzymes which is essential to their activity (31). The activated matrix materials (glass- or cellulose-NAP) can be stored in the dark without any damage. Then simply by photolysis the immobilization can be achieved.

This photochemical methodology gives a distinct improvement in yield of covalently bound catalytic activity over all the previous methods (Table VI). To see that the enzyme might not be noncovalently adsorbed on matrix material, a control experiment was run with glass under the same conditions as for immobilization but without photolysis. It was found that enzyme does adsorb on glass (Table VII). A NaCl concentration of 3 M was found necessary to remove all this adsorbed

TABLE VI

COMPARISON OF L-ASPARAGINASE ACTIVITY IMMOBILIZED BY DIFFERENT METHODS

Method	Activity*
Photochemical	1,200 IU/gm of cellulose 1,450 IU/gm of glass
Glutaraldehyde	270 IU/gm of glass
N-Hydroxysuccinimide	270 IU/gm of glass

* IU = One international unit of enzyme activity is defined as the release of 1 µmole of NH_3/min at 37°C under the assay conditions.

TABLE VII

ACTIVITY OF L-ASPARAGINASE NONCOVALENTLY ADSORBED ON GLASS

Wash Solution*	Activity Left on Glass (IU/gm)
0.02 M Potassium phosphate buffer pH 8	330
1 M NaCl	40
2 M NaCl	12
3 M NaCl	0

* Extensively washed with these solutions until activity on dried glass was constant. Also after NaCl washings, the glass was washed with phosphate buffer before drying.

enzyme. This was the reason for washing our immobilization reaction mixture with 3 M NaCl. Although many other enzymes have been immobilized on aminoalkyl glass (88-99), to our knowledge this washing step is not described in the literature. Activity was measured on matrix material because the activity level in the washings was very low.

It was noticed that the condition of the Nessler reagent affects the assay. Two lots gave different activity units on the same sample as well as for standard $(NH_4)_2\ SO_4$ curve (Figure 5). Therefore, as a precaution the same Nessler reagent should be used for determining standard curve and immobilized enzyme activity.

Within the experimental error there was no effect of drying glass immobilized Asnase in a dessicator under vacuum at room temperature (Table VIII). The dry immobilized Asnase, both on glass and cellulose, was found stable at 4°C and room temperature (Table IX). Glass immobilized enzyme retained its full activity after 6 days while cellulose linked enzyme lost less than 10 percent after a month at room temperature. The literature (158) indicates full retention of activity after a week when Asnase was immobilized on matrix supports by different methods. The data indicating the relatively greater stability of immobilized Asnase than the soluble one to temperature confirm the previous review (43). Provided a suitable support (hydrophilic and porous) (30) and mild coupling conditions are used, this property may be regarded as

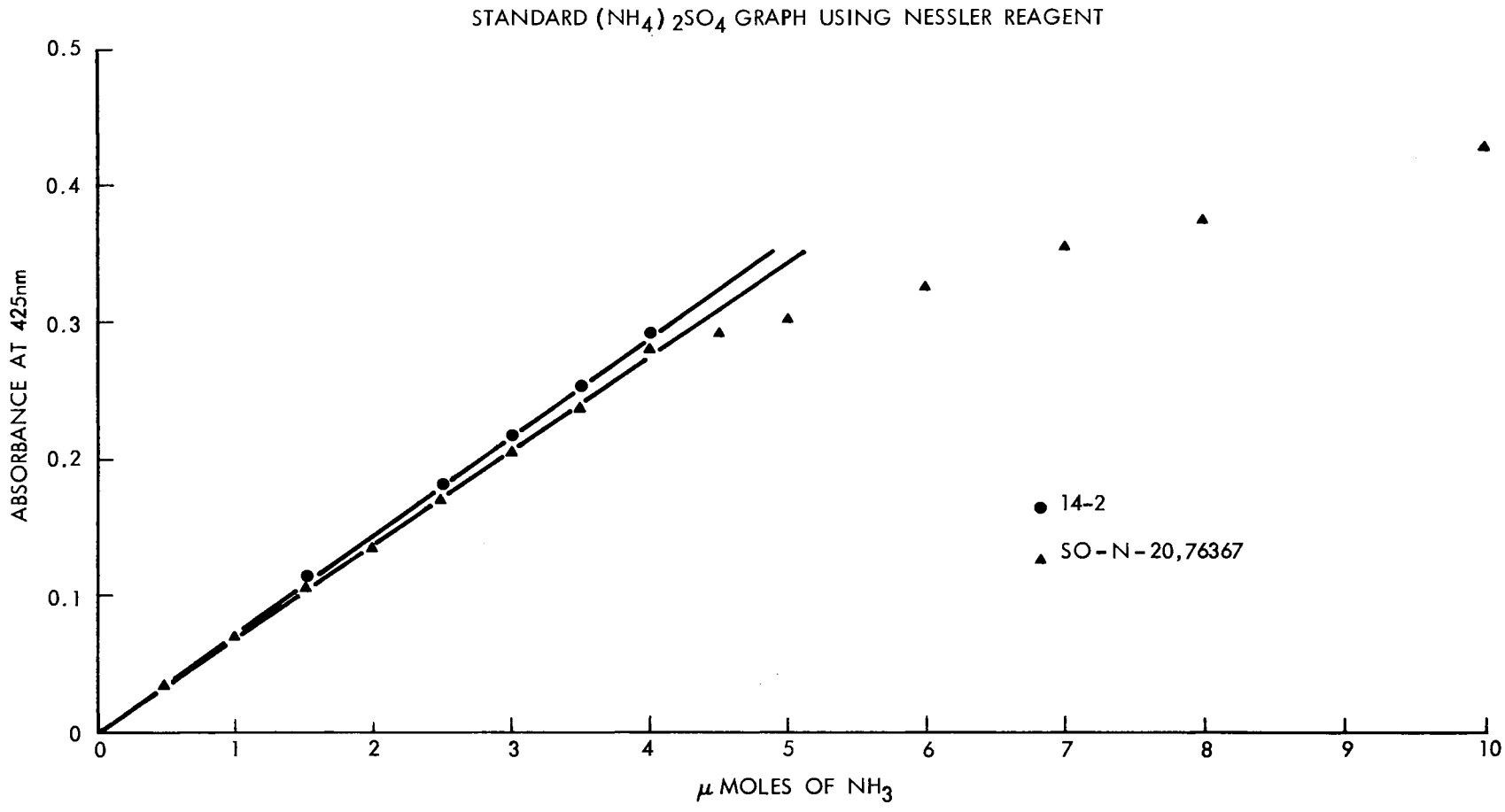

STANDARD $(NH_4)_2SO_4$ GRAPH USING NESSLER REAGENT
ABSORBANCE AT 425nm
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
8
9
10
μ MOLES OF NH_3
14-2
SO-N-20,76367

FIGURE 5

TABLE VIII

EFFECT OF DRYING ON GLASS IMMOBILIZED L-ASPARAGINASE

Wet Glass-Asnase		
Moisture Content	Activity on Dry Wt. Basis	Dried Glass-Asnase Activity
77.3%	1,480 IU/gm	1,450 IU/gm

The drying was done at room temperature in a dessicator under vacuum.

TABLE IX

EFFECT OF TEMPERATURE ON STABILITY OF IMMOBILIZED L-ASPARAGINASE

Time	Glass-Asnase 4°C	Cellulose-Asnase 4°C	Glass-Asnase Room Temp.	Cellulose-Asnase Room Temp.
	100% (1,450 IU/gm)	100% (1,200 IU/gm)	100%	100%
1 Day	100%	100%	100%	98%
6 Days	100%		100%	95%
15 Days				93%
30 Days				92%

Dried immobilized L-Asnase samples were stored in closed vessels at 4°C and room temperature.

characteristic of supported enzymes. Binding to an insoluble support evidently provides better protection from denaturing effects. With continuous use, the stability may further increase from the influence of the substrate.

Mostly we studied glass immobilized enzyme because it was described as a better support (27), for many uses. However, it is found not to be so good as thought because it hydrolysed under certain conditions (please see last part of discussion) and has adsorption property.

The activity of immobilized and soluble L-Asnase in buffer relative to temperature is shown in Figure 6. The data indicate activation of enzyme in the lower range of temperature and deactivation in the higher range. The activation in the case of immobilized Asnase may be due to breakage of noncovalent interactions of the enzyme with the matrix material, providing the enzyme with greater freedom of interaction in solution, which may result in greater availability of the active site to the substrate. This conformation may be stabilized in the presence of substrate during the assay time. The decrease in activity at higher temperature is assumed to be due to denaturation of soluble as well as immobilized enzyme. However, the data indicate that immobilized enzyme is more stable to denaturation than the soluble one. The immobilized L-Asnase retained activity up to 10°C higher than the soluble L-Asnase.

TEMPERATURE STABILITY OF SOLUBLE AND GLASS IMMOBILIZED L-ASNASE IN BUFFER
(30 MINUTES STORAGE, 37° ASSAY)

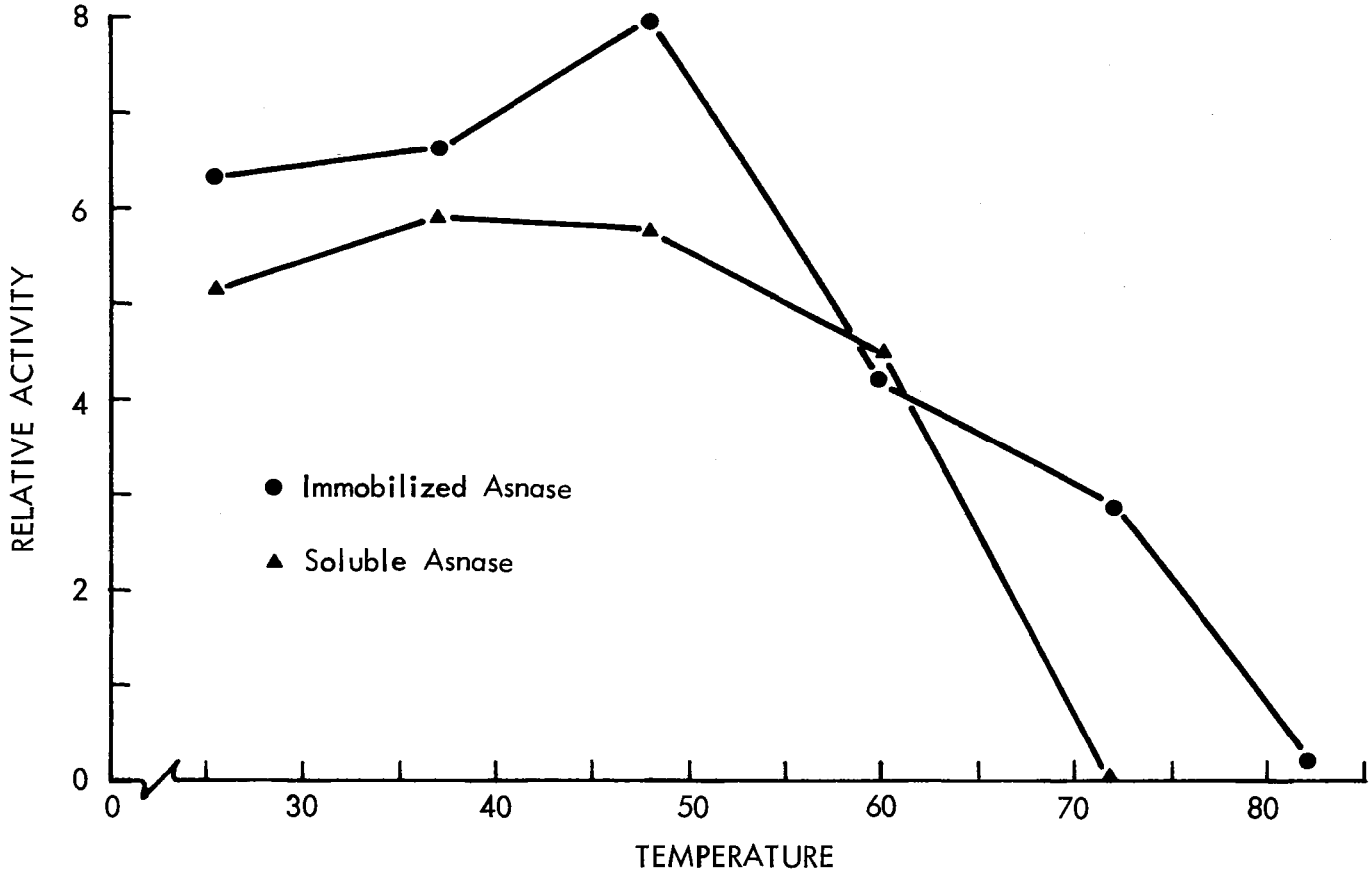


FIGURE 6

The stability to temperature in the presence of substrate (L-Asn) is shown in Figure 7. Temperature tends to increase the reaction rate while denaturation decreases it. At 60°C the increase in rate due to temperature is neutralized due to decrease in rate by denaturation of enzyme. At 82°C the soluble enzyme is relatively more active than the immobilized enzyme. The reason for this is not clear. Apparently the degree of substrate stabilization is less for the glass bound enzyme.

The pH profiles for soluble and immobilized Asnase are shown in Figure 8. The pH optimum of soluble Asnase is sharpened and slightly shifted to higher pH in immobilized Asnase. Katchalski (48,49), et al., have shown that when an enzyme is covalently attached to a charged matrix material, a microenvironment is created. A charged matrix material can accumulate oppositely charged ions causing a concentration of these ions at the carrier surface (169). Thus, the pH at the carrier surface is different from that in the external solution which causes a difference of pH profiles. The glass complicates the picture because of the presence of both free amino groups and silanol residues. The amines, however, near netural or alkaline pH values will not be protonated. Also many may be coupled to the enzyme, leaving mostly silanol residues having a negative charge (27). This negative charge can accumulate H^+ from external solution causing an acidic microenvironment around the immobilized Asnase which can cause the shift in pH

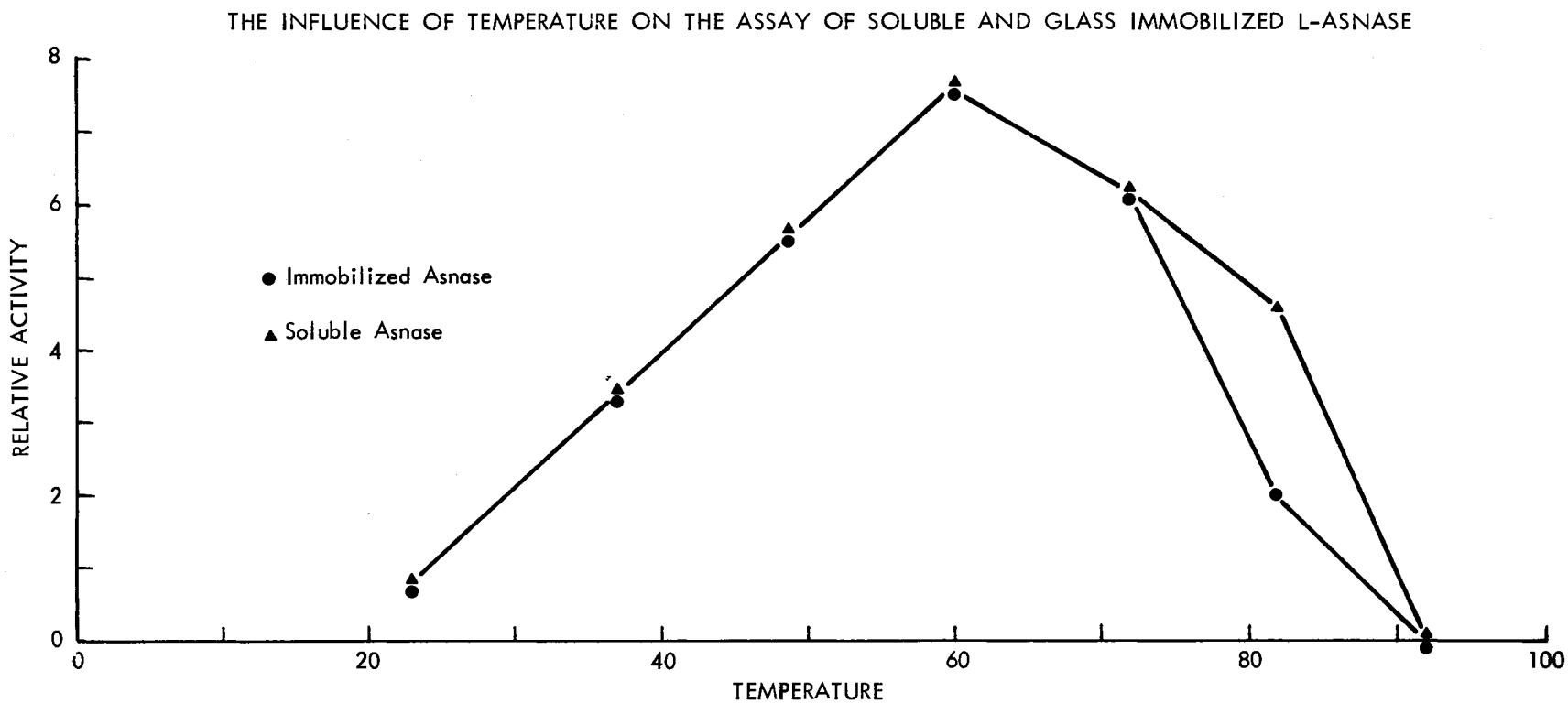

THE INFLUENCE OF TEMPERATURE ON THE ASSAY OF SOLUBLE AND GLASS IMMOBILIZED L-ASNASE
RELATIVE ACTIVITY
8
6
4
2
0
● Immobilized Asnase
▲ Soluble Asnase
0
20
40
60
80
100
TEMPERATURE

FIGURE 7

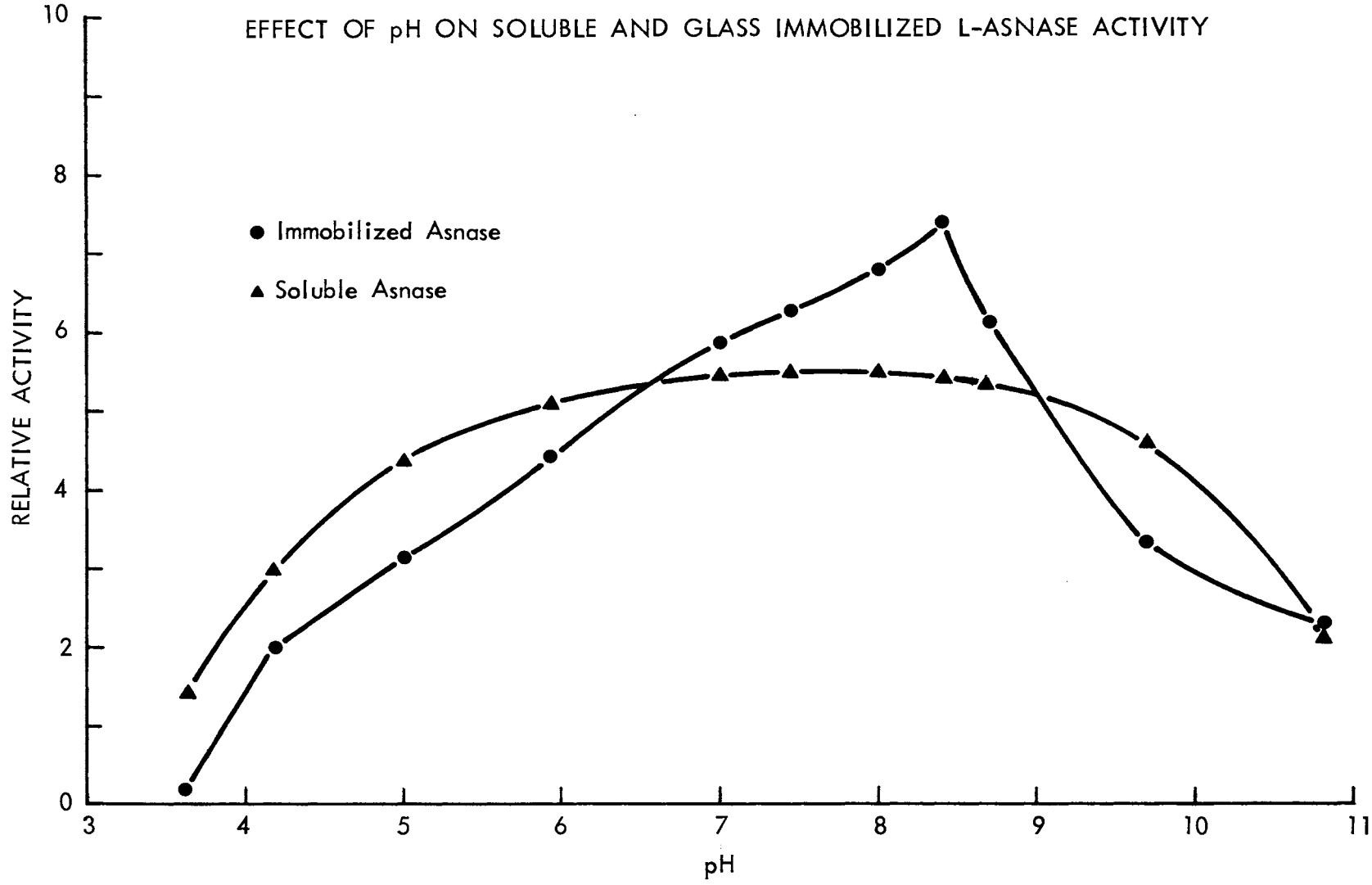

EFFECT OF pH ON SOLUBLE AND GLASS IMMOBILIZED L-ASNASE ACTIVITY
● Immobilized Asnase
▲ Soluble Asnase
RELATIVE ACTIVITY
0
2
4
6
8
10
3
4
5
6
7
8
9
10
11
pH

FIGURE 8

optimum. Thus, the nature of carrier has definite influence on the apparent pH optimum of the enzyme. This suggests the possibility of "tailoring" a carrier specifically to permit an enzyme to operate efficiently at apparent pH not optimal for that enzyme. This may have applicability in a variety of areas where the soluble enzyme cannot be utilized.

The effect of physiological saline at 37°C on stability of immobilized Asnase is shown in Table X. The activity after 24 hr is increased to 157 percent which after a week is retained up to 123 percent. This effect may be due to Na^+ from the solution which are attracted toward the negative charges of the silanol groups on glass. This creates a Na^+ microenvironment around the immobilized Asnase which may stabilize its conformation. The decrease (22 percent lower than the highest activity but still 23 percent more than the original) in activity after a week is probably the effect of temperature. These data support the review (49), that the microenvironment of the immobilized enzyme has strong influence on its activity.

Stability data of soluble and immobilized Asnase to proteolytic enzymes is shown in Table XI. The stabilizing effect of the matrix may be due to steric hindrance imposed by the matrix on large size proteolytic enzymes which cannot reach immobilized enzyme as easily as the soluble one. An enzyme molecule may be fixed to the matrix, not at one point of attachment, but at several (30). This results in a

TABLE X

EFFECT OF PHYSIOLOGICAL SALINE ON GLASS IMMOBILIZED L-ASPARAGINASE

Time	% Residual Activity Glass-Asnase in Saline at 37°C
0	100
1 Day (24 hr)	157
2 Days	156
7 Days	123

The dried immobilized L-Asnase samples were placed in autoclaved 1.25 ml physiological saline in 25 ml Erlenmeyer flasks in an incubation chamber at 37°C. The assays were done by standard procedure.

TABLE XI

STABILITY OF SOLUBLE AND GLASS IMMOBILIZED L-ASPARAGINASE TO PROTEOLYTIC ENZYMES

Proteolytic Enzyme	Soluble Asnase Residual Activity	Immobilized Asnase Residual Activity
None	100%	100%
Alpha Chymotrypsin	23%	97%
Trypsin	50%	72%
Protease (Streptomyces griseus)	0	47%

Five micrograms of proteolytic enzyme was added in 1.25 ml 0.02 M potassium phosphate buffer (pH 8) containing soluble or immobilized L-Asnase (equal on basis of activity) in 25 ml Erlenmeyer flasks. These flasks were stoppered, incubated at 37°C on a water bath shaker for 5 min. Then 1.25 ml 30 mM L-Asn solution was added. Assays were done for 10 min.

certain amount of cross-linking, which, together with intramolecular cross-linking of the matrix itself, hinders the access of substrate, inhibitor and other molecules to the bound or enclosed molecules. This type of argument is supported by Cresswell and Sanderson (170), who found a linear relationship between the residual activity of immobilized pronase and the logrithm of the molecular weight of substrate. This situation is similar to that in exclusion chromatography of proteins on cross-linked dextran gels, in which the fraction of pore volume accessible to a protein depends on the molecular weight of protein. In our experiment protease which quickly digests the free Asnase, is not so accessible to the immobilized Asnase. These data indicate that by choosing a suitable support the immobilized enzymes can be stabilized to proteolytic enzymes.

Another factor which may influence the proteolytic enzyme concentration in the microenvironment of immobilized Asnase, and hence its activity, is diffusion. The presence of a diffusion-limiting layer in the neighborhood of immobilized enzymes has been indicated by Lilly, et al. (171). Probably this is the reason that immobilized enzymes exhibit their maximum activity only at sufficiently high stirring speeds in the reaction mixture (172). This is why we carried out all assays at a stirring rate of water bath shaker which produced

maximum activity under the conditions of the assays. Recently the effect of diffusion on the properties of soluble and immobilized enzymes on theoretical basis has been described (173).

The stability of immobilized Asnase to human plasma is considerably higher than the soluble one under the experimental conditions (Table XII). A number of factors may be responsible for it. The metal ions present in plasma may attach to negative charge on glass by electrostatic interaction to produce microenvironment more suitable to immobilized Asnase activity than the soluble one. The matrix material may protect immobilized Asnase against enzymatic digestion. The other components of plasma may also be favorable to immobilized Asnase as it has been described (26), that the environment of immobilized enzymes resembles more the natural environment of stable intracellular enzymes.

The inherent Glnase activity of L-Asnase has antineoplastic activity (125,137). Therefore, it was of interest to see whether the Glnase activity of L-Asnase changes on immobilization. Data (Table XIII) show that immobilized Asnase exhibits Glnase activity equal to about 4 percent of its Asnase activity. This same ratio was found with the soluble Asnase. However, to increase Glnase activity of L-Asnase is not a problem. This is because variations in the Glnase activity in the matrix bound enzyme preparations would seem to be readily accomplished by the

TABLE XII

STABILITY OF SOLUBLE AND GLASS IMMOBILIZED L-ASPARAGINASE TO HUMAN PLASMA

Time	Soluble Asnase	Glass-Asnase
0 Hr	100%	100%
24 Hr	74%	325%

The flasks containing enzyme and human plasma were incubated for 24 hr at 37°C. The assays were done by modified assay procedure as described under the effect of human plasma in method section.

TABLE XIII

L-ASPARAGINASE:L-GLUTAMINASE ACTIVITY OF SOLUBLE AND GLASS IMMOBILIZED L-ASPARAGINASE

	L-Asn	L-Gln
Soluble L-Asnase	100%	4%
Glass Immobilized L-Asnase	100%	4.1%

The assays were done under standard conditions modified by substrate change.

choice of enzyme (126,174), to be immobilized. It is described (174), that such enzymes have activities against tumors other than leukemia and thus, may enlarge the potential for cancer chemotherapy by amino acids deprivation.

All these data indicate that photochemically immobilized L-Asnase has some useful properties of stabilized activity which should be useful for its in vivo study. This method is also useful for the immobilization of other enzymes (175).

Dissociation-reconstitution behavior of L-Asnase: L-Asnase is a tetramer having identical subunits (142). In studying the function of subunits in proteins an interesting question is whether individual subunits are active. A comparison of the properties of the active subunits with those of the oligomeric protein should further our understanding of the important effects of subunit interaction on protein function (176). However, the natural tendency of subunits to associate makes it difficult to study isolated subunits under conditions in which the oligomeric protein is functional.

Previous work has shown that the soluble tetrameric Asnase can be dissociated in 7 M urea into individual subunits (132). When urea is removed, the subunits associate to form active tetrameric molecules indistinguishable from native enzyme. Therefore, exhaustive washings of the matrix bound Asnase with 7 M urea should cause dissociation and removal of all subunits not covalently linked to the matrix (Figure 9). Dialysis (or placing in buffer) (132) would be expected to

SCHEME OF MATRIX BOUND L-ASPARAGINASE DERIVATIVES

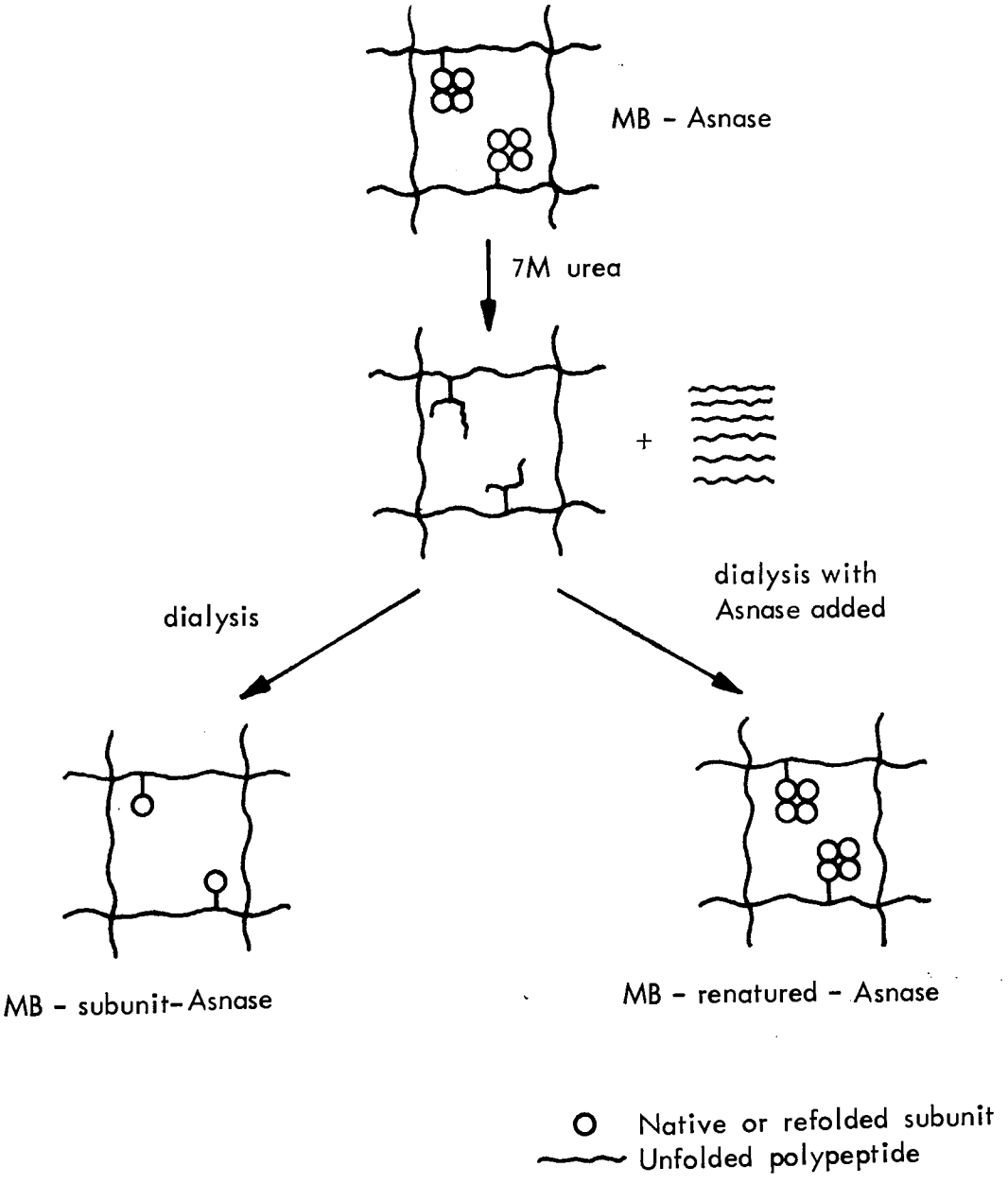


FIGURE 9

lead to refolding of each of the remaining subunits. These subunits, however, are prevented from reassociation by the rigidity of the matrix (82). The absence of autodigestion in immobilized emzymes has been attributed to the inability of the bound enzyme molecules to come into contact with one another (177). Therefore, the properties of subunit(s) (Figure 9, MB-subunit-Asnase) can be studied. Dialysis in the presence of soluble Asnase is expected to give matrix bound renatured Asnase. This derivative is useful for comparison as it has undergone the same treatment as covalently linked subunit.

Our activity measurement data are in Table XIV. This shows that subunit(s) left after urea wash and dialysis are active.

Attempts were made for the determination of glass and cellulose immobilized Asnase by recently described ninhydrin method (178), to know how many subunits are bound to matrix material after 7 M urea wash. While this method is useful for soluble protein determination, no reproducible data were obtained for immobilized enzyme. This is because amino groups on glass and cellulose as well as azide gave high background. It was found that alkaline conditions described in this method hydrolysed glass. Probably the Si-O-Si bond is not stable to these conditions. Ultraviolet absorbance method failed to give reproducible results as the protein concentration in washings was low.

TABLE XIV

ACTIVITY DATA OF MATRIX BOUND SUBUNITS OF L-ASPARAGINASE

Treatment	Glass-Asnase	Cellulose-Asnase
Immobilization	100% (1,450 IU/gm)	100% (1,200 IU/gm)
7 M Urea washed, dialysed, dried	17%	19%
Dialysis in presence of soluble Asnase	90%	92%

Dialysis was done overnight in 0.02 M potassium phosphate buffer, pH 8. Samples were dried and activity measured by normal method. After dialysis in the presence of soluble Asnase, extensive washings were given with 3 M NaCl, 0.02 M potassium phosphate buffer before drying the samples.

Elemental analysis also did not give reproducible data. Therefore, at present, we can only say that less than four subunits of Asnase are also active. The properties of single subunit of L-Asnase needs further experimentation.

Summary

A method is described for the preparation of nitrophenylazide derivatives of glass and cellulose. These activated matrix materials are stable for months when stored at room temperature in the dark. L-Asnase is immobilized with these matrix materials by a simple photochemical method under very mild reaction conditions. Procedures are described for studying the properties of immobilized L-Asnase. The immobilized Asnase is more stable to temperature, proteolytic enzymes and human plasma than the soluble Asnase. This photochemical technology has a wide range of applications in medicine, industry and environmental control as well as for analytical work.

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