# Purification of Rabbit Brain Enolase and Demonstration of an Active Immobilized α Monomer

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A Thesis

in

The Department

of

Chemistry

Presented in Partial Fulfilment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada

February 1985

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## ABSTRACT

Purification of Rabbit Brain Enolase
and Demonstration of an Active Immobilized α Monomer

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Rabbit brain enolase (2-phospho-D-glycerol hydrolase E.C. 4.2.1.11) was purified with an ion-exchange column, ammonium sulphate fractionation and was separated into three distinct isozymes on a DEAE Sephacel column. The first isozyme (αα) from the DEAE column was further chromatographed on a Polybuffer ion exchanger and on Sephadex G-150. This enolase was composed of two identical subunits of 45,000 dalton molecular weight and had an isoelectric point of 8.2.

When soluble rabbit brain enolase (αα)was exposed to 1M KBr and 4mM EDTA, at 37°C in the absence of $Mg^{2+}$, the enzyme dissociated into inactive subunits. The catalytically active dimer was recovered in virtually 100% yields by dilution of the salt with buffer containing $Mg^{2+}$ or by dialysing out the salt against the same buffer at 25°C. The dissociation procedure was then used for the isozyme immobilized onto a solid matrix in the presence of $Mg^{2+}$. The immobilized derivatives that remained on the matrix were reactivated in the presence of $Mg^{2+}$ and the recovered enzymatic activity and protein were analysed before and after incubation with soluble

subunits. The immobilized rabbit brain enolase reactivated in the absence of soluble subunits had about one-half the activity and protein content of the untreated isozyme. The specific activity of the reactivated immobilized isozyme after reassociation with soluble subunits was 95% of that of the initially immobilized αα isozyme.

The results obtained were consistent with a salt inactivation that was due to dissociation of the immobilized αα isozyme in the presence of magnesium ions. The immobilized monomer obtained after eliminating the dissociation conditions were found to be catalytically active.

Each person is an end in themselves
and not just the means to an end.

Dedicated to my family and

Yaw Boakye with my love.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my research supervisor, Dr. M.J. Kornblatt for her guidance and assistance throughout the entire work. I would also like to thank the other members on my research committee; Dr. R.K. Ibrahim and Dr. O.S. Tee.

I wish to thank Dr. J.A. Kornblatt, my fellow graduate students and the faculty and staff of the Chemistry Department for all their help.

A special note of my appreciation to my fellow graduate student; Mr. H.E. Khouri for his friendship and continued encouragement, during my stay at Concordia and to Ms. Lee Chin for her secretarial assistance.

The Natural Science and Engineering Council of Canada is also acknowledged for their financial support of this work.

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ABBREVIATIONS

| | |
|---|---|
| EDTA | Ethylenediamine tetraacetate |
| Tris | 2-Amino-2(hydroxymethyl)-1,3-propanediol |
| TEA | Triethylamine |
| PBE | Polybuffer exchanger |
| DEAE- | Diethyl aminoethyl- |
| CM- | Carboxymethyl- |
| HPLC | High performance liquid chromatography |
| pI | Isoelectric point |
| CNBr | Cyanogen bromide |
| Ve | Elution volume |
| Vo | Void volume |
| SH- | Sulfhydryl- |
| m- | mitochondrial- |
| K | Kilodalton |

# INTRODUCTION

## A. INTRODUCTION

The glycolytic enzyme enolase (2-phospho-D-glycerol hydrolase EC 4.2.1.11) catalyses the interconversion of 2-phosphoglyceric acid and phosphoenolpyruvate with an absolute requirement for divalent cations, preferably magnesium, for the maintenance of its dimeric structure as well as for its enzymatic activity.

Three features of the structure and function of enolase appear to have been preserved through the evolution of plants and animals alike; the catalytic site, the metal binding site(s) and the subunit interaction. An approach to help understand the need for the preservation of the features mentioned above and the mechanism of action of this enzyme has been to study the properties of the single subunits and to compare them with those of the native enzyme (oligomer).

The yeast and mammalian enzymes have long been reported to exist as dimers in vitro, composed of two apparently identical polypeptide chains (1-3). The activity of the separated polypeptide chains (monomers) has been a question of enormous interest. According to Brewer and Weber (4) when the enzyme was dissociated at low protein concentration the resulting monomers had no enzymatic activity. The same authors in another piece of

work (5), used 1M KCl to dissociate the enzyme and concluded that the monomers of this enzyme dissociated in the absence of magnesium ions have no catalytic activity. Evidence from frontal gel filtration (6) at neutral pH in the presence of magnesium and substrate demonstrated the existence of active enzyme monomers at 43°C. Furthermore, sedimentation coefficient measurements of the yeast enzyme (7) have indicated a monomer possessing catalytic activity. A similar picture has emerged from data on the dissociation of the mammalian enzyme. In a sedimentation coefficient study, Winstead and Wold (8) reported the resulting monomer of rabbit muscle enolase to be devoid of enzymatic activity when the dimeric enzyme was exposed to 20% dioxane or 20% acetone in the presence of ammonium sulphate and versene. In another study where pressure (9,10) was used to induce dissociation of the three isozymes of the yeast and mouse brain enzyme, the resulting α monomer was reported to be inactive and the γ monomer was found to be active.

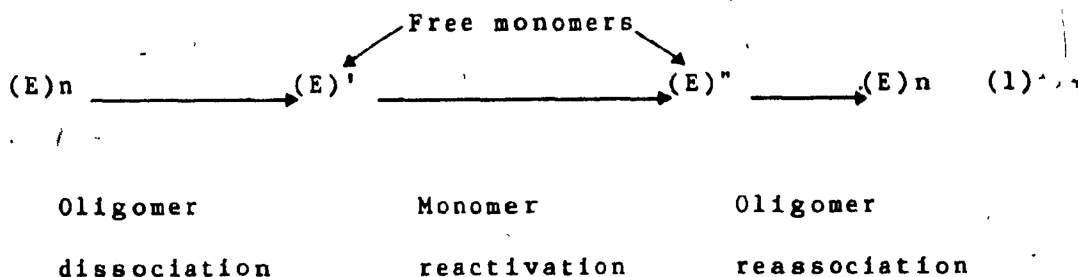
The possible catalytic ability of other enzymes has also been examined. Using sucrose density gradient centrifugation at 4°C the monomers of rat liver acetyl-coenzyme-A-hydrolase (11) were found to be inactive. Dissociation of fumurase, porcine m-malic dehydrogenase and glutamate dehydrogenase with chaotropic agents such as urea and guanidinium chloride have produced

inactive monomers of these enzymes (12-14). Evidence reported by Grossman et al (15) from cross-linking and fluorescence polarization analysis of N-dansylaziridyl creatine kinase have shown the monomeric forms of the enzyme to be active. Using target analysis the irradiation of dehydroshikimic reductase from Euglena gracillis produced catalytically active monomer (16).

In almost all of the above cases the dissociation has been carried out in solution where an active monomer could be recovered only after the elimination of the dissociating agent or condition. However, there is a natural tendency for the subunits to reassociate to form the native structure. A different approach is to first attach the enzyme and/or protein covalently onto a rigid matrix before the dissociation process.

This method (17-20) entails covalently coupling enzymes and/or protein to Sepharose beads (solid matrix) in a way such that each molecule of enzyme would be attached via only one subunit and the coupling sites on the Sepharose are sufficiently far apart to eliminate any interaction between adjacent subunits. Once an enzyme is coupled it can be dissociated and the free soluble subunits washed away leaving a product of an immobilized monomer. The dissociating agent can now be eliminated and renaturing conditions returned without spontaneous reassociation of the subunits. The following shows a

schematic representation of the process involved in the dissociation of both soluble and immobilized enzymes.




Where (E)n represents the oligomer, (E)' an inactive monomer (without cofactor), (E)" the reactivated monomer (after adding cofactor and removal of dissociating agent). In the dissociation of immobilized enzymes, there have also been conflicting reports on the catalytic ability of the resulting immobilized monomers. In many studies (21-26) where chaotropic agents were used to bring about dissociation of the enzyme the resulting monomers have been reported to have no catalytic activity. However, under milder conditions, such as low enzyme concentration, salt, pH and cofactor removal or even with chaotropic agents, the monomers, E", produced from these dissociation conditions of other enzymes have been found to be active (19, 27-30).

In the examples cited above where the activity of the monomer could not be restored, it is possible that the dissociating agent disrupted the tertiary as well as the

quaternary structure of the enzyme. It is also likely that the experimental conditions used did not allow the process E' $\longrightarrow$ E" to occur before the reconstitution of the oligomer. It is also possible that for some of the enzymes the intact quaternary structure is an absolute requirement for the expression of the catalytic activity.

This work was undertaken partly, to attempt to clarify the conflicting evidence on the activity of enolase monomers. Therefore, after successfully separating and purifying the three forms of rabbit brain enolase we sought the possibility of attaching the enzyme αα to a solid matrix. Then, using milder dissociating conditions, salt and/or cofactor removal, the question of whether the tertiary structure of the enzyme was sufficient for expressing the catalytic activity was adressed. The results obtained showed the reversible dissociation of the enolase isozyme αα . The immobilized monomer obtained after the dissociation of the dimeric enzyme in the presence of magnesium was catalytically active. Thus, for the αα isozyme of rabbit brain enolase subunit interactions are not essential for the expression of catalytic function.

# LITERATURE REVIEW

## B. LITERATURE REVIEW

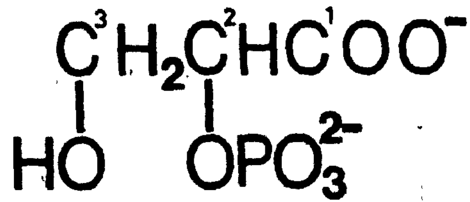
### B.1. Physiological aspects of enolase

The interconversion of 2-phosphoglyceric acid and phosphoenolpyruvate is an important step in glycolysis and as such the elucidation of the structural and functional properties of the enzyme that catalyse this step would help provide information as to the energy metabolism in the cells where the enzyme is located.

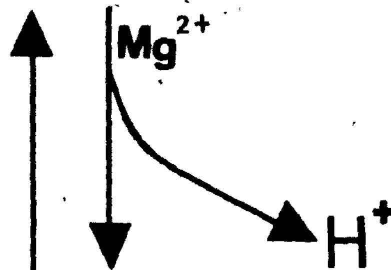
### B.2. Heterogeneity of glycolytic enzymes

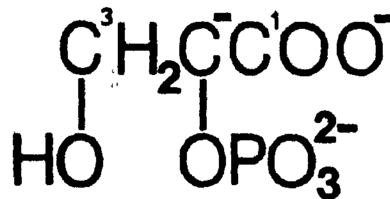
The enzymes involved in the glycolytic pathway have received enormous attention in research over the past decade. The discovery of the distribution and properties of isozymes of glycolytic enzymes have greatly increased our understanding of how various tissues have evolved control features to allow glycolysis to serve the different functions in the different organs. Both pyruvate kinase (31) and phosphofructokinase (32) have been found as one kind of isozyme in liver and another in muscle. A systematic survey of organs in different species (33,34) have shown that enolase, which catalyse the oxidoreductive step (Fig. 1) in glycolysis, to be present as one type of isozyme or other in the brain,

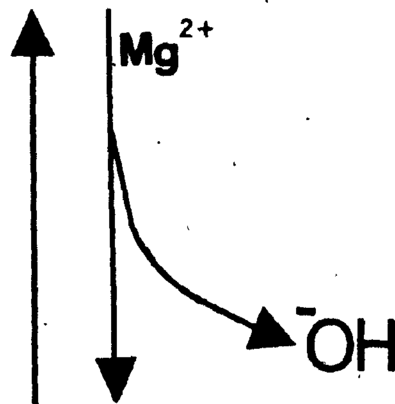
**Figure 1:** Proposed scheme of the intramolecular oxidoreduction of carbons 2 and 3 of 2-phosphoglyceric acid by rabbit brain enolase.

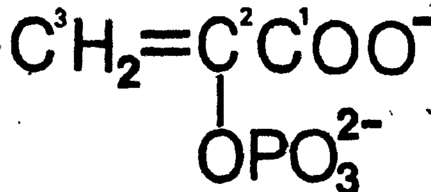



2–Phosphoglyceric acid




Phosphoenolpyruvate

liver, kidney, skeletal muscles, adrenal gland and the large intestine. Kinetic data on pyruvate kinase and phosphofructokinase showed that their isozyme distribution and their properties relate to the characteristic energy metabolism of the respective organs. The distribution of enolase isozyme in the various organs could also relate to the characteristics of the energy metabolism in these organs.

B.3. Different forms of enolase and their cellular location.

Ever since Taylor and Rider (33) discovered the brain specific forms of enolase all accumulating reports indicate that three forms of enolases: $\alpha\alpha$ , $\alpha\gamma$ and $\gamma\gamma$ exist in the adult mammalian brain. It has also been shown (35) that another type of isozyme, $\beta\beta$ exists in the skeletal muscles. Another eukaryotic organism where isozymes of enolase have been isolated is yeast (36,37).

The most acidic form of the three isozymes, usually designated as $\gamma\gamma$ , has been shown by sensitive immunological assays to be localized in the neurons (38,39), hence the name, neuron-specific enolase (NSE). This protein has been shown (40) to be homologous to the bovine 14-3-2 protein isolated by Moore (34). Recent immunocytochemical evidence has suggested NSE not to be

confined to neurons, but it is also present in islet cells in the pancreas, c-cells of the thyroid and chromaffin cells in the adrenal cortex (41).

The least acidic form, $\alpha\alpha$, has been shown to be identical to enolase in the liver (40,41); it was found only in the glial cells of the brain and has been designated non-neuronal enolase, NNE (39).

Immunological studies have shown $\alpha\gamma$ (42) to exist as a hybrid molecule consisting of one $\alpha$ subunit and one $\gamma$ subunit. This isozyme seems to be confined to the nervous sytem.

The $\alpha$ and $\gamma$ subunits are believed to be products of immunologically distinct, separate genes (43,44).

### B.4. Developmental profile of enolase isozymes

Not only are the locations of these metabolic isozymes different but there seem to be a changing profile of the isozymes in cells undergoing differentiation. The homodimer $\alpha\alpha$, seems to be the predominant form in immature (fetal) brain (45,46). Similarly, endoderm cells, formed by differentiation of embryonal carcinoma cells, contained only the $\alpha\alpha$ isozyme. In contrast, the $\gamma\gamma$ and $\beta\beta$ appear late in the embryonic development of the brain and muscle cells, respectively, and these isozymes

seem to reach their maximum levels after birth in mature functional brain and skeletal muscles (46,47,48).

The coinciding appearance of γγ with neuronal functional maturation (45,49) seems to indicate that neuronal enolase function is a highly differentiated one and it also suggests that γγ might have different functional properties than the non-neuronal enolase.

B.5. Enolases as cellular markers

The strict location of αα and γγ have earned them a role as cellular markers in neuroanatomic studies. They are a means of identifying various cell types in the brain (34).

It has been suggested (50) that γγ serves as a marker for cells of the amine precursor uptake and the decarboxylation system.

Other studies of the mammalian isozymes have also suggested that they would be particularly useful as markers where differentiation into muscle and nervous tissues is of concern, since the isozymes ββ and γγ are apparently specific for the muscle and nervous tissues respectively.

The three mammalian isozymes of enolase, αα , γγ and ββ have also been used as markers for differentiation in tetratocarcinoma cells and normal tissues (45). This

has come as a result of the expression of $\gamma$ and $\beta$ and the de-expression of the $\alpha$ gene in the developmental stages of the cell.

# MATERIALS AND METHODS

## C. MATERIALS AND METHODS

### C.1. Source of enolase

Rabbit brain was purchased from Pel-freez Biologicals (Rogers, Arkansas). The brains were stored at - 15°C.

### C.2. Enzyme Purification

#### C.2.1. Separation of the three forms of enolase

This part of the purification was based on the purification procedure used by Suzuki et al (51). All procedures were carried out at 4°C unless otherwise stated. One hundred grams of whole mature brain was homogenized in 200 ml of 15mM Tris-acetate buffer, pH 6.5, containing 5mM $Mg(OAc)_2$ and 0.1mM EDTA (buffer A) using a teflon-glass homogenizer. The homogenate was centrifuged at 19,000 rpm for 30. minutes and the supernatant was passed through a column (3.0 x 62 cm) of CM-Sephadex which had previously been equilibrated with Buffer A. The eluate was fractionated with 50-80% solid ammonium sulphate and the precipitate was dissolved in 15mM Imidazole-HCl buffer, pH 7.4, containing 5mM $Mg(OAc)_2$ and 0.1mM EDTA (Buffer R) and dialysed against

the same buffer for 24 hr. The sample was applied onto a DEAE-Sephacel column (2.5 x 50 cm) previously equilibrated with Buffer R. Enolase, αα was eluted with Buffer R and αγ and γγ were eluted with a 50-400mM NaCl concentration gradient in the same buffer. Fractions containing high enzyme activity of each form were pooled, concentrated with polyethylene glycol and then dialysed against Buffer R. Glycerol was added to each form of enolase to give a final concentration of 50% glycerol and the αγ and γγ forms stored at - 15°C.

C.2.2. Chromatofocusing

The above mentioned enolase, αα in 50% glycerol was applied onto a Polybuffer exchanger-94 column (0.7 x 30 cm) previously equilibrated with 0.025M ethanolamine-acetate, pH 9.4, containing 10% glycerol. The enzyme was eluted with polybuffer 96-HCl, pH 6.0, containing 10% glycerol which generated a linear gradient between pH 9 and 6. Thus the protein was eluted at its isoelectric point (pI).

C.2.3. Chromatography on Sephadex G-150

The above enzyme was chromatographed on a Sephadex G-150 superfine column (2.5 x 45 cm) previously

equilibrated with Buffer R and the enzyme was eluted in the same buffer. The enzyme was concentrated, dialysed against Buffer R containing 0.1M NaCl and stored at - 15°C in 50% glycerol.

C.3. Enolase assay

Two different assay buffers were used:

(a) Assay buffer for the purification consisted of 50mM Tris-HCl, pH 7.5, containing 5mM $Mg(OAc)_2$ and 0.3M KCl.

(b) Assay for all other analyses was done in Buffer R.

The reaction in both assay media were started by the addition of 1mM 2-phophoglyceric acid. The production of phosphoenolpyruvate was followed spectrophotometrically at 240nm in a Perkin-Elmer 552 with thermostated sample compartments at 25°C.

C.4. Preparation of Immobilized Enzyme

C.4.1. Activation of Sepharose 4B

Unless otherwise stated all chemicals used were of standard analytical grade. Preswollen Sepharose 4B (Pharmacia, Uppsala, Sweden) was neutrally activated using cyanogen bromide (Aldrich, Milwaukee) and Triethylamine (HPLC reagent grade, Fisher Scientific, New Jersey) by the

method of J. Kohn and M. Wilchek (52) with some minor modifications.

Twenty grams of wet Sepharose 4B was washed with 200 ml of 30% aqueous acetone at 25°C and the gel was collected on a sintered glass funnel. This was followed with 200 ml of 60% acetone at 5°C. The gel was then suspended in 20 ml of 60% acetone and the temperature brought to -3°C. 1.5M CNBr in 60% acetone was added, with constant stirring. Then 2M TEA in 60% acetone was added to the reaction mixture over a period of 3 minutes. The whole mixture was poured into 200 ml of ice cold washing medium (acetone: 0.1M NaCl = 1:1). The activated gel was filtered by suction on a sintered glass funnel. For immediate use, the activated gel was washed with 60% acetone followed by quick washings with cold 30% acetone and $H_2O$, all at 5°C. The activated resin was then washed with 0.1M $NaHCO_3$-HCl, pH 9.0, containing 5mM $Mg(OAc)_2$, 10% glycerol and 0.1mM EDTA (coupling buffer). For prolonged storage, the rest of the gel was extensively washed with storage medium (acetone: dioxane: $H_2O$ = 60: 35: 5) and stored air-tight at -15°C in the same medium. The resin was reswollen for 5 minutes in cold washing medium before use each time.

Two types of activation were investigated. Activation of Sepharose at neutral pH, outlined above, and

activation at basic pH, using NaOH and comparatively large amounts of cyanogen bromide (53-56).

### C.4.2. Coupling of enzyme

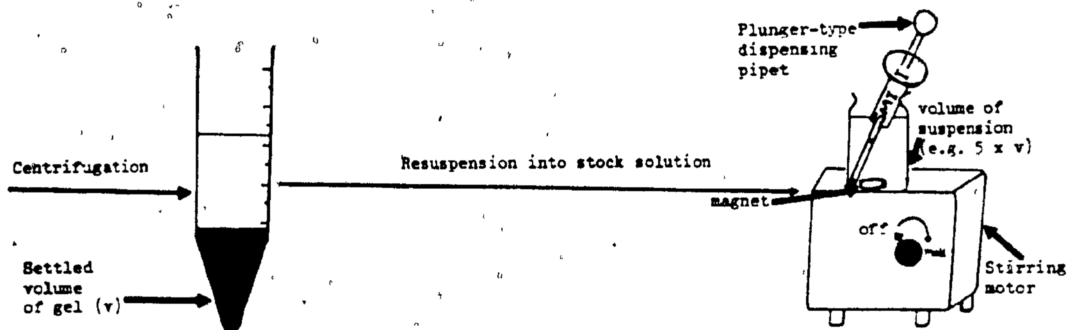
The enzyme, ( αα ), was coupled to the activated Sepharose 4B by the method of Chan (57). The enzyme was dialysed against the coupling buffer. The dialysed enzyme was then added to activated Sepharose. The suspension was incubated at 4°C with occasional gentle swirling, for 24 hr. Unreacted groups on the gel were blocked and unbound protein removed by washing the gel with 10 volumes of 0.1M ethanolamine-HCl, pH 7.5, containing 5 mM $Mg(OAc)_2$ and 10% glycerol, suspending the gel in 2.5 volumes of the same buffer and then incubating at 4°C with occasional gentle swirling. After 24 hr the slurry was washed by filtration on a sintered glass funnel, with 10 volumes of Buffer R containing 0.1M NaCl to remove any enzyme not tightly bound to the gel. The immobilized enzyme was stored in Buffer R containing 50% glycerol at 4°C.

A reference gel was treated the same way as above without enzyme and used as control in some experiemnts.

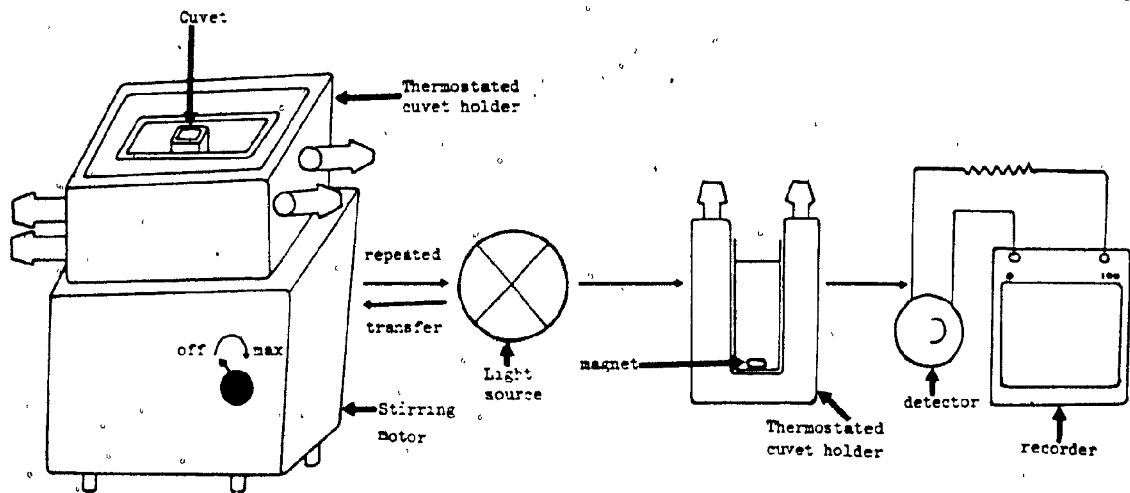
C.4.3. Immobilized enolase assay

The immobilized enzyme was assayed using the standard enolase assay mixture. Accurate aliquots were obtained, using SMI pipettes, from a stock solution of immobilized enzyme, kept homogeneous by stirring. A modified continuous spectrophotometric assay as decribed by Chan (58) was used for the measurement of the activity of the immobilized enzyme. A 3 ml cuvet containing the assay mixture with a magnetic bar was kept stirring in a thermostated cuvet holder mounted on a stirring motor (Fig. 2). At 30 seconds intervals the cuvet was placed in the spectromphotometer, a trace of the absorbance was recorded for 30 seconds and the cuvet was then returned to the cuvet holder for stirring. This process was repeated and the reaction was followed for 5 - 10 minutes (Fig. 3). Whenever possible the assay mixture was centrifuged and the supernatant was checked for soluble activity. The stored immobilized enzyme suspensions were also checked for soluble activity. Centrifugation of the suspensions followed by enzyme assays of the supernatant revealed no detectable solubilization.

Figure 2: A simple arrangement of a modified continuous spectrophotometric assay, used for the measurement of the enzymatic activity of the immobilized rabbit brain enolase.




Quantitative methods for measuring immobilized derivatives




Arrangement for spectrophotometric assay of immobilized enzymes

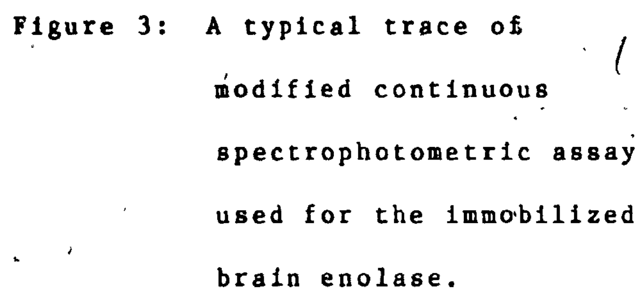
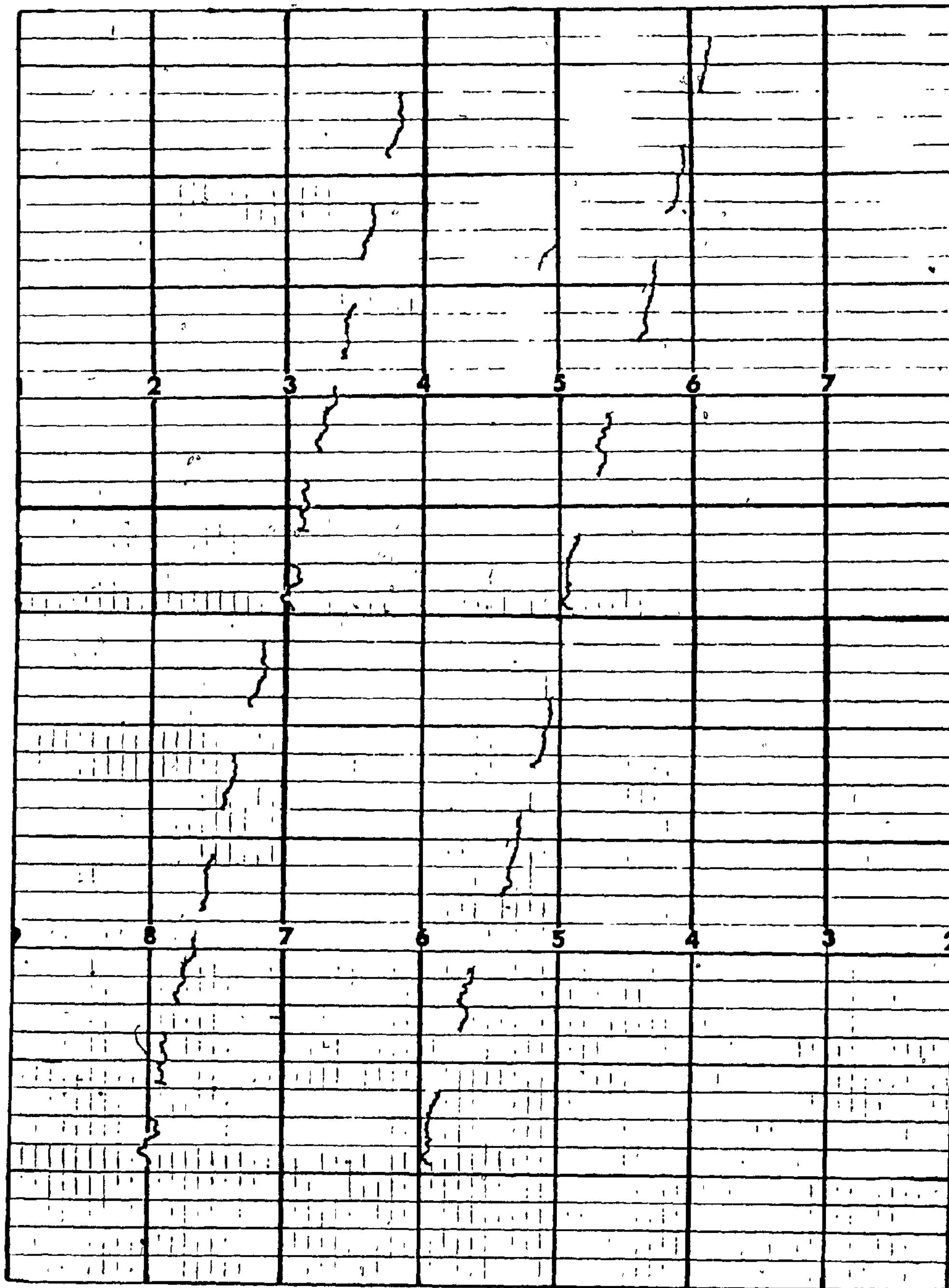


Figure 3: A typical trace of modified continuous spectrophotometric assay used for the immobilized brain enolase.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## C.5. Inactivation and Reactivation Experiments

### C.5.1. Inactivation of soluble enzyme

The procedure of Marangos (58) was adopted with some minor changes. Enzyme in solution was dialysed against 15mM Imidazole-HCl, pH 7.4, containing 0.1M EDTA, 0.1M NaCl and 10% glycerol for 20-24 hr. The partial $Mg^{2+}$-free enzyme was diluted to the desired salt concentration with 15mM Imidazole-HCl, pH 7.4, containing 2M KBr and 4mM EDTA (Buffer R). The sample was incubated at the desired temperature. The 37°C and 25°C experiments were carried out in a magni water bath. The 4°C experiment was done on ice. The samples and controls, which consisted of enzyme diluted to the same protein concentration as the samples with Buffer R, were given occasional gentle swirling. Some inactivation experiments were carried out in the presence of 2.5mM dithiothreitol or 2-mercaptoethanol. Aliquots were withdrawn at appropriate times for rate measurements.

Percent inactivation at the various temperatures was calculated based on the appropriate control.

C.5.2. Reactivation of soluble enzyme

Inactive enzyme was diluted 10 times in Buffer R and incubated at 25°C with occasional gentle swirling. Aliquots were withdrawn at appropriate times for rate measurement. The percent recovery was calculated based on the activity of the control. Reactivation was also achieved by gently dialysing out the salt against Buffer R at 25°C. Some of the reactivation experiments were carried out with 2.5mM dithiothreitol or 2-mercaptoethanol.

C.5.3. Inactivation, and reactivation of immobilized enzyme

The immobilized enzyme was washed three times by suspending the gel in Buffer R and then centrifuging at 1,100 rpm for 3 minutes. Then, the immobilized enzyme was diluted with Buffer I to give the desired salt concentration. The sample was incubated at 37°C with occasional mixing of the reaction medium. After inactivation, the supernatant was removed and the gel washed three times with Buffer I, each time collecting buffer after spinning down the gel. The supernatants were reactivated in the same way as described for the soluble enzyme.

The reactivation of the inactive enzyme on the gel was initiated by first washing gel three times with

Buffer R and collecting excess buffer for analysis. The gel was then suspended in about ten times its volume of Buffer R and incubated at 25°C for 12 hr. The gel was spun down, excess buffer collected for analysis, and the gel, suspended in Buffer R with 50% glycerol was stored at 4°C.

In other reactivation experiments the reactivated immobilized enzyme was incubated in Buffer R with a high concentration of inactivated soluble enzyme at 25°C for 1-2 hr. The gel was spun down and stored at 4°C.

C.6. Molecular weight determination

The molecular weight of the soluble inactive enzyme was determined by gel filtration at 37°C using Sephadex G-150 superfine column (2.8 x 30 cm) previously equilibrated with Buffer I. The molecular weight of the dimer and the reassociated enzyme were done at 4°C using the same size column as that for the inactive enzyme, but equilibrating the column with Buffer R. The flow rate on the columns were controlled by a monostaltic pump (bi Buchler). A three ml sample was applied onto the column and two ml fractions were collected. The columns were calibrated using proteins of known molecular weight from Sigma Chemical Co., (St. Louis, MO).

C.6.1. Inactivated enolase assay

Activity measurement of the monomer from the column was achieved with a slight modification of the standard enolase assay. Fractions from the column were diluted into Buffer R containing 1mM 2-phosphoglyceric acid (substrate) and the amount of phosphoenolphyruvate (product) produced after 4 hr of incubation was read at 240nM.

C.7. Definition of enzyme units

Enolase activity was expressed in enzyme units. One unit is defined as the amount of enzyme catalysing the conversion of 1 μmole of substrate per minute under the assay conditions.

C.8. Protein determination

The method of Bramhall et al (60) for protein determination was used during enzyme purification, using bovine serum albumin as standard.

The protein of the immobilized enzyme on the gel was determined by the method of Lowry et al (61) using bovine serum albumin as the standard protein, with the reference gel serving as a control.

Protein content of the enzyme from the molecular weight determinations were analysed by the method of Bradford (62) using Biorad reagent (Richmond, California) and bovine serum albumin as the standard protein.

C.9. Chemicals

All the gels used, CM-Sephadex, DEAE-Sephacel, Polybuffer ion exchanger-94, and Sephadex G-150 superfine were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); Tris, Imidazole and magnesium acetate were obtained from Fisher Scientific (New Jersey) and D(+)-2-phosphoglyceric acid from Sigma Chemical Co., (St. Louis, MO).

# RESULTS

## D. RESULTS

The work was carried out under four basic phases:

1. The enzyme was purified.
2. Conditions were found for reversible inactivation and dissociation of soluble enzyme.
3. After ascertaining that inactivation was equivalent to dissociation and reactivation to reassociation, these conditions were used for immobilized enzyme.
4. The activity of the resulting monomers was determined and proof of their existence was also established.

### D.1. Purification of enolase

The enzyme was purified (Fig. 4) by ion exchange chromatography followed by the fractional precipitation with ammonium sulphate and the three forms of enolase were resolved on a DEAE-Sephacel column (Fig. 5). The αα isozyme was further chromatographed on a Polybuffer ion exchanger (Fig. 6) and Sephadex G-150 superfine (Fig. 7) columns. The protein peak of αα was eluted at pH 8.2 from the former column and the latter column eluted αα as a 90,000 dalton molecular weight protein. The DEAE

Figure 4 PURIFICATION SCHEME OF RABBIT BRAIN ENOLASE

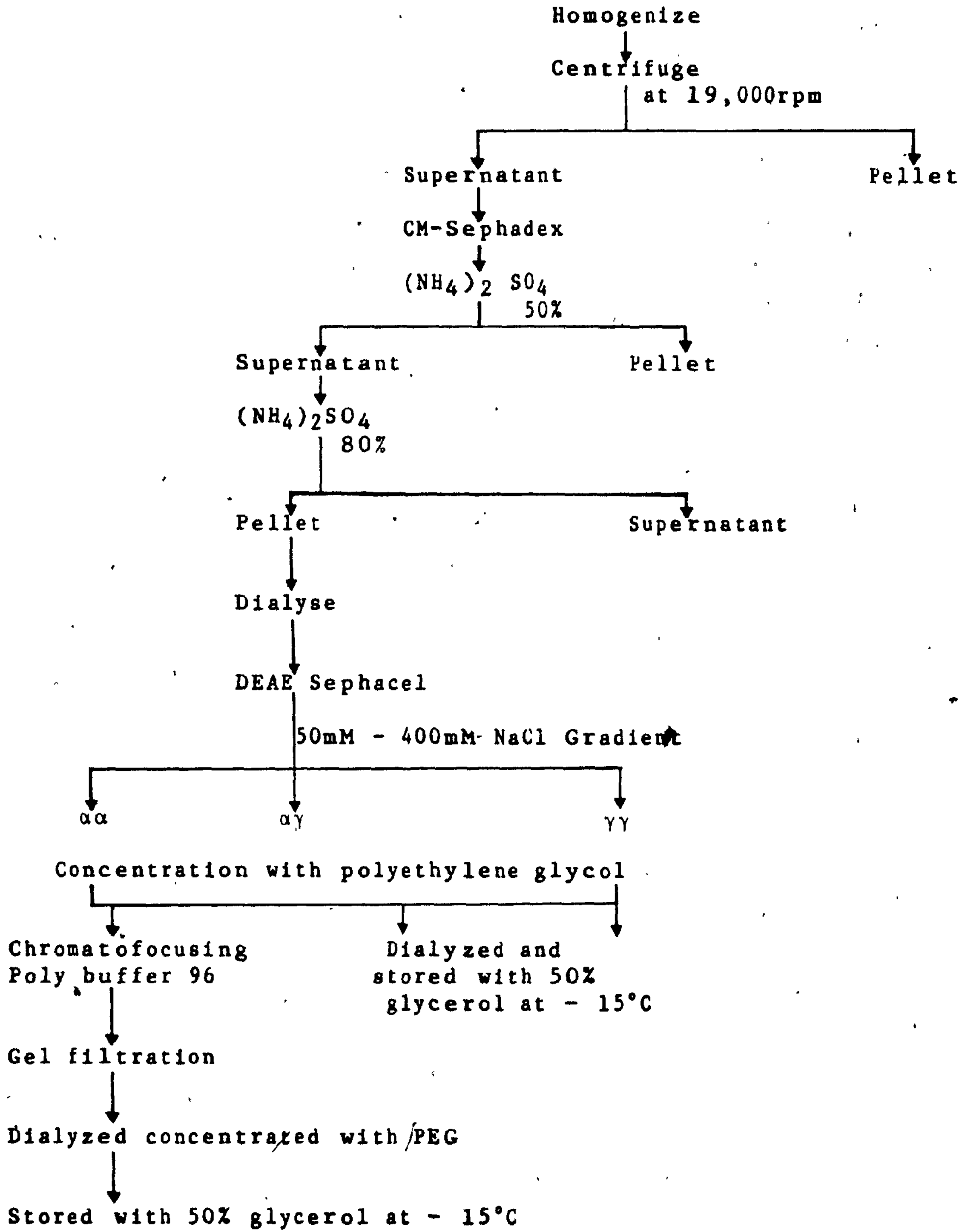
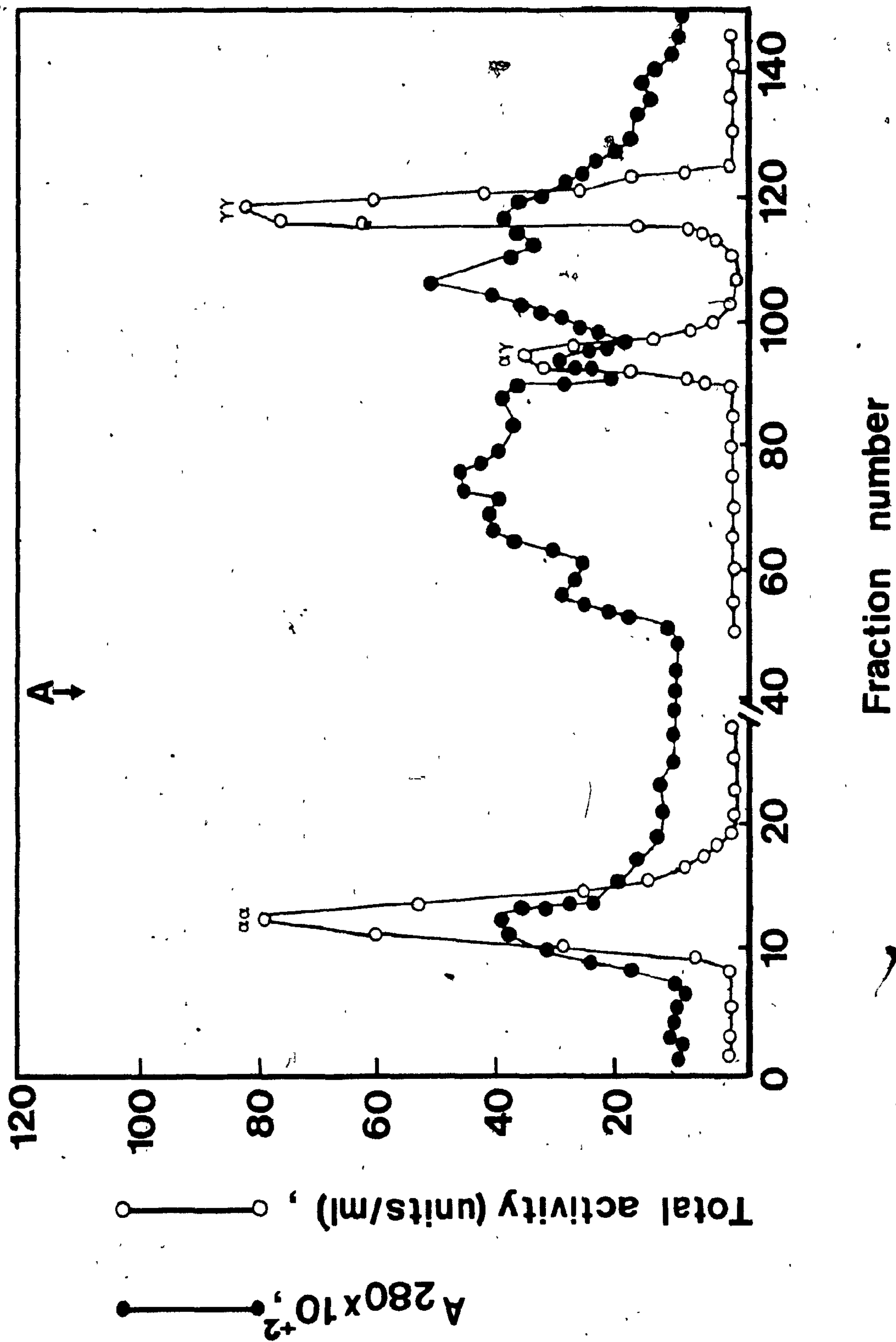
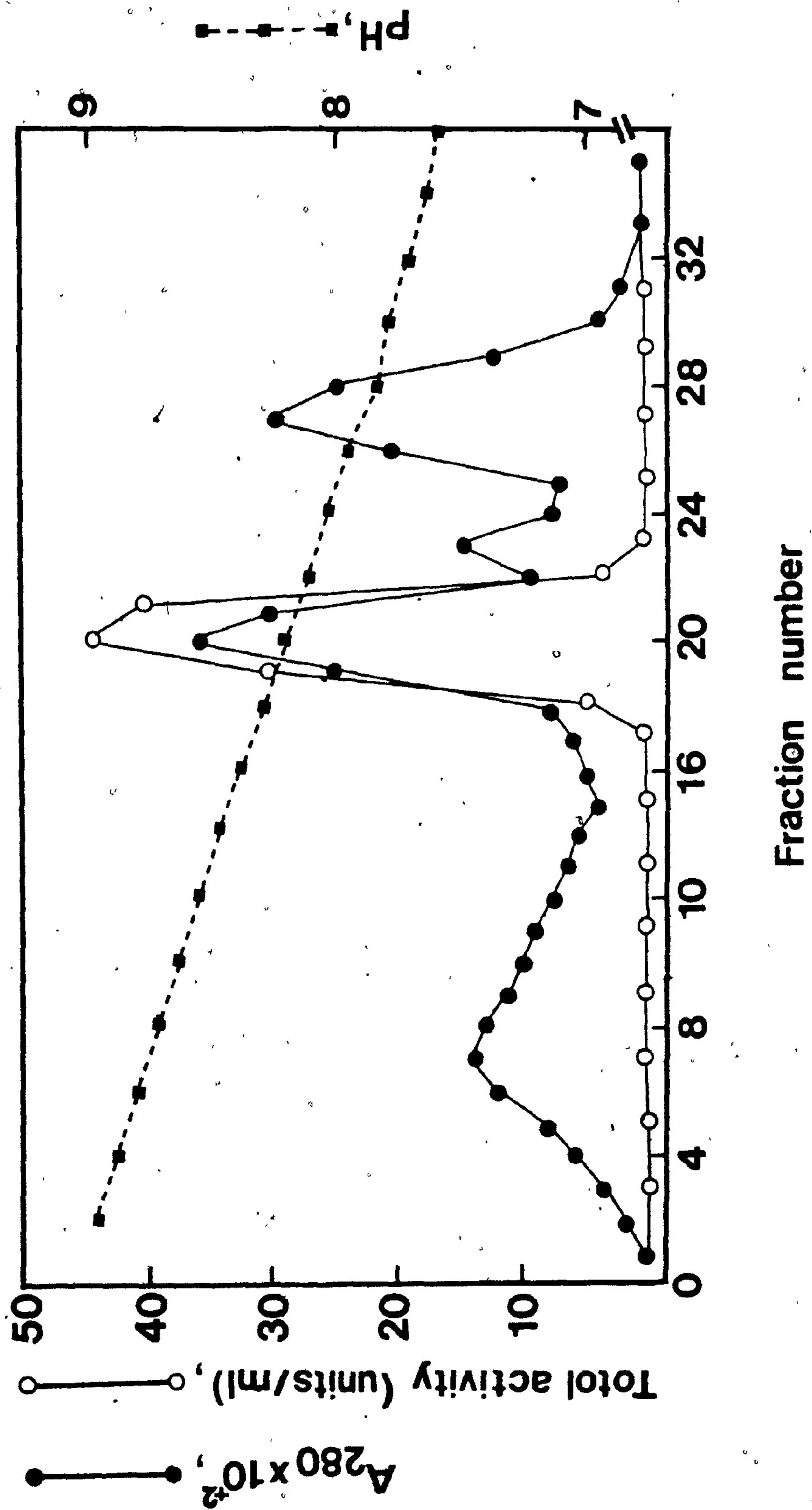


Figure 5: Elution profile of enolase protein and activity from DEAE-Sephacel column. The enzyme (after dialyzing out the salt from ammonium sulphate fractionation) was applied to a column pre-equilibrated with 15 mM Imidazole-HCl, pH 7.4, containing 5mM $Mg(OAc)_2$ and 0.1mM EDTA (Buffer R). The αα protein was eluted with Buffer R. The proteins of αγ and γγ were eluted from the column with a concentration gradient, 50-400mM NaCl (A) in the same buffer. The αγ and γγ proteins were eluted at on ionic strength of 105mM and 235mM respectively.



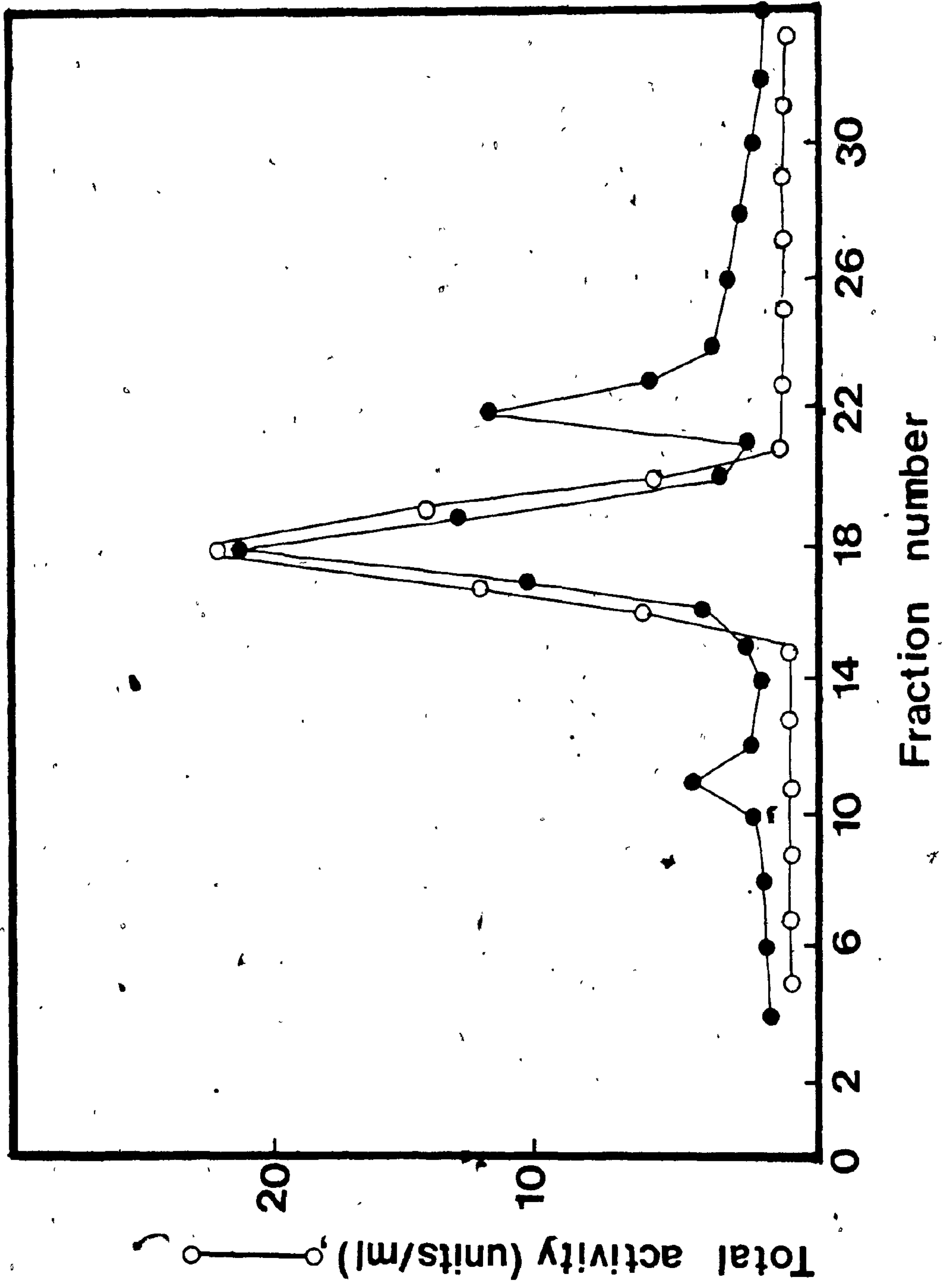
A
αα
αγ
γγ
0
10
20
40
60
80
100
120
140
Fraction number
0
20
40
60
80
100
120
Total activity (units/ml)
$A_{280} \times 10^{+2}$

Figure 6: Chromatofocusing of αα activity on polybuffer ion exchanger (PBE-94). The enzyme was applied to a column preequilibrated with 0.025M ethanolamine-acetate, pH 9.4, containing 10% glycerol. Using polybuffer 96-HCl, pH 6.0, containing 10% glycerol, a linear pH gradient between 9 and 6 was generated and the protein was eluted near its isoelectric point.



pH,
9
8
7
Fraction number
0
4
8
10
16
20
24
28
32
50
40
30
20
10
Totol activity (units/ml),
$A_{280} \times 10^{+2}$,

Figure 7: Elution profile of αα activity from Sephadex G-150 superfine column. The column was preequilibrated with 15mM Imidazole-HCl, pH 7.4, containing 5mM $Mg(OAC)_2$ and 0.1 mM EDTA and fractions were eluted in the same buffer.



Fraction number
0
2
6
10
14
18
22
26
30
10
20
Total activity (units/ml),
A280 x 10+2,

fractions of the three iosyzme, αα , αγ and γγ gave an enrichment of 8.64, 5.45 and 6.98 respectively; with the additional columns, αα's enrichment increased to 77.7 as compared with the crude enzyme preparation (Table 1).

D.2. Stability of the enzyme

The enzyme was found to be unstable, losing about 25% of its activity when it was concentrated by ultrafiltration. Therefore polythelene glycol was used to concentrate it with virtually no loss of enzymatic activity. In the absence of 0.1M NaCl and 10% glycerol, the enzyme lost about 40% of its activity after 24 hr of dialysis. The same amount of activity was lost when the enzyme was dialyzed in the presence of sodium chloride and glycerol but with no magnesium.

Almost all the enzymatic activity of enolase was lost in the presence of Tris-HCl buffer pH 7.4 at 4°C. The enzyme was stabilized by dialysing it over 24 hr against 15mM Imidazole-HCl buffer, pH 7.4, containing 5mM $Mg(OAc)_2$, 0.1mM EDTA, 0.1M NaCl and 10% glycerol. The enzyme was stored in the above mentioned buffer in 50% glycerol at -15°C. Such a preparation retained full enzymatic activity over 3 months of storage. As a result of these findings, that is, effect of glycerol on the

Table 1 PURIFICATION OF RABBIT BRAIN ENOLASE

| Fraction | Total Activity | Total Protein | Protein Recovery | Specific activity | Enrichment | Yield |
|---|---|---|---|---|---|---|
| | units | mg | % | units/mg | | % |
| Homogenate | 1500 | 1235 | 100 | 1.21 | ____ | ____ |
| CM-Sephadex | 1490 | 750.0 | 60.7 | 1.99 | 1.64 | ____ |
| $(NH4)_2$ $SO_4$ (50-80%) | 1255 | 320.0 | 25.9 | 3.92 | 3.24 | ____ |
| DEAE Sephacel | | | | | | |
| αα | 313.8 | 30.0 | 2.43 | 10.5 | 8.64 | 100 |
| αγ | 188.3 | 28.5 | 2.31 | 6.60 | 5.45 | 100 |
| γγ | 540.0 | 64.0 | 5.18 | 8.44 | 6.98 | 100 |
| Further purification of αα | | | | | | |
| Chromato-focusing | 160.0 | 2.30 | 0.186 | 70.0 | 57.9 | 50.9 |
| Gel filtration G-150 S | 141.0 | 1.50 | 0.121 | 94.0 | 77.7 | 44.9 |

The standard enolase assay (a), was used as described in "Materials and Methods".

stability of the enzyme, columns were run with 10% glycerol whenever possible.

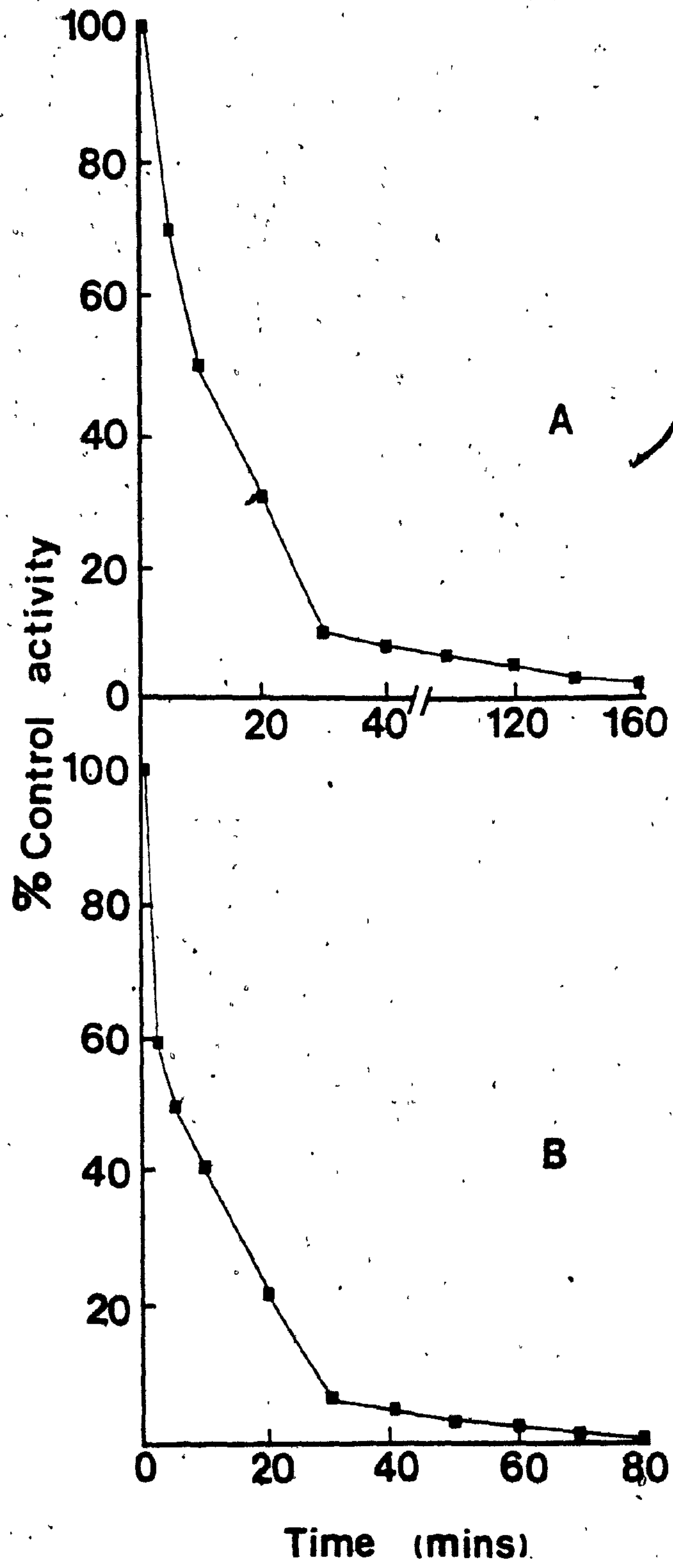
### D.3. Factors affecting inactivation of the enzyme

#### D.3.1. Effect of salt and enzyme concentration on inactivation

A detailed time course of the effect of salt on the enzyme is shown in Figure 8. The enzyme in 1M KBr and 4mM EDTA at 37°C (B) lost all its activity in about one hour; on the other hand, at the same temperature and EDTA concentration but with 0.5M KBr (A), the enzymatic activity decreased markedly with time reaching the residual activity of the enzyme at the same time as for high salt concentration. The residual activity thereafter, decreased slowly for over more than an hour before complete inactivation was achieved.

The inactivation of brain enolase was observed to be much faster in the presence of 1M KBr than in 0.5M KCl. The difference in the effectiveness of these two salts will be discussed later. The active component of the salt(s) is apparently the halide anion since the potassium salts of phosphate and sulphate (59) have no effect on the inactivation of the enzyme.

Figure 8: Kinetics of rabbit brain enolase ($\alpha\alpha$) inactivation by KBr and EDTA at 37°C. Magnesium was partially removed from 0.0125 mg/ml of purified $\alpha\alpha$ by dialysis against 15mM Imidazole-HCl, pH 7.4, containing 10% glycerol, 0.1M NaCl and 0.1mM EDTA. (A) was inactivated in 15mM Imidazole, pH 7.4, containing 0.5M KBr and 4mM EDTA at 37°C. (B) was inactivated at the same temperature in the same buffer with 1M KBr. The initial velocities were determined at regular time intervals as a percent of a control incubated in the absence of salt.



100
80
60
40
20
0
A
20
40
120
160
% Control activity
100
80
60
40
20
B
0
20
40
60
80
Time (mins)

Concentration of protein had a marked effect on the inactivation process. It took over 24 hr before 4mg/ml of soluble enzyme could be effectively inactivated as compared to the 70 minutes for the 0.0625mg/ml enzyme.

D.3.2. Temperature effect on inactivation

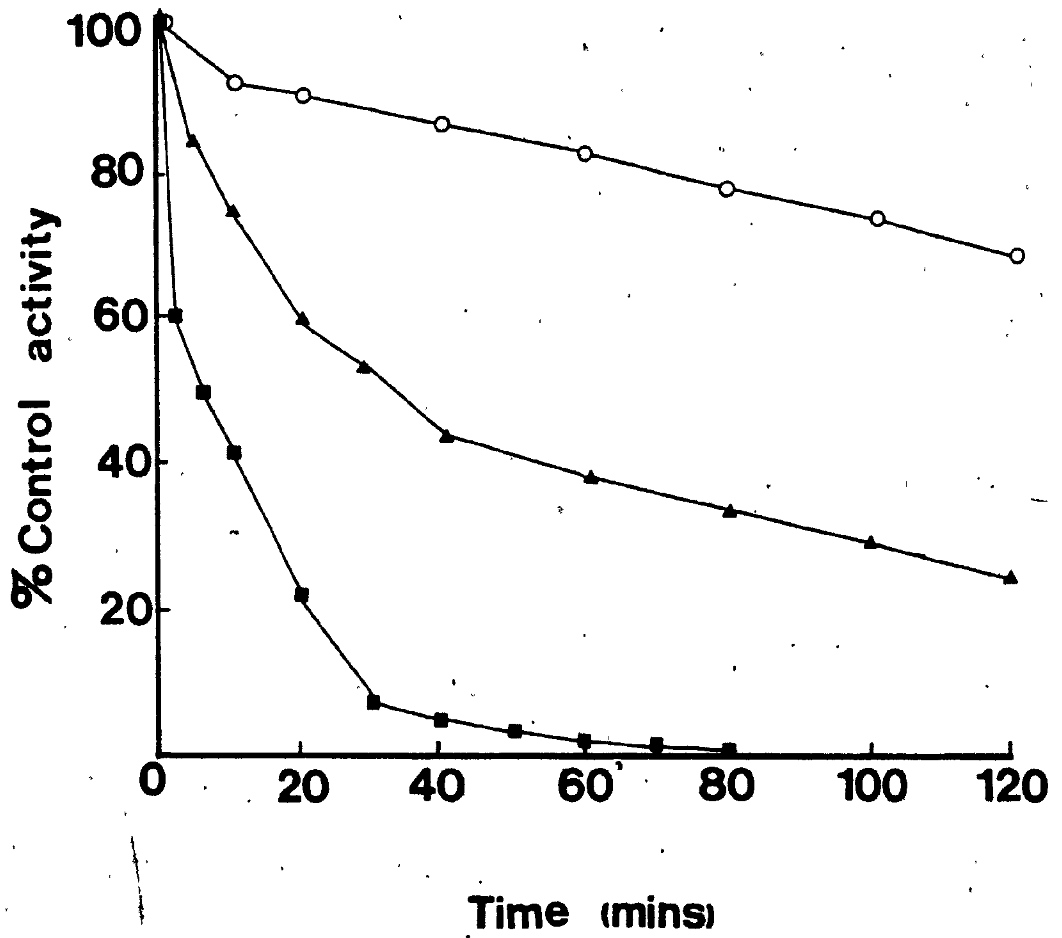
The inactivation of brain enolase by KBr was found to be a temperature dependent process (Fig. 9). It took only 10 minutes for the enzyme incubated at 37°C to reach 50% inactivation; while the enzyme being inactivated at 4°C reached the same percent inactivation in 30 minutes. The enzyme incubated at 25°C did not lose more than 25% of the controls activity within the whole two hours in the presence of salt.

D.4. Rate of reactivation of enzyme

D.4.1. Reactivation of soluble enzyme

The inactivation by salt and temperature was found to be reversible at 25°C. Reactivation was accomplished by either dialysing out the salt or by a 10 times dilution of the enzyme salt solution; thereby reducing the KBr concentration to 0.1M which has no effect on enolase activitiy. Total enzymatic activity was

Figure 9: The kinetics of rabbit brain enolase (αα) inactivation by 1M KBr and 4mM EDTA at various temperatures: 37°C (■), 25°C (o) and 4°C (▲). The inactivation kinetics at 37°C are shown here again to make comparison of the inactivation at the various temperatures easier.


100
80
60
40
20
0
% Control activity
20
40
60
80
100
120
Time (mins)

recovered in the presence of magnesium for samples previously incubated under inactivation conditions at all three temperatures. Under reactivation conditions, the activity of the enzyme inactivated at 37°C, was recovered in 80 minutes (Fig. 10). It took 60 minutes for the partially inactivated enzyme at 4°C to regain its full activity, while it took only 10 minutes for the enzyme inactivated at 25°C to recover its full enzymatic activity under reactivation conditions.

Reactivation was observed to be dependent on the period of inactivation. The enzymatic activity (100%) was restored in 80 minutes under reactivating conditions for the 0.0625 mg/ml enzyme inactivated at 37°C in 1M KBr, while it took more than 2 hr to reactivate the enzyme inactivated with 0.5M KBr (Fig. 8) and about 5 hr for the reactivation of the 4 mg/ml enzyme inactivated with 1M KBr at 37°C. In both of these cases only about 60-70% of the control activity was recovered regardless of time allowed for reactivation.

D.5. Molecular weight

Having found conditions for the reversible inactivation of enolase, the question of whether those conditions also gave reversible dissociation was then addressed. The question was answered by the determination

Figure 10: Time course of reactivation of enolase ($\alpha\alpha$) that had been inactivated by KBr at 37°C. The completely inactivated enzyme was reactivated at 25°C by diluting it into 10 volumes of 15mM Imidazole-HCl, pH 7.4, containing 5mM $Mg(OAc)_2$ and 0.1mM EDTA. The initial velocities were determined at various time intervals as a percent under the same reactivation conditions.

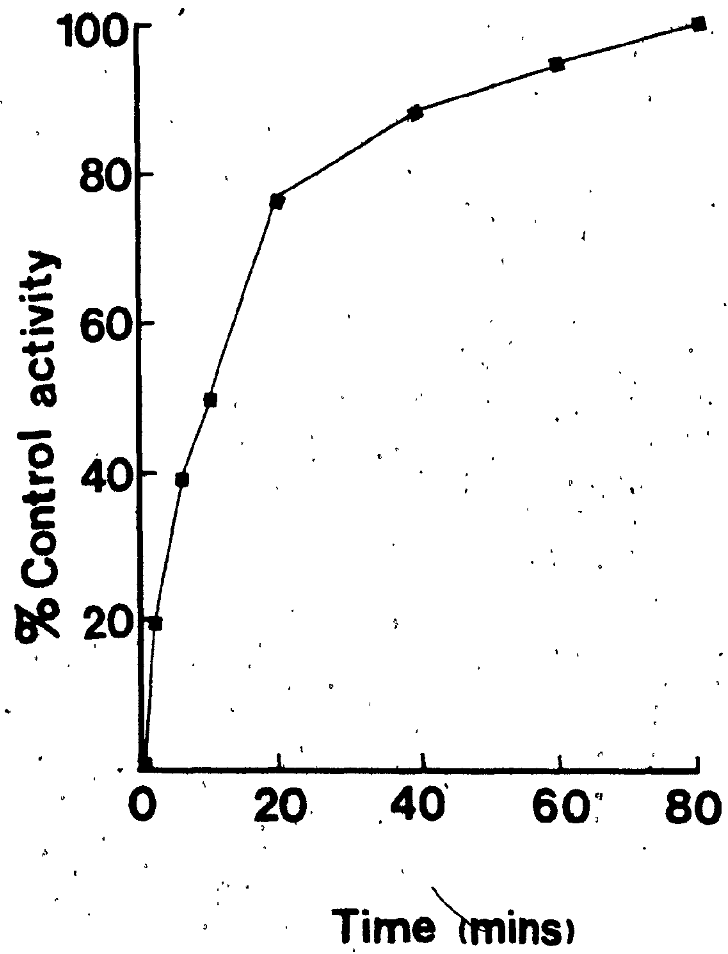
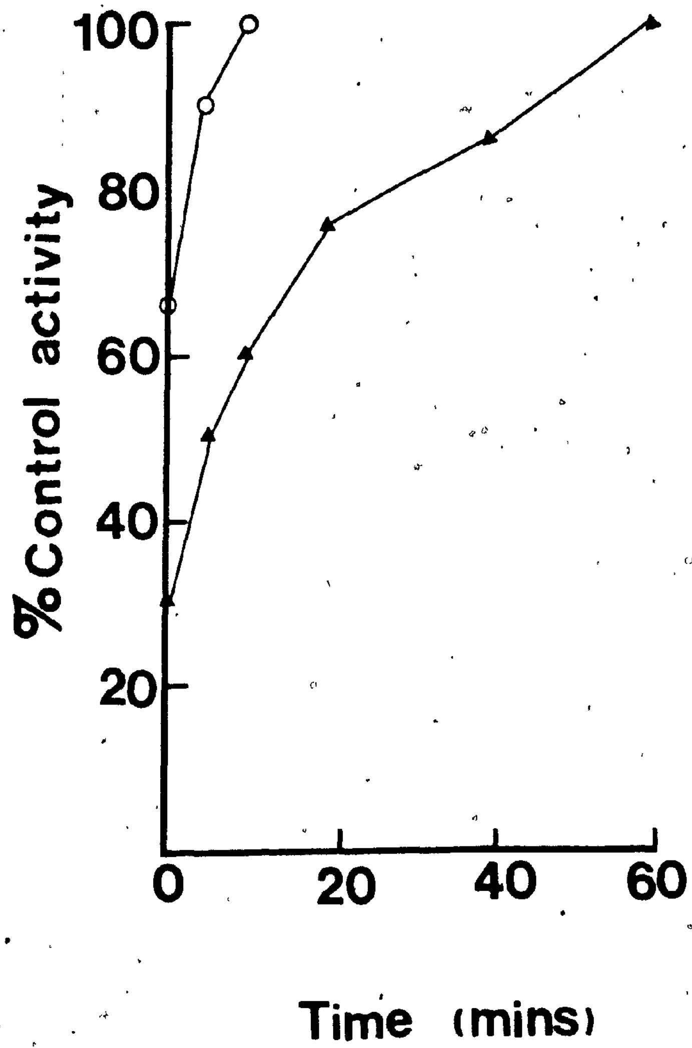

100
80
60
40
20
% Control activity
0
20
40
60
80
Time (mins)

Figure 11: The reactivation kinetics of αα at 25°C. The salt inactivation at 25°C (o) and 4°C (▲) and the reactivation procedure used was as described in "Materials and Methods".


% Control activity
100
80
60
40
20
0
20
40
60
Time (mins)

of the molecular weight, of the inactivated, reactivated and the native enzyme(s).

A slight modification of the standard enolase assay was made to measure the catalytic activity of the inactive enzyme from the Sephadex G-150 chromatographic column. After obtaining the inactive enzyme, inactivation conditions were maintained during gel filtration to avoid the reformation of the native structure on the column. This meant 12 more hours under inactivation conditions. Prolonged exposure of the enzyme to inactivation conditions had already been observed to cause a slow irreversible denaturation following the initial reversible inactivation. The rate of the reaction measured, upon reactivation of the inactive enzyme from the column from previous experiments, have also been found to be very low. In order to obtain detectable activity and also to avoid a complete loss of activity due to the irreversible denaturation, the reactivation was initiated by diluting the fractions into the assay buffer containing 1mM 2-phosphoglyceric acid (substrate) at time zero. The sample was allowed to incubate at 25°C for 4 hr, then the optical density of the phosphoenolpyruvate (product of the reaction) was read at 240nm, together with the standard proteins at their appropriate $\lambda$ max. The elution volume (Ve/Vo) of the inactive enzyme (Fig. 9) extrapolated very well on a Ve/Vo vs log. mol. wt. plot, to give a molecular

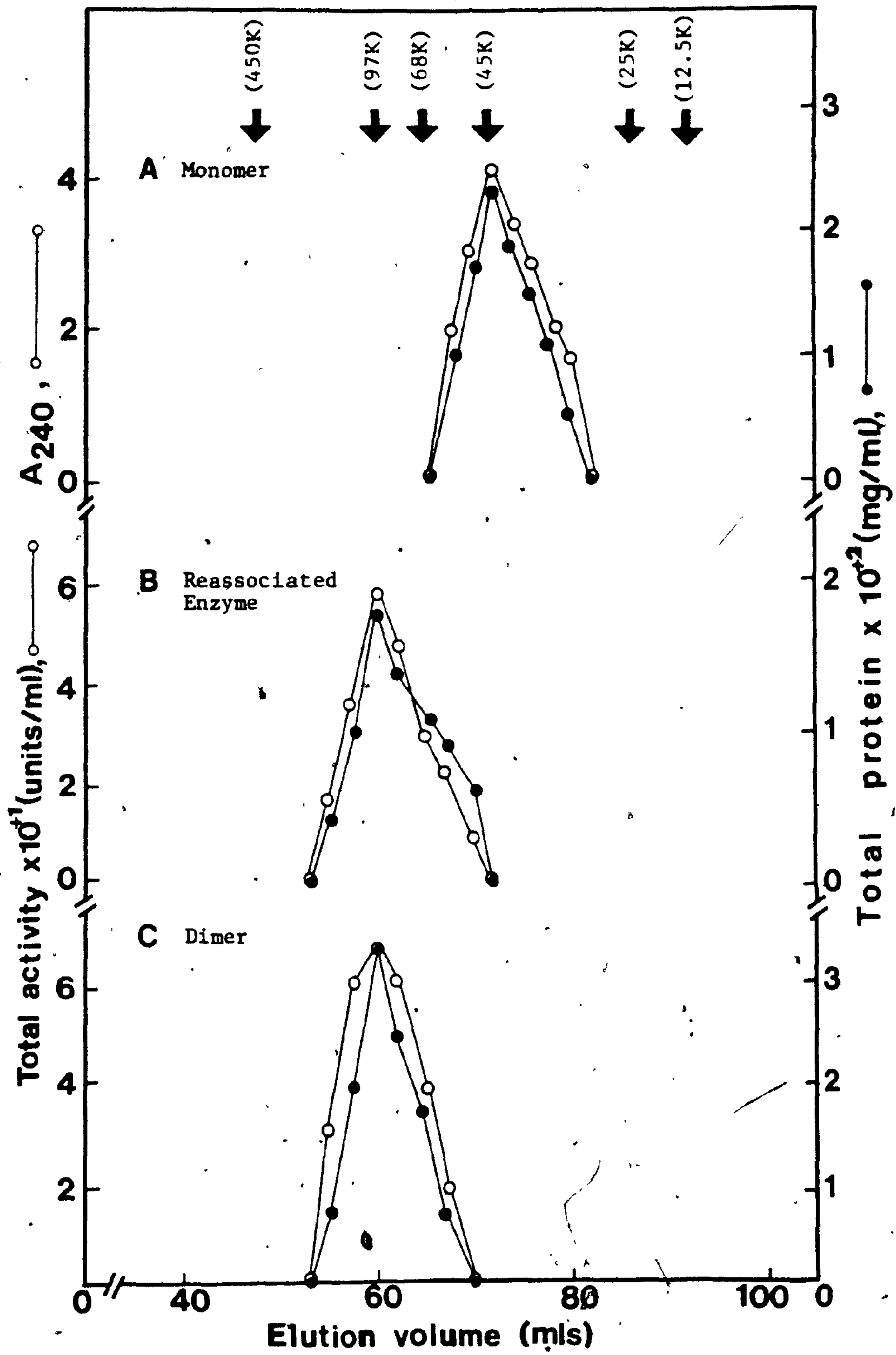
weight of 45,000. The reactivated enzyme, reactivated under the conditions described earlier by dialysis, gave the same molecular weight as the native enzyme (Table 2). The Biorad analysis of the inactive, reactivated and native enzyme(s) from the column gave protein recoveries of 100, 102 and 100 percent respectively. A 2% error is within the experimental error. The recoveries for activity of the reactivated and the native enzyme were 95 and 85 percent respectively.

The gel filtration results from the Sephadex G-150 column have been shown on Figure 12. Dissociated enolase should show an increased retention in the gel filtration, as is evident in the upper curve (A). The yeast enzyme has been shown (63,64) to be tightly coiled in nature and therefore no conformational change in the intact molecule should produce a retention in gel filtration. As mentioned earlier the structure of enolase has been preserved in both plants and animals, therefore the increased retention shown on Figure 12 (A) could be accounted for only by a dissociated enolase, evidently, of lower molecular weight. The middle and lower curves (B) and (C), were obtained from the same size column with the same marker proteins as (A), under undissociating conditions. Both the native and the reassociated enzyme (Table 2) were eluted at a volume corresponding to about 97,000 molecular weight. The difference between this

Table 2: Elution volumes, from Sephadex G-150 superfine column, of marker proteins, native and reassociated enolase at 4°C and that of the monomer at 37°C. The chromatographic conditions at the two temperatures have been described under "Materials and Methods".

| SAMPLE | Ve/Vo at 4°C | Log. Molecular weight | Ve/Vo at 37°C |
|---|---|---|---|
| Ferretin | 1.00 | 5.65 | 1.0 |
| Phosphorylase B | 1.25 | 4.987 | 1.25 |
| Bovine Serum Albumin | 1.35 | 4.833 | 1.35 |
| Ovalbumin | 1.50 | 4.655 | 1.50 |
| Chymotrypsin | 1.80 | 4.398 | 1.80 |
| Chychrome C | 1.90 | 4.097 | 1.92 |
| Native Enzyme (Dimer) | ___ | 4.987 | 1.25 |
| Reassociated Enzyme | ___ | 4.987 | 1.25 |
| Monomer | 1.50 | 4.655 | ___ |

Figure 12: Elution profile of brain enolase ($\alpha\alpha$) activity (o) and protein (●) from Sephadex G-150 superfine columns at 37°C (A) and 4°C (B & C). The experimental conditions for the elution of the monomer (A), reassociated enzyme (B) and the native enzyme (C) was as described in "Materials and Methods". The molecular weight of the enzyme(s) was determined from the elution pattern of the indicated ↓, marker proteins on the same column.


(450K)
(97K)
(68K)
(45K)
(25K)
(12.5K)
A Monomer
B Reassociated Enzyme
C Dimer
$A_{240}$,
Total activity x10$^{+1}$(units/ml),
Total protein x 10$^{+2}$(mg/ml),
Elution volume (mls)
0
40
60
80
100
0
2
4
6
1
2
3

value and that of the subunit is not significant, since the error usually quoted for this method is about 10%.

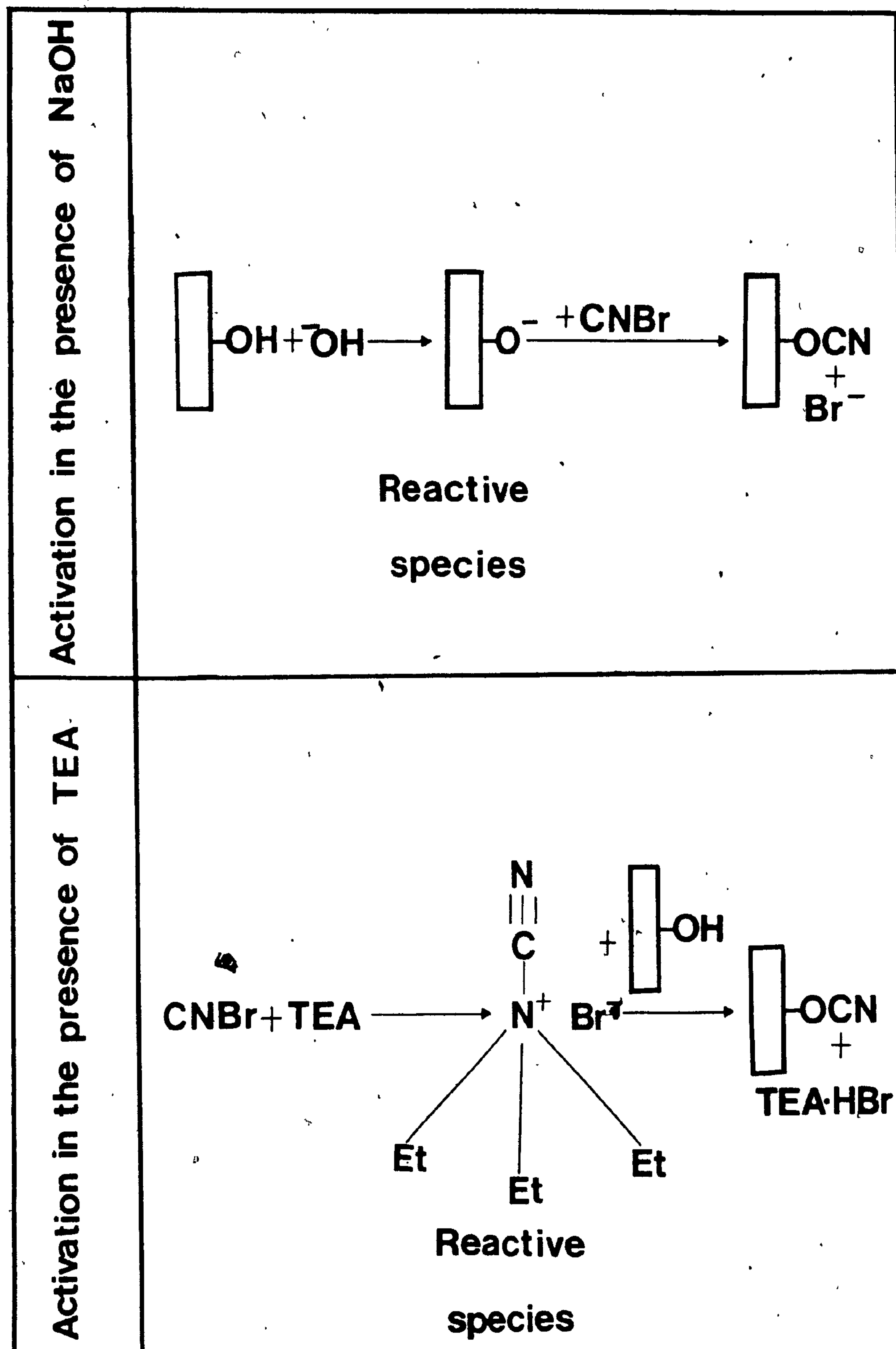
## D.6. Sepharose bound enzyme

The gel filtration analysis successfully demonstrated that the inactivation and reactivation process employed for enolase were due to dissociation and reassociation respectively of the enzyme. The next phase of the work was to covalently couple the enzyme to activated Sepharose. The dissociation process would then be used to obtain immobilized monomers for analysis. The reassociation process would also be used as one piece of evidence for the existence of enolase monomers.

### D.6.1. Cyanogen bromide activated Sepharose

Sepharose 4B was activated by two methods namely: neutral and basic pH activation. The former method has been outlined in "Materials and Methods" and in the latter procedure, a strong base, NaOH, serves to enhance the nucleophilicity of the resin to facilitate the reaction of the CNBr with the resin (Fig. 13). It has been shown (65) that in CNBr activation, cyanate esters and not imidocarbonates are the species responsible for coupling of enzymes (Fig. 14) on activated Sepharose.

Figure 13: A comparison of the mode of action of NaOH and TEA: NaOH acts on the resin, causing an increase in the resin's nucleophilicity by the formation of alkoxide ions. TEA is proposed to operate on CNBr, increasing the electrophilicity of the cyano-moiety by complex formation.


Activation in the presence of NaOH
OH + ⁻OH
O⁻
+CNBr
OCN
+
Br⁻
Reactive
species
Activation in the presence of TEA
CNBr+TEA
N
C
N⁺ Br⁻
Et
Et
Et
+
OH
OCN
+
TEA·HBr
Reactive
species

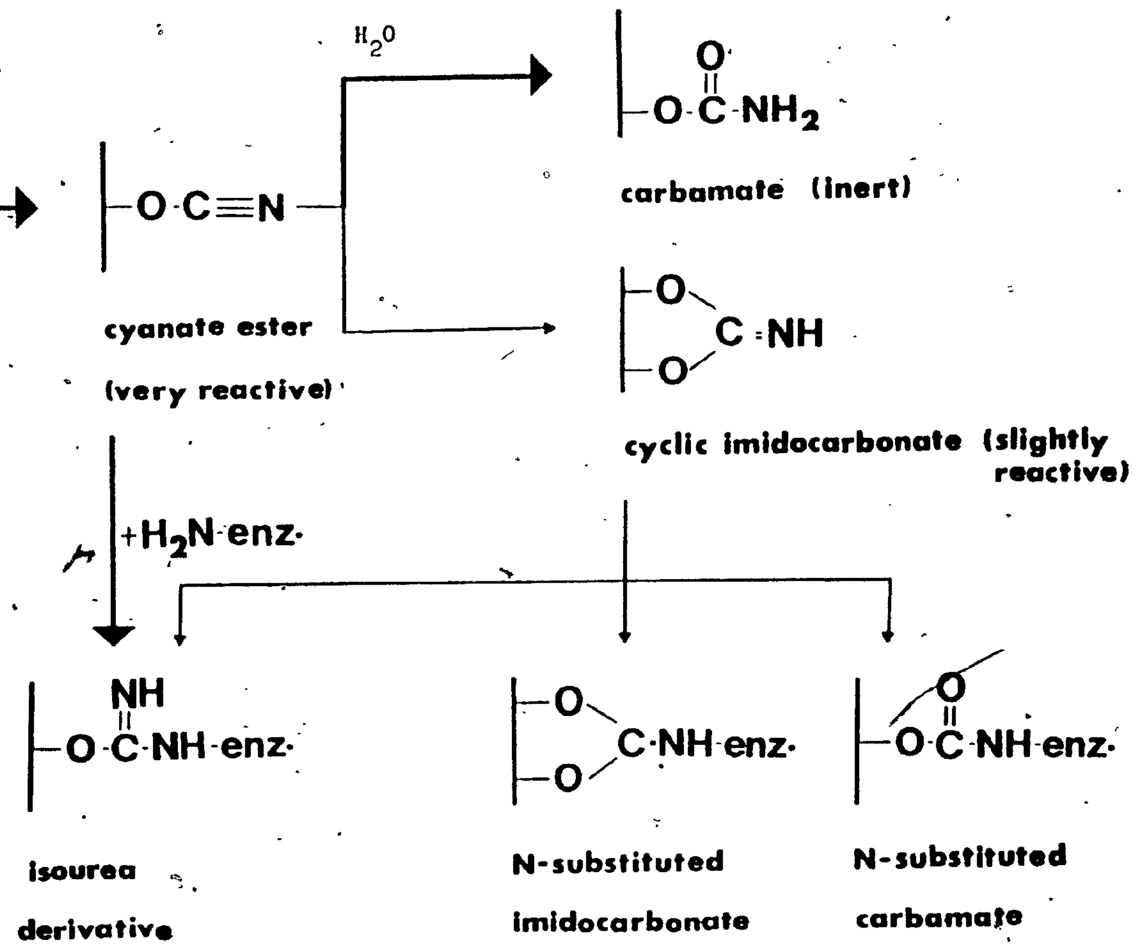



Figure 14: Proposed mechanism of activation of Sepharose by CNBr and subsequent coupling of ligand. Heavy lines indicate major reaction pathways.

These esters have been found to be very stable on gels activated at neutral pH. In strong basic reaction medium the cyanate esters are rapidly hydrolysed to inert carbamates (66).

The coupling results obtained using the two methods were in agreement with the predictions above.

D.6.2. Immobilization yields

The polysaccharide matrix of Sepharose 4B is non-ionic and hydrophilic by nature. These properties enable its use as a suitable matrix for the study of immobilized systems without any significant effect on the properties of these systems. It is also highly porous thereby allowing the enzyme to easily diffuse in and out of the gel during the immobilization process (19). Using the neutral pH activation, a low amount of CNBr; 10 mg per milliliter of Sepharose was used (as compared to the 200 mg of CNBr per milliliter of Sepharose used for basic pH activation of Sepharose) to activate the gel. This low amount of CNBr ensures that only one subunit of the bound enzyme molecule would be covalently linked to the polysaccharide matrix.

The properties of Sepharose 4B outlined above together with the low amount of CNBr used in neutrally activating the gel excludes the possibilities of any

interactions between adjacent subunits after dissociation. The properties of this type of gel also ensures a sufficient amount of bound enzyme units per volume of gel. Immobilization of the enzyme to neutral activated gel resulted in the incorporation of 3.0 $\pm$ 1.0 units of enolase per milliliter of packed Sepharose and a total of 65.6 μg of protein. This amounts to 74% of the specific activity of the enzyme in solution (Table 3).

D.7. Properties of the immobilized enzyme

The immobilized enzyme was found to be particularly stable under the storage conditions already outlined in "Materials and Methods". There was no loss of activity for the whole 6 week period over which each preparation was kept for analysis. Of prime importance to this study was to find out if the immobilized enzyme becomes solubilized under mild conditions of centrifugation during enzyme assay or upon storage. Any possibility of solubilization was checked by initiating an assay, and before all the substrate was used up, the immobilized enzyme was removed by low-speed centrifugation. The supernatant was then returned to the spectrophotometer to check whether any activity remained. No activity was found. Centrifugation of the immobilized enzyme suspension followed by enzyme assay of the

Table 3 The Enzymatic activity and Protein content of the immobilized Enolase derivatives

| Preparation | Activity (units) | % | Protein Content (µg) | % | Specific Activity units/µg | Specific Activity % |
|---|---|---|---|---|---|---|
| Native Enolase | | | | | 82.0 | 100 |
| Enolase-Sepharose | 4.0 | 100 | 66 | 100 | 61.0 | 100 |
| Monomer (Enolase-Sepharose) | 1.8 | 45 | 32 | 49 | 56.0 | 92.0 |
| Reassociated (Enolase-Sepharose) | 3.07 | 76.8 | 53 | 80.2 | 58.4 | 95.7 |

The standard enolase assay used here was as described in "Materials and Methods".

supernatant showed no solubilization, not even after 2 months of storage.

D.8. Dissociation of matrix bound enzyme

Unlike the soluble enzyme where partial removal of magnesium ion preceeded dissocation, the immobilized enzyme was dissociated in the presence of the ion. It took 24 hr to dissociate the same concentration of immobilized enzyme as that in solution, under the same dissociation conditions of temperature and salt.

D.9. Reactivation and reassociation of immobilized enzyme

D.9.1. Reactivation of immobilized enzyme

The inactive immobilized enzyme was reactivated by first removing the supernatant through centrifugation of the immobilized enzyme-salt suspension. Then the gel was washed three times with 15mM Imidazole-HCl buffer, pH 7.4, containing 5mM $Mg(OAc)_2$, 0.1M NaCl, 0.1mM EDTA and 10% glycerol. The washings were collected after centrifuging down the gel and the washings checked for activity, but no activity was detectable. The activity from the gel was recovered by making a 10 fold dilution of

the washed immobilized enzyme into the imidazole buffer mentioned above.

The protective effect of substrate against the inactivation of yeast enolase has long been reported (4,67). Therefore, an additional control apart from the one described under the reactivation of soluble enzyme was added. A fraction of the inactive immobilized enzyme was reactivated in the presence of 1mM 2-phosphoglyceric acid. This was done to ensure the protection of the enzyme against any further inactivation due to dilution inherent from the length of time the enzyme stayed under inactivating conditions. The initial velocity of the reaction was determined after the addition of 10 times the concentration of substrate. The rates of the reaction of the enzyme reactivated in the presence of substrate were low compared to those reactivated without substrate. The substrate was added in the reactivation medium to protect the enzyme against any further inactivation due to dilution inherent from the duration of the inactivation process. But in the presence of the substrate a reaction was occurring which was causing a decrease in the rates of the enzymatic reaction. This was contrary to the expected effect of substrate. To exclude the possibility that this peculiarity was due to the immobilization of the enzyme, substrate was added in the reactivation medium of soluble enzyme and the enzyme assayed after the addition of 10.0mM

2-phosphoglyceric acid. The same effect was observed for soluble enzyme. The possible explanation here is that of a product inhibition which is actually consistent with Gawronski's observation (68) of the yeast enzyme.

Table 4 shows the recovered activities after salt inactivation of both soluble and immobilized enolase. The data, shows that, there is indeed activity on the gel. Approximately 50% of the total activity recovered was on the gel. This gives evidence of monomers on the gel and that these monomers are indeed active. The recovery of activity in the supernatant was lower than that on the gel, about 40% of the total recovered activity was in the supernatant. The protein results on Table 3 gave further evidence of active enolase monomers. Approximately half of the total protein remained with the gel. The activity results together with the protein results from the gel gives strong evidence that there are monomers on the gel and that they are active.

D.9.2. Reassociation of immobilized enzyme

An additional experiment to rule out the possibility of an artifact in the results given above, was, to show the specific ability of the immobilized monomers, to pick up or reassociate with subunits in solution. Unlike the reactivation of the monomer, the

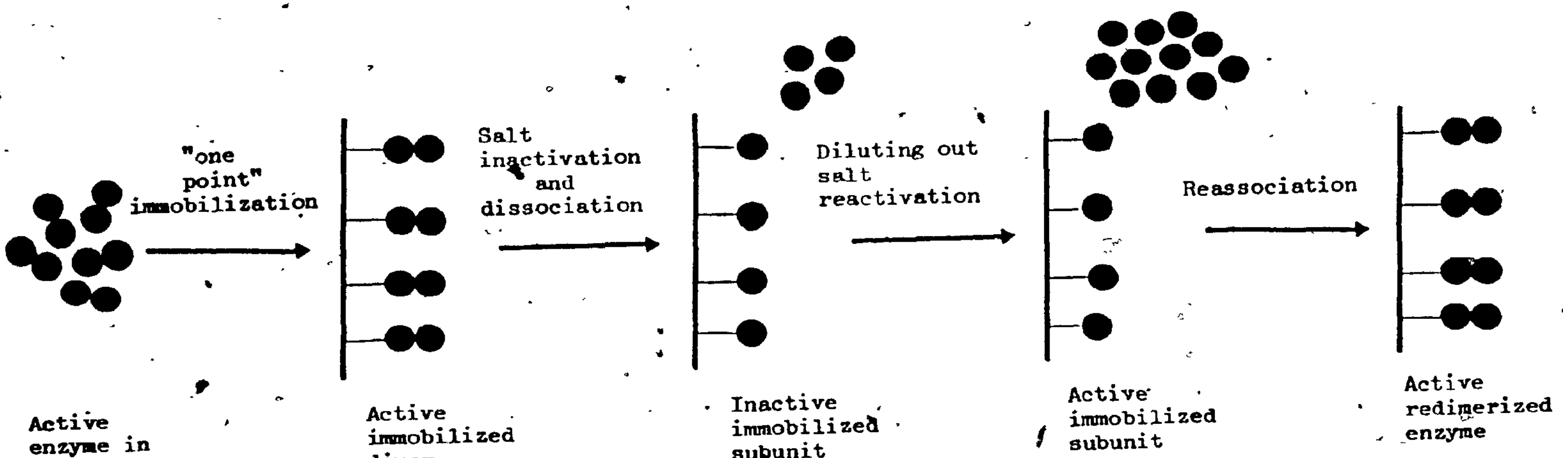
TABLE 4: The percent control activity before and after the inactivation and re-activation of soluble as well as immobilized enzyme. Activity measurement were performed at pH 7.4 (15mM Imidazole-HCL, 50mM $Mg(OAc)_2$, 0.1mM EDTA and 1.0mM 2-phosphoglyceric acid) and 25°C as was described for both enzyme systems under "Materials and Methods".

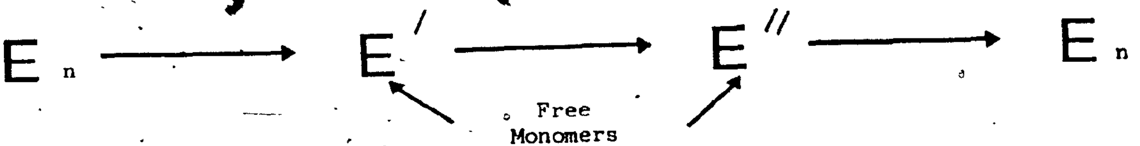
| Type of Enzyme | Preparation | % Initial Control Activity | % Final Control Activity |
|---|---|---|---|
| Immobilized Enzyme | | | |
| | Before Inactivation | | |
| | Control | 100 | ___ |
| | Sample | 100 | ___ |
| | After Inactivation and Reactivation | | |
| | Control | 74 | 100 |
| | Supernatant | 28 | 38 |
| | Sepharose | 33 | 45 |
| | Sepharose (reactivated with soluble inactive enzyme) | 57 | 77 |
| Soluble Enzyme | | | |
| | Before inactivation | | |
| | Control | 100 | ___ |
| | Sample | 100 | ___ |
| | After Inactivation and Reactivation | | |
| | Control | 40 | 100 |
| | Sample | 40 | 100 |

reassociation was achieved under slightly different conditions (Fig. 15). An aliquot of inactive subunits in solution was diluted into a large volume of imidazole buffer containing the immobilized monomer, E". To ensure reassociation of soluble monomers with the immobilized monomers and not among themselves small aliquots of the soluble monomer were added at 10 minute intervals. After an hour the gel was spun down and both the gel and supernatant were analysed for activity. For a control, an immobilized dimer was treated under the same conditions as the immobilized monomer.

If indeed monomers were obtained from the salt dissociation and these monomers were active, then the specific interaction between the immobilized monomer and soluble monomers described above, should result in increased bound activity and a specific activity that is not substantially lower than the original activity of the immobilized dimer. This was, indeed, what the data indicated. As can be seen from Table 3 about 77% of the original enzyme activity was recovered upon reassociation. The protein data in Table 3 also indicates an 80% recovery of the original total protein. A 95% recovery of specific activity of the original immobilized dimer goes to show that the immobilized monomers have indeed regained the required subunits to regenerate the original quaternary structure of the enzyme.

Figure 15: Schematic representation for the dissociation of immobilized enolase $\alpha\alpha$ with salt, followed by the reactivation of enolase activity and reassociation of the dimeric structure by diluting out the salt. The dissociation, reactivation and the reassociation procedures used were as described in "Materials and Methods".


"one point" immobilization
Salt inactivation and dissociation
Dissociated subunit
Diluting out salt reactivation
More subunits in salt in solution added to facilitate reassociation
Reassociation
Active enzyme in solution
Active immobilized dimer
Inactive immobilized subunit
Active immobilized subunit
Active redimerized enzyme


E n
E′
E″
E n
Free Monomers

Three out of seven criteria described by Chan (6) for assessing the possibility of an active immobilized enzyme were used in this work, namely; activity, protein content and the specific interaction of an immobilized monomer with soluble subunits. The data from all these results confirm the existence of active immobilized monomers of rabbit brain enolase.

# DISCUSSION

## E. DISCUSSION

The existence of the three forms of isozymes of enolase has been shown in the yeast (4) and in the rat brain (33).

Three forms of enolase in the rabbit brain enolase have been shown in this work. These forms were resolved on a DEAE Sephacel column after an initial purification by chromatography on a CM-Sephadex and fractional precipitation with ammonium sulphate. The least acidic of the three forms, $\alpha\alpha$, was purified 78-fold by chromatofocusing and gel filtration. It was found to be a dimeric enzyme with identical subunits of apparent molecular weight of 45,000 daltons and an isoelectric point of 8.2 $\pm$ 0.1. The enzyme was found to show some similarities in its general properties, molecular weight, $Mg^{2+}$ requirement and product inhibition, to the enolase isolated from different species and organs (4,33,38,68-71).

The addition of sulfhydryl reagents; dithiothreitol and 2-mercaptoethanol used in some of the experiments involving inactivation and reactivation of the enzyme, did not affect the enzymes reactivity under these conditions. This means that the protein's SH-groups may be well protected inside the protein moiety and hence may not be exposed to the inactivation and reactivation conditions the enzyme undergoes. On the other hand if the

SH-groups are exposed on the enzyme surface then it is possible that the conditions did not have any effect on them or that the oxidation-reduction state of the SH-groups has no effect on the activity of the enzyme.

The characteristics of salt inactivation at 37°C appear to be correlated with reversible dissociation of the oligomeric enzyme into one with smaller molecular weight. Dissociation of the dimeric enzyme into monomers appeared to be dependent on protein concentration, salt concentration and the presence of magnesium. This indicates that the dissociation of the enzyme may proceed through some equilibrium between the dimeric and the monomeric forms where high protein concentration, low salt concentration and the presence of magnesium favour the equilibrium towards the dimeric form. This result is consistent with studies done on the yeast enzyme (68).

The greater effectiveness of bromide ions over chloride ions in these studies can be best explained by a mass action model. This model gives a strong argument of the halide ion effect to be caused by direct binding to the protein and not by environmental effects such as water structure or charge shielding. This has been shown for collagen (72,73) and for acetyltetraglycine ethyl ester by Jencks (74). However, recent studies have shown that the predicted effectiveness of anions does not hold for all systems (74-76). Brewer observed relatively little

binding of yeast apoenolase with chloride and sulphate salts from Laser Raman Spectroscopy and titration studies (77). However, the paper also indicated that conformational changes subsequent to dissociation could have complicated the interpretation of the data. In this study, the potency of bromide ions over chloride ions in its dissociating capacity is best explained by the former binding effectively to the protein at more sites than does the latter. This observation is consistent with Gawronski and Westhead's data on yeast enolase (68).

The exact effect of cold (4°C) inactivation on the enzyme structure cannot easily be explained. However, it is quite obvious from the reactivation kinetics data (Fig. 11) that another type of inactivation (most probably, one equivalent to denaturation) different from the one which occurred at 37°C is occurring here. This suggestion is based on the length of time it took to recover the original activity of the enzyme previously inactivated (partially) at 4°C as compared to that of the enzyme previously inactivated (total) at 37°C.

The results show that salt inactivation of the soluble and matrix bound enzyme at 37°C is reversible with a quantitative recovery of protein and catalytic activity. The greater recovery of activity shown by the immobilized enzyme (Table 4) could well be due to greater stability of immobilized systems. Apart from this

difference in stability, the coupling of one subunit to the solid matrix did not seem to change the topography of the enzyme markedly, since the catalytic activity of the soluble and matrix-bound dimers was similar. The intersubunit contact sites did not seem to be affected by the matrix binding either, since soluble subunits stoichiometrically reassociated with matrix-bound subunits.

Even though matrix-bound monomers may represent a non-physiological system, a matrix bound dimer may be physiologically important (78-82) from the the point of view of compartmentalisation of metabolic systems (e.g. membrane bound and stable complexes of glycolytic enzymes). The greater stability of the immobilized enzyme as compared to the soluble enzyme (Table 4 ) further shows the importance of immobilized enzymes.

From the gel filtration data on Figure 12 (B), the reassociated enzyme shows a shoulder on the major protein peak to coincide with an activity peak at about 68,000 dalton molecular weight. This suggests that the restoration of the catalytic activity of the soluble enzyme was accompanied by association of the subunits to the dimeric form of the enzyme. It could also imply an intermediate structure on the reassociation pathway of the enzyme.

For most dimeric enzymes with identical polypeptide chains it has been established that each of

the subunits has a substrate binding site. This, together with the gel filtration result and the establishment of an active immobilized monomer from the three criteria examined in this work, gives a strong evidence that enolase has two catalytic sites. Furthermore, these sites are capable of acting independently in the dissociated enzyme.

Although there have been several studies on the yeast and rat enolase to demonstrate that it is a dimeric enzyme composed of identical subunits, very few studies on the dissociation of the enzyme have attempted to show the possibility of a catalytic active monomer (4-7). To our knowledge this is the first time active immobilized monomers of enolase have been reported.

As the enolase monomers have complete catalytic activity one cannot help but wonder why such structural difference, necessary for the expression of catalytic activity, has evolved. It definitely poses an interesting technical question and further studies of the enzyme are necessary.

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