FINAL REPORT

on

STUDIES ON OPTIMIZATION OF TECHNIQUES FOR ENZYME INSOLUBILIZATION (NAS 2-4890)

to

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
AMES RESEARCH CENTER
MOFFETT FIELD, CALIFORNIA 94075

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by

J. Lynn, D. Emmerling, and R. D. Falb

BATTELLE MEMORIAL INSTITUTE
Columbus Laboratories
505 King Avenue
Columbus, Ohio 43201

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INTRODUCTION

The insolubilization of enzymes through attachment to polymeric matrices permits a wide number of applications for enzyme-controlled processes. As a result of their attachment to polymers, modified enzymes can have properties differing significantly from those of the native enzymes. Among these properties, the most significant is the enhanced stability exhibited by many enzyme/polymer adducts. The physical form of the insolubilized enzymes makes them amenable to column processes in which the substrate can be passed through a bed, or to batch processes in which the enzymes can be easily recovered for reuse. In addition, the pH optima and K_m values can be altered depending on the type of polymer to which the enzymes are attached.

The ultimate goal of this research program is the application of insolubilized enzymes to the conversion of metabolic wastes to edible products. Toward this end, our effort during the last year has concentrated on exploring

and optimizing methods for insolubilization of three key enzymes in the Calvin cycle. The enzymes selected for study presented a particular challenge because of their complex structure and sensitivity to chemical reagents.

In the initial work on the attachment of the Calvin cycle enzymes, several standard methods used for simpler enzymes[1] were examined. As the work progressed, more recent techniques were adopted including those using glutaraldehyde as a cross-linking agent.[2,3,4] At the end of the first year, stable derivatives of aldolase, glutaraldehyde phosphate-3-dehydrogenase, and fructose-1,6-diphosphatase have been obtained and have been evaluated on a column. This report describes the methods used for preparation of these enzyme-polymer adducts and their resultant properties.

SUMMARY

The primary goal of this project is the atta aldolase, glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-diphosphatase to insoluble polymeric matrices to provide stable enzymic catalysts. In the initial phase of investigation, studies were performed on the solution stabilities of enzymes and on the reaction of enzymes with model compounds. The second phase of investigation concerned insolubilization of these enzymes/and the characterization of the enzyme-polymer adducts.

The effect of polar aprotic solvents, substrates and sulfhydryl protective reagents on the solution stabilities of the three enzymes was studied. No significant enhancement of the stability or of the specific activity of the enzymes resulted from placing them on a 25 percent solution of dimethylsulfoxide. Dimethylformamide significantly decreased the stability

of glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-diphosphate aldolase. The stability of the enzymes was affected very little by the presence of substrate.

Model compounds containing functional groups which would be used to attach the enzymes to polymeric matrices were reacted with the enzymes, and the effect on enzyme activity determined. Compounds investigated were maleic anhydride, benzenediazonium chloride, and potassium acetate-Woodward's reagent K adducts. The activities of glyceraldehyde-3-phosphate dehydrogenase stabilized by substrate and fructose-1,6-diphosphatase were enhanced by reaction with benzenediazonium chloride. No significant changes in activities resulted from reaction of the three enzymes with potassium acetate-Woodward's reagent K or maleic anhydride. Glyceraldehyde-3-phosphate dehydrogenase required protection by substrate during reaction with all of the reagents.

Three different carrier materials, ethylene maleic anhydride (EMA) p-aminobenzylcellulose (PAB) and aminoethyl cellulose (AEC) were used to form enzyme-polymer adducts. This work has demonstrated that large enzyme molecules such as aldolase, glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-diphosphatase may be attached to polymeric matrices with retention of a portion of their catalytic activities.

The stability of insolubilized enzyme derivatives is markedly different from that of soluble enzymes. For example, the aldolase-EMA in either powder or suspension form was stable over a period of twenty days at 4 C while soluble aldolase became deactivated more than 50 percent in 15 days. The aldolase-AEC in the suspension form was stable over a period of twenty-one days. While the soluble GAPD became completely deactivated overnight, the GAPD-AEC retained most of the enzymatic activity after 1 day.

The studies of pH optimum revealed significant alterations for the enzyme polymer adducts. In general, attachment of enzymes to basic polymers resulted in shifts of pH to lower values, and attachment to acidic polymers resulted in shifts to higher values. For instance, soluble aldolase exhibited optimum activity at pH 8, aldolase-PAB at pH 6.0, aldolase-AEC at pH 6.5 and aldolase-EMA at pH 9.0. While soluble GAPD exhibited optimum activity at pH 9.5, the GAPD-AEC exhibited an optimum at pH 7.0. As for FDPase-AEC, the pH was shifted to 8.5 from 9.2, the optimum of soluble FDPase.

Studies of the equilibrium constant K_m for the reaction between the insolubilized enzyme derivatives and their substrates demonstrated that the K_m value could be significantly altered depending on the type of polymers to which the enzyme was attached. When the enzyme was attached to basic polymers, the K_m value was lower than that of the soluble enzyme. Conversely, the K_m value was higher than that of the soluble enzyme when the enzyme was attached to acidic polymer.

The insolubilized enzyme derivatives formed between aminoethyl cellulose and each of the three enzymes contained the largest amounts of protein and retained much of the native enzyme activities. These adducts demonstrated remarkable stability at room temperature. A sequential reaction using columns of aldolase and GAPD was demonstrated. The substrate for the aldolase column was fed at the top, and the eluate became the substrate for the GAPD column. The result of the reaction was visually demonstrated by disappearance of the blue color of methylene blue in the presence of diaphorase activity.

These results obtained from the three different enzyme-polymer adducts should equip us to extend our studies to other enzymes of the Calvin

cycle so that columns packed with the enzymes of the Calvin cycle might eventually convert carbon dioxide into edible materials such as fructose or glucose.

EXPERIMENTAL PROCEDURES

Materials

Organic Compounds	Source
Fructose-1,6-diphosphate, DL-glyceraldehyde-3-phosphate Dithioerythritol Ethylenediamine tetraacetic acid L-cysteine, tris(methylhydroxy)-amino methane 5,5-dithiobis(2-nitrobenzoic acid)	Sigma Chemical Co.
Methyl sulfoxide N,N-dimethylformamide	Matheson, Coleman & Bell
Benzidine hydrochloride Maleic anhydride	Fisher Chemical Co.
Woodward-Reagent: K,N-ethyl-5-phenyl-isoxazolium-3'-sulfonate	Aldrich Chemical Co.
Methylene blue	Eastman Organic Chemicals
Pyridoxal-phosphate	Nutritional Biochemical Co

Inorganic Compounds	
Sodium arsenate, dibasic	Matheson, Coleman & Bell
Sodium dibasic phosphate Sodium tribasic phosphate	Fisher Chemical Co.
Sodium chloride Manganous chloride	J. T. Baker

Enzymes and Biochemicals	Source
Aldolase (Lot ALD 80DB)	
Glyceraldehyde-3-phosphate dehydrogenase (Lot GAPD 8GD)	Worthington Biochemicals Corporation
Fructose-1-6-diphosphatase (FDPase)	
Diaphorase Type 11-L	
α-Glycerophosphate dehydrogenase-triosephosphate isomerase	
Glucose-6-phosphate dehydrogenase Type X	
Phosphohexose isomerase Grade III	Sigma Chemical Co.
β-NADH, Grade III	
β-NAD, Grade III	
NADP, AMP	
Glycylglycine, DL-glyceraldehyde-3-phosphate (GAP)	
Glycine adenosine monophosphate	

Polymers	
Ethylene maleic anhydride copolymer (Lot 2516)	Monsanto
Cellex-PAB Control No. 4133 exchange capacity 0.18 meq/g	BioRad Lab.
Cellex-AE Control No. 5612, exchange capacity 0.27 meq/g	

Instruments

Radiometer pH meter 26 and Zeiss monochromator equipped with the Gilford absorbance expander together with the Varian recorder were used through this investigation.

Methods

Enzyme Assay

The aldolase activity was assayed according to the procedure by Rajkumar, et al.[5] and GAPD activity by Allison,[6] and FDPase by Pontremoli.[7]

Reactions of Enzymes with Model Compounds

GAPD. One hundred ml solutionof GAPD (0.155 mg/ml) was mixed with 0.05 ml of 0.0147 M GAP, and the solution was adjusted to pH 8.4, containing 0.03 M phosphate 0.004 M L-cysteine and 0.001 M Versene. One ml of this enzyme solution was then treated with 0.2 ml of Woodward reagent K (2.0×10^{-4} M).

Benzenediazonium Chloride. A solution of $NaNO_2$ (10.0 g) in 20 ml of cold water was added slowly to a mixture of 150 g ice, 41 ml concentrated HCl and 12.8 g of aniline. The resulting yellow solution was diluted to 500 ml with cold water to give a solution 0.27 M in benzenediazonium chloride. One hundredth ml of this diazotized solution was added to 1.0 ml GAPD solution (0.2 mg enzyme/ml) already containing some GAP.

Maleic Anhydride. One-tenth ml of maleic anhydride solution was added to 1.0 ml GAPD solution (0.3 mg enzyme/ml) already containing GAP.

Aldolase. Woodward Reagent K. To a solution of aldolase 0.12 mg/ml) was added 0.2 ml (4×10^{-5} M) Woodward reagent K solution, and the resulting solution was diluted to 10 ml with glycylglycine buffer 0.5 M, pH 7.5. The same procedure was repeated with aldolase stabilized with FDP. Both reactions were performed at 2 C.

Benzenediazonium Chloride. One-hundredth of a ml of benzenediazonium chloride solution, prepared as previously described, was added to 4.0 µl of aldolase solution (0.11 mg/ml) to give a colorless solution which slowly turned yellow on standing.

FDPase. Woodward Reagent K. To 50 ml samples of FDPase, (5.1 mg/ml) was added respectively 0.01 ml, 0.2 ml, and 1.0 ml Woodward reagent K solution (2.0×10^{-4} M). The samples were diluted to 10.0 ml with glycine buffer, 0.04 M, pH 9.2, before assay.

Benzenediazonium Chloride. One-tenth ml of fructose-1,6-diphosphate (7×10^{-5} g/ml) was added to 1.0 ml of FDPase (0.5 mg/ml). To this solution was added 0.01 ml benzenediazonium chloride, and the solution turned yellow instantaneously.

Maleic Anhydride. One-tenth ml of maleic anhydride solution (7.9×10^{-6} M) was added to 1.0 ml FDPase (0.5 mg/ml) solution. The resulting solution was assayed as described.

Attachment of Aldolase to Ethylene Maleic Anhydride Copolymer

The procedure employed is essentially the one described by Levin, et al.,[8] except for a few changes. Thus, 100 mg of ethylene maleic anhydride (EMA) suspended in 10 ml of tris buffer (0.2 M), pH 7.5, was cross-linked by treatment with 1.0 ml of 1 percent aqueous hexamethylenediamine at room temperature for 10 minutes. In a separate flask, a mixture of 4.0 ml of

fructose diphosphate (10^{-4} M) and 1.0 ml of aldolase (37 mg/ml) was stirred at 4C for 5 minutes and then poured into the suspension of EMA. The whole was stirred at room temperature for 30 minutes and then at 4 C overnight, and finally designated as 26255-1A.

After reaction at 4 C overnight, the pellet was centrifuged at 6,000 rpm, the supernatant discarded, and fresh buffer added for resuspension of the pellet. Centrifugation at 6,000 rpm was repeated to remove the unreacted protein, the sediment washed several times with buffer containing 0.1 N NaCl to insure elimination of protein not attached to the polymer support. Every third washing was assayed for activity, and washing was repeated until the supernatant was free of aldolase activity.

Attachment of Aldolase to Cellex-PAB Resin

Cellex-PAB, a cellulose-based anion exchanger bearing p-aminobenzyl groups, was diazotized as described in the BioRad catalogue (1961).

Then, 100 mg of the diazotized Cellex-PAB was suspended in a mixture of 3.0 ml of 0.1 M sodium pyrophosphate, pH 7.6, and 3.0 ml of solution containing 111 mg of aldolase. The whole was stirred at 4 C for two days, and designated as 24539-96A. The isolation procedure was similar to that described for isolation of aldolase-EMA.

Attachment of Glyceraldehyde Phosphate Dehydrogenase (GAPD) to Cellex-PAB Resin

One hundred milligrams of diazotized Cellex-PAB were suspended in 3.0 ml of 0.1 M of sodium pyrophosphate, pH 7.6, containing 0.04 M of L-cysteine, 0.01 M of EDTA and 30 mg of GAPD in 1 ml of water. The suspension was stirred at 4 C overnight, then centrifuged and repeatedly washed with

phosphate buffer 0.1 N NaCl and centrifuged until all unreacted protein was removed. The preparation was labeled 26255-20A.

Attachment of Aldolase to Aminoethyl Cellulose Resin (AEC)

Two and one-tenth g of AEC that had been washed first with 500 ml phosphate buffer 0.5 M, pH 7.5, and then 500 ml distilled water followed by methanol and then thoroughly air dried were suspended in 11.0 ml phosphate buffer, 0.5 M, pH 7.5, containing 2 percent glutaraldehyde. Three and five-tenth ml aldolase, straight from the stock bottle in 52 percent saturated ammonium sulfate, was introduced into the suspension. The whole suspension was stirred at room temperature for 1 hour and then at 4 C overnight. The brownish enzyme-polymer adduct was then isolated by filtration, followed by thorough washing with phosphate buffer, 0.5 M, pH 7.5, containing 0.5 N NaCl. About 3 liters of phosphate buffer were used to remove all the soluble aldolase. Another liter of phosphate buffer, 0.5 M, pH 7.5, was used to remove the sodium salt.

Attachment of GAPD to Aminoethyl Cellulose Resin

Two and one-tenth g of AEC that had been treated as previously described was suspended in 11.0 ml phosphate buffer, 0.5 M, pH 7.5, containing 10^{-3} M dithioerythritol and 2 percent glutaraldehyde. Five ml GAPD stock solution in 75 percent saturated ammonium sulfate were introduced into the suspension with stirring. After the whole suspension was stirred for 1 hr at room temperature, the brownish enzyme polymer adduct was isolated via filtration. After 3 liters of phosphate buffer, 0.5 M, pH 7.5 containing

0.5 N NaCl had to be used to remove all soluble GAPD. The sodium salt was diluted with another liter of phosphate buffer, 0.5 M and pH 7.5.

Attachment of FDPase to Aminoethyl Cellulose (AEC) Resin

To 250 mg of AEC were added 3.4 ml of 0.05 ml sodium phosphate buffer, pH 7.5 and 0.6 ml of 25 percent glutaraldehyde. The suspension was stirred at room temperature for 5 minutes after which time 10.0 ml cold FDPase solution (5.1 mg/ml) were mixed with the suspension. The whole suspension was then stirred at room temperature for an hour and then at 4 C overnight. The brownish yellow enzyme polymer adduct was isolated via filtration. The enzyme-polymer adduct was thoroughly washed with phosphate buffer, 0.05 M, pH 7.5, containing 0.5 M NaCl and then with the same buffer less salt to remove soluble FDPase.

Determination of Protein Attached to the Polymeric Matrix

For aldolase-EMA and aldolase-PAB derivatives, the protein content of the insolubilized enzyme products was determined by the Folin method essentially as described in Methods of Enzymology, Vol III (1957). Bovine serum albumin was used as a standard, and a calibration curve of the amount of protein versus the optical density at 750 mμ was established.

All protein assays were compared to blanks based on an amount of carrier equivalent to that of the enzyme-carrier product, thereby nullifying the light scattering imparted by the carrier. The optical density readings thus obtained were attributed to the protein alone.

For all the enzyme-AEC derivatives, the total nitrogen was determined by the ninhydrin technique as described by Hirs[9] with employment of a modified buffer system described by Moore.[10] Soluble aldolase and GAPD of $E^{1\%}_{280\ m\mu} = 9.38^{*}$ and $E^{1\%}_{280\ m\mu} = 10.2^{*}$ respectively were used as standards, and calibration curves of the amount of protein versus the optical density at 570 mμ were established. Since the calibration curves for aldolase and GAPD were very similar, it was assumed that FDPase would have a similar calibration curve; hence, the GAPD calibration curve was used to determine the amount of FDPase.

Activity Assay of Insolubilized Enzymes

The activities of aldolase-EMA, aldolase-PAB, and GAPD-PAB were determined using the same procedure as was used for the corresponding unmodified enzymes. Small samples (2.0 mg) of aldolase-EMA and aldolase-PAB were used in the assays. It was assumed that the fine suspension of insolubilized enzyme would have a sufficiently slow settling rate to produce a slurry with a uniform might scattering. The rate of change of optical density was uniform throughout the initial phase of the assay although a skew portion of the line was occasionally observed.

In the case of insolubilized GAPD, the low activity made it necessary to employ 50 to 100 mg of material. Unfortunately, these amounts interfered with spectrophotometric measurements. However, assay of GAPD-PAB was possible by the use of a Sweeny adapter containing a Teflon filter (mean pore size 5 micron) connected to a 5.0 ml syringe. The substrate solution, together with GAPD-PAB inside the syringe was allowed to react

* Obtained from Worthington Biochemicals catalogue (1968).

one minute, the mixture filtered by suction, and the NADH content of the filtrate determined spectrophotometrically at 340 mμ. The filtered GAPD-PAB was resuspended in the substrate solution in the syringe, and the reaction/processing cycle repeated several times. The rate of change of optical density at 340 mμ thus recorded for a cumulative reaction period of 25 min was uniform and linear for the first 5 minutes.

For AEC-enzyme derivatives, a small column, 9 mm x 100 mm, was packed with the insolubilized enzymes. After equilibration with the buffer, the substrate in the buffer solution was passed through the column, and the solution was recycled if necessary. Three different enzyme columns were used to assess the enzyme activity, as follows:

Aldolase-AEC. Ten mg aldolase-AEC were packed in a 9 mm column to a height of 5 mm. Two and six-tenth ml of 0.02 M FDP solution in phosphate buffer 0.5 M, pH 7.4, containing 10^{-3} EDTA and 10^{-3} diethioerythritol was passed through the column in 5 min. Two and four-tenth ml of the collected eluate was mixed with 0.05 ml of NADH (8.5 μmole/ml) and 0.05 ml of mixture of α-glycerophosphate dehydrogenase and triosephosphate isomerase (0.1 mg/ml) The reaction rate was then followed spectrophotometrically at 340 mμ for the disappearance of NADH.

GAPD-AEC. One hundred mg of GAPD-AEC were packed in 9 mm column to a height of 20 mm. A solution consisting of 0.5 ml NAD ($3. \times 10^{-3}$ M), 0.5 ml sodium arsenate (0.4 M) and 0.5 ml GAP (0.015) and 1.5 ml pyrophosphate buffer 0.1 M, pH 7.0 containing 10^{-3} M EDTA and 10^{-3} M dithioerythritol was passed through the column in 2.6 min. The optical density was read at 340 mμ

after the solution had completely gone through the column. The $O.D._{340\ m\mu}$ was plotted against the number of cycles, and this plot yields the activity curve for GAPD-Cellex AE.

FDPase-AEC. Fifty mg of FDPase-AEC were suspended in a 4.5 ml solution containing FDP (0.01 M), NADP (0.01 M), 0.01 ml phosphohexose isomerase (2 mg/ml) and 0.05 ml glucose-6-phosphate dehydrogenase (1 mg/ml). To this suspension was then added 0.5 ml of $MnCl_2$ (0.01 M). The mixing was done with a vortex mixer, and the suspension centrifuged to yield a supernatant whose optical density was measured at 340 mμ. The increase in optical density as a function of the mixing time constituted the rate curve.

Heat Stability Study

A series of five tubes each containing 2.0 ml of glycylglycine buffer (0.5 M), pH 7.6 and 0.1 ml of aldolase (0.16 mg/ml) were immersed in a bath at 62 C. At one minute intervals, a tube was pulled out and then cooled to room temperature. Aldolase activity was assayed by adding respectively 0.25 ml of 0.02 M solution of NADH and α-glyceraldehyde dehydrogenase-triosephosphate isomerase mixture, and 0.25 ml FDP (0.02 M) to the tubes and determining the rate of change of optical density at 340 mμ. The heat stability of aldolase-EMA was assessed in the same manner except that 2.0 mg of insolubilized aldolase was used in each tube.

Inhibitor Studies on Enzyme Columns

In general, studies of inhibitory effects of certain compounds on enzyme activity were performed by introducing the inhibitor solution directly into the insolubilized enzyme column. For instance, 5,5'-dithiobis(2-nitro-

benzoic acid) (DTNB) solution was introduced at various concentrations onto a GAPD-AEC or aldolase-AEC column. The column was washed thoroughly to remove unreacted DTNB. The substrate was then passed through the column, and the $O.D._{340\ m\mu}$ was read per cycle to determine the enzyme activity. For the studies of the inhibitory effects of AMP and pyridoxal phosphate on aldolase-AEC columns, the inhibitor solutions were soaked in the column for a while, and then the FDP solution was introduced into the column. The eluate was recycled once more before being assayed for the enzymic activity.

RESULTS

Enzyme Stability Studies

In order to devise effective techniques for attachment of aldolase, GAPD, and FDPase to polymeric matrices, it was essential to know under what conditions these enzymes retain their catalytic activities. To obtain this information, studies on the solution stability of the unmodified enzymes in the presence of solvents, substrates, and other protective reagents were performed. In addition, the retention of activity of the enzymes after reaction with the functional groups involved in the attachment to polymers was measured.

Solution Stability Studies of Unmodified Enzymes

The solution stabilities of the unmodified enzymes were studied with the following different categories of reagents:

(1) Polar aprotic solvents,[11] DMSO, and DMF

(2) Substrate and coenzymes (NAD and FDP)

(3) Sulfhydryl protective reagents (dithioerythritol and L-cysteine).

Activities of the three enzymes in solution with the various reagents at 4 C were measured over a time period and compared to the values obtained in the absence of any protective reagents. The results of these studies are shown in Tables I to III. In no case was stability enhanced or an increase in specific activity obtained from either DMSO or DMF. In fact, DMF greatly decreased the stability of GAPD and FDPase. Aldolase and FDPase were quite stable in solution while GAPD lost most of its activity after 8 days.

Reactions with Model Compounds

Functional groups of polymers to be used for attachment of enzymes include: carboxylic acids, acid anhydrides, and diazonium salts. As a first step in enzyme attachment, model compounds containing these groups were reacted with aldolase, GAPD, and FDPase, and the effect of these reactions on the enzymatic activity was determined. The effect of protection of the active site by substrate in the reactions was also studied. Table IV summarizes the results of reactions of the enzymes with Woodward's reagent K, N-ethyl-5-phenylisoxazolium-3'-sulfonate)-potassium acetate adduct, benzenediazonium chloride, and maleic anhydride. GAPD, stabilized with substrate GAP, retained its activity after treatment with ten times the amount of WRK-potassium acetate adduct required to react with every free amine group on the GAPD molecule. GAPD without substrate stabilization lost its activity when treated with WRK-potassium acetate adduct. An indication of reaction of amino groups with WRK was obtained by titration of the enzyme with trinitrobenzenesulfonic acid (TNBS). This method showed that eight out of the thirty-seven

TABLE I. SOLUTION STABILITY OF ALDOLASE

Age, days	Activity in μM FDP/mg Enzyme/min		
	Buffer	DMSO, 25 %	DMF, 25 %
0	4.75	3.18	0.58
1	--	3.50	0.99
2	--	3.55	1.31
3	--	3.55	--
6	3.49	3.77	--
8	3.67	3.77	--
10	3.67	--	--
13	3.76	--	--
15	--	3.77	--
17	--	--	--
20	3.49	--	--
22	--	3.77	--
23	4.49	--	--
29	3.67	--	--

TABLE II. SOLUTION STABILITY OF FDPase

Age, days	Activity (μM/mg/min)		
	Buffer	DMSO, 25 %	Buffer With FDP
0	2.75	2.18	1.95
1	1.84	2.22	1.95
2	1.81	2.04	1.95
8	--	2.12	--
10	--	1.88	1.64

TABLE III. SOLUTION STABILITY OF GAPD

Age, days	Activity, μM/min/mg			
	25 % DMSO	25 % DMF	Buffer Containing Dithioerythritol	Buffer Containing NAD
0	19.7	2.08	15.8	17.1
1	11.4	--	8.18	11.8
2	9.23	--	9.97	9.16
5	7.37	--	1.61	5.6
6	4.62	--	1.56	5.3
7	4.78	--	0.924	3.81
8	4.44	--	0.577	4.96

free amine groups available on the GAPD molecule had reacted with WRK-potassium acetate.

Reaction of GAPD with benzenediazonium chloride approximately doubled the enzymatic activity. The bright yellow color of these reaction products was assumed to arise from the formation of the azo derivative, and hence was interpreted to signify the feasibility for covalent attachment of this enzyme to polymer supports via diazonium coupling. Reaction of GAPD with maleic anhydride left enzymatic activity unaffected. Aldolase protected by FDP reacted with WRK-potassium acetate adduct and benzenediazonium chloride to form products which had little change in enzymatic activity.

The addition of WRK-potassium acetate adduct to FDPase resulted in a product with approximately the same activity as the control. It was

TABLE IV. REACTION OF ENZYMES WITH MODEL COMPOUNDS

Enzyme	Reagent	Stoichiometric Ratio Moles Reagent/Moles Enzyme Functional Group	Activity, %
GAPD	WRK-KOAC	10 (a)	22
	Benzenediazonium chloride	40 (b)	174
	Maleic anhydride	1 (a)	90
	Maleic anhydride	5 (a)	82
Aldolase	WRK-KOAc	2×10^4 (a)	75
	Benzenediazonium chloride	100 (b)	85
FDPase	WRK-KOAc	17 (a)	100
	WRK-KOAc	1700 (a)	77
	Benzenediazonium chloride	15 (b)	130
	Maleic anhydride	9 (a)	66

(a) Ratio based on the amount of free amino groups available on the enzyme.

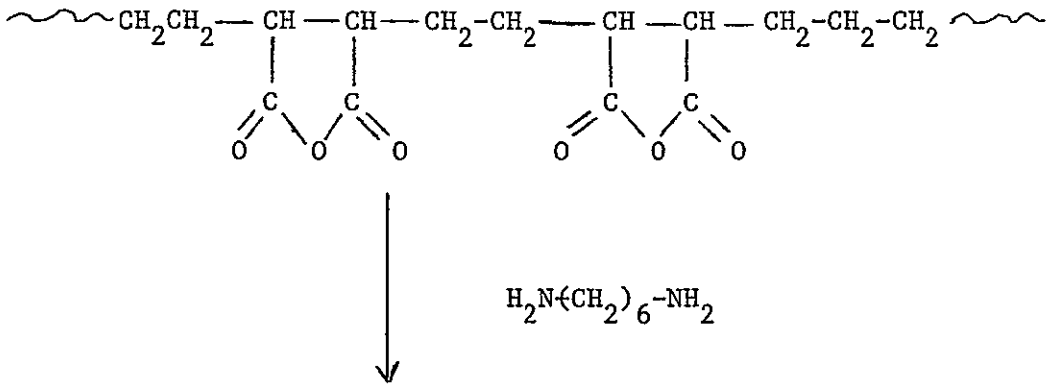
(b) Ratio based on the amount of phenolic groups available on the enzyme.

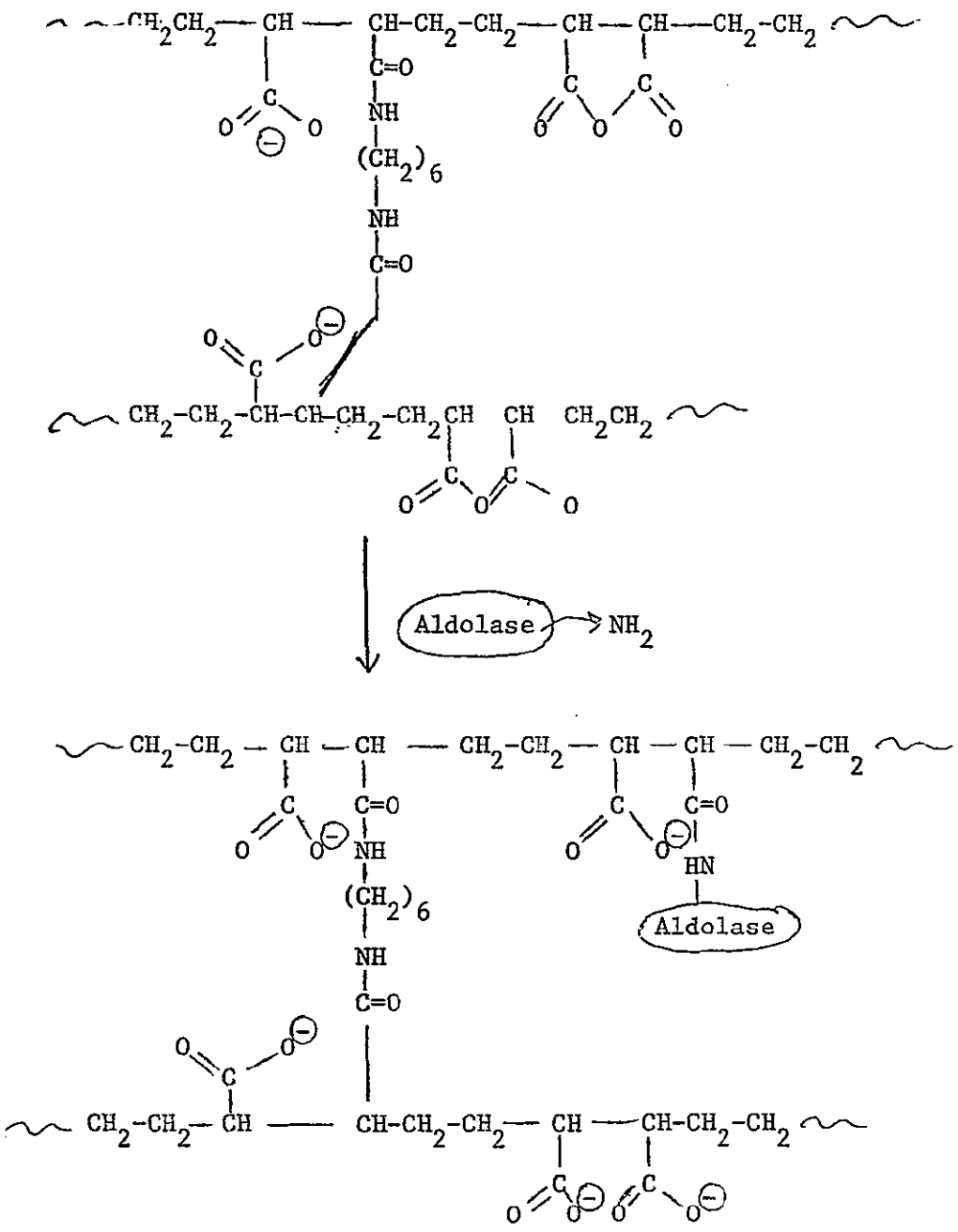
necessary to stabilize the enzyme with its substrate, FDP, to obtain an active benzenediazonium chloride adduct. Reaction of FDPase with maleic anhydride resulted in a loss of one half of its original activity.

Enzyme-Polymer Attachment Reactions

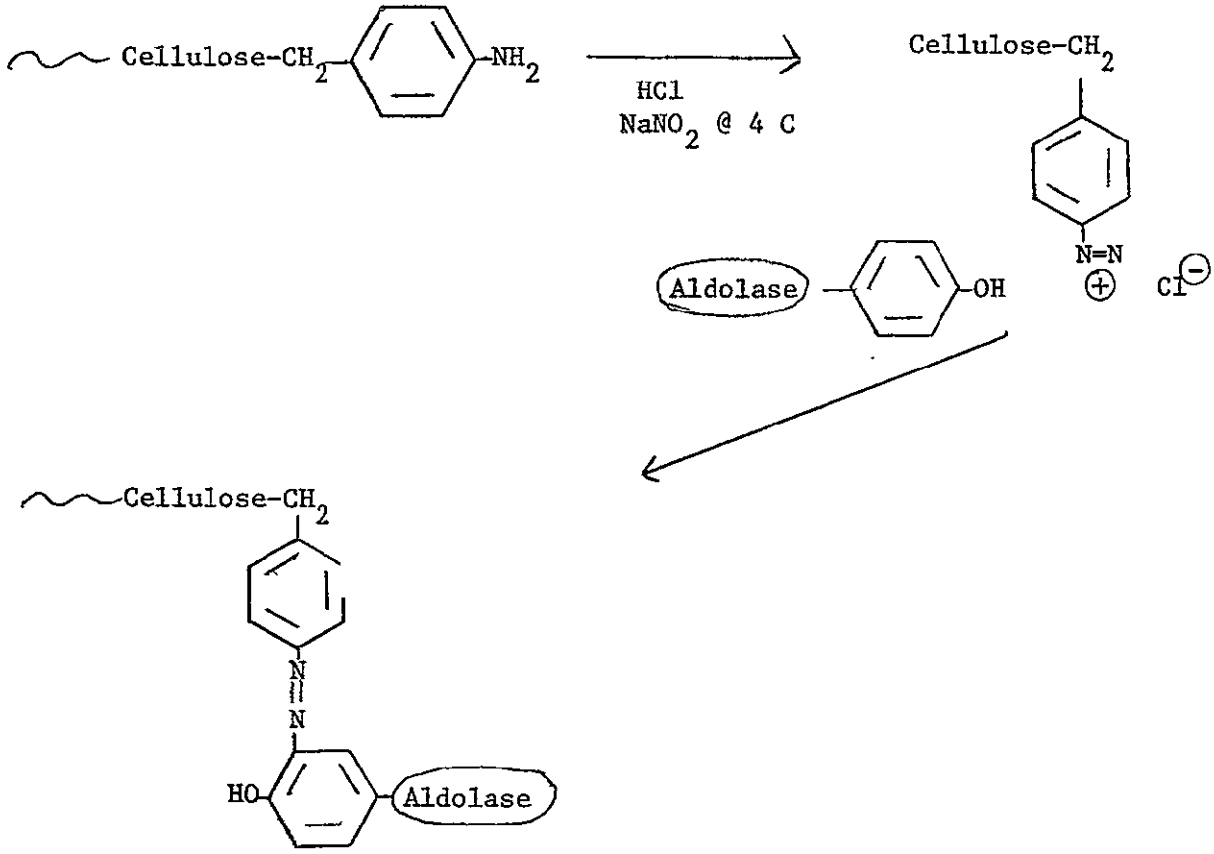
Functional groups such as the highly nucleophilic ε-amino groups and phenolic groups of tyrosine on the protein molecule are capable of reacting with polymers containing either anhydrides or activated carboxyl groups or diazonium salts. Of course, to attain high specific activity the active site of the enzyme should not be in the attachment. To avoid this, substrate or certain anions may be used to protect the essential functional groups from reacting with the polymer so that an active enzyme-polymer adduct may be obtained.

The attachment of enzyme to ethylene maleic anhydride polymer to yield an active enzyme-polymer adduct has been documented. Aldolase was attached to EMA in order that the effect of negative charges due to the carboxylates of the polymer on the pH optimum and K_m could be assessed. The reaction chemistry for obtaining aldolase EMA is as follows:

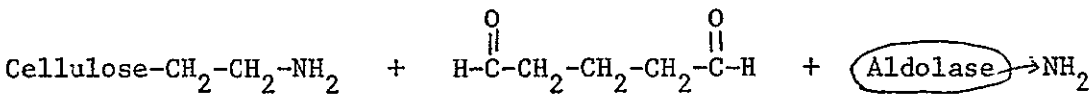




To study the effects of positive charges, due to the polymer, on the pH optimum and K_m of aldolase, the attachment of aldolase to p-aminobenzyl cellulose was undertaken. The reaction was based on the coupling of tyrosine and/or histidine groups on the enzyme molecule with the diazonium salt of polymer, as illustrated in the following:



The third type of polymer used was a cellulose resin containing amino groups. The enzyme-polymer adducts were obtained by coupling with glutaraldehyde. A possible mechanism for the reaction between aldolase and the amino containing cellulose resin could be through the Michael addition of the amine groups to the aldol condensation product of glutaraldehyde as shown below:



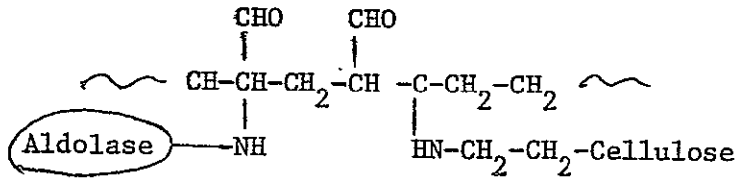


Table V shows the various amounts of proteins attached to different polymeric matrices and their corresponding specific activities for each insolubilized enzyme. The specific activity of insolubilized aldolase is about 16 percent of that of its native, soluble form. The specific activities of insolubilized GAPD and of insolubilized FDPase are much lower, about 0.1 percent of those of soluble forms, indicating that some enzyme molecules become inactivated after attachment to polymer matrices.

TABLE V. ACTIVITIES AND PROTEIN CONTENT OF INSOLUBILIZED ALDOLASE, GAPD, AND FDPase

Prep. No.	Enzyme Product	Maximum Specific Activity, moles NADH/min/mg Protein	mg/Enz/mg Carrier
24539-96-A	Aldolase-PAB	3.05×10^{-7}	4.9×10^{-2}
26255-1-A	Aldolase-EMA	1.08×10^{-7}	1.4×10^{-2}
26255-20-A	GAPD-PAB	3.66×10^{-9}	4.1×10^{-2}
26642-96-A	Aldolase-AEC	4.8×10^{-7}	6.0×10^{-2}
26823-2-A	GAPD-AEC	2.0×10^{-8}	3.6×10^{-2}
26823-5-A	FDPase-AEC	5.5×10^{-9}	7.0×10^{-2}
26255-80-B	Aldolase-AEC	9.0×10^{-7}	4.2×10^{-2}
26255-80-B	GAPD-AEC	3.0×10^{-9}	7.6×10^{-2}

Characterization of Enzyme-Polymer Adducts

pH Effects

Since an enzyme molecule is made up generally of acidic, basic, and neutral residues, its ionic character is sensitive to the pH of the environment. Any perturbation in pH normally affects catalytic activity. The polymeric matrices used for the attachment of the Calvin cycle enzymes contain charged groups and therefore would be expected to change the pH in the region of their active sites. For this reason, the pH optimum of the various enzyme polymer adducts were determined.

Insolubilized Aldolase Derivatives. Figure 1 demonstrates optimal pH for maximum activity of soluble aldolase, aldolase-EMA, and aldolase PAB, and aldolase-AEC. Although aldolase is shown to have a pH optimum at about 8.0, this curve differs from the broad pH optimum reported by Rutter.[12] Attachment of aldolase to EMA an acidic polymer, resulted in a shift of the pH optimum to a higher value. On the other hand, attachment to basic polymers such as PAB and AEC shifted the optima to the lower pH values of 6.0 and 6.5 respectively. This observation confirms that the pH optimum of an enzyme can be significantly altered by the type and nature of the polymeric matrix.[1]

GAPD-AEC Adducts. Figure 2 demonstrates the marked difference in the pH optima for GAPD and GAPD-AEC. The pH optimum of the soluble GAPD was around pH 9.5 while that of GAPD-AEC shifted to pH 7.0. This shift is consistent with that observed for aldolase-Cellex-AE. It was also noticed

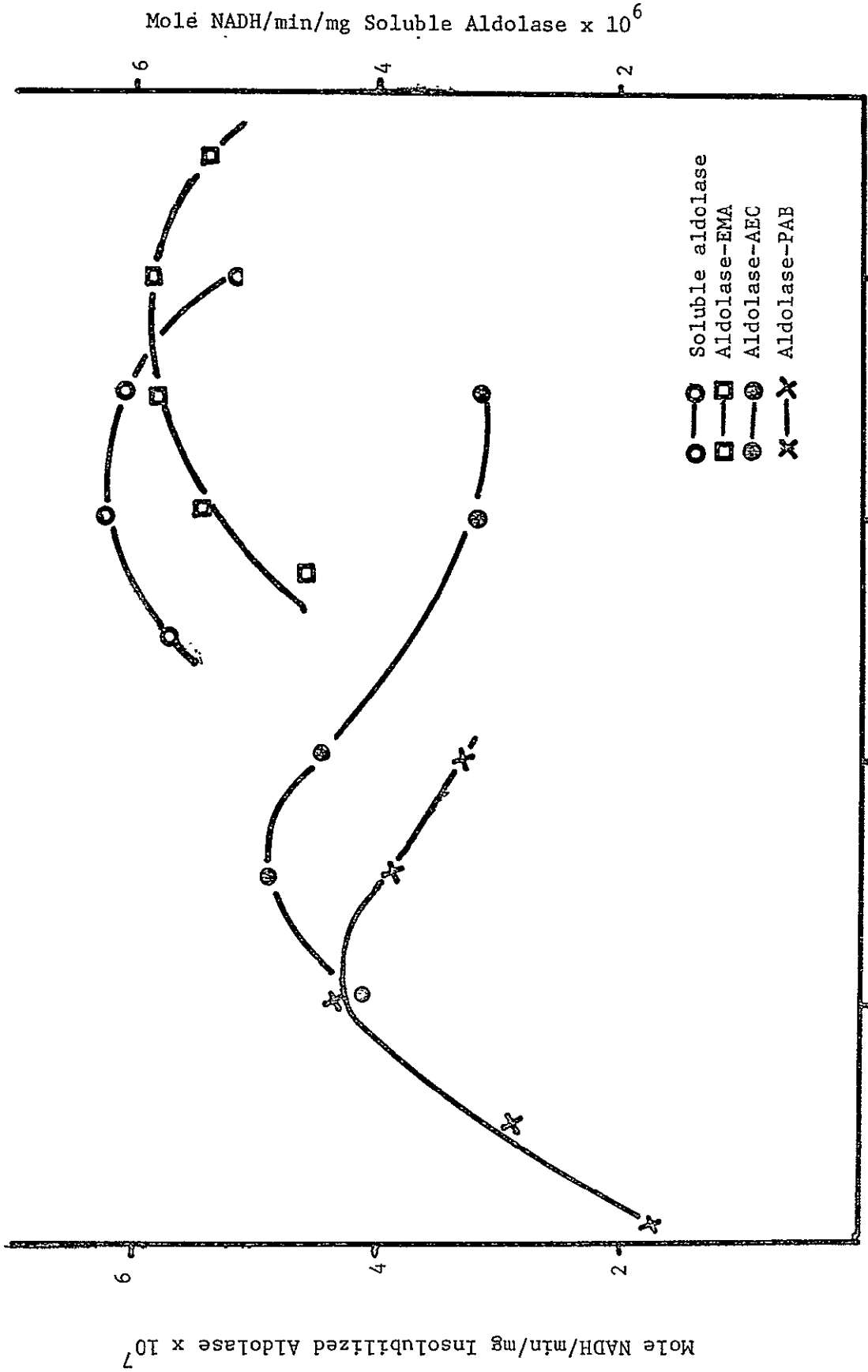


FIGURE 1. THE EFFECT OF pH ON THE ACTIVITY OF ALDOLASE AND INSOLUBILIZED ALDOLASE DERIVATIVES

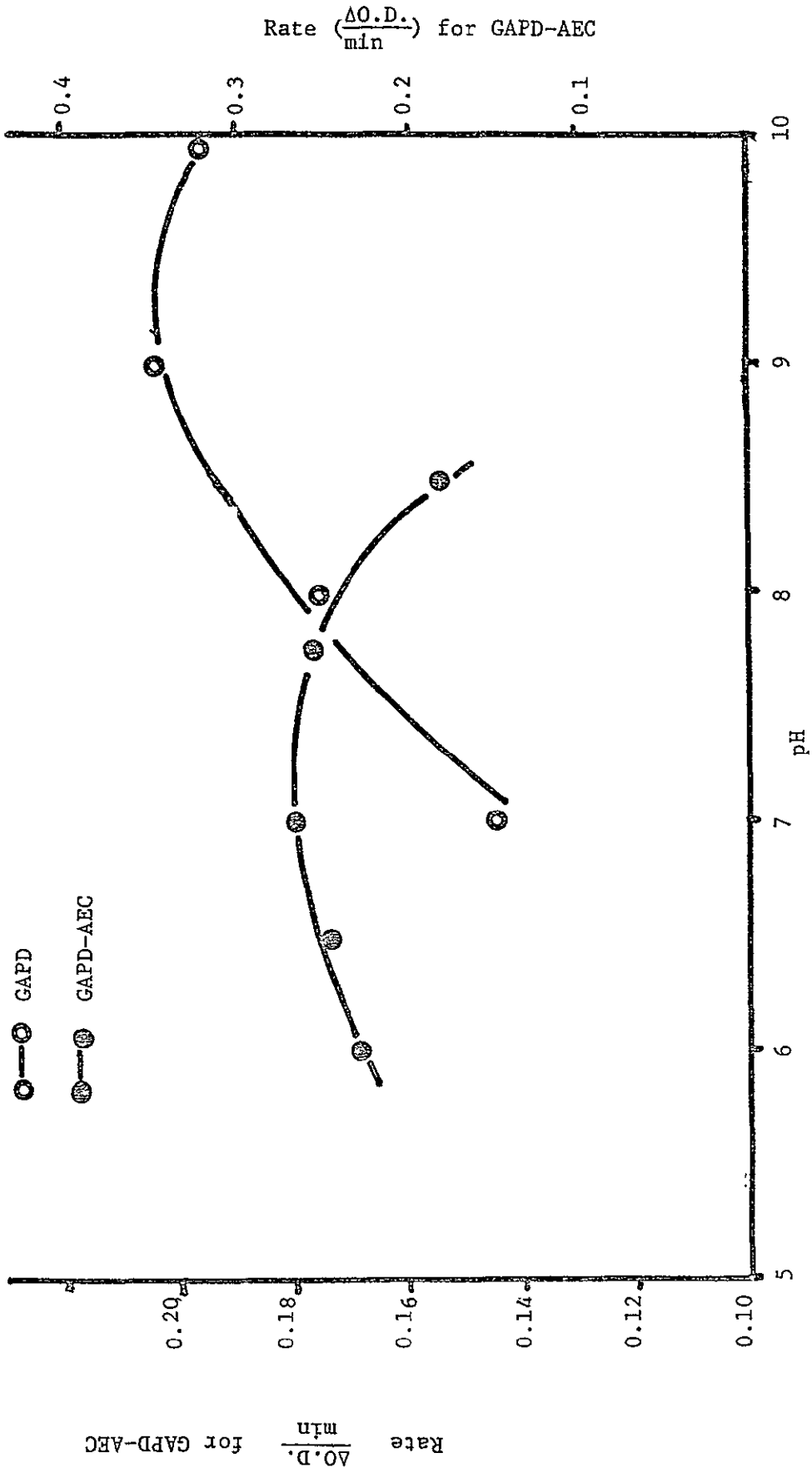


FIGURE 2. THE EFFECT OF pH ON THE ACTIVITY OF GAPD AND GAPD-AEC

that the incubation period necessary to initiate enzymic catalysis with soluble GAPD was not required for insolubilized GAPD.

FDPase-AEC Adducts. As shown in Figure 3, FDPase showed only a slight shift in the pH optimum toward the acid side after its attachment to AEC, a phenomenon quite similar to those observed with aldolase-Cellex-AE and GAPD-AEC. These data are not unequivocal however, because the glucose phosphate dehydrogenase used in the assay showed reduced activity at pH 7.0

Effect of Insolubilization of Attached Polymer on K_m Values

The charged nature of the polymeric materials also affects the concentration of charged substrates at the actual sites of the enzyme. For this reason, the K_m values of enzyme-polymer adducts can be altered depending on the type of polymer to which the enzymes are attached.

The Michaelis-Menten kinetic treatment was assumed to hold for the insolubilized enzymes. When the fine suspension was assayed spectrophotometrically as described in the Methods section, the velocity increased with increasing FDP concentrations for the insolubilized aldolase systems. Table VI records the initial conversion velocities with soluble aldolase, aldolase-EMA, and aldolase-PAB. Figure 4 shows the Lineweaver-Burk plots for soluble aldolase and aldolase-PAB. The K_m values for aldolase, aldolase-PAB, and aldolase-EMA were 4.3×10^{-5} M, 6.0×10^{-6} M, and 3.6×10^{-3} M, respectively.

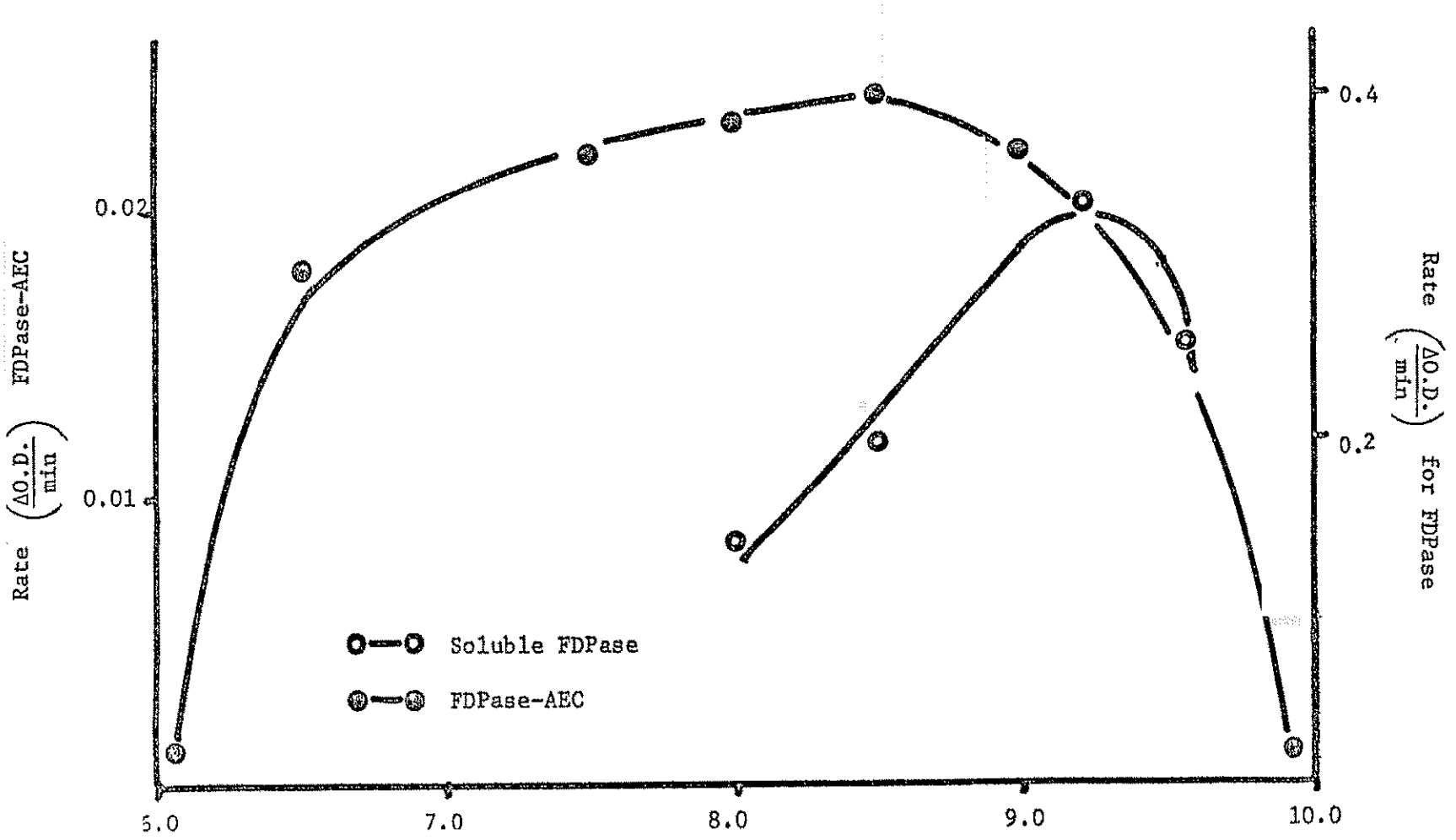


FIGURE 3. THE EFFECT OF pH ON THE ACTIVITY OF FDPase AND FDPase-AEC

TABLE VI. INITIAL CONVERSION VELOCITIES WITH ALDOLASE DERIVATIVES AS A FUNCTION OF FDP CONCENTRATION

Substrate Concentration, x 10^4 M	Moles, FDP/minute, pH 7.6		
	Soluble Aldolase	Aldolase-EMA	Aldolase-PA
3.9	3.3×10^{-7}	0.22×10^{-8}	0.8×10^{-8}
7.8	3.6×10^{-7}	0.51×10^{-8}	--
15.7	3.7×10^{-7}	0.77×10^{-8}	1.2×10^{-8}
19.6	--	--	1.1×10^{-8}

When the aldolase column was used to study the initial conversion velocities of FDP at various concentrations, the typical hyperbola rate curve was obtained as shown in Figure 5. The Lineweaver-Burk plot of $\frac{1}{V}$ versus $\frac{1}{S}$ shows that the Michaelis kinetics treatment is still applicable (Figure 6). The K_m of FDP as performed in the aldolase-Cellex-AE column was determined to be 5.4×10^{-4}. This value is higher than that of the soluble enzyme, although aldolase was attached to the basic polymer. This discrepancy is explained by the fact that the mixture of enzyme of α-glycerophosphate dehydrogenase and triosephosphate isomerase did not work in the presence of aldolase. The K_m value was essentially for dihydroxyacetone phosphate.

For the GAPD-AEC column, the relationship between initial rates and the substrate concentrations is shown in Figure 7. The Lineweaver-Burk plot for the same rate curve is illustrated in Figure 8, and the K_m for GAP

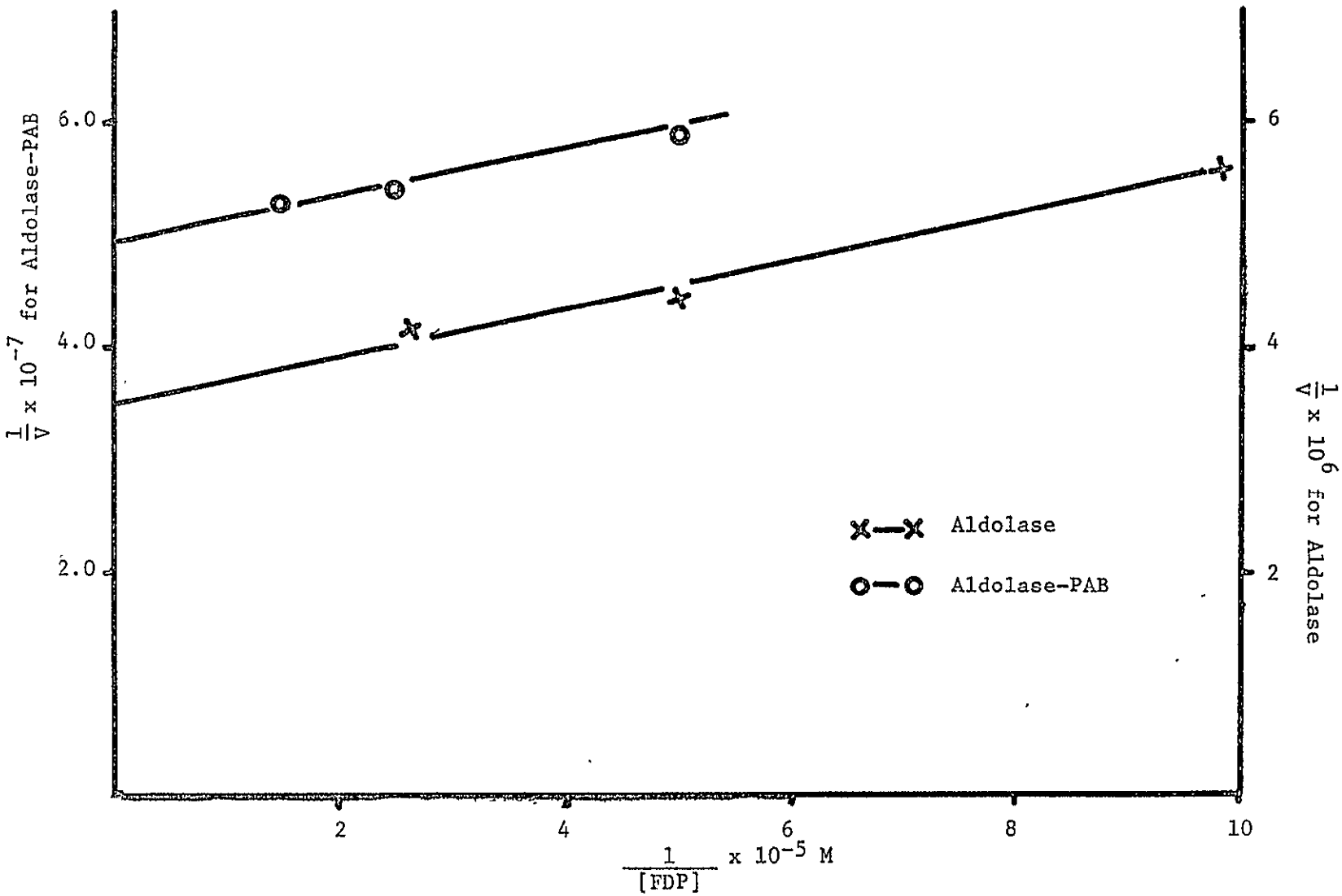


FIGURE 4. THE LINEWEAVER-BURK PLOT FOR ALDOLASE AND ALDOLASE-PAB

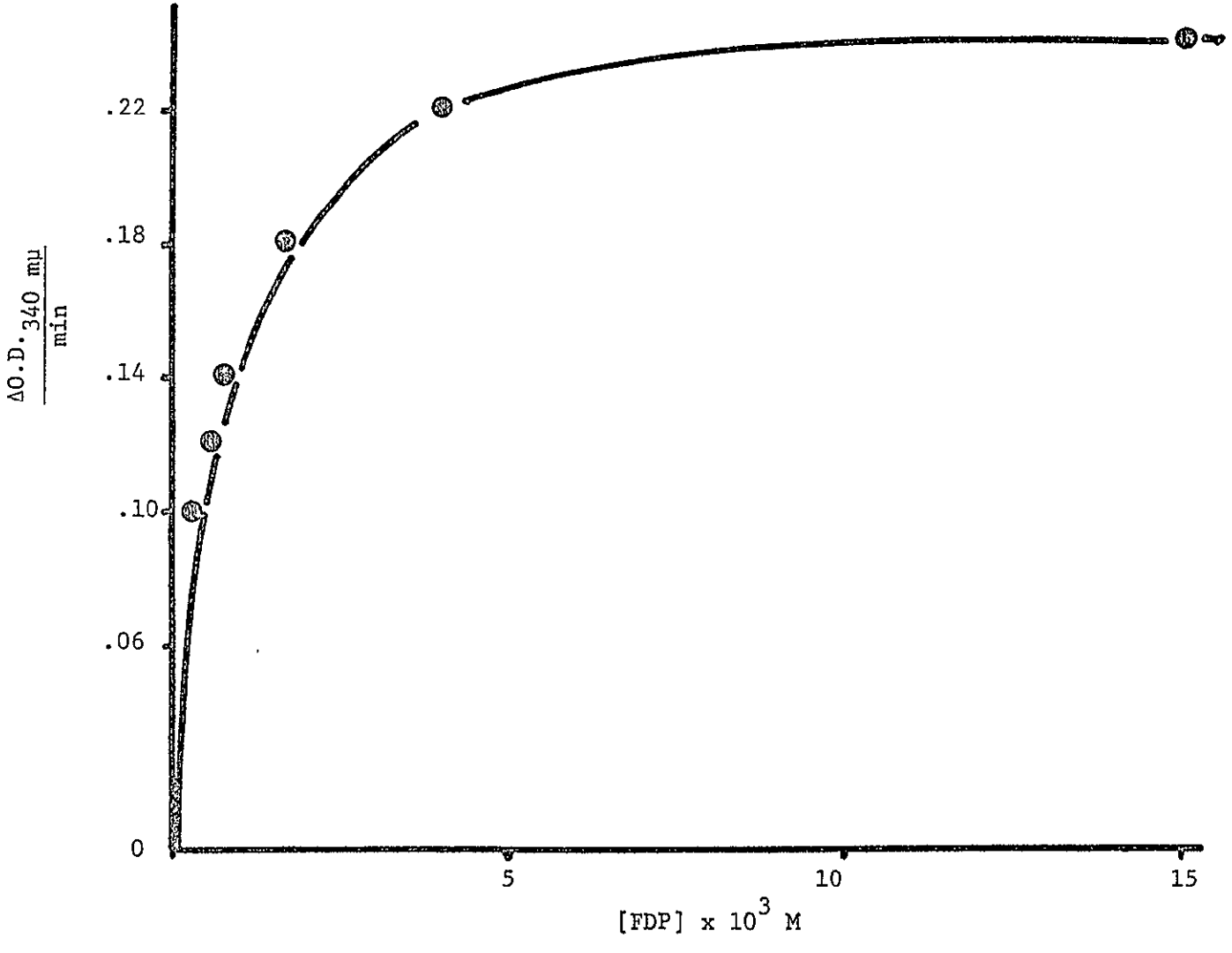


FIGURE 5. THE RELATIONSHIP OF INITIAL RATE OF SUBSTRATE CONVERSION AND FDP CONCENTRATION

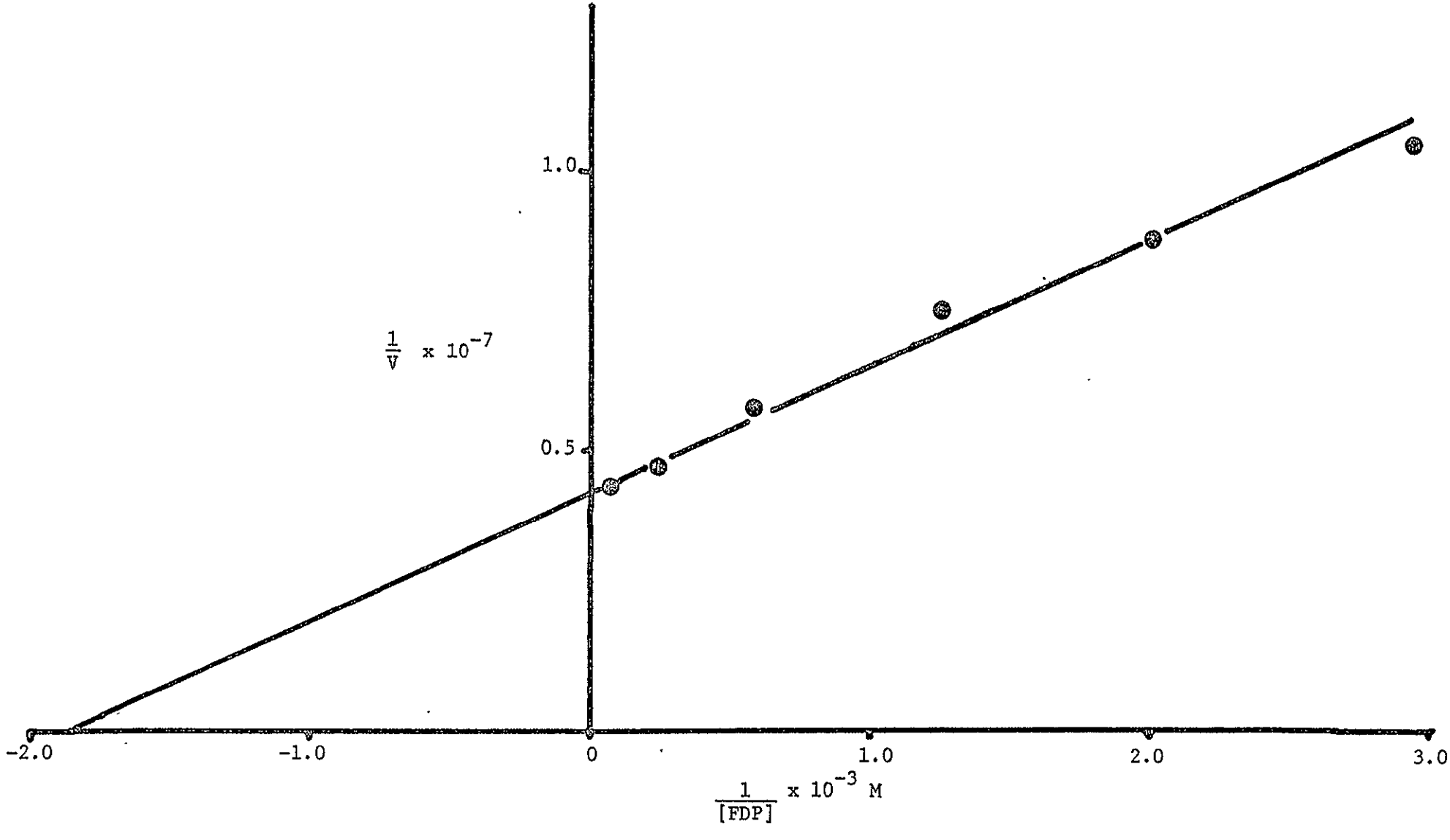


FIGURE 6. THE LINEWEAVER-BURK PLOT FOR FDP ASSAYED WITH ALDOLASE-AEC

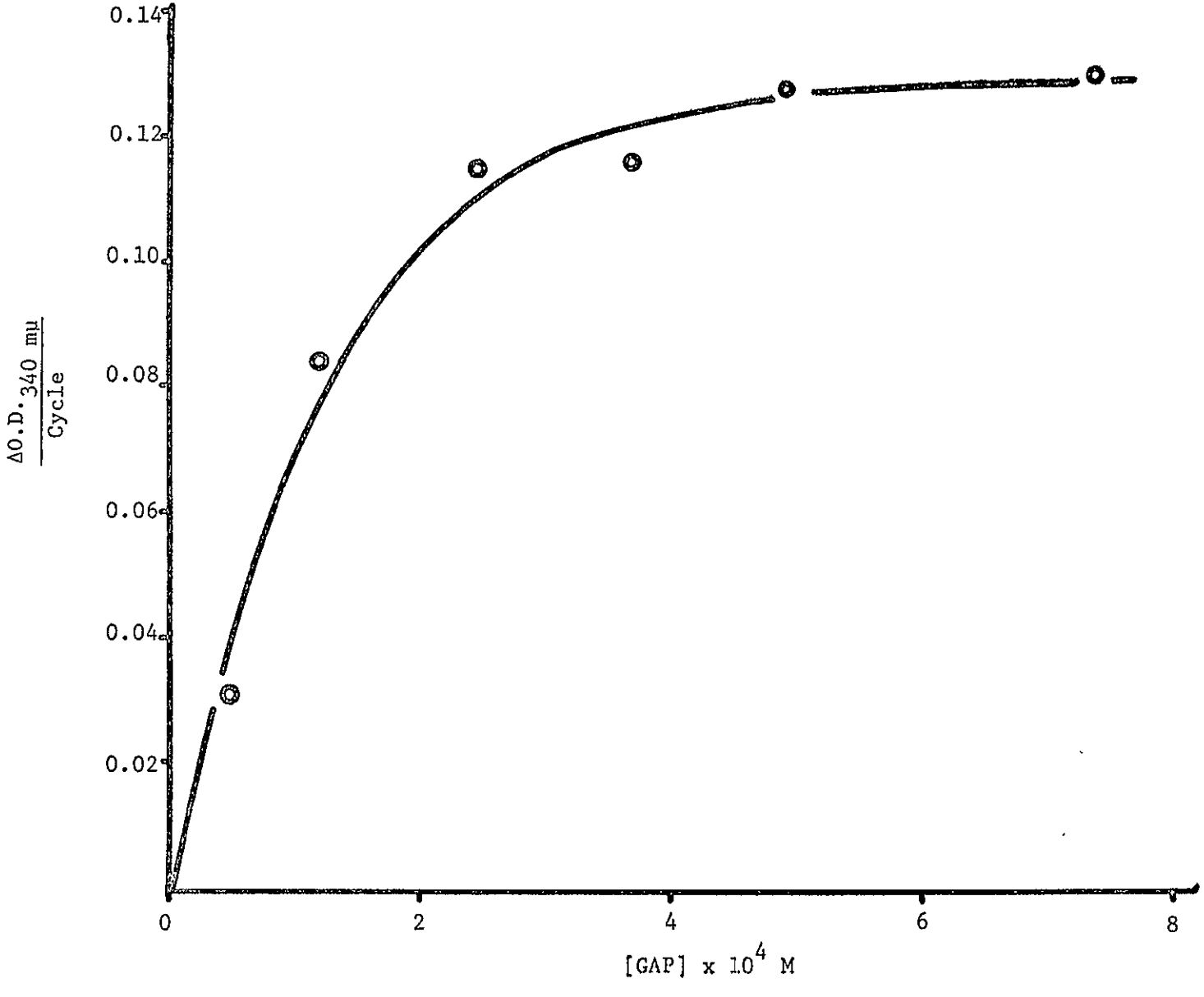


FIGURE 7. THE RELATIONSHIP OF RATE CONVERSION AND GAP CONCENTRATION

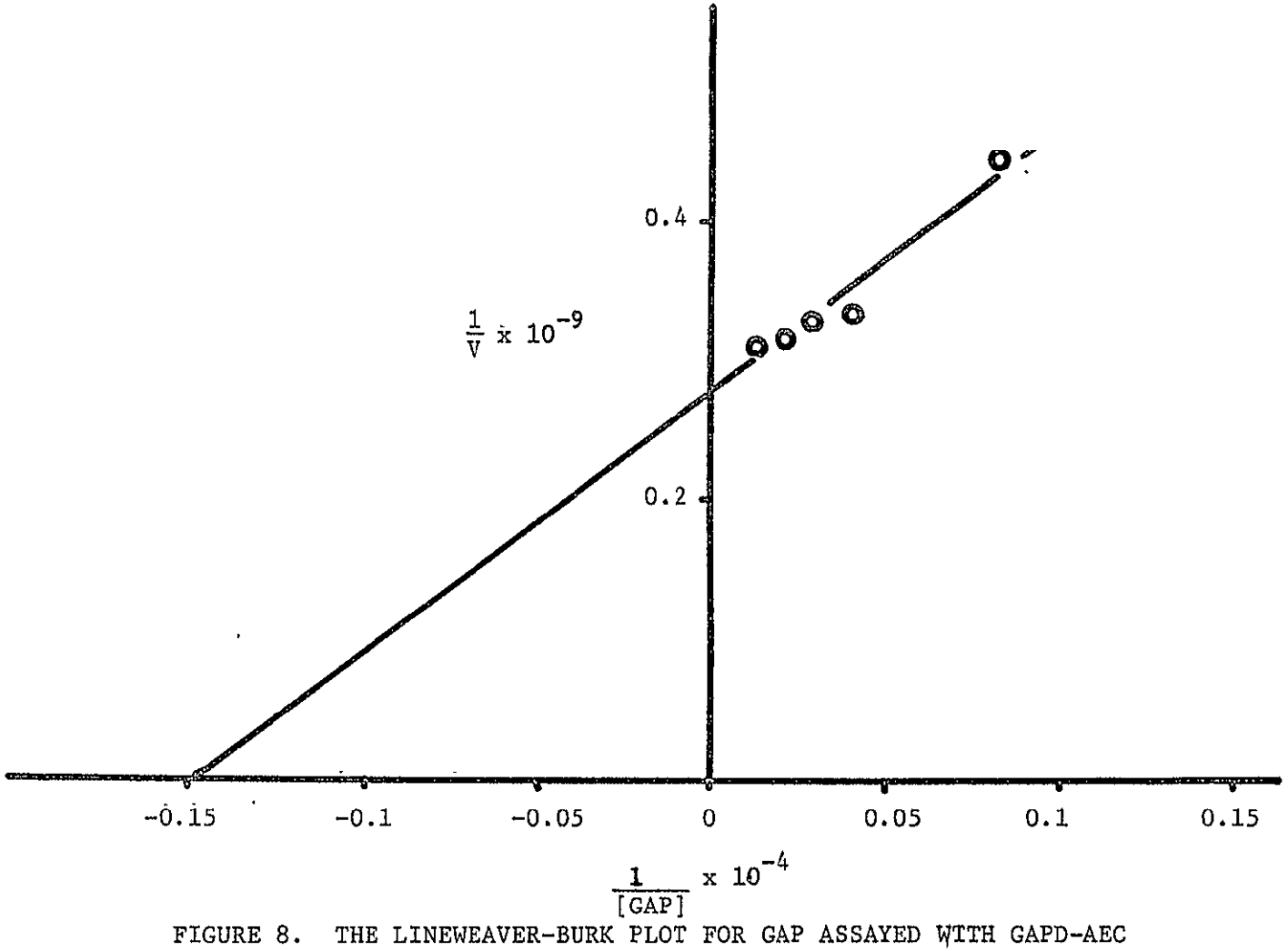


FIGURE 8. THE LINEWEAVER-BURK PLOT FOR GAP ASSAYED WITH GAPD-AEC

as determined to be 6.6×10^{-4} M. The K_m of GAP using soluble GAPD was letermined to be 6.4×10^{-3} M, though the literature value has a much lower K_m value at 2.5×10^{-6} M. This marked difference could only be explained by he fact that we did not purify our GAPD. Since GAPD has been shown to form complex with NAD,[13] and also shown to have a Michaelis constant, it would be valuable to determine the Michaelis constant of NAD for insolubilized APD. With the concentration of GAP, sodium arsenate, and GAPD constant, he initial rate curve as a function of NAD concentration was obtained as hown in Figure 9. The corresponding Lineweaver-Burk plot for NAD with a APD-AEC column is shown in Figure 10, and the K_m for NAD was determined t e 2.8×10^{-4} M. Although this K_m was higher than the literature value, yet ie value obtained was smaller than that of soluble GAPD, indicating the harge effect of the attached polymer on the binding of coenzyme of NAD.

Effect of Inhibitors, Sulfhydryl Reagents, Salt, and Urea

Reaction of 5,5'-Dithiobis-(2-nitro-benzoic Acid (DTNB) With Aldolase-AE and GAPD-AEC

DTNB being a disulfide compound can readily exchange with the free sulfhydryl groups on the protein. Indeed, this compound has been used to titrate the number of sulfhydryl groups on the protein.[14]

Since the modification of sulfhydryl groups on aldolase has been shown to increase the specific activity of aldolase,[15] a study of the effect of DTNB on aldolase-CAE was undertaken to assess the sulfhydryl groups on insolubilized aldolase. As seen in Figure 11, the activity of aldolase-CAE

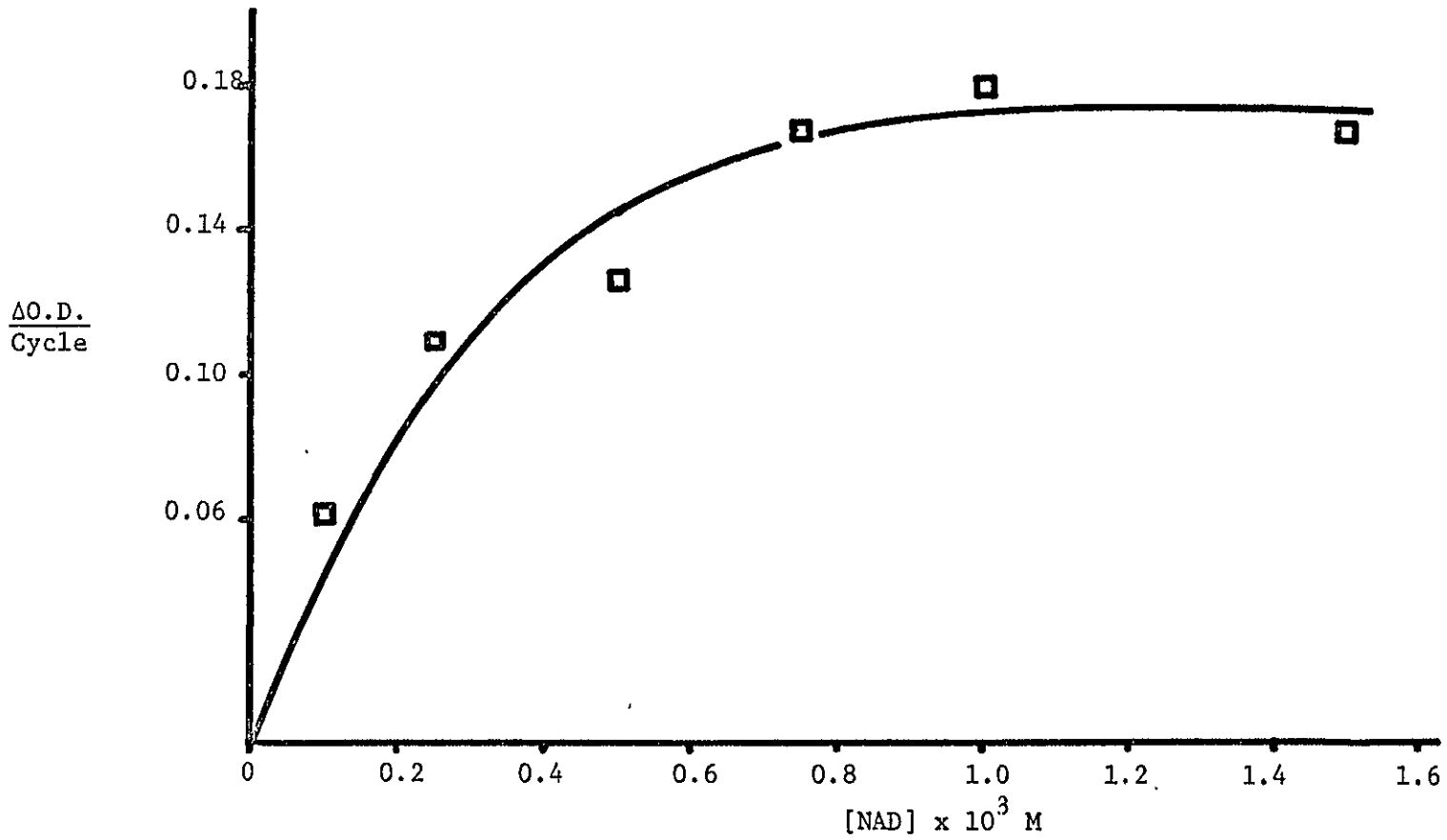


FIGURE 9. THE RELATIONSHIP OF THE RATE OF REDUCING NAD AND NAD CONCENTRATION

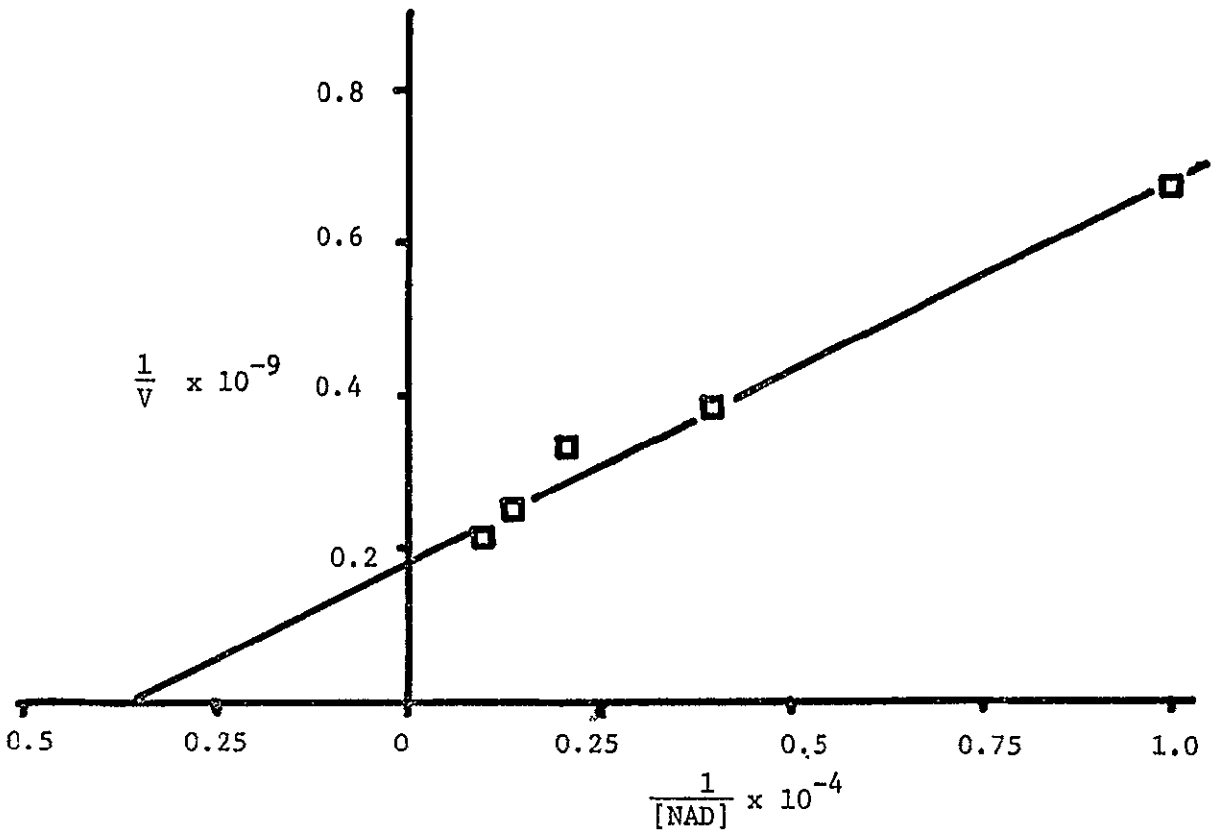


FIGURE 10. THE LINEWEAVER-BURK PLOT FOR NAD, ASSAYED WITH GAPD-AEC

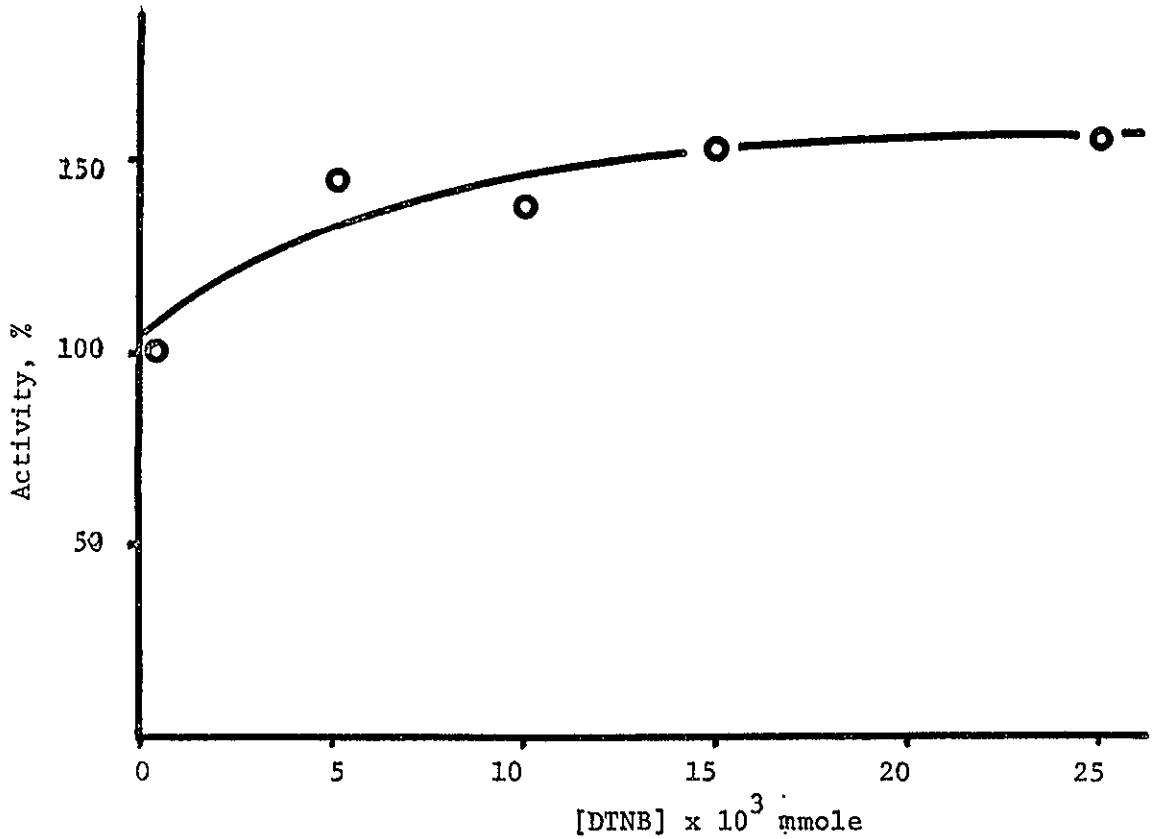


FIGURE 11. THE EFFECT OF DTNB ON THE ACTIVITY OF ALDOLASE-AEC

increased with increasing DTNB concentration. This phenomenon is very similar to that reported for soluble aldolase, although sulfhydryl groups of the soluble enzyme were reacted with dinitrofluorobenzene.[16]

A different effect was noted in the reaction of GAPD with DTNB because of the requirement of free sulfhydryl groups for the activity of this enzyme.[13] To assess the role of sulfhydryl groups in insolubilized GAPD, DTNB was reacted with GAPD-CAE. As seen in Figure 12, the activity of GAPD decreased with increasing DTNB. However, after soaking the column with phosphate buffer containing 10^{-3} M dithioerythritol and 10^{-3} M EDTA, the activity of GAPD was restored to about 80 percent of the original value.

Inhibitors of Aldolase-AEC. AMP has been shown to inhibit the aldolase molecule,[5] and also a recent study has demonstrated the competitive inhibition of pyridoxal phosphate on aldolase.[17] Figure 13 shows the inhibitory effects of pyridoxal phosphate and AMP on aldolase-CAE. Apparently, pyridoxal phosphate had a slight inhibitory effect on the aldolase-CAE, but the degree of inhibition was not as marked with the soluble aldolase. A similar effect was observed with AMP in that its action on aldolase-CAE was only minimal.

Salt Effects. In general, the presence of salts, such as NaCl and KCl, in a protein solution helps to neutralize charge-charge interactions, thus stabilizing the conformational structure of the protein, However, as

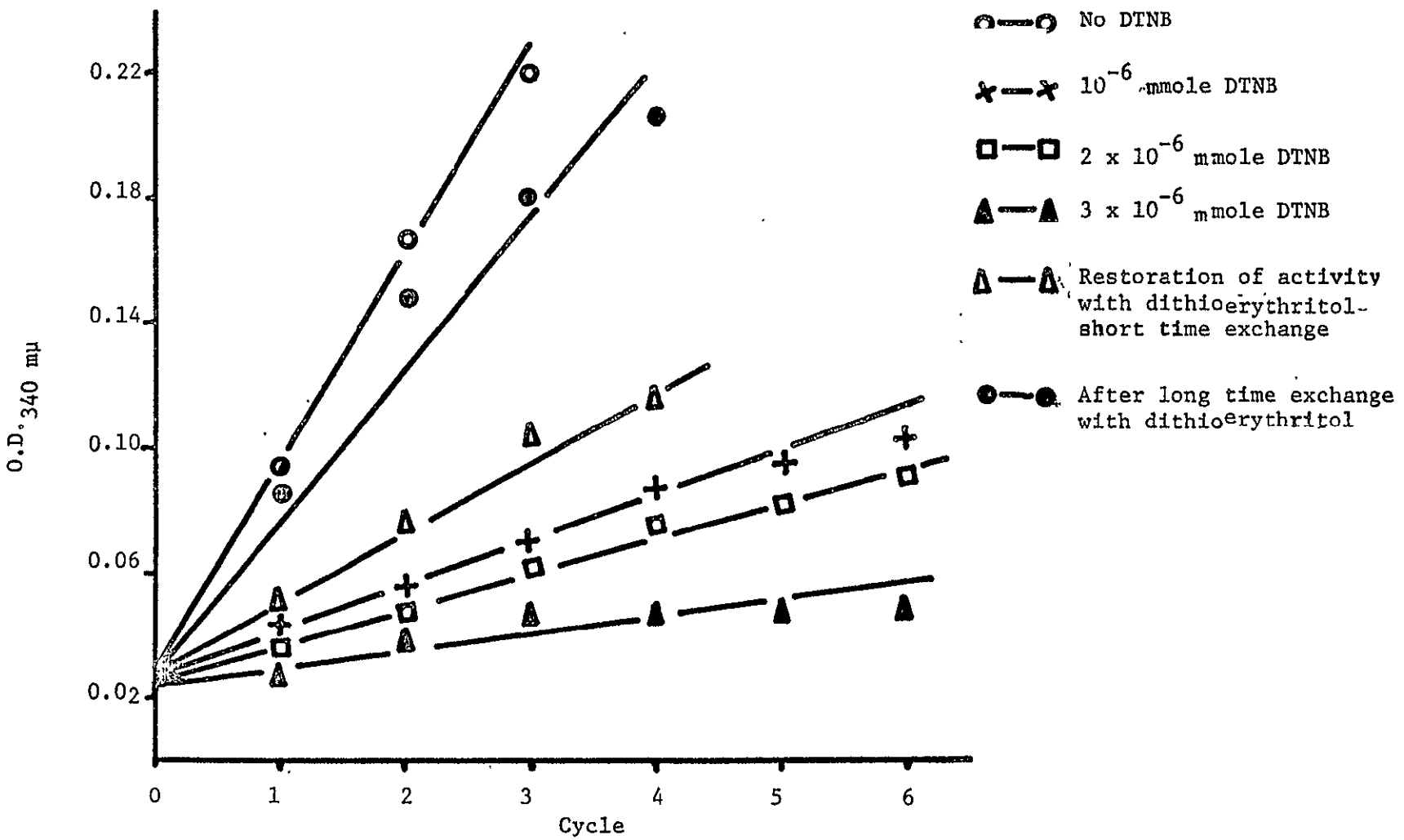


FIGURE 12. THE EFFECT OF DTNB ON THE ACTIVITY OF GAPD-AEC AND RESTORATION OF ACTIVITY WITH DITHIOERYTHRITOL

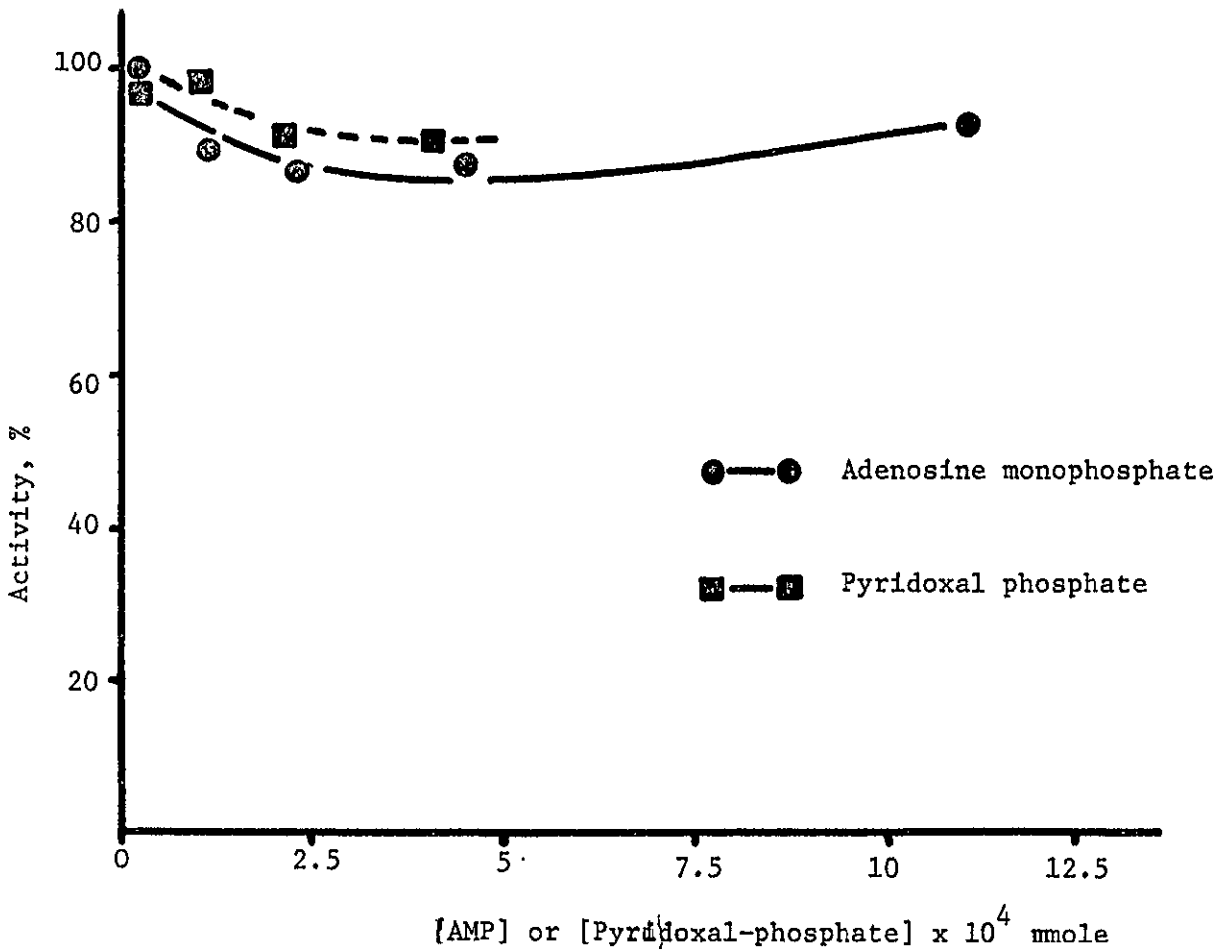


FIGURE 13. THE EFFECT OF AMP AND PYRIDOXAL PHOSPHATE ON THE ACTIVITY OF ALDOLASE-AEC

the salt concentration is increased, the conformational character is perturbed and dissociation of subunits of the protein molecule occur.

Because of the additional charges introduced in the attachment of enzymes to ionic polymeric matrices, charge-charge interaction may destabilize the protein. Hence, the activity of insolubilized aldolase as a function of sodium chloride was studied in order to assess the role of the added electrostatic interactions. Figure 14 demonstrates the activity of aldolase-EMA as a function of various concentrations of sodium chloride. For comparison, the soluble aldolase was treated in exactly the same way as was the insolubilized aldolase. As seen in Figure 14, the activity of solubl aldolase dropped to 80 and 53 percent of its original activity in the presenc of 0.3 and 0.5 M, respectively, of sodium chloride. However, 0.1 M sodium chloride did not significantly affect the activity of aldolase. While there was a similar trend of decreasing the activity of aldolase-EMA with increasing sodium chloride concentration, the insolubilized enzyme tolerated higher concentrations of salt than did the soluble aldolase.

Using the GAPD-CAE column, we studied the effect of NaCl on the activity of GAPD. The result showed a definite sensitivity of the enzyme to the NaCl concentration as shown in Figure 15. The 1.0 M NaCl solution reduced the GAPD activity by more than 50 percent, and 3.0 M NaCl solution completely inactivated the enzyme.

At the end of 3.0 M NaCl solution experiment, the GAPD column was soaked with a phosphate buffer pH 7.5 containing 10^{-3} M dithioerythritol

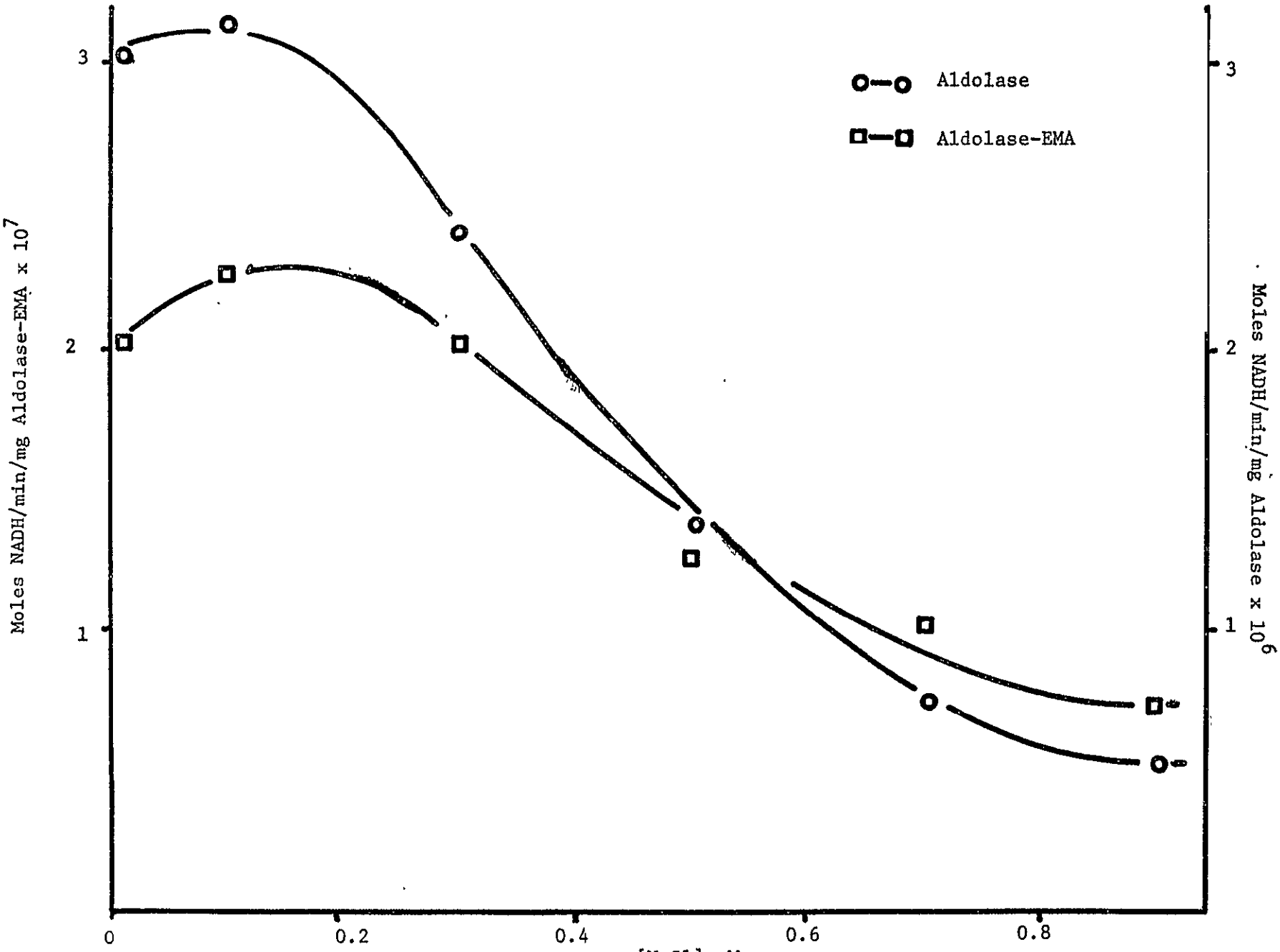


FIGURE 14. THE EFFECT OF NaCl ON THE ACTIVITY OF ALDOLASE AND ALDOLASE-EMA

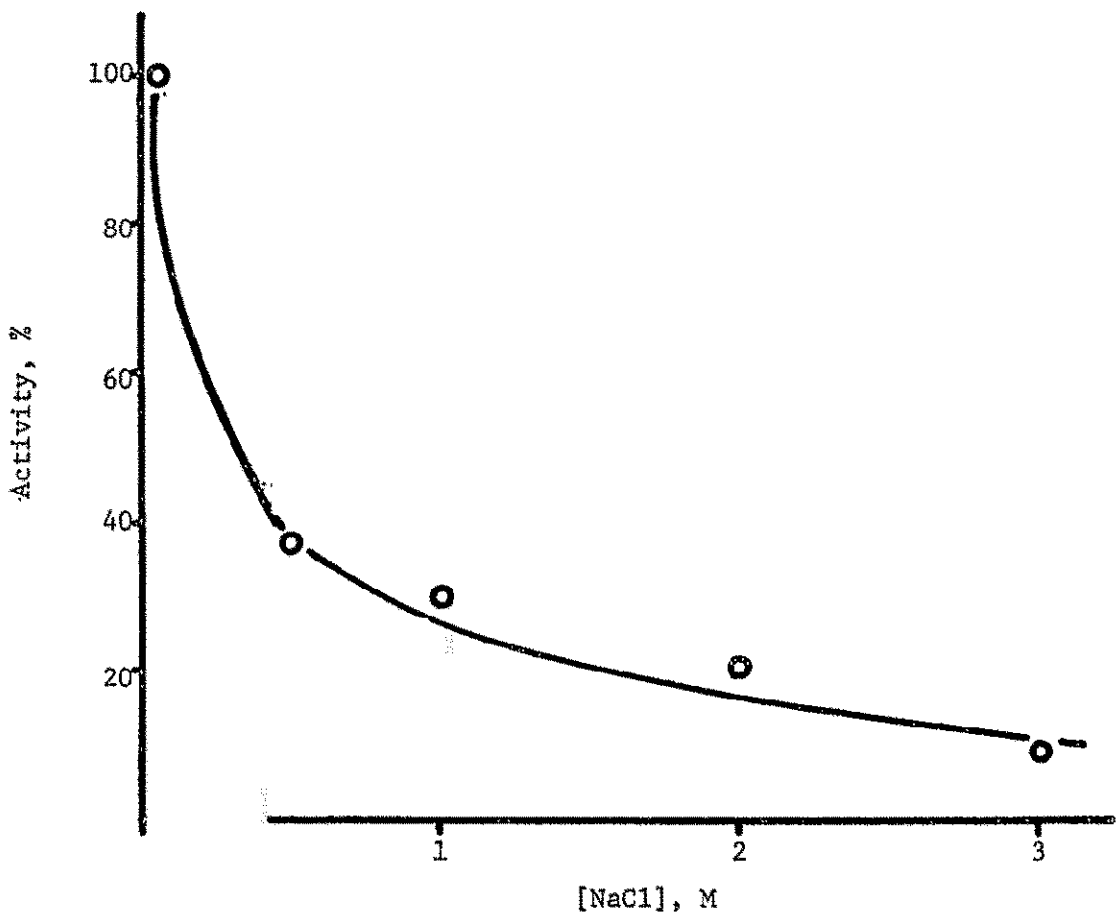


FIGURE 15. THE EFFECT OF NaCl ON THE ACTIVITY OF GAPD-AEC

and 10^{-3} M EDTA, followed by washing the column with 25.0 ml of the buffer. The GAPD activity was restored to about 50 percent of its original value. Further washing the column with the buffer restored another 10 percent of the original activity.

Urea Denaturation. Urea is known to have a disrupting effect on hydrogen bonds and/or hydrophobic bonds. Since the tertiary and quaternary structures of a protein molecule are governed in part by these specific interactions, any perturbation of the protein structure with urea, or the similar denaturing agent, may affect the catalytic property of the enzyme. The effect of urea on the stability of soluble and insolubilized aldolase was therefore examined, since such studies could be informative relative to the interactions characteristic of the "native form" of the enzyme. Figure 16 illustrates the results of these studies. Apparently, urea destabilized both soluble and insoluble aldolase to essentially the same extent. In 4.0 M urea solution, soluble aldolase exhibited 42 percent of its original activity, and insolubilized aldolase 58 percent of its origianl activity.

The effect of urea on the GAPD activity is not seemingly as marked as that of NaCl. However, the direction of destabilization for GAPD was clearly indicated, as shown in Figure 17. At 3.0 M urea solution, the GAPD had about 80 percent of the original activity and at 5.0 M urea solution, the activity had been reduced to about 50 percent of the original activity

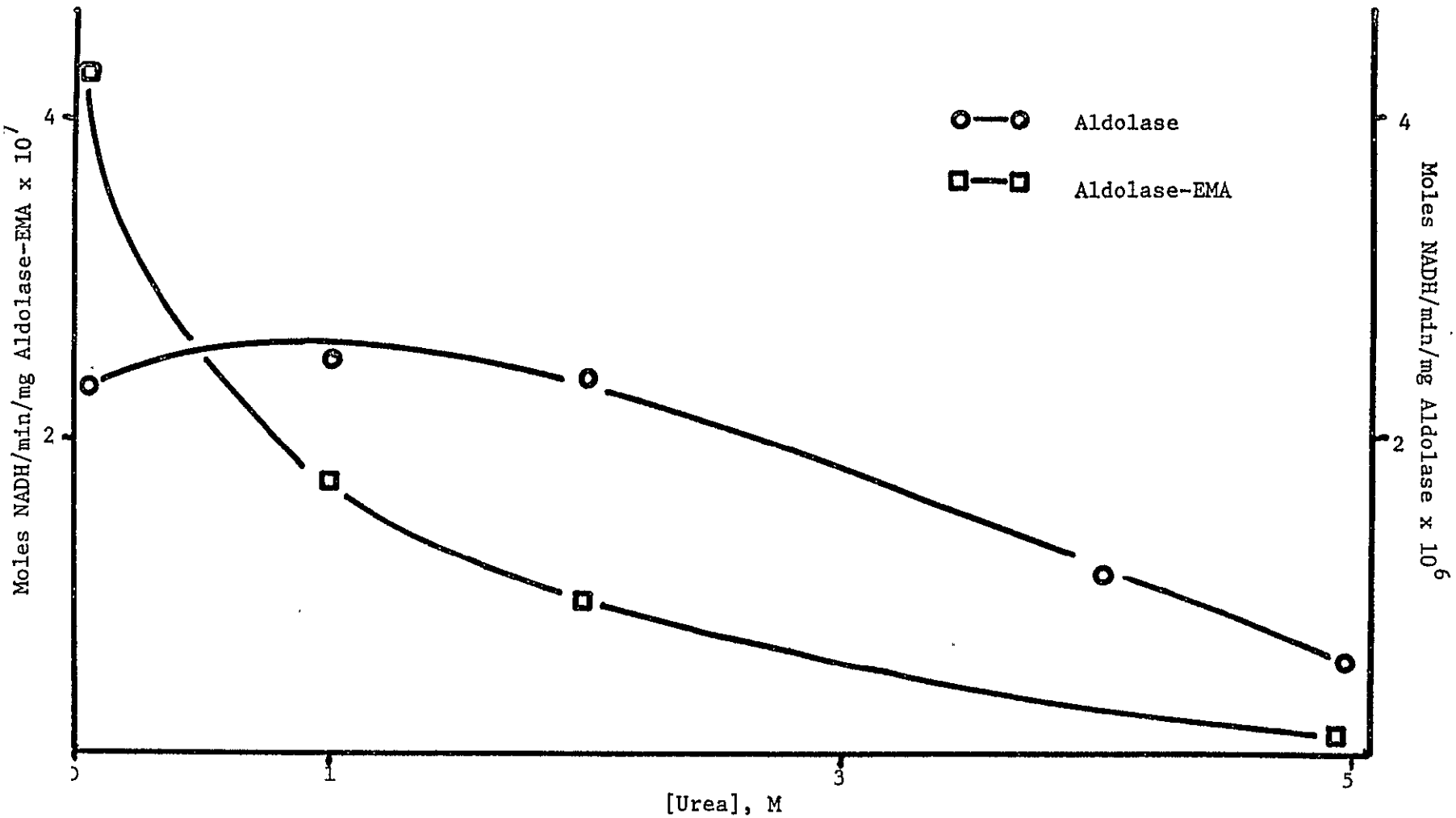


FIGURE 16. THE EFFECT OF UREA ON THE ACTIVITY OF ALDOLASE AND ALDOLASE-EMA

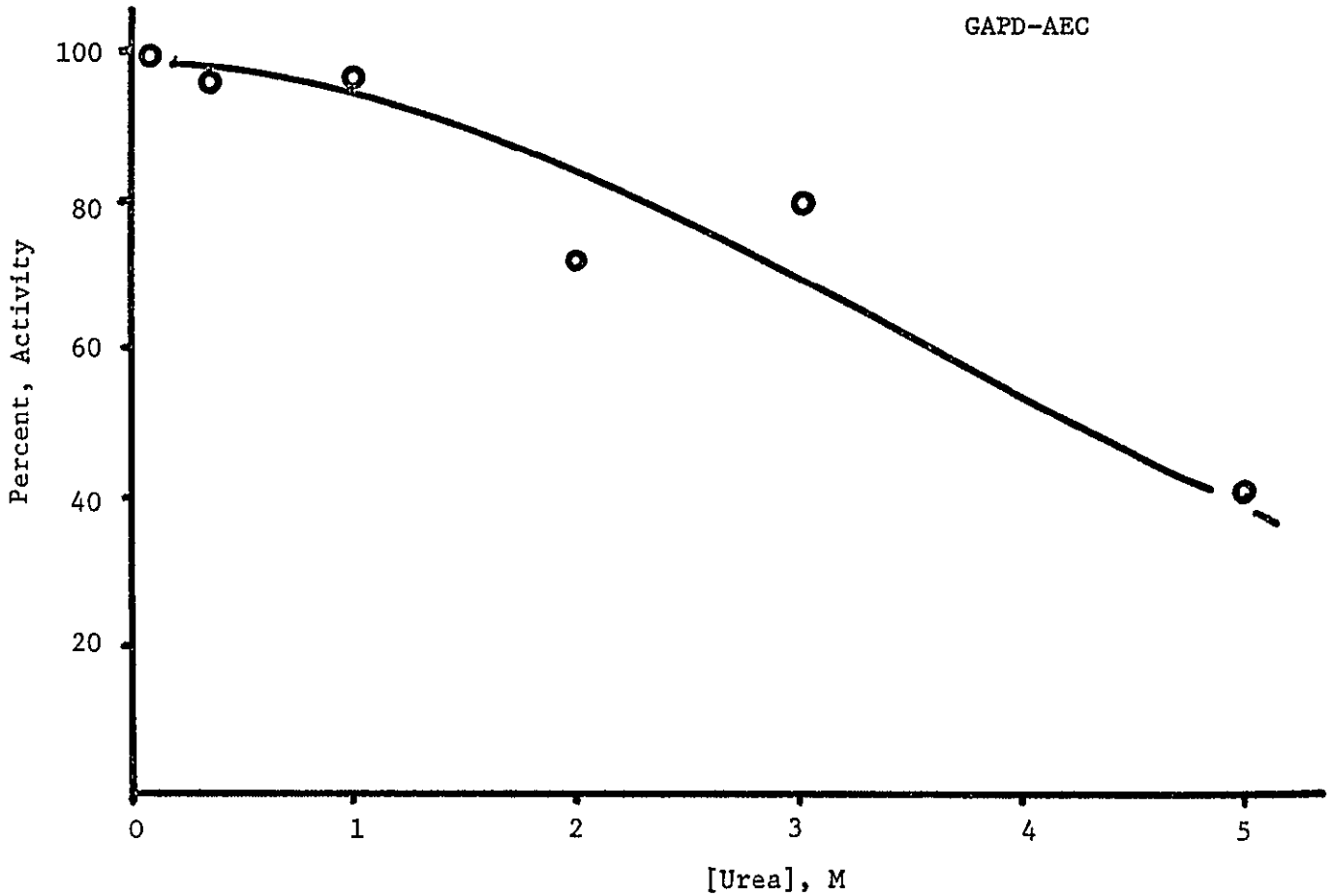


FIGURE 17. THE EFFECT OF UREA ON THE ACTIVITY OF GAPD-AEC

At the end of 5.0 M urea solution testing, the column was soaked with buffer as previously described. The GAPD activity was restored to slightly more than 55 percent of the original activity. After further washing, the column regained slightly more, to approximately 60 percent of the original activity. From these results, the delineation of the mode of action between NaCl and urea on the GAPD-CAE is clearly defined.

Stability of Enzyme Polymer Adducts

Heat Stability of Aldolase-AGC. The structural conformation of proteins may also be affected by heat. In most cases, enzyme molecules are not stable to heat and undergo thermal denaturation with the accompanying loss of catalytic activity. Figure 18 illustrates the heat sensitivity of both soluble aldolase and aldolase-EMA. The effect and extent of thermal denaturation were similar in both of these forms of aldolase. For example, upon heating soluble aldolase to 62 C for 2 minutes, 75 percent of the original activity was lost; whereas a similar heat treatment of insolubilized aldolase destroyed 67 percent of its original activity. Aldolase-CAE was also subjected to the heat stability study. Included in the same figure is the plot of the heat sensitivity of aldolase-CAE. Although the first minute of heating appeared to have reduced 50 percent of the original activity, further heating did not inactivate very much. At the end of the fifth minute of heating, the aldolase activity had 10 percent left.

Stability of Suspension and Powder Form of Insolubilized Enzymes. Since the stability of enzymes after chemical attachment to a polymeric matrix is of utmost interest and importance to the current work, we assayed repeatedly the activity of aldolase-EMA suspended in a pH 7.6 phosphate buffer. Under these conditions, aldolase-EMA retained its activity throughout a twenty day period (Table VII). Even after the suspension had been lyophilized to yield aldolase-EMA in powder form, the specific activity of aldolase-EMA was very much the same, and remained stable for twenty days.

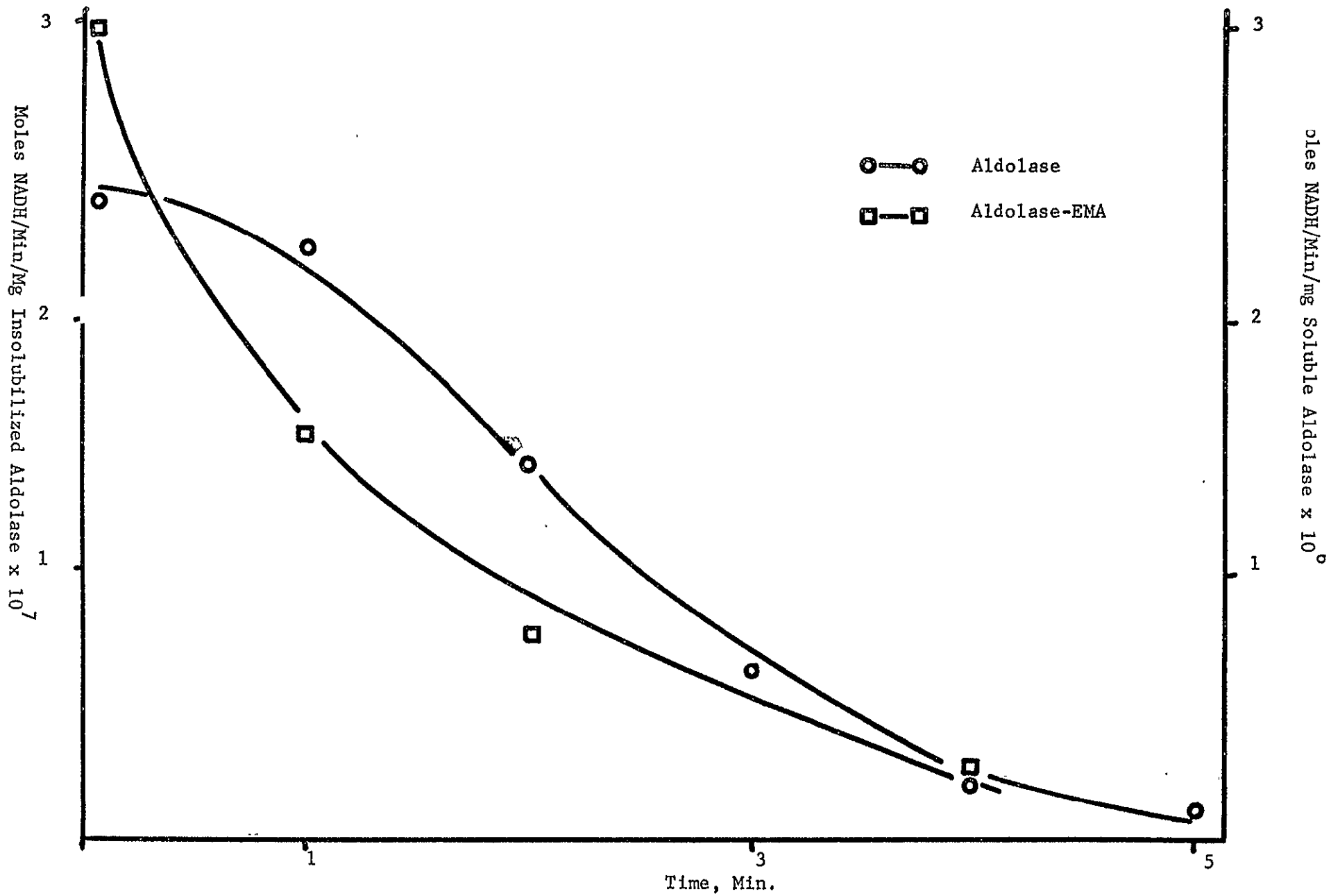


Figure 18. The Effect of Heating at 60° as a function of Time on the Activity of Aldolase and Aldolase-EMA

TABLE VII. STABILITY OF ALDOLASE-EMA

Days of Storage at 4 C	Form	Specific Activity x 10^7 Mole NADH/min/mg at pH 7.6
0	Wet in suspension	1.8
30	" "	1.9
0	Dry powder	0.9
20	" "	1.1

In view of the apparent stability of aldolase-EMA, it became of interest to determine its potential reusability against fresh substrate. Thus, the activity of aldolase-PAB against fresh batches of fructose diphosphate was determined for three cycles of enzyme recovery. Each cycle consisted in measuring the rate change of optical density at 340 mμ, separating the enzyme by centrifugation, removing the supernatant and adding fresh substrate to the enzyme residue. After three such cycles, the rate change of $O.D._{340\ m\mu}$ was 0.095 O.D. units per minute as compared to 0.097 O.D. units for the first cycle. It was concluded from these data that recycling of aldolase-EMA seems feasible.

Attachment of aldolase to AEC resulted in a marked increase in stability at room temperature. The insolubilized enzyme remained fully active after three weeks at room temperature, while the soluble aldolase became deactivated after 2-1/2 weeks. Since long exposure of the enzyme columns at room temperature might invite bacterial growth, a 0.02 percent sodium azide solution was soaked in the column, and this appeared to arrest some bacterial growth. Sodium azide at this concentration did not inhibit enzyme activity.

Of the three enzymes studied, GAPD was the least stable, possibly because of its requirement of free sulfhydryl groups for catalytic activity. Because of this instability, GAPD provided a crucial test for determining the effects of attachment to polymeric carriers. The stability at room temperature of a GAPD-CAE column was compared to a GAPD solution containing approximately the same amount of protein. Table VIII shows that 30 percent of the activity of GAPD-CAE remained after a period of several days, whereas the soluble GAPD solution became completely inactivated overnight at room temperature.

TABLE VIII. STABILITY OF GAPD-CAE

Days in the column at Room Temperature ~ 22 C	Percent Activity Remaining
0	100
1	95
4	58
4 (Repeat)	32
5	30

From the above data, it is possible that the GAPD-CAE column became deactivated via a different mechanism than the soluble GAPD. Probably the GAPD-CAE was deactivated by some impurity which was not washed away easily.

The FDPase derivatives resembled the aldolase derivatives in that the insolubilized enzymes were more stable than the soluble FDPase.

Sequential Column Reactions of Insolubilized Aldolase and GAPD

A demonstration of a sequential reaction through the use of enzyme columns would be a major step toward achieving the goal of conversion of metabolic wastes into edible materials, since there are a few intermediate steps in this series of reactions. As already shown, GAPD catalyzes the oxidation of glyceraldehyde-3-phosphate in the presence of NAD, and GAP can be formed from the enzymatic cleavage of FDP by aldolase.

A FDP solution containing NAD was first passed through the aldolase column and part of the eluate was then assayed with soluble GAPD. The rate of increase of O.D. $_{340\ m\mu}$ indicated that there was aldolase activity. The other part of the eluate, after being mixed with sodium arsenate, was passed through the GAPD column, and the eluate recycled twice. To this eluate 3 drops of methylene blue (10^{-4} M) and 25 λ of diaphorase were then introduced. The blue color became colorless instantaneously, indicating that NADH had been formed, due to the catalysis of oxidation of glyceraldehyde-3-phosphate by GAPD-AEC. This sequential reaction could be repeated after a week, signifying that the two enzyme columns were still active. The whole arrangement for the sequential reaction is illustrated in Figure 19.

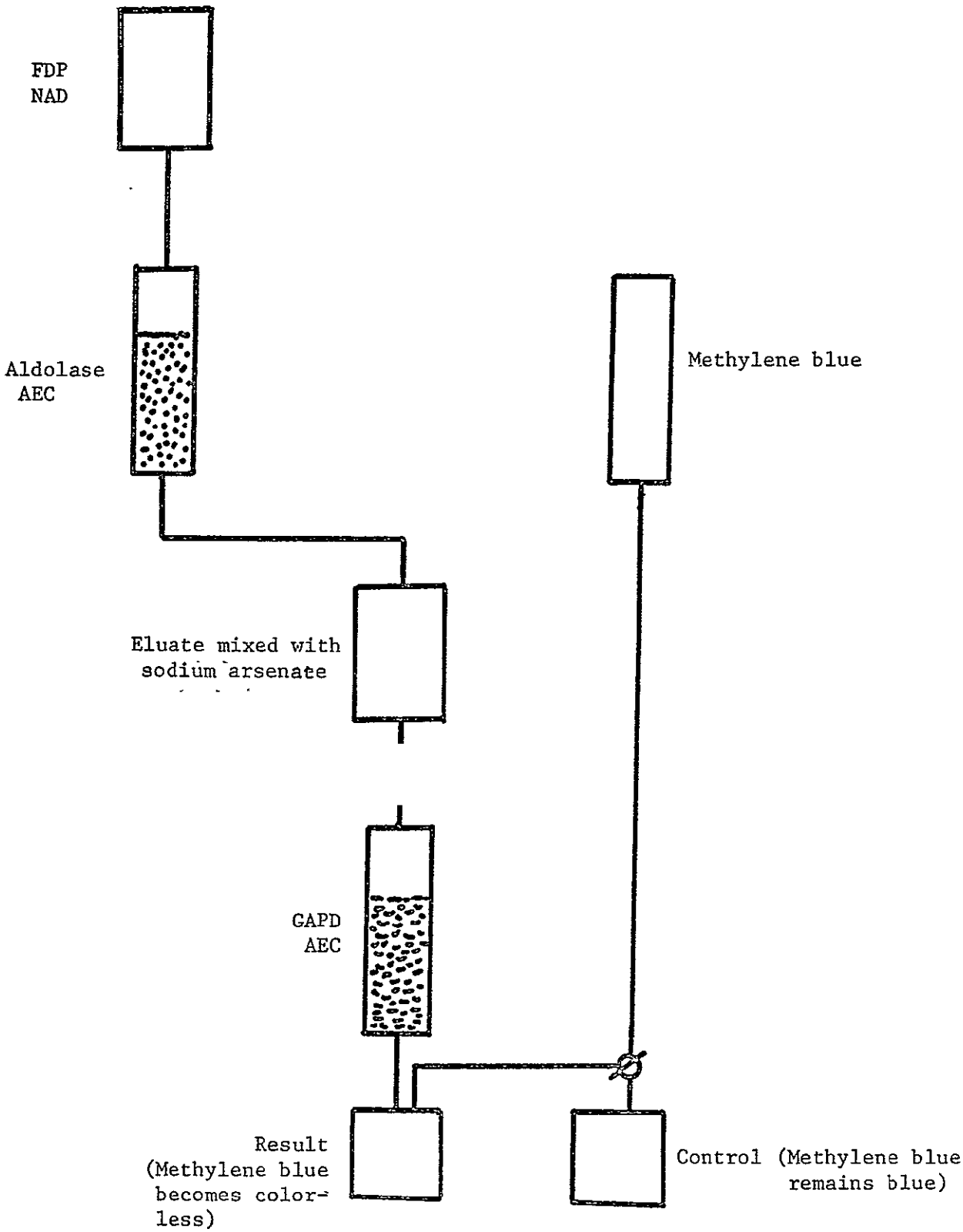


FIGURE 19. THE ARRANGEMENT OF INSOLUBILIZED ALDOLASE AND GAPD COLUMNS FOR SEQUENTIAL REACTIONS

DISCUSSION

The work reported herein has demonstrated that insolubilization of large enzymes containing multiple forms such as aldolase, GAPD and FDPase can be achieved. Although the activities of such insolubilized enzymes have not exceeded 10 percent of the activity of the native enzyme, the findings suggest that the molecular integrity of the enzyme was not altered in the course of forming enzyme-polymer adducts.

It is interesting to note the activity of the soluble aldolase decreases with increasing sodium chloride concentration, as did the activity of aldolase-EMA and GAPD-CAE. This destabilization phenomenon may be attrib uted to the dissociating effect of sodium chloride on the subunit structures which interact with one another through ionic linkages. The shielding of charges by sodium chloride may disrupt the integrity of subunit structures.

Thermal and urea denaturation of soluble and insolubilized aldolase, and insolubilized GAPD seemed similar in effect. This observation indicates the same degree of disruption, and hence the molecular integrity of aldolase and GAPD must remain unperturbed after attachment to polymeric matrices.

Additional evidence which strongly supports the supposition that the molecular integrity of subunit structured enzymes remain unperturbed is the chemical modification of aldolase-CAE and of GAPD-CAE with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Aldolase-CAE was activated with DTNB treatment, a phenomenon not uncommon with the soluble aldolase. GAPD-AEC was also inactivated with DTNB, which presumably reacted with the free sulfhydryl groups of GAPD.

Because aldolase is unusually stable in solution, assessment of enhanced stability of insolubilized aldolase would not be simple. Nevertheless,

the present work has shown that:

(1) Aldolase-EMA and aldolase-AEC remained active in the suspension for a period of some thirty days

(2) Its activity was not altered appreciably by lyophilization

(3) Its powder form remained as active as the suspension over a period of twenty days.

The insolubilized GAPD derivatives demonstrate most vividly the stabilization resulting from attachment to a polymeric matrix. Soluble GAPD became completely inactivated overnight at room temperature, while GAPD-AEC still retained more than 90 percent of its original activity. Although the enzyme derivative lost some activity with time at room temperature, the enzymatic activity had more than 30 percent of the original value after a period of five days. The FDPase-polymer derivatives also had the same degree of stability as the aldolase-polymer adducts. We therefore conclude that enzyme stability can be greatly enhanced by attachment to polymer matrices.

One of the significant results from the work is the fact that the K_m and pH optima of the enzymes can be affected by the choice of polymeric matrix. Insolubilization of aldolase by attachment to p-amino-benzyl cellulose shifted the pH optimum of aldolase toward lower pH values. Ethylene maleic anhydride copolymer exerted an opposite effect and thus shifted the pH optima slightly to the basic side. The K_m value for aldolase-PAB is slightly smaller than that for the soluble aldolase. Aldolase attached to ethylene maleic anhydride copolymer had a somewhat higher K_m than that of the soluble aldolase.

The above results can be explained in terms of a vicinal effect from charges residing on the polymeric matrix. For example, with aldolase-PAB, the pH of the immediate environment around the active site of aldolase becomes higher than the pH of the milieu under the influence of the amino groups. As a result, the pH optimum shifts to a lower value as compared to that of soluble aldolase. By a similar process, the positively charged environment can attract the negatively charged substrate fructose diphosphate. The high local concentration of substrate can lower the K_m value, so that low-level substrate concentrations can be catalyzed by the enzyme more readily. In multiple step enzymatic processes, such as the conversion of metabolic wastes into edible materials, not only can the catalytic activities be regulated, but also the stable enzyme-polymer adducts can offer flexibility, manageability, and reusability.

In summary, the encouraging data obtained in this year of investigation have assured us the feasibility of preparing stable insolubilized enzymes. Continued work on the preparation of the rest of the enzymes of the Calvin cycle should help us attain the goal in converting carbon dioxide into fructose or glucose.

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