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FINAL REPORT

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STUDIES ON STABILIZATION OF ENZYMES

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SUMMARY

1. Dilute, aqueous solutions of beef heart lactate dehydrogenase (LDH), (the model enzyme) have been stabilized under standardized conditions permitting a retention of 100% enzymatic activity for over 20 months.
2. Significant stabilization of E. coli B asparaginase and pig heart malate dehydrogenase was also achieved in dilute aqueous media.
3. Lactate dehydrogenase was insolubilized in an enzymatically active form, by being covalently bound to the insoluble support, diazotized Cellex PAB. Optimum binding ratios were derived and studies completed permitting the regeneration of bound and unbound enzyme.
4. Antibodies to LDH were successfully produced in rabbits. Such antibodies were to be used as enzyme stabilizers.
5. Other studies, including the selective alkylation of non essential sulfhydryl groups of LDH by reaction with γ-chloropropylthiosulfate were performed.

Two communications and three papers describing this work have been prepared for publication.

I. Introduction

Considerable success has been achieved during the two year life of this contract (1967-1969). After a consideration of a number of model enzymes during the first contract year (beef heart lactate dehydrogenase, E. coli B asparaginase, and pig heart malate dehydrogenase), we focused our efforts during the second year on beef heart lactate dehydrogenase.

During the first contract year, the major portion of our work was devoted to the stabilization of the model enzymes in liquid solution. Different criteria of stabilization were established: (a) stability towards inactivation when stored in dilute solutions at $4 \pm 2^{\circ}$ for extended periods of time (1-12 months) and (b) stability towards short term thermal or dilution inactivation. Such studies were designed to determine whether suitable additives and media (different from the classical aqueous media) could be used for enzyme stabilization, as well as to identify any solvent limitations that could impose restrictions on the use of media for the synthesis of solid supports or the preparation of selective sulfhydro derivatives. The approach used in these initial studies was based on the fact that enzymes in their natural or intracellular environment, do not exist free in aqueous solution. Rather, they are both bound and stabilized by such components as the proteins, carbohydrates, lipids and other structures that constitute their natural environment. We sought to determine whether enzymatic reactions could occur in mixtures of organic-aqueous systems, such as water-dioxane, water-tetrahydrofuran, water-ethanol, water-methanol, etc. In addition, we wanted to be able to compare the enzyme's activity and its stability in the organic solvent mixture to the corresponding activity and stability in classical aqueous solution. Our choice of enzyme system was based on the following criteria: (a) The enzyme must be involved in synthesis of some biologically

important product at the expense of ATP; (b) The enzyme must use a cofactor in its reaction, other than merely metal ions which are involved in simple hydrolytic processes: (c) The enzyme must be available in sufficient quantities and in a high degree of purity.

Many of these requirements were met by the model enzymes herein described. During the two years covered by this report, our efforts and accomplishments can be summarized as follows:

YEAR I

1. The chemical synthesis of the sodium salts of β-chloroethylthiosulfate and γ-chloro-N-propylthiosulfate to serve as potential selective alkylating agents for non-essential sulfhydro groups on the enzyme. Upon subsequent biochemical evaluation of these compounds, the initial results appeared promising, however budgetary restrictions did not permit an intensive study of these compounds.

2. Two different criteria of enzyme stabilization were established: stabilization to thermal inactivation and stabilization to long-term storage.

3. Selective additives and solvent stabilization studies were performed on three model enzymes, lactic dehydrogenase, pig heart malate dehydrogenase, and E. coli B asparaginase.

YEAR 2

1. The use of solvent-protective colloid mixtures for the stabilization of a model enzyme - LDH.

2. The preparation of enzymatically active, insolubilized enzyme derivatives, by means of the covalent bonding of beef heart lactate dehydrogenase onto the solid support diazotized Cellex PAB. Considerable attention was given to studies concerning both the mechanism of the reaction and the stability of the insolubilized product.

II. Methods

A. Enzyme Assays

1. Assay for Lactic Dehydrogenase[1]

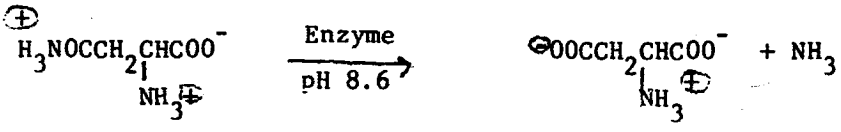
Lactic dehydrogenase was measured by following the oxidation of $NADH_2$ at 340 mμ, as illustrated by the following equation:

$$\text{pyruvate} + H^+ + \text{NADH} \xrightarrow[\text{pH 7.4}]{\text{Enzyme}} \text{L-Lactate} + NAD^+$$

The reaction mixtures contained pyruvate (1 μmole); $NADH_2$ (0.2 μmole); potassium phosphate buffer (80 μmoles, pH 7.4) and enzyme (0.3 μg) in a final volume of 3.0 ml. The reaction was run at room temperature (~25°) and was followed by measuring the decrease in absorbance at 340 mμ (absorption maximum for $NADH_2$) as a function of time for 1 minute. During this interval, the reaction is linear and produces a change in absorbance of about 0.1.

2. Assay for Asparaginase[2]

Asparaginase activity was measured by determining the amount of ammonia formed (as ammonium sulfate) according to the following equation:



The reaction mixtures contained: asparagine (4 μmoles); potassium phosphate buffer (25 μmoles, pH 8.6) and enzyme (10 μg) in a final volume of 0.55 ml. The reaction was run at 37° for 10 minutes and terminated by the addition of 0.10 ml of 6% TCA. The progress of each reaction was determimed by measuring the amount of ammonium ions present in solution as follows: (1) 3 ml H_2O was added to each tube, and the NH_3, evolved after the addition of 1 ml of saturated Na_2CO_3, was distilled over into a trap, containing 2.5 ml of 1% H_2SO_4, by bubbling compressed air through the system; (2) each sulfuric

acid trap was then brought up to 10 ml (with H_2O); (3) 2.5 ml Nessler's reagent was added to each (the contents mixed well), and the absorbance was immediately measured at 480 mμ. Under these conditions, the reaction is linear and produces about 20 μmoles of ammonia/mg/min.

3. Malate Dehydrogenase - Enzyme activity[3] was measured by studying the rate of oxidation of $NADH_2$, as illustrated by the following equation:

$$NADH + H^+ + \text{oxaloacetate} \xrightarrow[\text{pH 7.4}]{\text{Enzyme}} NAD^+ + \text{L - malate}$$

The reaction mixture[3] contained: potassium phosphate buffer (280 μmoles: pH 7.4); $NADH_2$ (0.2 μ mole); oxaloacetate (0.3 μmole) and enzyme (0.24 μg) in a final volume of 3.0 ml. The reaction was carried out at room temperature (25 ± 2°) and was followed by measuring the decrease in absorbance at 340 mμ (absorption maximum for $NADH_2$) as a function of time for 1 minute. During this interval, the reaction rate is linear and produces a change in absorbance of about 0.14 O.D. unit.

B. Measurement of Stabilizing Effect.

Introduction - The ultimate success of such an enzyme stabilization study will be influenced to a large part by the methods available for the measurement of the degree of stabilization produced relative to the control. Successful stabilization procedures should be readily adaptable for use with the various model enzyme systems envisioned. It is precisely such a procedure that we feel we have developed in the measurement of the effect of various additions towards the thermolability of the enzyme under certain standard conditions.

1. Thermal inactivation assay for LDH

a. Principle - The thermolability assay for beef heart lactic dehydrogenase is based on the observation that under appropriate conditions of

enzyme concentration, pH and buffer ionic strength, the thermal inactivation at 37° appears to be a function of the incubation time. Approximately 30 minutes is required for 50% inhibition of lactic dehydrogenase activity. The extent to which the enzyme is protected from 50% thermal inactivation is then a measure of the efficacy of the additive under study. By such means, one can readily quantitate and hence correlate both the stabilizing and labelizing effects of various diverse compounds.

b. Procedure - Stock dilutions of a crystalline suspension (in 55% saturated ammonium sulfate) of beef heart lactic dehydrogenase (Worthington Biochemical Corp.) containing 90 μg enzyme/ml can be stored in the cold (4 to 7°) for at least 1 month with no detectable loss in enzymic activity. This as well as all subsequent enzyme dilutions are prepared with 0.1 M potassium phosphate buffer, pH 7.0. All dilutions of the stock enzyme solution are used on the day of preparation. To test tubes containing 0.10 ml of enzyme (3 μg/ml in 0.1 M potassium phosphate buffer, pH 7.0) were added 0.10 ml of water of the compound to be tested (dissolved in water), the contents mixed and incubated at 37° (final enzyme concentration 1.5 μg/ml). Thermal inactivation is stopped by the addition of 2.6 ml of 0.03 M potassium phosphate buffer (pH 7.4) that is kept at room temperature. Under the conditions described above, when water is added to the enzyme (the control), about 50% of enzyme activity is lost in 30 minutes (Figure 1) as compared to the t_o (zero incubation time at 37°) sample. The amount of enzymatic activity remaining after such treatment is then determined by the addition of 0.20 ml of "pooled reaction mixture" (containing $NADH_2$ plus pyruvic acid), mixing and measuring the decrease in absorbance at 340 mμ as previously described. During this period, the extent of thermal inactivation is a function of the

time of incubation (Figure 1). By this procedure direct measurement of the efficacy of an added reagent over that of the control is readily made.

2. Thermal Inactivation for E. coli B Asparaginase

Stock dilutions of purified (lyophilized powder) E. coli B asparaginase (Worthington Biochemical Corp.) containing 2.5 mg enzyme/ml (in 0.14 M NaCl) can be stored frozen (-20°) for at least several months with no detectable loss in enzymic activity. All dilution of the stock enzyme solution are used on the day of preparation. To test tubes containing 0.10 ml of enzyme (250 µg/ml in water) are added 0.10 ml of water or the compound to be tested (dissolved in water), the contents mixed and incubated at 60° (final concentration 125 µg/ml). Thermal inactivation is terminated by plunging the tubes into an ice bath. Under the conditions described above, when water is added to the enzyme (the control), approximately 50% of the enzyme activity is lost in 5 minutes (Figure 2) as compared to the t_o (zero incubation time at 60°) sample. The amount of enzymatic activity remaining after such treatment is then determined by the addition of 0.35 ml of reaction mixture (containing asparagine and potassium phosphate buffer), mixing and incubating at 37° for 10 minutes. The reaction is stopped by the addition of TCA; the amount of NH_4^+ formed is then determined by Nesslerization as previously described. During this period, the extent of thermal inactivation is a direct function of the time of incubation (Figure 2). By this procedure, a direct measurement of the efficacy of an added reagent over that of the control is readily made.

3. Dilution Inactivation Assay for Pig Heart Malic Dehydrogenase

a) Principle

The dilution inactivation assay for pig heart malic dehydrogenase is based on the observation that under appropriate conditions of enzyme

concentration, pH and buffer concentration, the ice-bath temperature inactivation appears to be a function of the incubation (dilution) time. Approximately 30 minutes is required for 50% inhibition of pig heart malic dehydrogenase activity. The degree to which the enzyme is protected from 50% dilution inactivation is then a direct measure of the efficacy of the additive or solvent under study. By such means, one can quantitatively measure and hence compare, the usefulness of various additives and solvents as enzyme stabilizers.

b) Procedure

Stock dilution of a crystalline suspension (in 70% saturated ammonium sulfate) of pig heart malic dehydrogenase (Worthington Biochemical Corp.) containing 24 μg enzyme/ml. can be stored in the cold (4 to 7°) for at least one week with no detectable loss in enzymic activity. This as well as all subsequent enzyme dilutions was prepared with 0.1M potassium phosphate buffer, pH 7.4. To test tubes containing 0.010 ml. of enzyme (24 μg/ml. in 0.1M potassium phosphate buffer, pH 7.4) were added 0.190 ml. of water or the compound to be tested (dissolved in water), the contents mixed and incubated at $4^{\circ} \pm 2^{\circ}$ (final enzyme concentration 1.2 μg/ml.). Under these conditions, when water is added to the enzyme (control), about 50% of the enzyme activity is lost in 30 minutes (Figure 3) as compared to the t_0 (zero incubation or dilution time at $4 \pm 2^{\circ}$) sample. The amount of enzymatic activity remaining after such treatment is then determined by the addition of 2.6 ml. of 0.10 M potassium phosphate buffer, pH 7.4 and 0.20 ml. of a "pooled reaction mixture" (containing $NADH_2$ plus oxaloacetic acid), mixing and measuring the decrease in absorbance at 340 mμ at 15 second intervals for a total elapsed reaction time of one minute. During this period the extent of dilution inactivation is a function of the elapsed time after the dilution is made (Figure 3). By

this procedure, the efficacy of an added stabilizer over that of the control (water) is readily evaluated.

C. Preparation of Enzyme Coupled to Solid Support

1. Diazotization of p-aminobenzyl cellulose (Bio-Rad Cellex PAB)

A cold solution of sodium nitrite (5 ml., 14% w/v) was added to a suspension of Cellex PAB in cold hydrochloric acid (1 gm/25 ml 2N). The mixture was allowed to react for 1 hr at 0°C. The diazotised adsorbent was collected by filtration and washed with portions (50 ml) of a solution containing sodium acetate (5%, w/v) and urea (5%, w/v) and finally deionized water. The product was stored moist at 4° in a tightly sealed container until used.[4]

2. Coupling of Lactate Dehydrogenase to Diazotized Cellex PAB

Diazotized Cellex PAB (1 g.) was added to LDH (5 mg.) in a final volume of 5 ml. The mixture was stirred at 4° for 2 1/2 hours, centrifuged and the precipitate washed with portions (3-5 ml) of sodium chloride solution (1.0 M).[4] The supernatants were found to possess no detectable enzymatic activity and were discarded. The filter cake containing the LDH coupled to the diazotized Cellex PAB was stored moist and cold (4°) until assayed (see results).

D. Binding of IDH to Carboxymethyl Cellulose (Bio-Rad Cellex CM)

1. Preparation of CDI Activated Cellex CM Support

A 5% suspension (w/v) of carboxymethyl cellulose (CMC) was washed twice with distilled water, twice with 0.03M potassium phosphate buffer, pH 7.4 and stored as a 5% working suspension in 0.03M phosphate buffer, pH 7.4 (4°) until used. To replicate 500 mg samples of the 5% working suspension of CMC, was added 0.5, 2.5, 10, 25, 62, 125, 250 or 1250 mg of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate (CDI). The mixture was reacted,

with stirring, at room temperature for 30 minutes; then chilled to 4° and immediately used for the enzyme coupling step.

2. Coupling of Lactate Dehydrogenase to CDI Activated CMC Support

To each CDI activated CMC support mixture, 2.5 mg of dialyzed LDH was added, and the contents reacted with stirring at 4° for from 2 to 18 hours. At the conclusion of the reaction, the mixture was filtered, and washed successively with portions (6-10 ml) of cold sodium chloride solution (1.0 M) and potassium phosphate buffer (0.03M, pH 7.4). The washed filter cakes were suspended in 10 ml of additional buffered and immediately assayed (see results).

E. Procedure for the Production of Anti-LDH Sera in Rabbits

1. Preparation of Antigenic Suspension

a) Preparation of the adjuvant. The adjuvant was obtained by mixing 9 parts of light mineral oil (Drakol 6 VR - Pennsylvania Refining Co.) and 1 part Mannide Monooleate (Arlacel A-Atlas Chemical Co.)

b) Emulsification - to each ml of salt-free LDH (2 mg/ml) was added an equal volume of the adjuvant. Emulsions were prepared by forcing the contents back and forth through a 1.0 ml glass tuberculin syringe fitted with a 20 gauge needle. The process was continued until a drop of the emulsion expelled through the hypodermic needle into a beaker of cold water retained its shape.

2. Immunization Protocol - three subcutaneous injections spaced at 1 month intervals, the dose rate being 0.2 mg LDH (1 mg/ml emulsion) per injection.

3. Serum Collection - animals are to be bled 1 week after injections one and two, and three weeks and three months respectively after injection three.

PART III Biochemistry - Results

A. Beef Heart Lactate Dehydrogenase

1. Stabilization of Beef Heart Lactate Dehydrogenase to Long Term Storage by the Presence of Various Additives

Dilute aqueous solutions of beef heart lactate dehydrogenase (LDH) were stabilized by the use of various organic additives. Continuing long term studies of such combinations held for prolonged storage in the cold (4^{o}) have been followed. (Figures 4-7). Final concentrations of from 20 to 40% DMSO (w/v) were excellent stabilizing agents for LDH under our experimental conditions. LDH when stored in the presence of 40% DMSO possessed approximately 65% of its original activity after storage for 20 months. Lower concentrations were found to be less effective stabilizers, i.e. LDH in the presence of 30% DMSO had about 30% of its initial activity after 20 months storage while LDH lost all of its activity in 16 months when stored in the presence of 20% DMSO. However, all concentrations of DMSO investigated were far superior to the control which lost 80% of its activity in 3 months (Figure 4).

The success of these experiments, namely the stabilizing property of DMSO, led us to study other solvent mixtures as is shown in Figure 5. Dilute solutions of LDH (1.5 mg/ml) stored in the presence of from 10 to 40% DMSO plus 0.5% gelatin have retained virtually 100% of their original activity after 19 months storage. The control (water rather than DMSO-gelatin additives) possessed approximately 10% of its original activity under otherwise identical conditions.

As a result of systematic investigations involving the effectiveness of protein additives (gelatin and bovine serum albumin) as well as selective organic compounds (ethyl alcohol, methyl alcohol, isobutanol, etc.,) we have been able to devise "optional" storage conditions for LDH. Utilizing our rapid

thermal inactivation screening technique, organic compounds were chosen for long term stabilization studies. As can be noted in Table I dimethyl formamide which originally demonstrated initial stabilizing properties for LDH in the thermal dilution assay, was not effective in prolonged storage. After one month formamide containing samples had deteriorated to approximately 10% of their original activity while the control retained 29%. Studies involving ethyl acetate (Table I) demonstrated values approximating the control figure of 29% after one month of storage (4°), while both of the aforementioned samples had no LDH remaining at the end of six months (control = 15%).

Other solvents, such as methyl and ethyl alcohol were only partially effective in stabilization of LDH (Figure 7, Table I). At six months the aqueous control had only 15% of its original activity while the alcohol samples averaged approximately 40%; in ten months the activity of the alcohol samples was about 25% versus 13% for the control. Final twenty months samples showed values of 10% to zero while controls were zero.

Isobutanol in our initial experiments was shown to be an effective stabilizing agent. However, upon prolonged storage higher concentrations demonstrated less stabilizing capabity than lesser amounts. (Table I). For example, at the end of one month, samples containing one and five percent isobutanol retained 70% of the original LDH activity while 20% had 46% activity. Subsequently, in 20% isobutanol the LDH fell to 18% in 6 months, 12% in 10 months and zero at 20 months, while the samples containing one and five percent decreased to 50% in 6 months, 40% in 10 months and 25% in 20 months.

We have successfully extended the activity of dilute aqueous solutions of LDH to 16 months by using the proteins, gelatin and bovine serum albumin as

protectants. Gelatin proved to be an excellent agent for stabilizing LDH towards long term storage; the LDH retained approximately 70% of its original activity after 16 months storage. However, lower concentrations though less effective were nevertheless superior to the control. (Table I, Figure 6).

Bovine serum albumin used under identical storage conditions demonstrated little advantage over the use of water. (Table I).

2. Preparation and Properties of Insolubilized LDH

a. Preparation

LDH has been successfully coupled to diazotized cellex PAB (an insoluble support) resulting in an insoluble derivative possessing enzymic activity. Under standard binding conditions, activities in the range of 0.02 to 0.4 O.D. units/min./mg. were obtained. Maximum enzymic activity for the insolubilized coupled LDH was obtained when a binding ratio of 0.5 g of adsorbent was reacted with 0.2 mg of LDH (Fig 8). As evident from this figure, when the proportion of LDH is increased with respect to the diazotized cellex, the activity of the produce decreases, indicating possibly the formation of excessive covalent bonding. This optimum relationship is further demonstrated by a second series of experiments (Fig. 9) where, when the amount of adsorbent was held constant and progressively lower levels of LDH were reacted, lower levels of enzymic activity were observed.

b. Properties - Ability to carry out sustained enzymic reactions

When coupled LDH was compared to free LDH with respect to its ability to carry out sustained enzymatic activity in the presence of sequential substrate (pyruvate + NADH) additions, it was found to be far superior to the native enzyme as can be seen in figure 10 - report VII. Whereas the rate of the reaction with the uncoupled LDH rapidly decreased with successive additions of substrate (curve b), the reaction rate as catalyzed by the PAB coupled

enzyme was virtually independent of the number of substrate additions after the first addition (curve a).

Initial experiments, studying the effect of drying on LDH coupled to diazotized cellex PAB did not appear promising. For when replicate samples of coupled enzyme were dried by the following methods; freeze drying, Rinco evaporator or solvent drying by ethanol, methanol or acetone in the presence or absence of DMSO, the dried products possessed no detectable activity.

c. Stability of LDH coupled to diazotized Cellex PAB stored in aqueous solution at various temperatures.

Insolubilized LDH-PAB cakes, having an initial activity of ΔO.D. = 0.260/min. were suspended in 0.03 M potassium phosphate buffer, pH 7.4 and stored at 37°, 28°, 4° and -20°. The cakes were assayed after various periods of storage, and the results are shown in Table II. As is illustrated, the cakes and their residual buffer supernatants maintain a high degree of activity for at least 2 weeks. The washed cakes on the other hand, retain only about 50% of the initial enzyme activity after 2 days storage. In all cases, storage of insolubilized enzymes at 37° resulted in complete inactivation after 24 hours. After storage for 7 days, only the sample stored at -20° was found to possess activity. From Table II it appears that when bound LDH is stored in an aqueous medium it generally uncouples, with the degree of this reversal being dependent upon the storage conditions.

3. Binding of LDH to other insoluble supports

A number of attempts were made to bind LDH to carboxymethyl cellulose (Cellex CM*) in the presence and absence of a water soluble carbodiimide**.

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* 1 - Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-Toluenesulfonate Aldrich Chemical #112451).

2 Cellex CM-Bio-Rad Company

To date, none of our preparations have shown significant enzymic activity.

4. Studies on the Effect of 1-Cyclohexyl-3(2-morpholinoethyl)-carbodiimide methc-p-toluene sulfate (IDC) on BHLDH Activity

The water-soluble carbodiimide derivative (IDC) is known to produce cross-linking in proteins[5] and to modify carboxylic acid groups in enzymes by producing acylureas.[6] As we had the intention of using this reagent in our experiments with BHLDH, it was necessary to investigate, first of all, its effect on this enzyme. Therefore, ICD was reacted with BHLDH in 50, 250, 500 and 5000 excess (on a molar basis) with BHLDH in the cold (4 to 7°) for 18 hours and then the enzyme was assayed. The results, summarized in Table III, although only preliminary at that time, nevertheless show that BHLDH could safely tolerate up to about a 250 fold molar excess of ICD with no apparent inhibition of enzyme activity. BHLDH suffered an approximately 50% loss in enzyme activity by treatment with 500 fold molar excess of ICD. Thermostability studies had been planned for the ICD reacted BHLDH.

5. Stimulation of LDH Antibodies in Rabbits

Antibodies to LDH have been produced in rabbits following the protocol described in the Methods Section. The antibodies appeared in the serum in trace amounts after the second injection and in significant quantities after the third injection. Using Ouchterlony Agar Diffusion techniques, a positive band of precipitating antigen-antibody was found with 90 γ LDH/ml antigen level.

Although our plan was to prepare isolated enzyme-antibody complexes and to examine them for their stability and enzymatic activity under various storage conditions, time and budgetary restrictions did not permit this.

B. E. coli B. Asparaginase

In order to extend our model enzyme studies, the effect of a series of organic compounds in protecting E. coli B asparaginase against thermal inactivation was investigated. The standardized conditions for the thermal inactivation of the asparaginase are described in complete detail in the methods section of this report. The compounds chosen for our initial studies were those whose effects on the thermostabilization of beef heart lactic dehydrogenase had already been investigated. The reagents investigated were: methanol, ethanol, n-propanol, n-butanol, acetone, glycerol, tetrahydrofuran, dioxane, dimethylsulfoxide, formaldehyde, acetic anhydride, dimethylformamide, ethylacetate, isopropanol, bovine serum albumin and gelatin. A summary of the results is presented in Table IV and Table V. The most interesting of these complunds, as judged by their ability to protect the enzyme against thermal inactivation, were ethylacetate and acetone.

C. Pig Heart Malate Dehydrogenase (MDH)

1. Stabilization to Dilution Inactivation

A third model enzyme system and one that was intrinsically more labile, pig heart malate dehydrogenase (MDH), was chosen in order to extend the scope of our studies on enzyme stabilization. A slightly different type of assay was developed for MDH, namely a dilution inactivation procedure; this assay is described in complete detail in the methods section of this report.

A systematic investigation was made of the effect of the previously investigated organic compounds on reducing the susceptibility of MDH towards dilution inactivation. The reagents investigated included the following; methanol, ethanol, n-propanol, isopropanol, n-butanol, iso-butanol, acetone, dioxane, tetrahydrofuran, dimethylformamide, ethylacetate, dimethylsulfoxide, dimethylsulfone, hexamethylphosphortriamide and glycerol. A summary of the

results is presented in Table VI. The most promising of these compounds, as judged by their ability to protect MDH against dilution inactivation, were ethanol, i-propanol, acetone, dioxane and glycerol. All of these compounds exhibited concentration ranges over which they preserved 90% or more of the original activity. With i-propanol, acetone and glycerol, there was even the suggestion of enzyme activation, i.e. greater than 100% of the original activity being present.

2. Stabilization of MDH to Long-term Storage. Dilute solutions of MDH (1.2 μg/ml) can be stored in the presence of gelatin, glycerol and ethanol mixtures (4 ± 2^{o}) with retention of enzyme activity. A summary of results showing long-term stabilization of MDH (16 weeks) in the presence of gelatin-glycerol-ethanol mixtures is presented in Figure 11. It is evident that the enzyme samples that remained in contact with the additives gelatin, glycerol and ethanol mixtures have retained a considerable portion of their original activity; whereas the control (water) lost virtually 100% of its activity during overnight storage. The most effective additive for the long-term stabilization of dilute solutions of MDH appears to be a mixture of 0.1% gelatin plus 70% glycerol; where dilute MDH solutions retained a significant portion of their original activity (25%) for over 35 weeks.

Part IV Chemistry Results

One of the objectives of our program was to determine whether the selective blocking of sulfhydryl groups, non-essential for enzymatic activity, would be of potential value for the stabilization of enzymes. Our approach was to prepare an alkylhalothiosulfate that would be capable of reacting selectively with nonessential-SH groups to yield a stable S-substituted derivative. We chose to synthesize[7] a haloethylthiosulfate, which after transformation to the disulfide, would undergo an intramolecular nucleophilic displacement as follows, yielding an intramolecular cyclic sulfonium salt. With such a reagent $ClCH_2CH_2CH_2SSO_3Na$, sulfonium-type cyclization involves the protein S exclusively and cyclization to the haloalkyl S is prevented by virtue of an unfavorable ring-size. The reactions between such an alkylating reagent and an enzyme leading to a stable thiocyanide can be represented as follows:

$$RSH + ClCH_2CH_2CH_2SSO_3^{\ominus} \longrightarrow RSSCH_2CH_2CH_2Cl$$

I

$$RSSCH_2CH_2CH_2Cl \longrightarrow RS^{\oplus}\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH_2$$

II

$$R\text{-}S^{\oplus}\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH_2 + KCN \longrightarrow RSCH_2CH_2CH_2SCN$$

II III

where RSH represents a protein with a free -SH function.

Before reacting the γ-chloropropylthiosulfate with LDH (or other enzymes), we felt it was necessary to become acquainted with the properties of this

compound and to study its reaction with simple -SH compounds. After initial studies with cysteine, which appeared to be too susceptible to oxidation, we chose glutathione (GSH) as the model compound.

The thiosulfate I was found to be stable in water and in aqueous solutions of sodium acetate for as long as 24 hours. The compound was a strong alkylating agent as indicated by its vigorous reaction with γ-(nitrobenzyl)pyridine (the so-called NBP reagent[8]). Alkylating agents such as di(β-chloroethyl) amine give an intense blue color with this reagent under appropriate conditions, both in solution and on paper chromatograms. On the other hand, the thiosulfate I was rapidly converted to another alkylating compound (IV) of undetermined structure in the presence of sodium hydroxide or sodium bicarbonate.

GSH reacted with I only sluggishly in aqueous solution, and paper chromatography revealed the formation of several by-products. One of these was apparently GSSG, formed by oxidation of GSH. On the other hand, the desired reaction (formation of II) readily occurred in the presence of 3 or 6 equivalents of sodium acetate. When 1 ml of 0.1 M GSH was treated first with 1 ml of 0.1 M aqueous thiosulfate I, and then with 1 ml of 0.3 M sodium acetate, and the resulting solution chromatographed on thin layer plates (after standing for 1 hr at room temperature), a new compound giving a ninhydrin-positive reaction was formed at the expense of both GSH and I. This compound was presumed to be compound II, however budgetary and time restrictions prevented its isolation and characterization by conventional means. (For chromatography data, see Table VII).

Employing conditions analogous to those found to be successful for the alkylation of glutathione, namely one hour incubation of 1 equivalent of LDH with 1 equivalent of aqueous thiosulfate in the presence of 3-6 equivalents of sodium acetate, followed by conversion to a stable thiocyanide by incubation

with an equivalent of KCN, LDH reacted only sluggishly (if at all) with the thiosulfate. Further work along these lines, in order to optimize conditions, was curtailed because of budgetary cutbacks.

Part V - Appendix

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Figure 1

Effect of Preincubation at 37° on BHLDH Activity

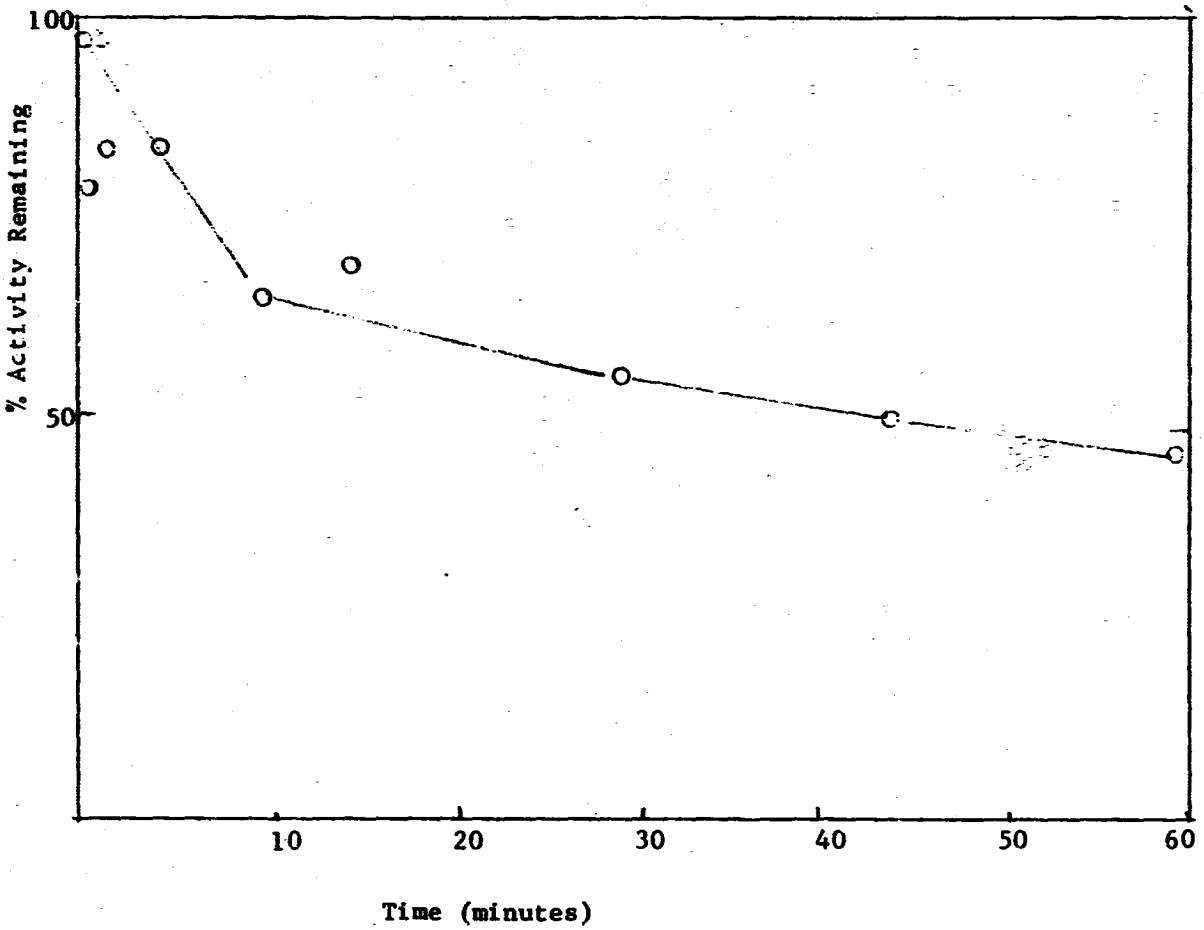


Figure 2

Thermal Inactivation Curve - Asparaginase

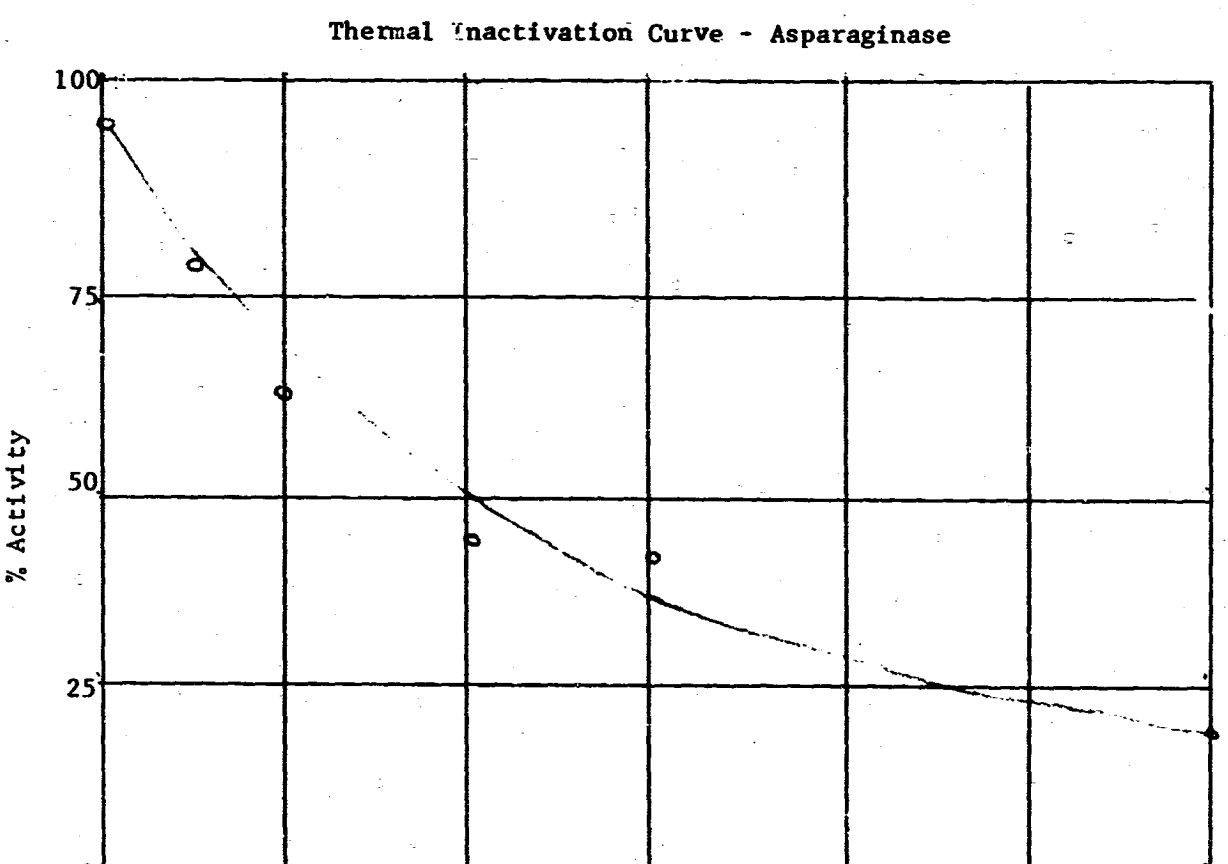


Figure 3

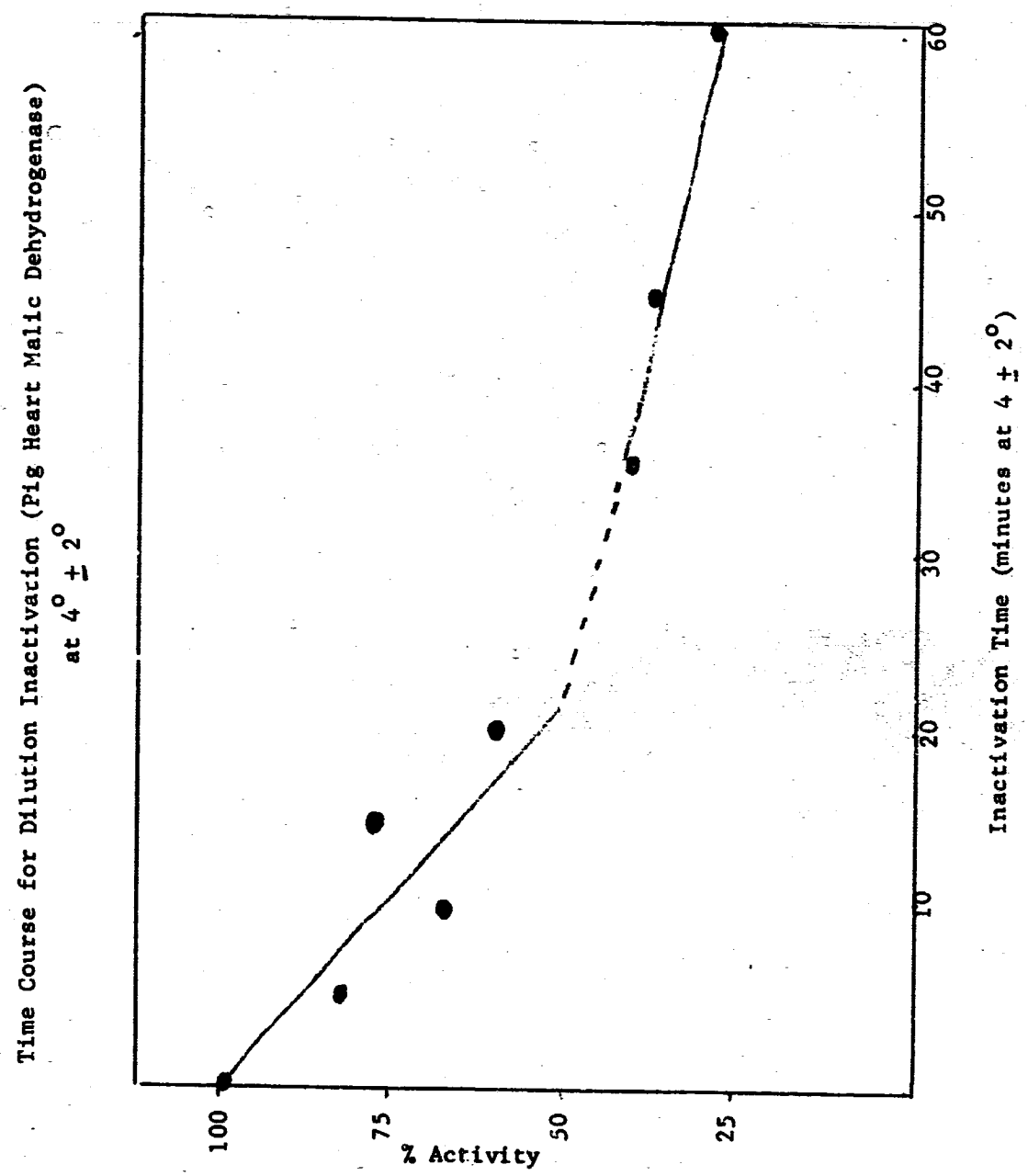
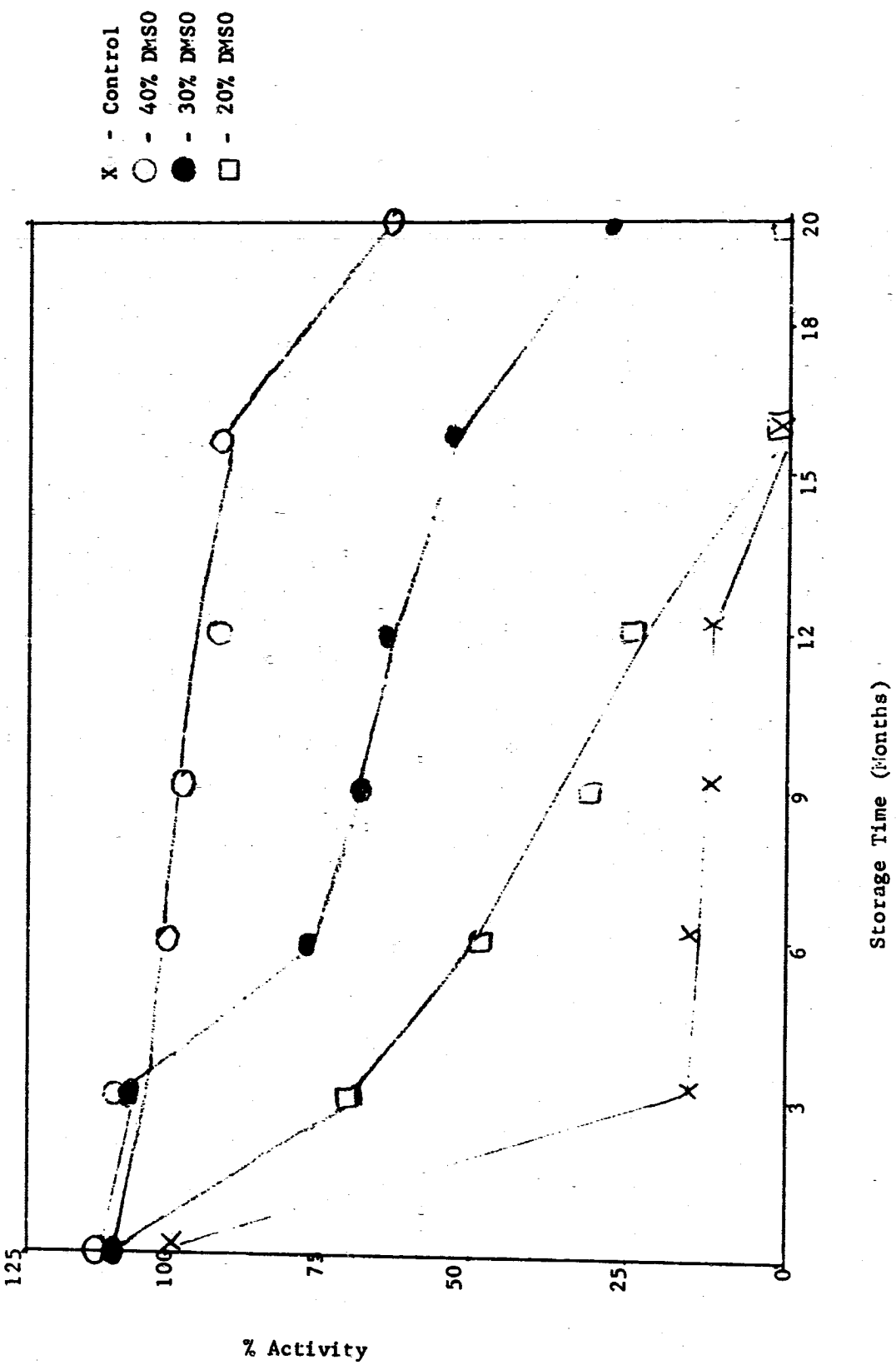

Time Course for Dilution Inactivation (Pig Heart Malic Dehydrogenase) at 4° ± 2°
% Activity
100
75
50
25
Inactivation Time (minutes at 4 ± 2°)
10
20
30
40
50
60

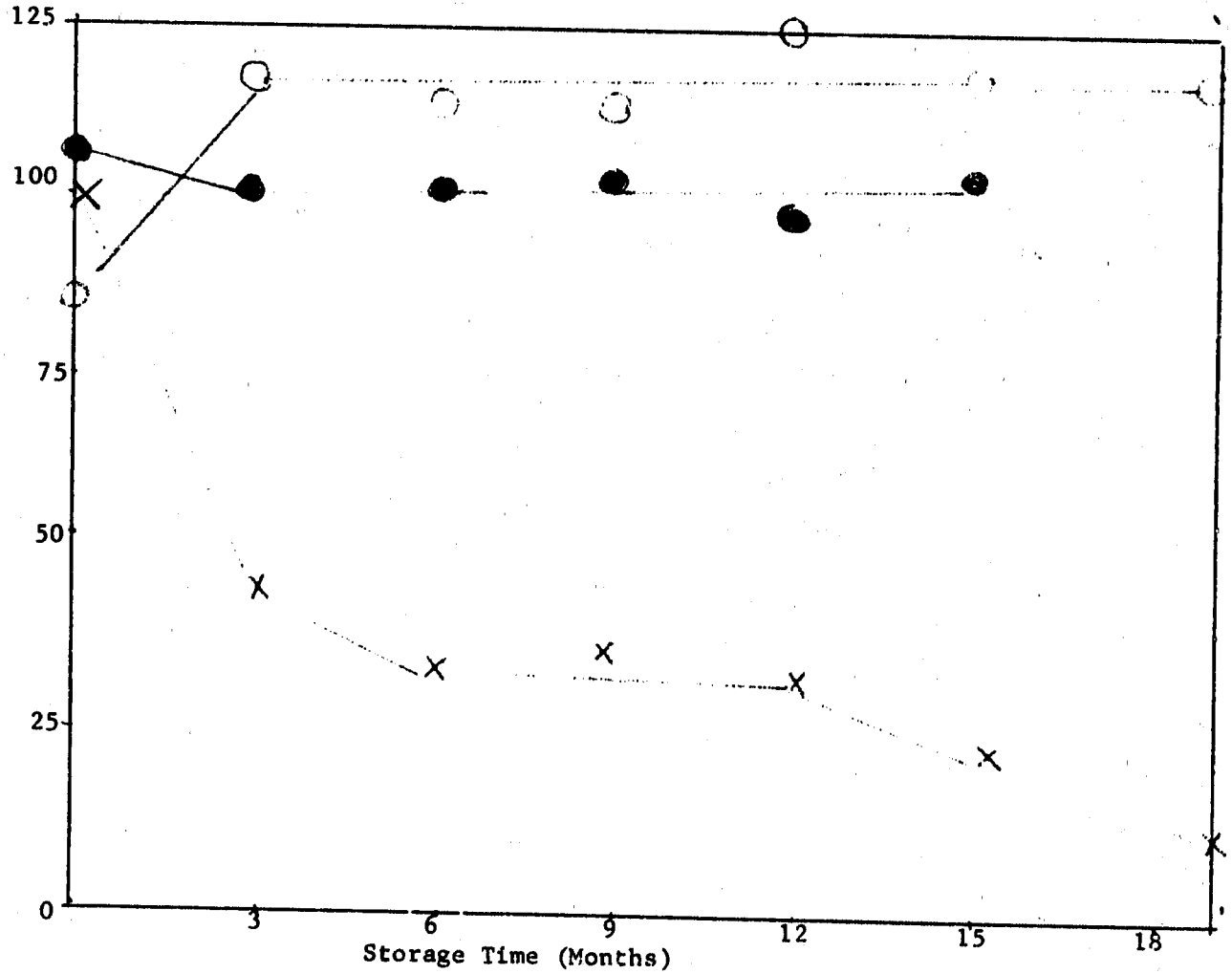
Figure 4

Stabilization of Lactate Dehydrogenase by Dimethylsulfoxide



Stabilization of Lactate Dehydrogenase by Combination of Dimethylsulfoxide and Gelatin

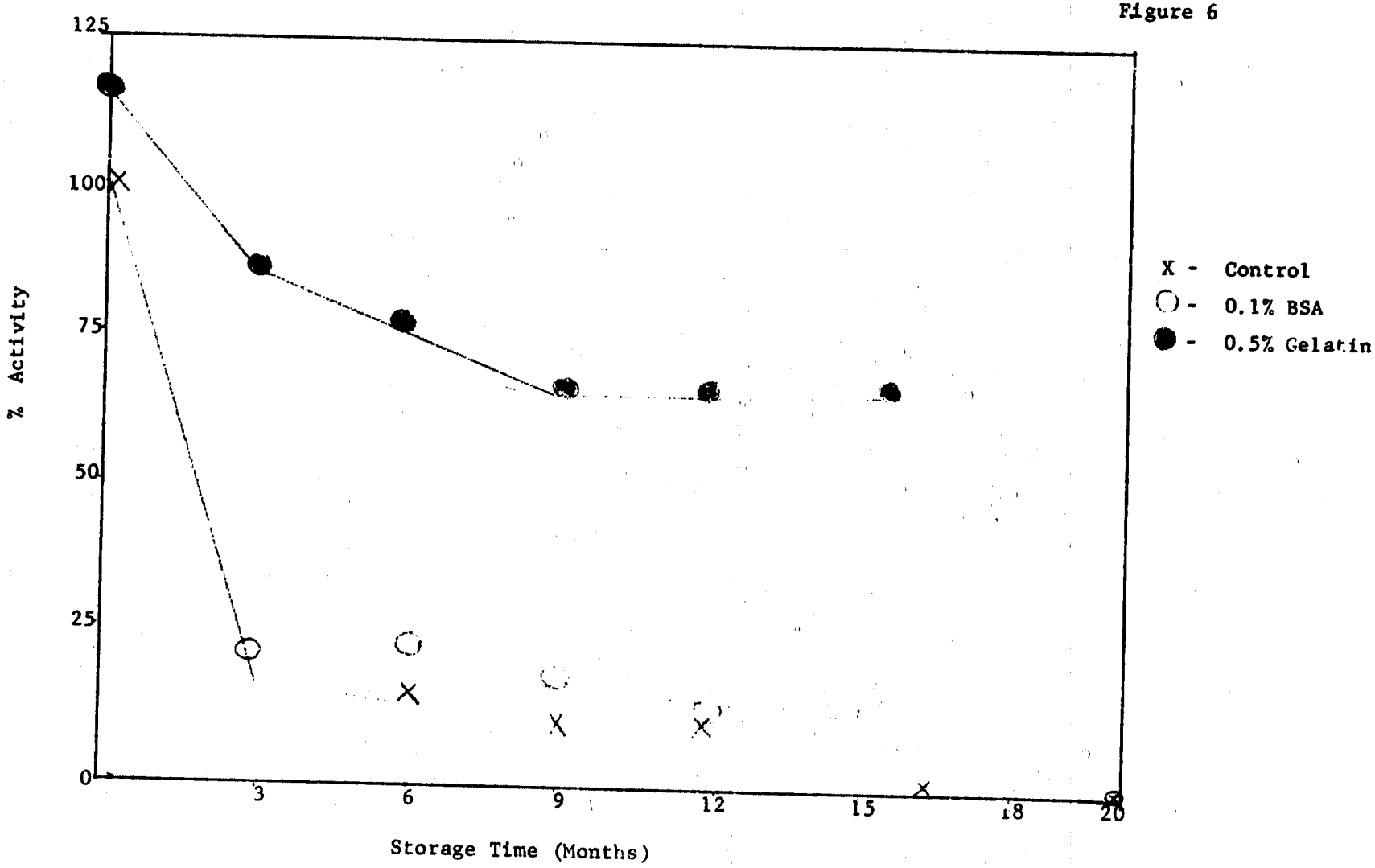
Figure 5



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Stabilization of Lactate Dehydrogenase by Bovine Serum Albumin and Gelatin

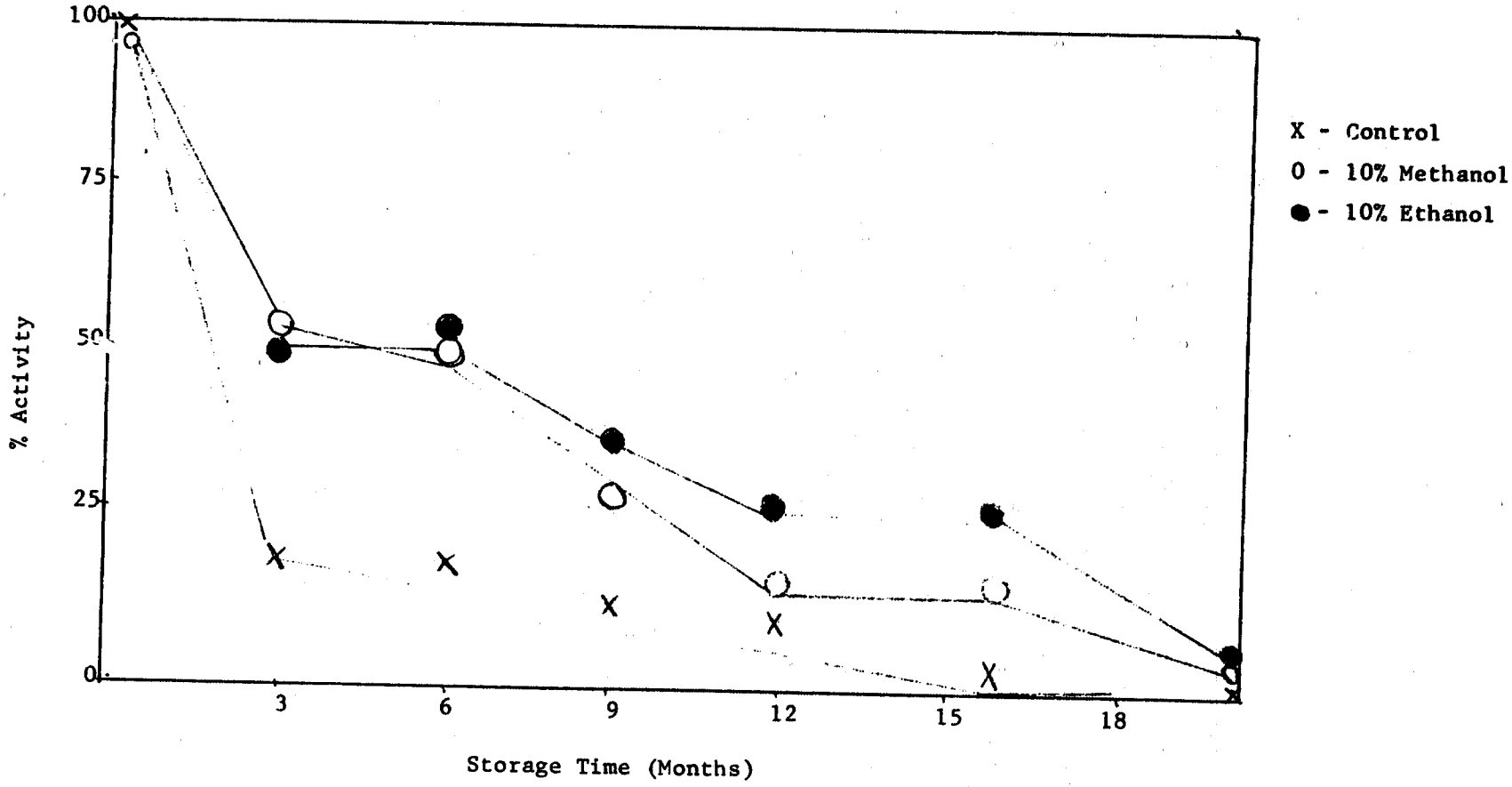
Figure 6



COLLABORATIVE RESEARCH, INC.

Figure 7

Stabilization of Lactate Dehydrogenase by Methanol and Ethanol



COLLABORATIVE RESEARCH, INC.

Figure 8

Activity of Insolubilized Lactate Dehydrogenase (Coupled to Diazotized Cellex PAB) as a Function of Increasing Amounts of LDH Reacted

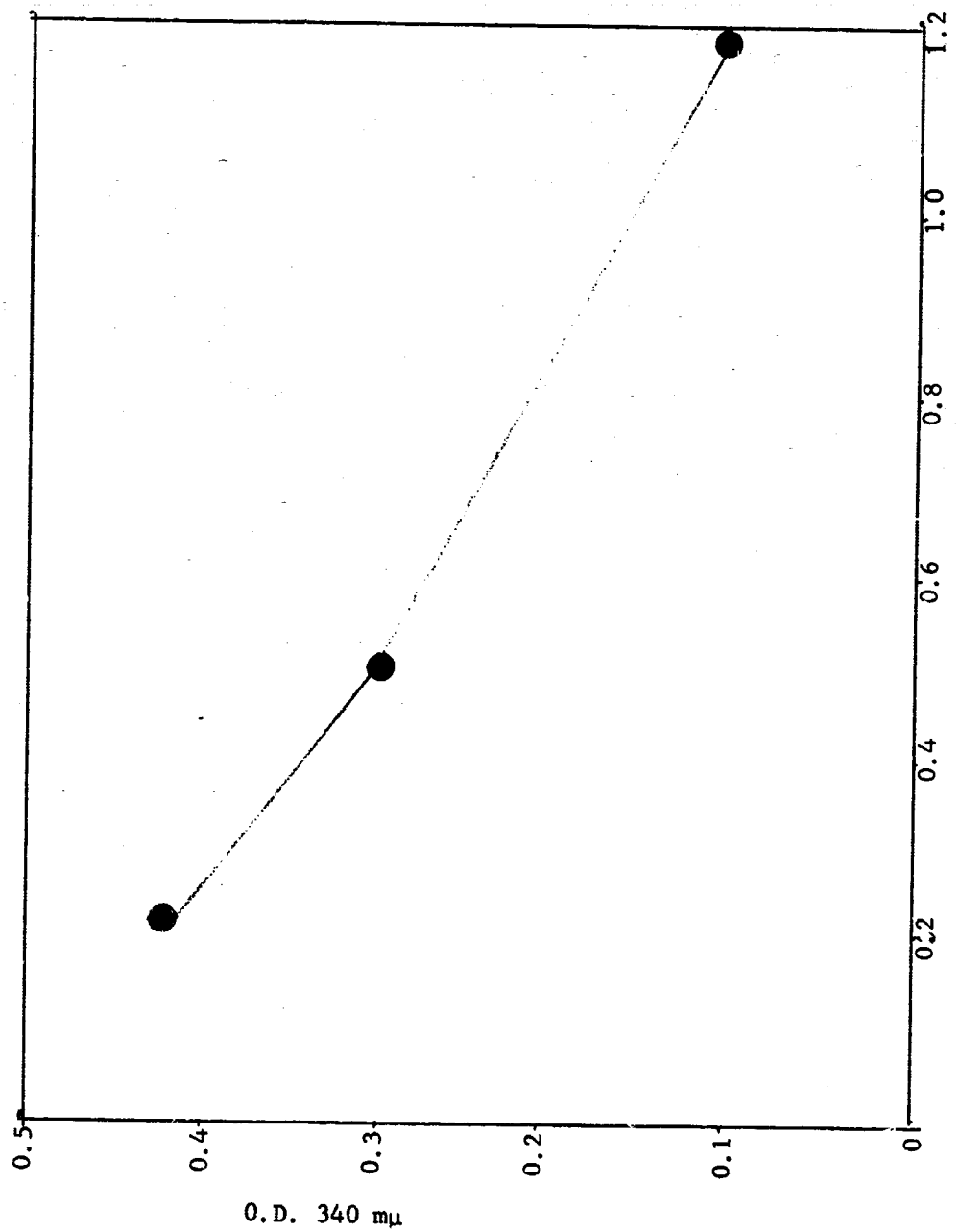


Figure 9

Activity of Insolubilized LDH (Coupled to Diazotized Cellex PAB as a Function of Decreasing Amounts of LDH Reacted

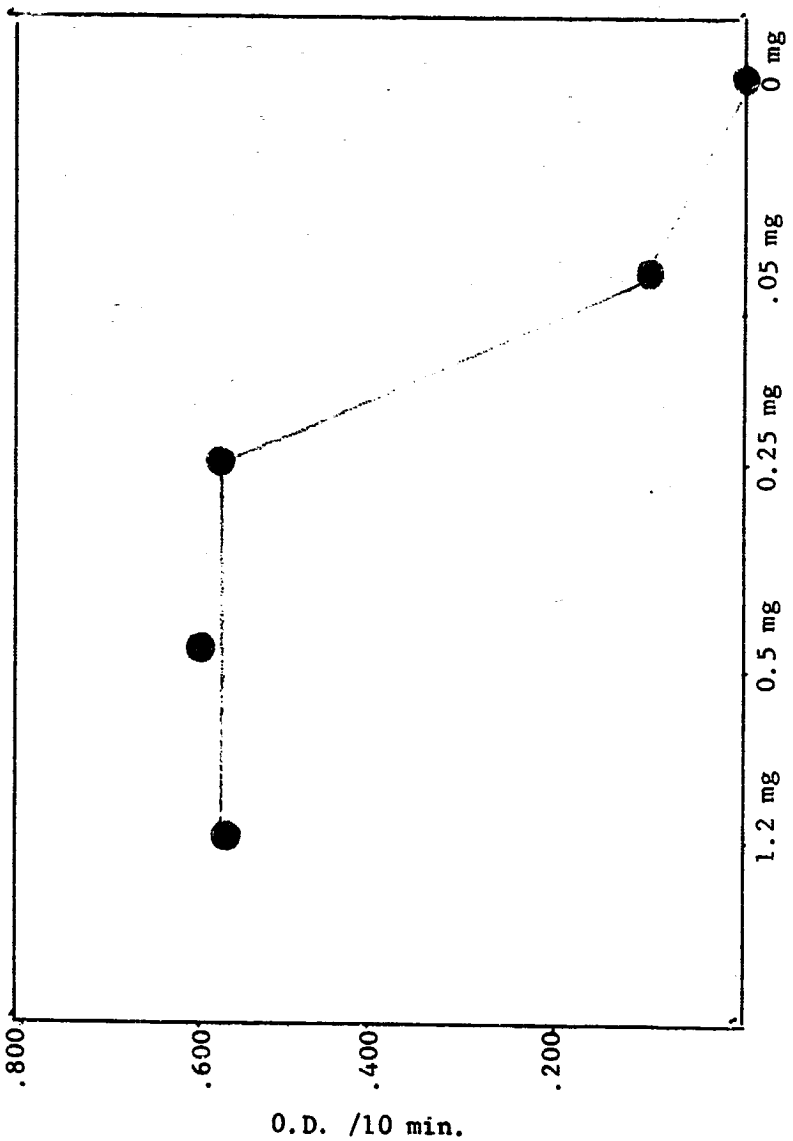


Figure 10

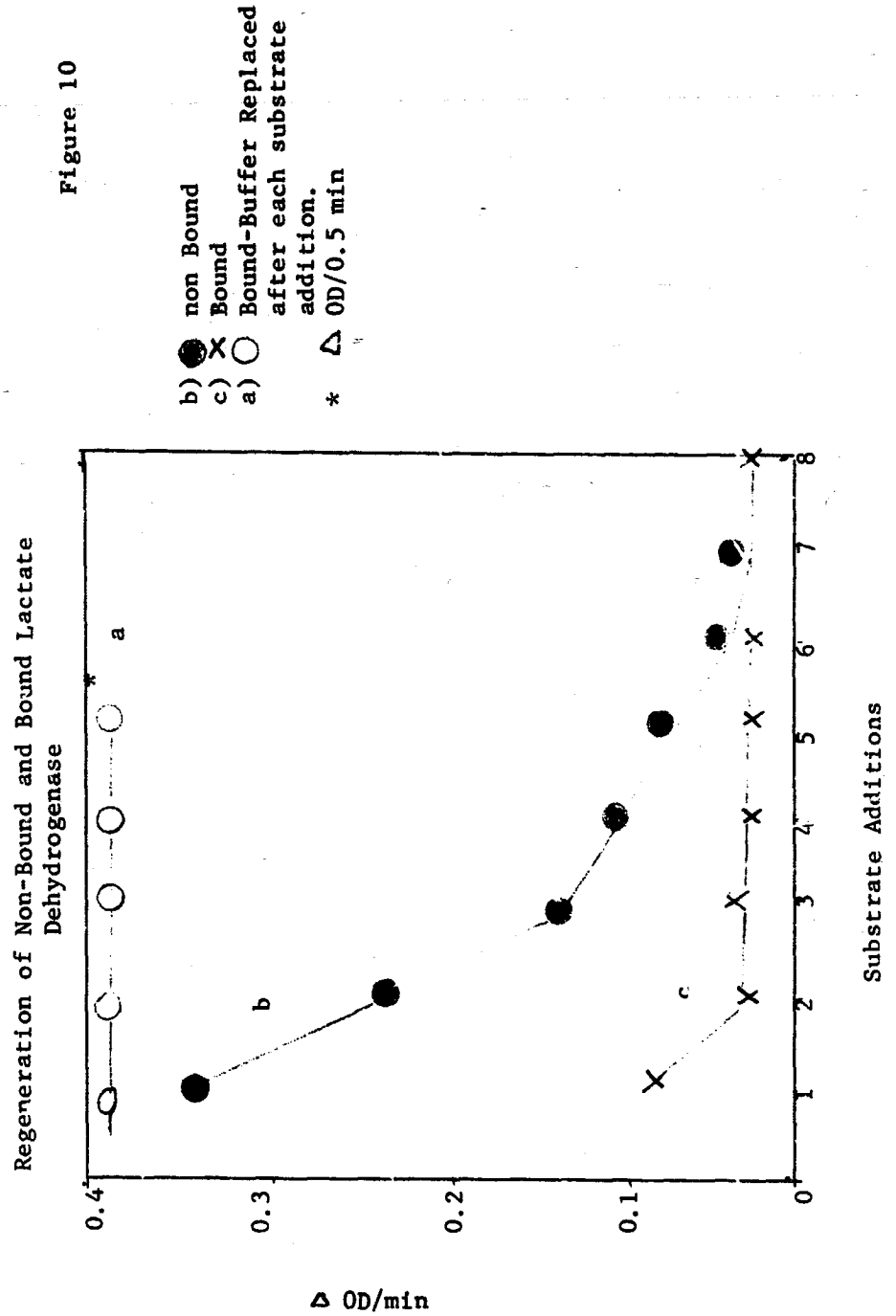

Regeneration of Non-Bound and Bound Lactate Dehydrogenase
b) non Bound
c) Bound
a) Bound-Buffer Replaced after each substrate addition.
* Δ OD/0.5 min
a
b
c
Δ OD/min
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
Substrate Additions

Figure 11

Effect of Organic Compounds on Stabilization of PHMDH to Long Term Storage at 4°C

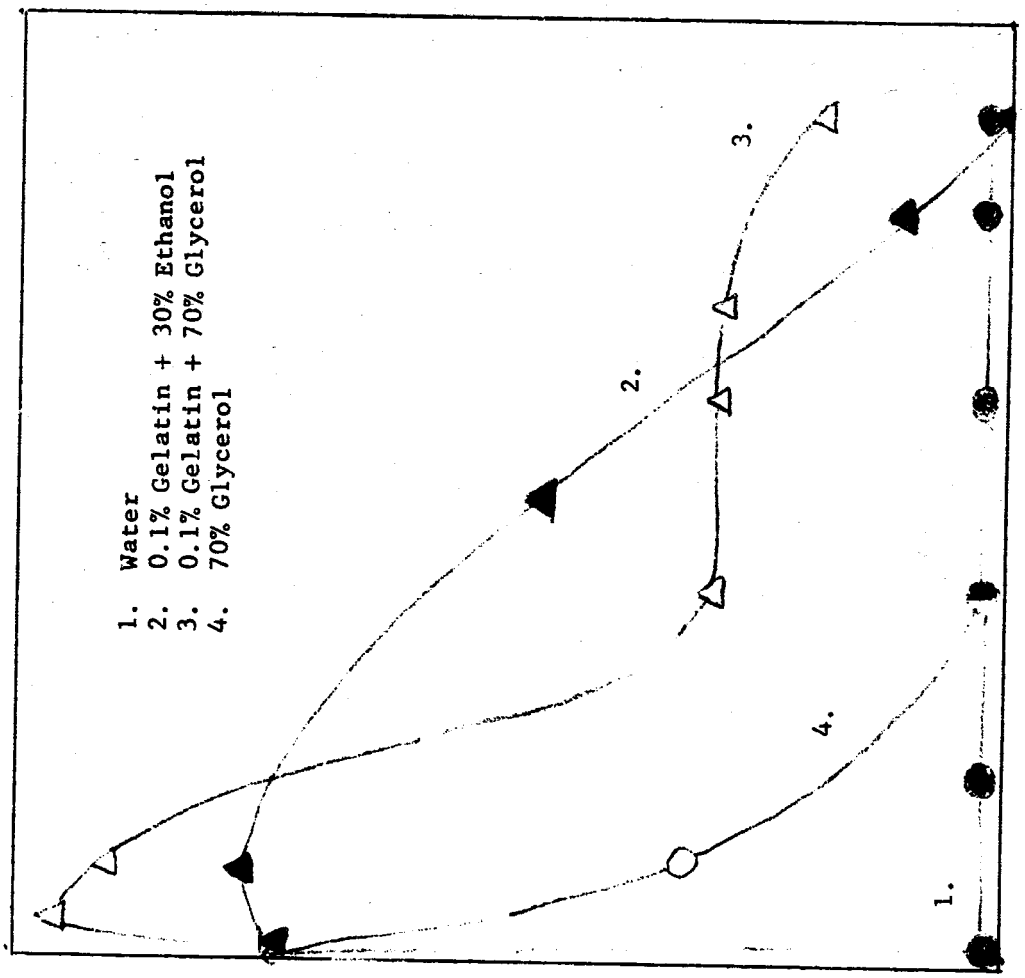


TABLE I

Stabilization of Lactate Dehydrogenase

Summary - Percent Activity

Compound (w/v)		Time (Months) 1	6	10	20
5	Dimethyl	9	0		
10	Formamide	0	0		
20		12	0		
0.3	Ethyl	36	0		
0.6	Acetate	29	0		
1.2		16	0		
2.5	Methyl	50	37	26	11
5.0	Alcohol	55	35	11	0
10		51	48	26	9
2.5	Ethyl	50	30	9	0
5.0	Alcohol	60	48	39	0
10		60	52	35	11
0.025	Bovine	42	30	15	0
0.05	Serum	37	28	17	0
0.10	Albumin	28	24	12	0
0.25	Gelatin	86	81	90	58*
0.5		88	76	70	70*
1.0		60	59	-	70*
1.0	Isobutanol	70	57	43	25
5.0		70	53	37	25
10		14**	-	-	-
20		46	18	12	0
10	DMSO+0.5 Gelatin	101	114	108	67**
20	DMSO+0.5 Gelatin	116	110	110	110
30	DMSO+0.5 Gelatin	110	110	106	98
40	DMSO+0.5 Gelatin	118	103	120	111
20	DMSO	91	50	28	0
30		95	76	62	28
40		104	100	100	65
Water Control		29	15	13	0

* 16 months

** Contaminated Sample

TABLE II

Stability of LDH Coupled to Diazotized Cellex PAB
Stored in 0.03M PO_4 pH 7.4 at Various Temperatures (^{o}C)

	% Activity - Cake + Supernatant				% Activity - Washed Cake			
	37^{o}	28^{o}	4^{o}	-20^{o}	37^{o}	28^{o}	4^{o}	-20^{o}
2 hrs	160	140	120	160	93	100	100	100
1 day	65	-	-	-	0	-	-	-
2 days	77	120	140	69	0	46	62	34
7 days	0	250	210	35	0	0	0	35
14 days	0	77	35	31	0	0	0	0

TABLE III

Effect of ICD[1] on BHLDH Activity

Molar Conc. ICD	Molar Ratio $\frac{ICD}{LDH}$[2]	Apparent Activity
0	0	100
3.5×10^{-4}	50	100
1.8×10^{-3}	250	100
3.5×10^{-3}	500	72
3.5×10^{-2}	5000	20

1 1-Cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-p-toluen sulfate

2 Molecular weights of BHLDH and ICD are 140,000 and 423.6 respectively.

TABLE IV

Effect of Organic Compounds on Asparaginase Thermostabilization

Compound	% of Activity at Solvent Concentration (V/V)[1]			
	0%	5%	10%	20%
Methanol	50	68	55	45
Ethanol	50	56	38	35
n-propanol	50	17	9	9
i-propanol	50	43[2]	20[3]	41[4]
n-butanol	50	52[5]	--[6]	
Acetone	50	71	52	44
Glycerol	50	56	46	50
Dioxane	50	55	34	10
Dimethylsulfoxide	50	--	31	10
Dimethylformamide	50	63	71	39
Ethylacetate	50	97[7]	89[8]	13[9]
Tetrahydrofuran	50	65	25	12

[1] Enzyme activities normalized to 50% activity for zero concentration organic compound present in control. Values shown are observed E. coli B asparaginase activities after preincubation (60°-10 min.) at concentrations of organic compound shown and assayed as described (Cf. Assay for Enzymic Activity).

[2] 1.25% (V.V) Isopropanol

[3] 2.5% (V/V) Isopropanol

[4] 5.0% (V/V) Isopropanol

[5] 1.25% (V.V) n-butanol

[6] 2.5% (V.V) n-butanol

[7] 0.625% (V/V) Ethylacetate

[8] 1.25% (V/V) Ethylacetate

[9] 2 5% (V.V) Ethylacetate

TABLE V

Effect of Organic Compounds on Asparaginase Thermostability

Compound	% Activity at Compound Concentration (W/V)			
	0%	.005%	.05%	.5%
Bovine Serum Albumin	50	55	50	60
Gelatin	50	50	47	50

TABLE VI

Effect of Organic Compounds on Pig Heart Malic Dehydrogenase Dilution Inactivation

Compound	% of Activity at Compound Concentration (V/V)[1]									
	0%	1.5%	2.5%	5%	10%	20%	30%	40%	50%	60%	70%
Methanol	50	-	-	51	63	62	59	63	66	40	20
Ethanol	50	-	-	75	72	87	87	76	79	68	62
n-Propanol	50	-	-	74	78	77	31	11	-	-	-
i-Propanol	50	-	-	85	-	99	105	-	-	-	-
n-Butanol	50	84	67	-	-	-	-	-	-	-	-
i-Butanol	50	79	88	-	-	-	-	-	-	-	-
Acetone	50	-	-	76	77	95	99	109	101	81	-
Ethylacetate	50	57[2]	-	4	11	-	-	-	-	-	-
Dioxane	50	-	-	86	94	77	77	-	-	-	-
Dimethyl-formamide	50	-	-	65	-	58	62	-	-	-	-
Dimethyl-sulfoxide	50	-	-	50	32	42	38	-	-	-	-
Dimethylsulfone	50	-	-	48	25	83	-	-	-	-	-
Hexamethyl-phosphortri-amide	50	-[3]	-	-	-	-	-	-	-	-	-
Glycerol	50	-	-	59	57	82	96	109	94	107	109
Tetrahydrofuran	50	-	-	59	63	73	78	21	-	-	-
Formaldehyde	50	-[3]	-	-	-	-	-	-	-	-	-
Acetaldehyde	50	-[3]	-	-	-	-	-	-	-	-	-

[1] Enzyme activities normalized to 50% activity for zero concentration organic compound. Values shown are observed pig heart malic dehydrogenase activities after dilution elapse time (4 ± 2^{6}) of 30 minutes at concentration of organic compound shown and assayed as described. (Cf. Assay for Pig Heart Malic Dehydrogenase Activity.) All values are ±5% error.

[2] 1.0% (V/V) ethylacetate.

[3] These compounds inhibited the enzyme at zero time, and hence were eliminated from further consideration.

TABLE VII

Rf Values on TLC Plates (Cellulose F, Merck) (x)

Compound	Solvent: n-BuOH-AcOH-H_2O 6 : 2 : 2	Solvent: methanol-pyridine-water 4 : 2 : 1
$Cl(CH_2)_3$ SSO_3 Na (I)	0.54[1]	0.76[1]
GSH	0.14[2]	--
Reaction product (II)	0.53[2]	--
GSS G	0.01[2]	--
IV	--	0.92[1]

x Run at 4^{o} for 5 hr.

1 Detected by the NBP reaction

2 Detected with ninhydrin

VI

Publications and Communications to Scientific Meetings

The following Communications have been made on the work reported in this document:

"Stabilization of Lactic Dehydrogenase by Organic Solvents"*
156th National Meeting, Am. Chem. Soc., Atlantic City, 1968.

"Stabilization of Malic Dehydrogenase by Organic Solvents"*
1st Northeast Regional Meeting, Am. Chem. Soc., Boston, 1968

The following paper has been published:

"Stabilization of Lactate and Malate Dehydrogenase by Organic Solvents". Biochem. Biophys Acta 191, 466 (1969). *

The following paper is in preparation:

"The Effect of Dimethylsulfoxide on Beef Heart Lactic Dehydrogenase"

* Abstracts and paper form part of this report, see pp. 42-46

STABILIZATION OF LACTIC DEHYDROGENASE BY ORGANIC SOLVENTS.* Harvey George, Joan McMahan, Katherine A. Herrington, Collaborative Research, Inc., Waltham, Mass. 02154

Beef heart lactate dehydrogenase has been found to be partially stabilized by a number of diverse organic solvents, the most promising of which is dimethylsulfoxide (DMSO). Two different parameters were established by the authors for the measurement of enzyme stabilization, namely, thermostability and long term storage in the cold (4°). With the first, a thermostability criterion was established, whereby in the absence of any stabilizing agent, BHLDH was 50% inactivated by a 30 minute incubation at 37°. The second procedure involved long term storage at 4°, where in the absence of DMSO the enzyme was completely inactivated in eight weeks; in the presence of 20 to 30% DMSO (V/V), the enzyme possessed complete activity. The former procedure proved very effective for the rapid screening of many compounds, only the most promising of which were tested by the second method. Some of the other organic solvents which were tested and found to be partially effective include the following: methanol, ethanol, propanol, n-butanol, i-butanol, acetone, dioxane, and dimethylformamide.

*This work herein reported is supported by the National Aeronautics and Space Administration and is based on Research Reports prepared for the Ames Research Center, NASA, under Contract No. NAS2-4525.

STABILIZATION OF MALIC DEHYDROGENASE BY ORGANIC SOLVENTS. * Harvey George, Mary Elizabeth Elliott, Joan McMahan, Collaborative Research, Inc., Waltham, Mass. 02154

The stability of pig heart malic dehydrogenase has been found to be enhanced by a number of different organic solvents. The efficacy of enzyme stabilization by a given solvent was determined by two different parameters, namely, pulse inactivation and long term storage (both procedures carried out in the cold, 4°). With the first, a pulse criterion was established, whereby in the absence of any stabilizing agent, PHMDH was 50% inactivated by a 30 minute incubation at 0-2°. The second procedure involved long term storage at 4 to 7°. The former procedure proved very effective for the rapid screening of various potentially interesting compounds, only the most promising of which were tested by the second method. Some of the organic solvents which were tested and found to be partially effective include the following: methanol, ethanol, propanol, n-butanol, i-butanol and dioxane.

*This work herein reported is supported by the National Aeronautics and Space Administration and is based on Research Reports prepared for the Ames Research Center, NASA, under Contract No. NAS2-4525.

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Stabilization of lactate and malate dehydrogenase by organic solvents

Many enzymes are rather unstable and exhibit a tendency to denature upon storage, even at low temperature. There is thus a great need to develop methods for preserving enzymes in a stable and fully active form. Much work has been done on the preparation of insoluble derivatives of various enzymes, beginning with the work of NELSON AND GRIFFIN[1], who adsorbed invertase on charcoal and alumina and found that the bound enzyme was active. KAY[2] has recently reviewed the methods of enzyme insolubilization which have been thus far used. Observations on the stabilizing effects of various additives have been published from time to time. A number of enzymes can be stabilized by adding reducing agents, chelating compounds, competitive inhibitors, co-enzymes and substrates or their analogues. Recently, TAKEMORI *et al.*[3] reported that benzoyl alcohol dehydrogenase from Pseudomonas sp., homogentisicase from bovine liver and alcohol dehydrogenase from yeast are stabilized by low concentrations of acetone and ethanol. The present communication describes a phase of our current work on the stabilization of enzymes in which organic additives have been used in an effort to stabilize lactate dehydrogenase (EC 1.1.1.27) and malate dehydrogenase (EC 1.1.1.37).

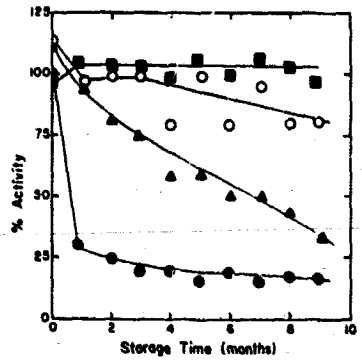
Lactate dehydrogenase (beef heart) was observed to have a half-life of from 2 to 3 weeks when stored in the cold (4 ± 2°) at an enzyme concentration of 1.5 μg/ml[4].

TABLE I

EFFECT OF ORGANIC COMPOUNDS ON STABILITY OF BEEF HEART LACTATE DEHYDROGENASE

Values expressed are for beef heart lactate dehydrogenase after long-term storage in the cold (4 ± 2°). After storage for the time periods indicated, 0.20-ml aliquots were transferred to the assay reaction mixtures and assayed as described in Fig. 1. Higher enzyme concentrations (*e.g.* 50–100-fold) also have a stabilizing activity, providing approximately a 50% increase in enzyme stability over the time intervals shown. All values are ± 5% error. Methanol and ethanol concentrations; % (v/v).

Compound	*Concn. (%)*	*% Activity after storage time (weeks)*					
		0	*1*	*4*	*12*	*24*	*36*
Control		100	65	29	18	18	16
Gelatin	0.25	104	118	86	84	81	63
	0.5	106	118	88	86	76	70
	1.0	100	97	60	60	59	60
Bovine serum albumin	0.025	96	76	42	29	30	11
	0.05	95	76	37	28	28	18
	0.10	100	72	28	22	24	18
Methanol	2.5	100	70	50	44	37	26
	5.0	100	80	55	53	35	16
	10	100	65	51	51	48	28
Ethanol	2.5	100	72	50	39	30	8
	5.0	96	80	60	46	48	36
	10	93	93	60	51	52	37



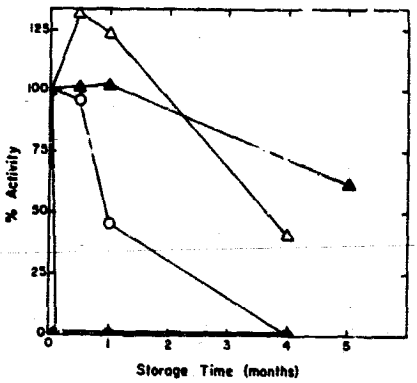


Fig. 1. Stability of lactate dehydrogenase in organic solvents. Enzyme (1.5 μg/ml) was stored at 4 ± 2° in 0.002 M potassium phosphate buffer (pH 7.0) containing different concentrations of additive. At the intervals indicated, 0.20-ml aliquots were transferred to the assay reaction mixture. The assay reaction mixtures contained: pyruvate (1 μmole); NADH (0.2 μmole); potassium phosphate buffer (80 μmoles; pH 7.4) and enzyme (0.3 μg) in a final volume of 3.0 ml. The reaction was at room temperature (25 ± 2°) and was followed by measuring the decrease in absorbance at 340 mμ as a function of time for 1 min. Dimethylsulfoxide concentrations (v/v): ■—■, 40%; ○—○, 30%; ▲—▲, 20%; ●—●, none.

Fig. 2. Stability of malate dehydrogenase in organic solvents. Enzyme (1.2 μg/ml) was stored at 4 ± 2° in 0.005 M potassium phosphate buffer (pH 7.4), which contained different concentrations of additive. At the times indicated, 0.06-ml aliquots were transferred to the assay reaction mixtures and assayed as described in Table II. The malate dehydrogenase samples were made up as follows: ▲—▲, 0.1% gelatin *plus* 30% ethanol; △—△, 0.1% gelatin *plus* 70% glycerol; ○—○, 70% glycerol; ●—●, none.

The addition of dimethyl sulfoxide, ethanol or methanol caused significant stabilization of lactate dehydrogenase to long-term storage in the cold (4 ± 2°)[4]. Thus, in the presence of 40% dimethylsulfoxide, the enzyme retained 100% of its original activity after storage for 9 months. Experiments shown in Fig. 1 indicate that after 1-month storage, lactate dehydrogenase (1.5 μg/ml) at 4 ± 2° and pH 7.0, lost 50% of its original activity but that no inactivation occurred in the presence of 40% dimethyl sulfoxide.

As shown in Table I, whereas gelatin protects against inactivation for 7 months, bovine serum albumin does not; in addition, methanol and ethanol also offer some stabilization.

Pig heart malate dehydrogenase had a half-life of about 30 min when stored in the cold (4 ± 2°) in a final concentration of 0.12 μg/ml[5]. Of the compounds tested, ethanol, *i*-propanol, *n*-butanol, *i*-butanol, acetone, dioxane and glycerol were the most effective in partially stabilizing this enzyme (Table II). Mixtures of glycerol and gelatin were found to result in significant stabilization of malate dehydrogenase not only in short-term studies but also during long-term storage in the cold (4 ± 2°)[5]. In the presence of 70% glycerol and 0.1% gelatin, the enzyme (1.2 μg/ml) was found to possess 100% of its original activity after storage for 7 weeks as shown in Fig. 2. The control (no additive) had a half-life of 5–10 h.

The mechanism by which these enzymes are stabilized by solvents and added proteins requires study. It seems possible that stabilization is associated with mainte-

TABLE II

EFFECT OF ORGANIC SOLVENTS ON STABILITY OF PIG HEART MALATE DEHYDROGENASE

Enzyme activities are expressed as 50% activity for samples containing zero concentration of organic compound. Values expressed are for pig heart malate dehydrogenase after 30-min incubation. The reaction mixtures contained: oxaloacetate (0.3 μmole); NADH (0.2 μmole); potassium phosphate buffer (280 μmoles; pH 7.4) and enzyme (0.24 μg) in a final volume of 3.0 ml. The reaction was run at room temperature (approx. 25°) and was followed by measuring the decrease in absorbance at 340 mμ (absorption maximum for NADH) as a function of time. All values are ± 5% error.

Compound	*% of activity at solvent concentration (v/v)*								
	0%	5%	10%	20%	30%	40%	50%	60%	70%
Methanol	50	51	63	62	59	63	66	40	20
Ethanol	50	75	72	87	87	76	79	68	62
***n*-Propanol**	50	74	78	77	31	11	—	—	—
***i*-Propanol**	50	85	—	99	105	—	—	—	—
***n*-Butanol**	50	84*	67**	—	—	—	—	—	—
***i*-Butanol**	50	79*	88**	—	—	—	—	—	—
Acetone	50	76	77	95	99	109	101	81	—
Dioxane	50	86	94	77	77	—	—	—	—
Dimethylsulfoxide	50	50	32	42	38	—	—	—	—
Dimethylsulfone	50	48	—	83***	—	—	—	—	—
Glycerol	50	59	57	82	96	109	94	107	109
Tetrahydrofuran	50	59	63	73	78	21	—	—	—

* 1.5% (v/v).
** 2.5% (v/v).
*** 15% (v/v).

nance of the enzymes in the "native" or "active" conformational state, by exclusion or replacement of water molecules.

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