C. 1

PROTEIN CHEMISTRY OF TRIOSE PHOSPHATE ISOMERASE

by

HELEN DIANA BURGESS

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Department of Chemistry

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

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ABSTRACT

The protein, triose phosphate isomerase (TIM) has been isolated from fresh chicken breast muscle and purified by anion exchange chromatography on DEAE Sephadex A50 column. Further purification proceeded via Biogel A DEAE resin. The TIM fractions of both chromatographies were contained in two adjacent protein peaks, A and B.

The separation of the two peaks was found to be based upon isozymic differences in the TIM active protein. Isoelectric focusing, both column and gel, showed one isozyme in the Peak A protein with pI=7.65 while Peak B protein contained two isozymes of pI 7.56 and 7.49. Focusing of old Peak B protein yielded a fourth isozyme with pI=7.62. No isozymic separation was observed with disc gel electrophoresis at pH 8.5. Amino acid analysis which was carried out on purified Peak A protein showed substantial deviations from literature values.

The Peak A isozyme was modified via the reaction of the thiol of cysteine with the maleimide N-ethylmaleimide (NEM), or trifluoro N-ethylmaleimide (FEM), as well as with the disulfides 4,4' dithiopyridine (4-PDS) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Two equivalents of reagent per molecule reacted but a kinetic non-equivalence of the two sites to modification was observed. ^{19}F NMR of the FEM labeled protein was performed.

The final chapter of this thesis deals with the kinetic analysis at several temperatures of the modification of TIM by 4-PDS and DTNB both in the presence and in the absence of sub-

strate glyceraldehyde 3-phosphate. Biphasic Arrhenius plots with a break at approximately 25°C were observed for the 4-PDS modification. In the presence of substrate, the activation energy for T>25°C was 7.2 kcal/mole while for T<25°C it was 50.0 kcal/mole. In the absence of substrate, the activation energy for T>25°C was 4.4 kcal/mole while for T<25°C, it was 39.9 kcal/mole.

Lastly, a segment discussing the importance of the results described in this thesis, in terms of the current TIM leterature is included.

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CHAPTER I

GENERAL INTRODUCTION

The purpose of this introduction is to provide, 1) the literature basis for the present understanding of the presence of multienzyme forms of triosephosphate isomerase (TIM) from different sources, 2) the mechanism of the catalytic process, and 3) some chemical and structural aspects of the enzyme. The TIM used for the work presented in this thesis was isolated from chicken breast muscle; however much of the early investigations have involved enzyme from rabbit muscle (4) and yeast (5). In addition definitive studies have been preformed on both human (6) and bacterial (7) triose phosphate isomerase. These studies from the various sources, formed the background for the isolation and purification of the protein, as well as the characterization and the chemical modifications which were performed and reported in this thesis.

The enzyme, triose phosphate isomerase (TIM) plays a central role in glycolysis, as well as in glucneogenesis and has one of the highest catalytic rates of all the glycolytic enymes. It catalyzes the reverse aldoseketose isomerization of glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). This bifunctional protein has been considered to be an unlikely point for metabolic regulation since its its high catalytic turnover rate ability and in vivo concentration has led to the concept that it is not metabolically rate limiting. However, there is evidence (1, 2) of genetically transmitted isomerase deficiencies in humans which leads to 5-20% the normal levels and can result

in a severe metabolic block, even though the anticipated levels of TIM would suggest that it still should not be rate limiting. The isomerase has been shown by various groups to be composed of several isozymes (3). A few examples (6) of the TIM isozymic content of various species are:

Species	Number of Isozymes
Human	
Erythrocytes	3
Skeletal muscle	3
Brain	3
Liver	3
Cardiac muscle	3
Spleen	3
Rhesus	3
Bovine	3
Porcine	3
Dove	3
Turtle	3
Frog	3
Catfish	
Muscle	3
Liver	3
Kidney	3
Brain	3
Heart	3
Crab	2
Lobster	2
Shrimp	2
Beetle	1
Cricket	1
Grasshopper	1
Clam	1
Snail	1
Squid	1
Ascaris suum	1
Sea Anemonae	1
Euglena gracilis	1
Escherichia coli	1
Bacillus sulitilis	1
Pseudomanas aeruginosa	1
Staphylococcusaureus	1

1.1 Purification and Characterization

In recent years interest has centered on the TIM obtained from rabbit muscle and chicken muscle. Preparation procedures for the rabbit muscle enzyme have been described (9,10) as well as for the chicken TIM (11). Considerable evidence has been presented to show that the native enzyme is composed of two subunits (9-16). Values for the native dimer molecular weight of rabbit TIM obtained prior to 1970, range from 43000-60000 (summarized by Norton et al (3)) although later molecular weights were calculated to be approximately 53,000 (3,11). Chicken muscle TIM was found to be 48,400 (11) but more recent amino acid sequence and x-ray crystallographic results place it nearer to that of the rabbit muscle with a molecular weight of 54,400 (17,18). It has been demonstrated that there are no disulfide bridges linking the two subunits, or within a single subunit (5).

The heterogeneity of triose phosphate isomerase has been reported for a wide variety of animal (19,20) and human tissues (3,4). Suggestions for the multiple nature of the enzyme have included it being the result of an artefact of the isolation procedure (23), the presence of conformational isoenzymes (13) or the existence of non-identical subunits (5,24). The isozymes to date have been identified through polyacrylamide (5,25) and starch gel electrophoresis (5,20).

In addition, a chromatographic separation for the isozymes of rabbit muscle TIM has been reported by Krietsch et al (16). A rechromatography of purified rabbit muscle TIM by a DEAE-cellulose column eluted by a very shallow gradient succeeded in sep-

arating the material into three major forms; α, β, and γ (97%) and two minor components, δ and ε (3%). Hybridization studies showed the α and γ isozymes to be homodimers AA and BB respectively and β the heterodimer AB. The heterogeneity of rabbit TIM was therefore found to be largely due to the combination of two chemically non-identical subunits A and B. Hybridization of the δ and ε forms was more complex with reassociation resulting in the formation of all five bands as well as two (in the case of δ) or four (in the case of ε) additional more electrophoretically mobile bands. Amino acid analyses of the three major isozymes were able to reveal small differences but the titration of thiols with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) gave identical numbers for the number of sulfhydryl (cysteine) residues.

A degree of ambiguity concerning TIM isozymes has existed in the literature for some time. For example Lee and Snyder separated commercial rabbit TIM into five bands in acrylamide electrophoresis (26) with a pattern of relative activities and protein concentrations of 7(α): 15(β): 6(γ): 2(δ): 1(ε) with 90% of the activity and protein in the first 3 bands α, β, and γ; however Burton and Waley (23) found only the three α, β and γ bands with 85% activity in the slower migrating α band and 15% in the second (β) and third (γ) faster migrating forms, but Coulson and Knowles (25) could detect just one sharp zone in the polyacrylamide electrophoresis of commercial enzyme. The experimental conditions of electrophoresis, most notably pH, were

probably the controlling factors since Krietsch (16) observed that in polyacrylamide electrophoresis with a 7.5% gel at pH 9.5 in the presence of mercaptoethanol TIM migrates as one single band, while at pH 6.6 the protein showed the same heterogeneity pattern as in starch gel electrophoresis.

Scopes (20) observed a similar phenomenon with chicken muscle TIM, namely a single band resulted upon acrylamide electrophoresis (pH 8.5) while a minor contaminating band was seen to separate out on starch gel electrophoresis. These results have encouraged the belief that the protein from chicken is relatively free from isozymes. Thus, much of the most recent work, most notably x-ray crystal structure studies, has centered on the chicken muscle enzyme. The implications of this in light of the observations reported in this thesis, will be discussed in the main body of this work.

A third source of TIM for which the enzyme heterogeneity has been characterized is human erythrocytes (6). Three isozymes have been found to exist for the human TIM as a result of an AA, AB and BB distribution of dimers. Amino acid analyses and peptide fingerprints have been able to indicate that the two types of subunits are very similar but contain several differences in their primary structures. They show similar catalytic properties and are found in all human tissues.

1.2 Enzymatic Inhibition and the Catalytic Process of TIM

Characterization of an enzyme's active site may be aided by chemical modification if the reagent's specificity allows inter-

pretation with respect to the reaction specificity (amino acid modified) and topographical specificity. The problem of reaction specificity may be solved by taking advantage of known reaction types while the problem of topographical specificity may be resolved by the procedure of "affinity labeling" in which a protein reagent is designed to resemble the substrate and thus have an affinity for the substrate binding site. The inactivation of triose phosphate isomerase has been achieved by haloacetal phosphates and epoxides (glycidol phosphate) (25,27) which are reactive analogs of the substrate dihydroxyacetone phosphate and have been proven to be a successful example of affinity labeling.

A highly selective modification of only one residue was observed with loss of activity proceeding pseudo first order upon addition of high reagent to enzyme molar ratios. It has been found that α-glycerophosphate, a competitive inhibitor of TIM, protects the enzyme against inactivation which suggests a competition of haloacetol phosphates and glycerophosphate for the same active site.

The inactivated enzyme was found to contain 1 mole of covalently bound reagent per mole of catalytic subunit (15). Therefore it was possible for the first time to determine with some certainty the presence of 2 active sites (one/monomer) for TIM.

The initial modification of the peptide chains was found to proceed via an esterification of glutamic acid. A hexapeptide containing the haloacetal phosphate has been isolated from both chicken (28) and rabbit muscle (52) with the sequence:

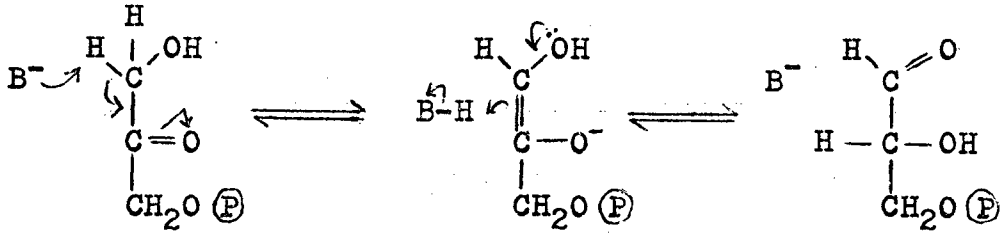
Ala-Tyr-Glu*-Pro-Val-Trp

TIM from a wide variety of species has been found to be inactivated by haloacetol phosphates, which increases the likelihood that a common essential region is the site of modification. If the glutamyl carboxylate is functional in catalysis, it may be expected to be an invariant feature among triose phosphate isomerases. The above mentioned hexapeptide was found to be conserved during evolution (30) and is therefore probably critical to function. A comparison of yeast and rabbit muscle TIM was chosen as an indication of the constancy of the active site glutamyl residue (and adjacent amino acid sequence) since there is a wide evolutionary separation between the two organisms. The hexapeptide containing the active site glutamic acid was found to be identical in both species.

The problem exists as to whether the enzymatic inactivation results from modifying a catalytically functional residue or merely from preventing substrate binding. However, several observations suggest that the glutamyl residue is functional in catalysis. For example, the only functional group in model compounds for proteins which reacts with haloacetol phosphates is the sulfhydryl functionality (27), but the group in TIM that reacts is a carboxyl. The rate of esterification is rapid. In addition, if the esterified glutamyl residue of rabbit TIM is not catalytically essential but merely located in the area of the active site, it would be expected that in some species, residues not susceptible to esterification would occupy the corresponding position. However, the findings that TIM from evolutionarily di-

verse species are inactivated by haloacetol phosphates with inactivation proceeding at similar rates, is consistent with modification of a catalytically functional, invariant residue.

The possibility of the catalytically important residue being a glutamate fitted well with previous mechanism which were postulated as the chemical pathway for the aldol-keto isomerism. The mechanism which was first formulated by Rose (31,32) involved the abstraction of a proton from C-3 of dihydroxyacetone phosphate to give an enediolate anion which is able to pick up a proton at C-2 to yield glyceraldehyde-3 phosphate:

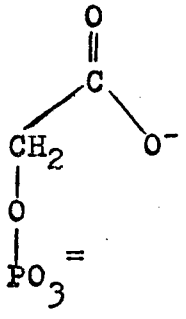


Any conjugate base, including a glutamyl γ-carboxylate, could function in this proton transfer. It has been established that carboxylates can promote enolization via general base catalysis, similar to the mechanism above (33). From the pH dependence of the rate of inactivation of yeast TIM, the apparent pKa of the active-site carboxyl group was estimated by Hartman to be 3.9 ± 0.1 (34).

Affinity labeling of TIM was carried out further by the preparation of a reagent designed to mimic the cis-enediol which is the postulated intermediate in the reaction catalyzed by triose phosphate isomerase. This reagent is glycidol phosphate (2,3-

epoxypropanol) which was found to label the glutamyl group in the isolated hexapeptide: Ala-Tyr-Glu*-Pro-Val-Trp (35). This is the same residue that was labeled by the haloacetol phosphates. The labeling of TIM by this reagent strongly supports the single general base mechanism as proposed for the aldose-ketose isomerization of TIM (36).

The most powerful competitive inhibitor of TIM is 2-phosphoglycollate whose structure is given below:



The inhibitor's structural similarity to the ene-diol and its anionic centre is important for transition state binding. The ultraviolet spectrum of TIM in the 280nm region may be changed by binding of 2-phosphoglycollate and 2-glycerophosphate (another reagent which inhibits due to its ability to bind to the active site) but not by the anionic inhibitors sulfate or inorganic phosphate (37). The shift, which was most marked in the 280nm region which corresponded to tyrosine and tryptophan absorptions was related to the presence of each of these amino acids in the hexapeptide which was found to be esterified upon inactivation of TIM by glycidol phosphate (37). In addition to the change in the U.V. spectrum, there was observed to be a change in the crystal structure of the enzyme upon binding of glycidol phosphate

which was able to suggest that there are two or more important sites of binding. The binding of the inhibitor and transition state analogue 2-phosphoglycollate to the rabbit muscle enzyme was able to effect a similar sort of change as observed by the 4% contraction in the crystal structure. At the same time, a 6% contraction has been noted for binding of either triose substrate to rabbit or chicken muscle TIM (37,38). It appears likely then that the magnitude of the change upon binding of inhibitor, represents a structural change in the protein conformation which mimics the conformational change which occurs upon binding of the two substrates dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The catalytic role of this substrate-induced conformation change is unknown and our understanding of this phenomonen is a long term goal of this laboratory.

The dependence of the rate of enzyme-catalyzed reactions on pH is an important parameter that must be accommodated by any complete proposal of mechanism. There is fundamental information to be gained from a knowledge of the pH-dependence of the individual kinetic parameters. They must be consistent with the mechanistic proposals and be interpretable with respect to the knowledge of the three-dimensional structure of the enzyme and the functional catalytic residues.

The most comprehensive series of pH rate studies were performed by Plaut and Knowles (39). One of the most useful studies was the determination of the stability of the chicken muscle enzyme as a function of pH. Incubation of enzyme in buffers of

various pH's was carried out at 38°C for 6 hours before determination of the enzymatic activity. The results showed a plateau (100% activity) between pH 6.5 and 7.5 with a fairly rapid decrease in stability at lower or higher pH's. An interesting contrast to this was observed in the TIM isolated from a psychrophilic organism (optimum growth below 20°C), clostridia, in which a 70% reduction in enzyme activity with a half hour heat treatment of cell-free extracts at 32°C was seen (pH 7.3) (40). This can probably be explained by the evolutionary adaptation of this microorganism.

Other pH studies showed a pH dependence of k_{cat}/K_m which allowed calculation of the pKa of two kinetically important functionalities: values of 6.05 and 9.05 were derived with dihydroxyacetone phosphate (DHAP) as substrate and 6.0 and 9.2 when glyceraldehyde 3-phosphate (G3P) was used. The apparent pKa values in k_{cat}/K_m may relate to ionizations in the free enzyme or in the free substrate. Under the experimental conditions, the pKa values of substrate alone are 6.0 for DHAP and 6.3 for G3P. Therefore, there is a possibility that the lower observed pKa's of the k_{cat}/K_m profiles have arisen from ionization of substrates. The upper pKa value of about 9 cannot be readily assigned but possibilities are lysine or arginine residues. On the other hand, there is the possibility that the ionization at pH 9 governs a large-scale conformation change resulting in loss of enzyme activity owing to a loss of the structural integrity or the active site (39).

1.3 Structural Aspects

A. The Subunit Monomer

When studying the relationship between the quaternary structure of a multi-subunit enzyme and its activity, it is often of interest to know the enzymatic activity of the monomer, if indeed association of the subunits is not necessary for catalytic ability. A useful approach is to attach the enzyme to a solid support via a single subunit, and to remove the other(s) so that the properties of the isolated subunit may be studied under conditions where reassociation is not possible.

Such a procedure was performed recently by Fell and White (43). They found that the kinetic properties of chicken TIM were altered by immobilization on Sepharose. The activity, which represented that of the bound monomer gave a K_m of 1.7mM while that of the soluble enzyme was reported to be 0.35mM. Upon denaturing with guanidine hydrochloride and subsequent rehybridization with rabbit muscle enzyme, an active hybrid of chicken and rabbit muscle enzyme could be formed. It is therefore possible to demonstrate that the 32 differences in amino amino acid sequence between these two enzymes (86% homology) has not significantly altered the subunit interface region (44).

It is important to exercise caution when evaluating evidence obtained in such experiments. For example, a contrasting report of the possibility of directly linking TIM to an agarose support (ie. no spacer arm between enzyme and agarose was inserted) via one subunit was reported by Sawyer and Gracy (45) when they ob-

served that both subunits of human TIM were linked to the matrix and that the double linkage was preventing dissociation. In contrast, the protein bound to the agarose via an acetamidoethyl linkage had kinetic as well as stability properties closer to the native enzyme. This data challenges the validity of the experiments of Fell and White who used direct linkage of the protein to the support.

The possibility that the monomer of TIM is inactive has been suggested by Waley (46) who performed experiments in which rabbit or chicken muscle TIM was denatured by guanidinium hydrochloride and then followed the kinetics of the renaturation. His scheme involved first of all the refolding of monomer and then the association of the two folded monomers to form a dimer. At low concentrations dimerization was a rate-determining step (kinetics found to be second order at low enzyme concentrations) and since the reappearance of dimers was followed by the increase in activity, this was taken to indicate that the monomers showed little or no activity. If this indeed is true, it would mean that the active site enzyme conformation induced by the subunit association is essential for the catalytic activity of TIM.

It is clear therefore that this issue is still unresolved and any probe of the inter-subunit interface when related to enzymatic activity will be of considerable value.

B. Protein Modifications

There exists very little information about the particular amino acid residues, other than the catalytic site hexapeptide,

which are most intimately involved in the TIM active site and therefore essential to enzymatic catalysis. However, recent studies have investigated the role of the sulfhydryl groups and there are conflicting reports in the literature concerning the effect of thiol-specific reagent on enzymatic activity (23,29).

One of the more comprehensive surveys of thiol reactivity was presented by Davis et al (47). Investigation of SH reactivity by reaction with maleimides resulted in a threefold greater Michaelis constant for the modified rabbit enzyme. Three thiols/mole enzyme reacted. However, mercurials were found to cause a greater change in K_m and reacted with 6 SH's/mole rabbit enzyme.

It was reported that the rabbit muscle and liver enzyme appear to have similar properties but that the chicken muscle enzyme is less reactive (47). The yeast enzyme does not become inactivated upon sulfhydryl modification. The enzyme from chicken muscle has been found (48) to contain one thiol group per subunit that is more reactive than the others to the reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). It was observed that the accessible thiol group was unnecessary to the catalytic activity of the enzyme (48) which is in direct contrast to results reported in this thesis. However, it has been suggested that for the rabbit muscle protein, one thiol per dimer can be modified by DTNB with a 50% loss of activity and that a second essential SH group which corresponds to the other active site is still fractionally intact but is inaccessible through the association of

the two subunits (16). However, there is a real deficiency of knowledge regarding the reactivity of the thiols of TIM and it is in this area that the results of this thesis attempt to expand the knowledge of the modified protein.

C. Amino Acid Sequence and X-Ray Crystal Structure of TIM

The amino acid sequence of the rabbit muscle enzyme was first reported by Corran and Waley (49) who found the polypeptide chain had 248 amino acid residues and that the molecular weight of the dimer was 53,257. The tryptic peptides of the rabbit muscle TIM was compared with that of chicken (51). Each chain of the chicken has 247 amino acid residues and there is one deletion in each chain. Apart from these gaps, there are 32 differences (86% homology). 22 of the 32 interchanges are consistent with a change of one nucleotide in the DNA codon. In addition, the amino acid sequence of the 15 residue tryptic peptide that contains the active-site glutamyl residue as determined by Corran and Waley (53) was found to be different than the analogous rabbit peptide reported by Hartman (52) in that valine in chicken was substituted for tryptophan in rabbit.

The amino acid sequence data for the chicken breast muscle was published in 1975 (54). The sequence data facilitated the determination of the 2.5 A resolution crystal structure which was reported in the same paper. It was found that each subunit of the chicken muscle TIM is composed of alternate segments of polypeptide chain in the α- and β-conformations that are arranged to form an inner cylinder of parallel-pleated sheet and a largely

helical outer shell. They were also able to indicate the residues participating in the subunit interface as well as those making up the active site. The two subunits were related by a two fold axis. Interaction between the subunits involves, in part, the loops 70-80 which form hydrophobic pockets around the Met 14 residue from adjacent subunits. The pockets lie on the edges of the interface. The active sites of the protein consists of residues from both subunits as has been found for another glycolytic enzyme, G3PD (89). This finding is particularily interesting in light of the previously mentioned observation that the monomer of TIM is inactive.

The x-ray structure as reported by Banner et al is particularily interesting in light of the striking resemblance which it has to other glycolytic enzymes which are composed largely of alternating segments of α- and β-structure which are folded similarily. There is the suggestion by Rao and Rossman that similar super-secondary structures may be found in many protein molecules with widely different amino acid sequence as a result of convergent evolution (55).

The gap between crystallography and kinetics can often, in part at least, be bridged by spectroscopic studies which make use of techniques such as NMR. NMR has the particular advantage of being able to observe individual residues; also changes in conformation can often be observed and characterized in detail.

A paper published by Browne et al in 1976 (56) has made considerable progress towards assigning the histidine resonances in rabbit and chicken TIM to individual residues and towards char-

acterising the change in conformation when ligands bind. This work, which was made possible through knowledge of the x-ray structure of Banner et al (75), was able to observe the proton resonances of five histidines in the chicken muscle enzyme and one histidine in the rabbit enzyme which were observed to titrate in the pH 5.4 to 9 range.

The extreme sensitivity of NMR techniques as observed by Browne et al (56) is supported by the preliminary F^{19} NMR experiments presented in this thesis. We have been able to collaborate the ability of magnetic resonance to detect changes in the environment of the observed nucleus ^{19}F when the two most reactive cysteines of chicken TIM were modified by a trifluro N-ethylmaleimide label (FEM) and compared to a model compound consisting of the addition product of FEM and N-acetyl cystein. Clues to the position of the site of modification of the chicken TIM by FEM are given by examination of the crystal structure of Banner et al (55).

The x-ray structure places the two most reactive cysteines, to 2-chlormercuri-4 nitrophenol, at residue 217 which would therefore be a likely site for the FEM modification of TIM. Cys 217 is situated on the outer surface of the enzyme as part of the mainly helical segments of polypeptide chain which form the cylindrical outer surface of each subunit. It is 5 amino acid residues away from the active site Val 212 and thus is about 1.4 complete turns of the α-helix away from the active site. Therefore Cys 217 is not in the intersubunit area of contact nor in

the immediately adjacent active site area. However, Cys 217 is close enough to the active site that it is possible to envision the possibility of its chemical modification affecting the catalytic ability of the active site if a conformational change occurs upon reaction of the thiol. However, since Banner et al also observed modification of a second less readily accessible pair of thiols at Cys 41 when using the smaller and more hydrophobic ethyl mercury phosphate, it cannot be assumed with certainty that FEM modification of chicken TIM proceeds at Cys 217. Cys 41 is located on the surface of the inner cylindrical β-pleated sheet structure which is not adjacent to either active site or intersubunit contact areas. It is fairly clear that studies on the actual FEM labeled protein must be carried out before any concrete assumptions may be made concerning the actual site of FEM modification.

CHAPTER II

ISOLATION AND PURIFICATION

2.1 Materials

The extracting buffer made use of ethylenediaminetetra-acetate, disodium salt (EDTA-$2Na^+$) from Fisher Scientific Company, certified ACS grade, and 2-mercaptoethanol from Matheson, Coleman and Bell. Buffers contained Trisma Base from Sigma, Reagent Grade, and sodium chloride, BDH Analar grade. Special Enzyme Grade (ultra pure) ammonium sulfate from Schwarz/Mann was used in the precipitations.

DEAE Sephadex A50 from Pharmacia Fine Chemicals and DE52 and Biogel A DEAE from Bio-Rad were the resins used in the chromatography experiments.

The assay made use of the diethylacetal barium salt of DL-Glyceraldehyde 3-Phosphate, glycerol-3 phosphate dehydrogenase, Dowex-50 Hydrogen Form Resin, and nicatinamideadeninedinucleotide, reduced form (NADH) from the Sigma Chemical Company.

2.2 Methods

A. Extraction and Isolation

A typical protein preparation used 500 gms of muscle dissected from the breasts of four chickens. The fresh muscle was obtained either from a local butcher shop or from freshly killed chickens (Department of Poultry Science, UBC). All procedures were carried out at 4°C or on ice (unless otherwise noted) using a modified procedure of McVittie's (11).

The defatted breast muscle was minced in a Waring blender for 1 minute with an extraction buffer which consisted of 0.2% mercaptoethanol and 1.5mM ethylenediaminetetraacetic acid (to pH 7.0 at 22°C with NaOH) using 1 ml of cold buffer per gram of muscle. The homogenate was centrifuged in the GSA rotor of a Sorvall RC-2B Centrifuge at 1200Xg for 45 minutes. The pellet was resuspended in 0.5 ml of extraction buffer per gram of muscle used, stirred for 30 minutes and recentrifuged as above. The combined supernatants were filtered through cheese cloth three times, and brought to 65% saturation with solid $(NH_4)_2SO_4$. 430.4 grams of $(NH_4)_2SO_4$ per liter of supernatant (65%) were added slowly with gentle stirring, all the while the extraction buffer being packed in ice. After standing in the cold for at least 18 hours, the 65% $(NH_4)_2SO_4$ saturated solution was centrifuged at 5500Xg for 75 minutes. The pellet was discarded and the supernatant brought to 90% $(NH_4)_2SO_4$ (182.5 gm/liter) saturation. Upon standing for about 24 hours, the protein precipitate was spun down and then dissolved in a minimum amount of 20mM Tris:HCl buffer, pH 7.2. (All buffers were adjusted to pH

at 22°C and then stored at 4°C.) The solution was dialyzed against the Tris buffer with at least 8 changes over 3 days and then applied to a DEAE Sephadex A50 column (5X70 cm) pre-equilibrated with the dialysis buffer. The column was eluted with a 4 liter linear gradient (ie. 2 l of low salt and 2 l of high salt buffer) of 20 to 110mM Tris pH7.2 at a flow rate of not more than 20 ml per hour. Fractions were collected on a LKB-Produkter fraction collector and the absorbance at 280 nm determined on a Zeiss PMQ II UV-Visible spectrophotometer. Initially, activity assays were performed on center protein fractions from each eluted peak. Activity assays were then performed at 2 or 3 fraction intervals across those protein peaks found to have triose phosphate isomerase activity.

B. Rechromatography

The first rechromatography experiments were performed with DEAE Sephadex A50. Initially, the first TIM peak off the column (Peak A) was rechromatographed using a 3 liter 60 to 90 mM Tris pH 7.2 gradient on a 110X2.5 cm column with a flow rate of less than 20 ml/hour. The eluted protein was assayed, pooled and precipitated for storage as before. The 'B' TIM peak was rechromatographed using a 3 liter 50 to 70 mM pH 7.2 Tris gradient on a 2.5X92 cm column, flow rate less than 20 ml/hour. Treatment of eluted protein was as before.

The isolated A and B peaks from a different protein preparation were pooled together and rechromatographed, this time by means of a gradient of 0 to 35mM NaCl in 5mM Tris pH 7.2 over

4 liters on a 2.5X70 cm column, flow rate less than 20 ml/hour. The considerable shrinkage of the gel over this salt concentration range necessitated the interruption of the gradient periodically, in order to allow the buffer to go down to the top of the bed, followed by resumption of the gradient.

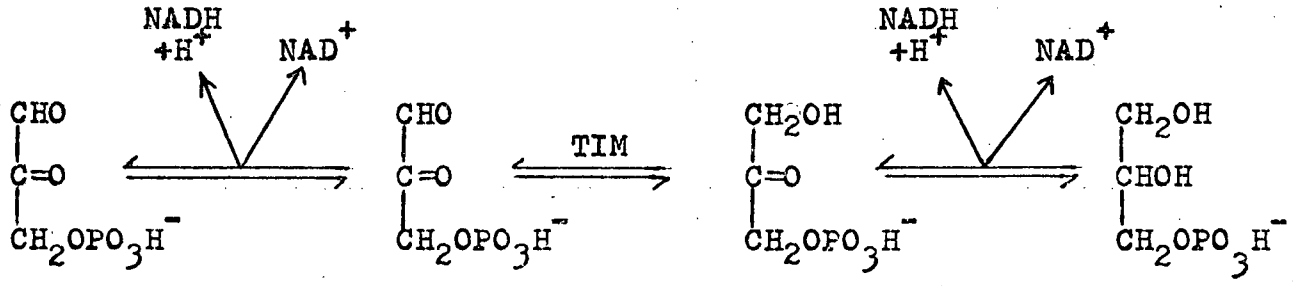
The rechromatography of still another protein preparation utilized DE52 cellulose resin. The pooled A and B peaks were reseparated with a 0-30mM NaCl gradient in 5mM Tris pH 7.8 over 3.5 liters, on a 2.5X60 cm column with a flow rate of less than 18 ml/hour. DE52 cellulose had the advantage of not shrinking with an increase in the salt concentration of the buffer. However, its protein binding capacity is lower than the Sephadex anion exchanger. A fairly high (7.8) pH was required to make the triose phosphate isomerase adhere to the column. Plaut and Knowles (39) have demonstrated a decrease in TIM's stability in buffers of pH greater than pH 7.5 with the trend increasing even more sharply after pH 8.0.

With these factors in mind, a third resin, Biogel A DEAE, was utilized in the rechromatography experiments. This gel has the advantage of the high capacity of the Sephadex resin but does not shrink or expand with salt concentration changes. The pH at which the resin could be used for the rechromatography (7.5) also makes it a better choice than the DE52 cellulose.

The pooled A peak was rechromatographed using a 3 l. 0 to 20mM NaCl gradient in 5mM Tris pH 7.5 on a 2.5X30 cm column, and flow rate of about 18 ml/hour. The B peak was similarily rechromatographed.

C. Assay

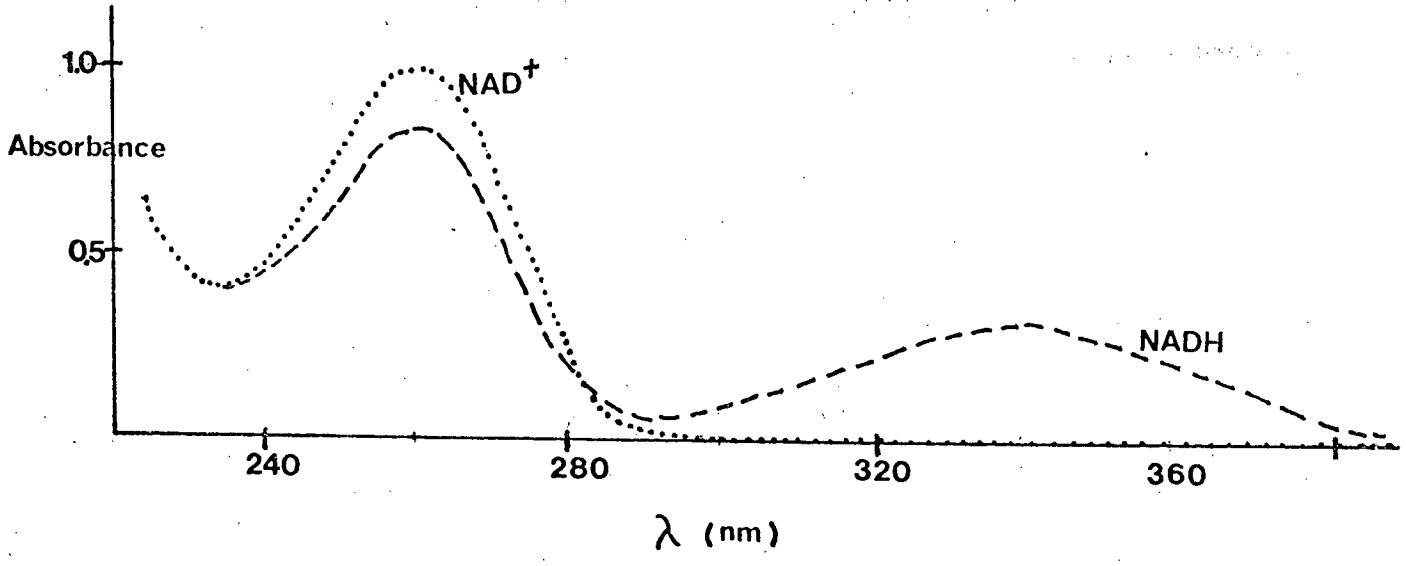
The triose phosphate isomerase assay, which was used in the work described in this thesis, is based upon a coupled enzyme system. A substrate of triose phosphate isomerase, glyceraldehyde-3 phosphate (G3P) is first converted to dihydroxyacetone phosphate (DHAP) by TIM; DHAP is next enzymatically reduced by α-glycerol phosphate dehydrogenase (GPD) to glycerol 1-phosphate. The coupled enzyme reaction of GPD proceeds via a nicotinamide adenine dinucleotide coenzyme oxidation ($NADH \rightarrow NAD^+$) with concomitant decrease in A_{340}. A similar assay to the G3P/GPD system is conversion of the substrate dihydroxyacetone phosphate by TIM to G3P, followed by the enzymatic oxidation of G3P by the NAD requiring enzyme glyceraldehyde-3 phosphate dehydrogenase (G3PD). The G3PD oxidation of G3P proceeds via the reduction of NAD^+ to NADH. A schematic representation of the central reactions in the two assays is as follows:



Thus, two assay procedures are possible, the first being the use of G3P as substrate and GPD as the coupling enzyme with the activity of TIM observable by the rate of decrease in the absorbance at 340nm as NADH disappears; the second uses DHAP as substrate, G3PD as coupling enzyme and the activity of TIM is fol-

lowed by an increase in the absorbance (at 340nm) as the coenzyme NADH is formed. The spectra shown below demonstrates that at 340nm, NADH is at its maximal absorbance while NAD^+ is non-interfering. Therefore, the convenient wavelength 340nm, makes it possible to follow the disappearance or appearance of NADH and hence follow the initiatory triose phosphate isomerase reaction.

FIGURE 1: Ultraviolet Spectra of NAD^+ and NADH



In order to accurately determine the activity of TIM, it is necessary that the coupling system not be rate determining. Plaut and Knowles (39), in an excellent series of experiments, demonstrate the conditions underwhich the overall reaction was limited by the triose phosphate isomerase catalysis. In order to check the validity of the assays (ie. that the observed rates of production or oxidation of NADH represents the rate of the TIM reaction), it was necessary to ensure the linear dependence of

the observed initial rate on the concentration of TIM under the highest substrate concentrations used (for G3P the range of 0.2-1.0mM had been investigated, while for DHAP the range was 0.2-2.0mM). This was accomplished by studying the initial rate as a function of isomerase concentration (at the highest concentration of substrate to be used).

Variation in the rate of G3P or DHAP isomerization was studied as a function of coupling enzyme concentration. The experiments ruled out any possibility of complications arising from enzyme-enzyme interactions and, with the evidence of the other studies, allowed one to determine 'safe' concentrations of all species in solution. It was therefore possible to establish the validity of the assay for the conditions used in this account. The G3P/GPD system was used exclusively in the work presented in this thesis.

Procedure:

The substrate, G3P, was prepared by the hydrolysis of the diethylacetal barium salt of DL-glyceraldehyde 3-phosphate. Manufacturer's preparative instructions were followed: Dowex 50 Hydrogen Form Resin (1.5 gm) was suspended in water (6.0 ml); the barium salt (100 mg) was added, mixed thoroughly, and then the mixture was placed into a boiling water bath for 3 minutes. The G3P solution was chilled quickly by transferring it to an ice bath and then it was poured through a funnel with a glass wool plug. The resin was washed with 2 ml aliquots of water until the supernatant fluids measured 17.5 ml. The resultant stock was 12mM in substrate (100 μmoles of enzymatically active D-isomer)

and had a pH of 2.4. Storage involved dividing the solution into separate 2 ml portions and freezing until use. The substrate is very stable when frozen at -20°C and could be kept for several months without any loss.

The following stock assay solutions were used in a 3 ml cuvette: 2.6 ml of 100mM triethanolamine pH 7.5 buffer with addition of EDTA to 5mM, 100 µl of 6mM NADH, 100 µl of 12mM G3P and 100 µl of α-glycerolphosphate dehydrogenase. The coupling enzyme was usually protein with a specific activity of about 200 units/mg and stock solutions for the assay had concentrations of about 0.5 mg protein per ml. Therefore, final concentrations in the 3 ml volume were: 100mM triethanolamine pH 7.5, 5mM EDTA, 0.2mM NADH, 0.4mM G3P, and 17 µg/ml GPD. The reaction was initiated by addition of about 6—10 ηg of TIM in a 5 or 10 µl volume. The decrease in absorbance at 340nm was followed for 1 minute and usually did not exceed 0.1 absorbance units. The small volume increase did not significantly change absorbance so activities were calculated upon a 3 ml volume. At the pH used, there is no detectable decay of NADH over one minute so the reaction was usually followed without a blank.

2.3 Results

High activity triose phosphate isomerase was eluted from the DEAE Sephadex A50 column in two peaks at approximately 75mM Tris (fig. 2). The results of a typical live chicken preparation are summarized in Table I. The considerable (36 X) purification, as determined by specific activities, can be mainly attributed to the initial DEAE chromatography step.

FIGURE 2: Initial DEAE Sephadex A50 Chromatography

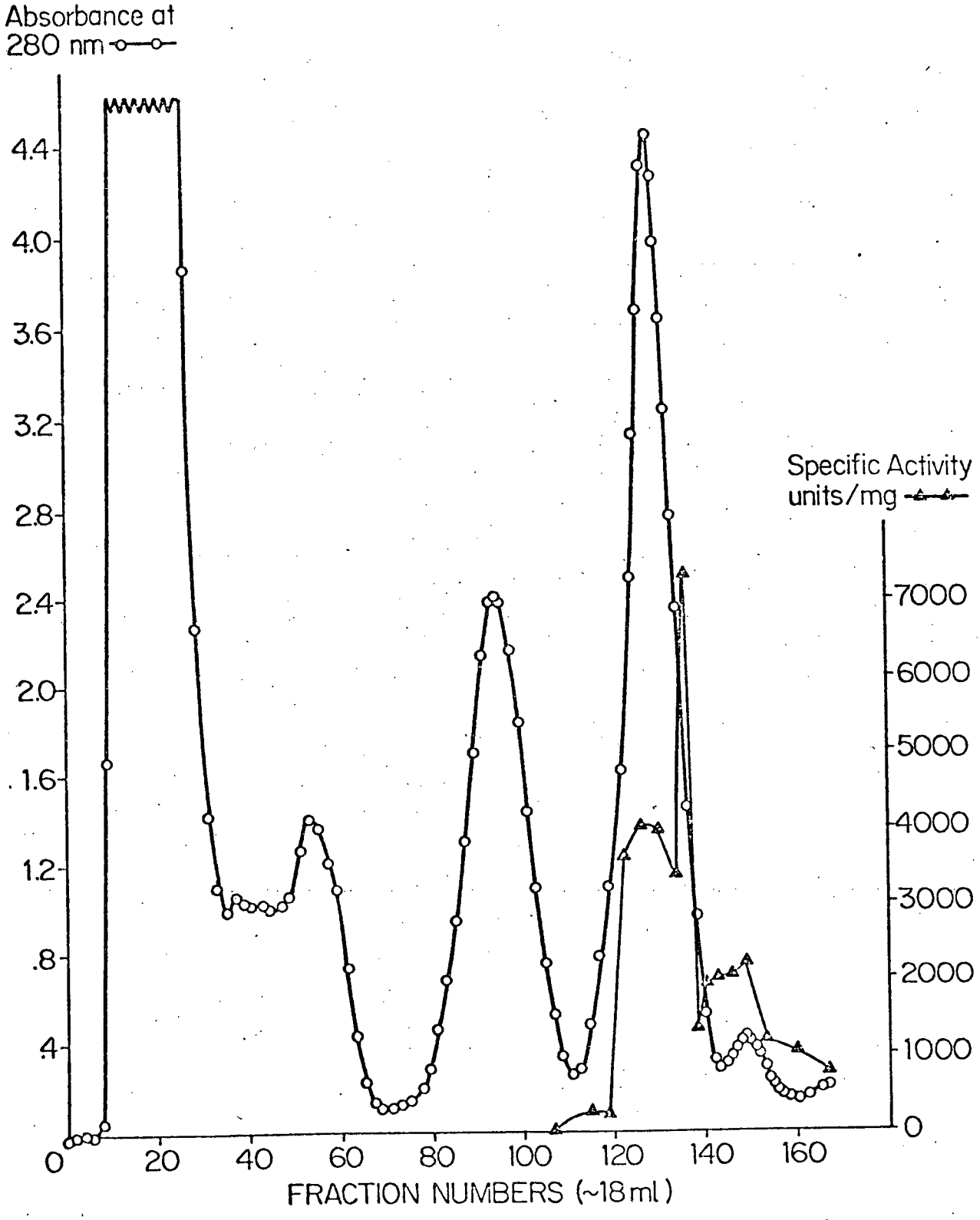


TABLE I: Determination of activity units followed throughout a typical live chicken preparation of chicken breast muscle

For each kilo of tissue:

Fraction	OD's protein	Units/OD	Total units	% recovery	Purification
A. Crude extract	63,585	142.2	9,041,591	100	0X
B. 65% ppt.	19,436	310.1	6,026,933	66.7	2.2X
C. 90% ppt.	10,064	340.8	3,429,661	37.9	2.4X
D. Peak A+B of initial:					
DEAE Seph. A50	971.7 (714.5mg)	5152.9	5,006,879	55.4	36.2X
Peak A	898.3 (660.5mg)	5147	4,623,323		
Peak B	73.4 (540.0mg)	5225	383,557		

In order to further purify and separate the two peaks, the protein was rechromatographed. The results are variable but did result in a protein up to twice as active. The fact that no other protein was observed as separating out in subsequent steps indicates that the lowering of specific activity in the one time chromatographed material is probably due to small amounts of inhibitory protein or other unknown contaminating substances which have a similar effect.

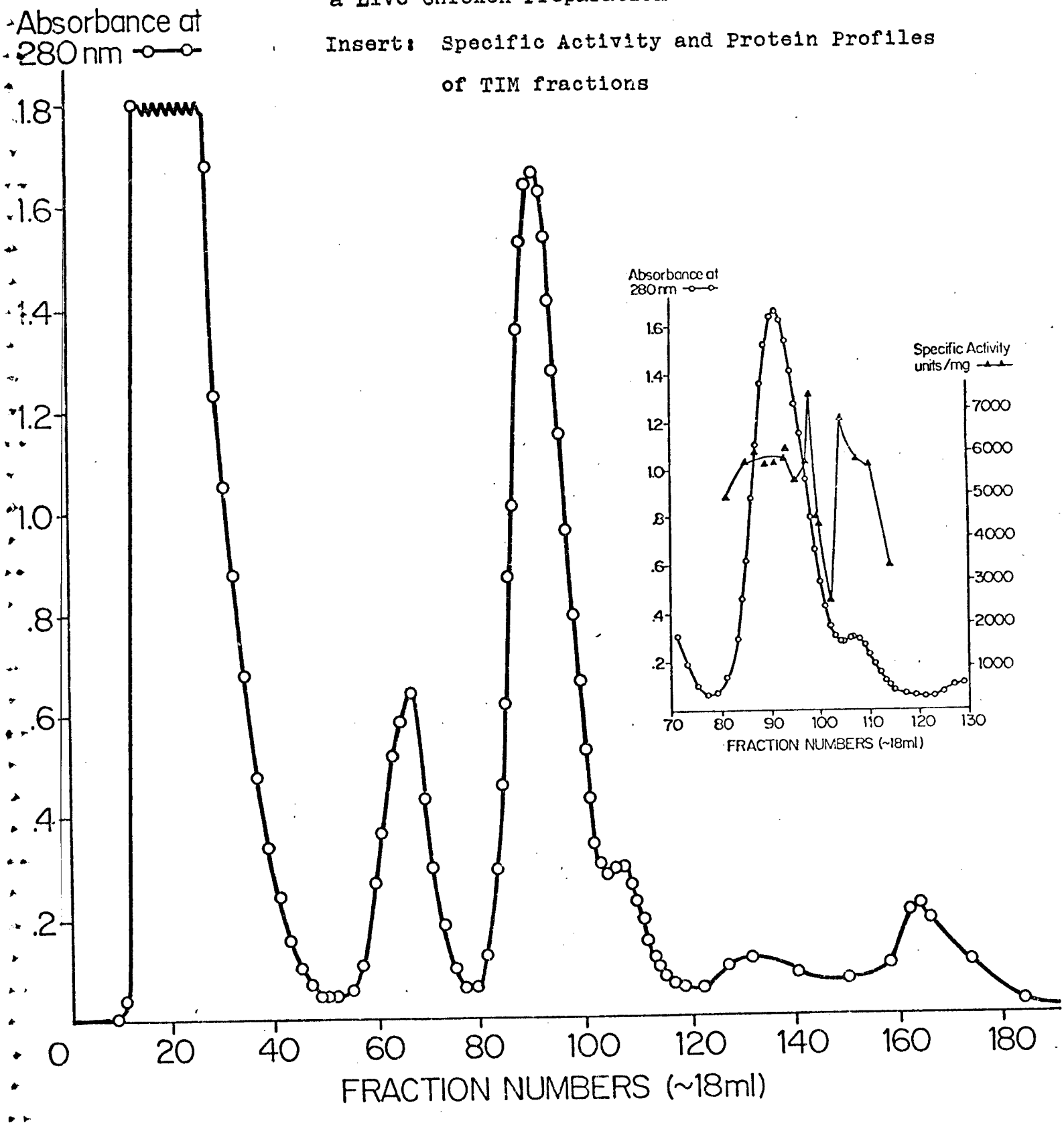
The difference in specific activity between the A and B peaks appears to vary with individual preparations. However, the live chicken preparation did usually yield B protein higher in specific activity than that from chickens obtained from local butcher shops. The B protein from the latter source often yielded enzyme with specific activity considerably lower than that of the A (fig. 2). Figure 3 shows the initial DEAE chromatography of a live chicken preparation.

Since specific activity is calculated as the rate of conversion of substrate per unit of enzyme (usually per OD_{280} or per mg), it is an indication of the efficiency of the catalytic process. If the protein is completely homogeneous throughout an eluted peak, it is expected that a constant specific activity would be observed. The chromatographic studies on TIM showed changes in specific activity which indicated that the protein is not completely homogeneous.

Contamination of the TIM peaks by protein (which can be eluted within the same salt concentration range as triose phosphate isomerase) could be a factor in the observed specific activity

FIGURE 3: Initial DEAE Sephadex A50 Chromatography of a Live Chicken Preparation

Insert: Specific Activity and Protein Profiles of TIM fractions



profile. If the contaminating protein(s) is inhibitory (or activating), minute amounts could produce substantial changes in TIM's ability to catalyze the isomerization of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (and hence in the specific activity profile).

Contaminating protein(s) which has no effect upon the enzymatic capabilities of TIM would have to be present in rather large amounts to produce the profile changes observed. Evidence to be presented in the next section indicates that the protein is homogeneous as to molecular weight. This would tend to rule out the possibility of substantially contaminated protein being eluted from the column. The only other possibility is that there is an inherent molecular or conformational difference in the triose phosphate isomerase at various points in the eluted TIM peaks. The possibility of isozymes (proteins of slightly different amino acid sequence and/or conformation which catalyze the same reaction) with differing triose phosphate isomerase activity capabilities is a possibility to be discussed in the protein characterization section to follow. The variation found in multienzyme forms of TIM (ie. number of charges, shape, protein aggregation, etc.) could be the basis for a chromatographic separation of the various isozymes. The presence of isozymes in the chicken breast muscle fits well with the observed protein and specific activity profiles of the DEAE chromatography experiments.

The characteristic specific activity profile was observed throughout the various chromatographies. The double peak observed for the A protein and single one for the B might seem to indicate

two enzyme forms in the A with only one in the B. However, as will be shown in the characterization work to follow, this does not seem to be the case, in fact, there is strong evidence to the contrary. The physical basis for the double activity peak of the A protein is obscure at this point since in the characterization methods used, no similar separation occurred.

The frequently incomplete separation of the A and B TIM peaks, as well as the observed specific activity profile, indicated that further purification and isolation of the possible molecular forms of the enzyme should be attempted. To this end, separate rechromatography experiments were performed.

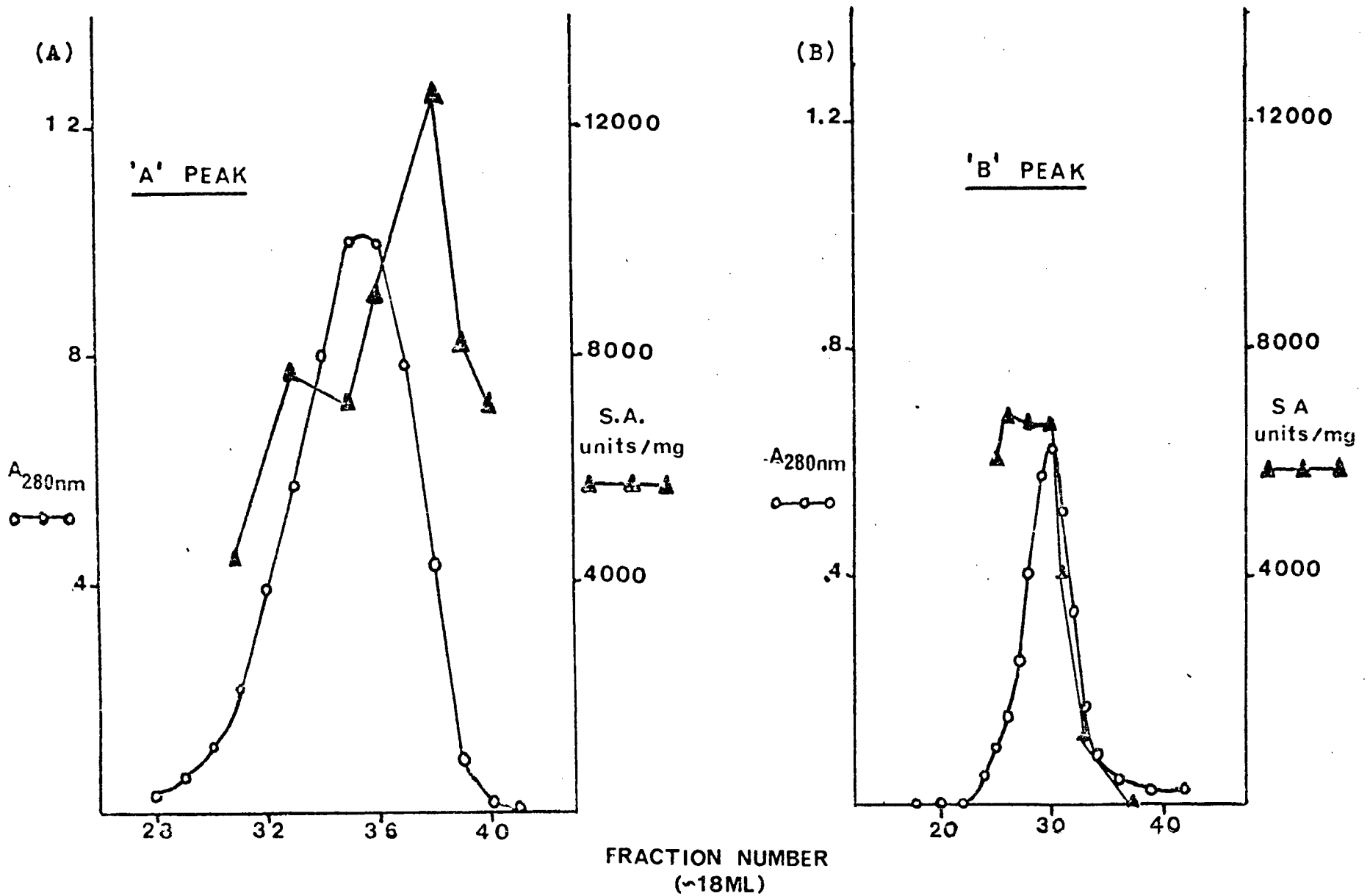
The first experiment utilizing DEAE Sephadex A50 resin resulted in a single peak being eluted for the A protein (fig. 4A) The peak came off very early in the gradient (but not with the void volume) and showed the characteristic specific activity profile for the A protein. The early elution of the A protein indicated that the rechromatography of the B protein on the DEAE Sephadex A50 should be attempted using a lower salt concentration and shallower gradient (in order to allow the protein to adhere more strongly to the column). However, an elution of B protein early in the gradient, was observed (fig. 4B). The specific activity profile of the rechromatographed B material was similar to that obtained in the initial chromatography.

The attempts to rechromatograph triose phosphate isomerase at salt concentrations in the same range (about 75mM) as that of the initial Sephadex-DEAE chromatography seemed to be inadequate. The adherance of the purified enzyme to the anion exchange col-

Figure 4: Rechromatography of TIM Fractions on DEAE Sephadex A50

(A) Protein from Peak A

(B) Protein from Peak B



umn appeared to be different from that of the impure protein. The interactions, which TIM has with the other proteins in the 90% $(NH_4)_2SO_4$ saturation cut, could well affect the manner and strength with which TIM is able to adhere to the column.

Therefore, with the considerable gradient changes (from 60-90 and 50-70mM Tris to 5mM Tris with 0-35mM NaCl) of the recombined peak A and B rechromatography (fig. 5), it was found that the protein was adhering much more strongly to the column. However, because of the interruptions in the gradient, the first peak did not come off in a smoothly symmetrical peak but rather in a 'step-like' manner. The protein profile obtained (fig. 5) illustrates the problems attached to this kind of resin. The specific activity profile of the rechromatography of pooled A and B (double peak for A and single peak for B) allowed one to identify with some certainty, the A and B proteins. The combining of fractions by the individual arrows of the individual A and B peaks is shown in figure 5. The second activity peak of the A protein was lower in specific activity than the first peak in the profile. This was unusual in terms of the profiles observed in the initial DEAE chromatography but has been observed in other rechromatographic experiments (eg: figure 7B).

The next resin to be tried, in an attempt to get over the Sephadex shrinkage problem, was cellulose DE52 (fig. 6) which, however, has a lower capacity. A good separation was observed of recombined A and B peaks from the first DEAE Sephadex A50 chromatography although the specific activity profile did not show its usual definition (although it was generally the same).

FIGURE 5: Rechromatography of Peak A and Peak B TIM Fractions on DEAE Sephadex A50

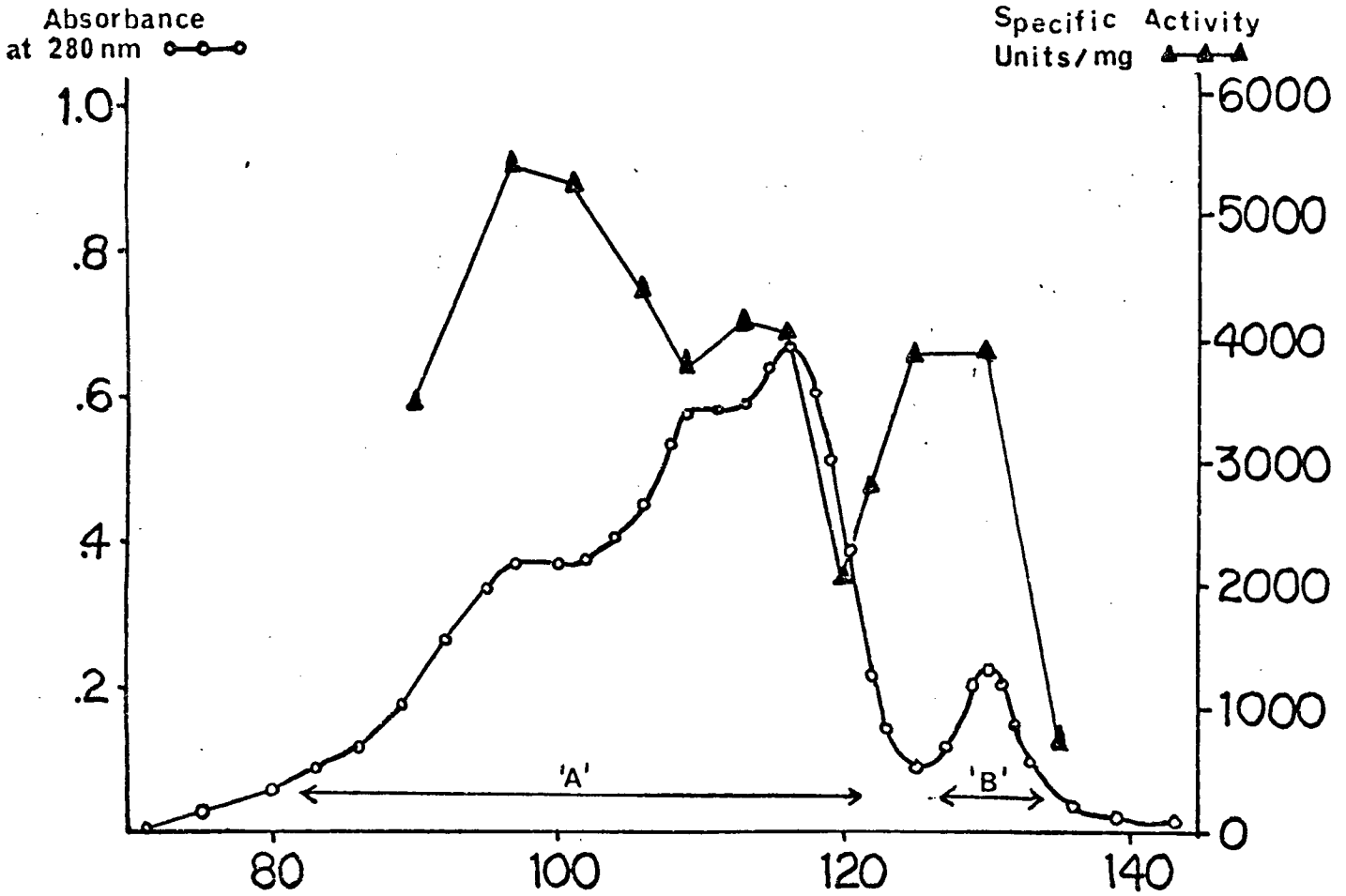
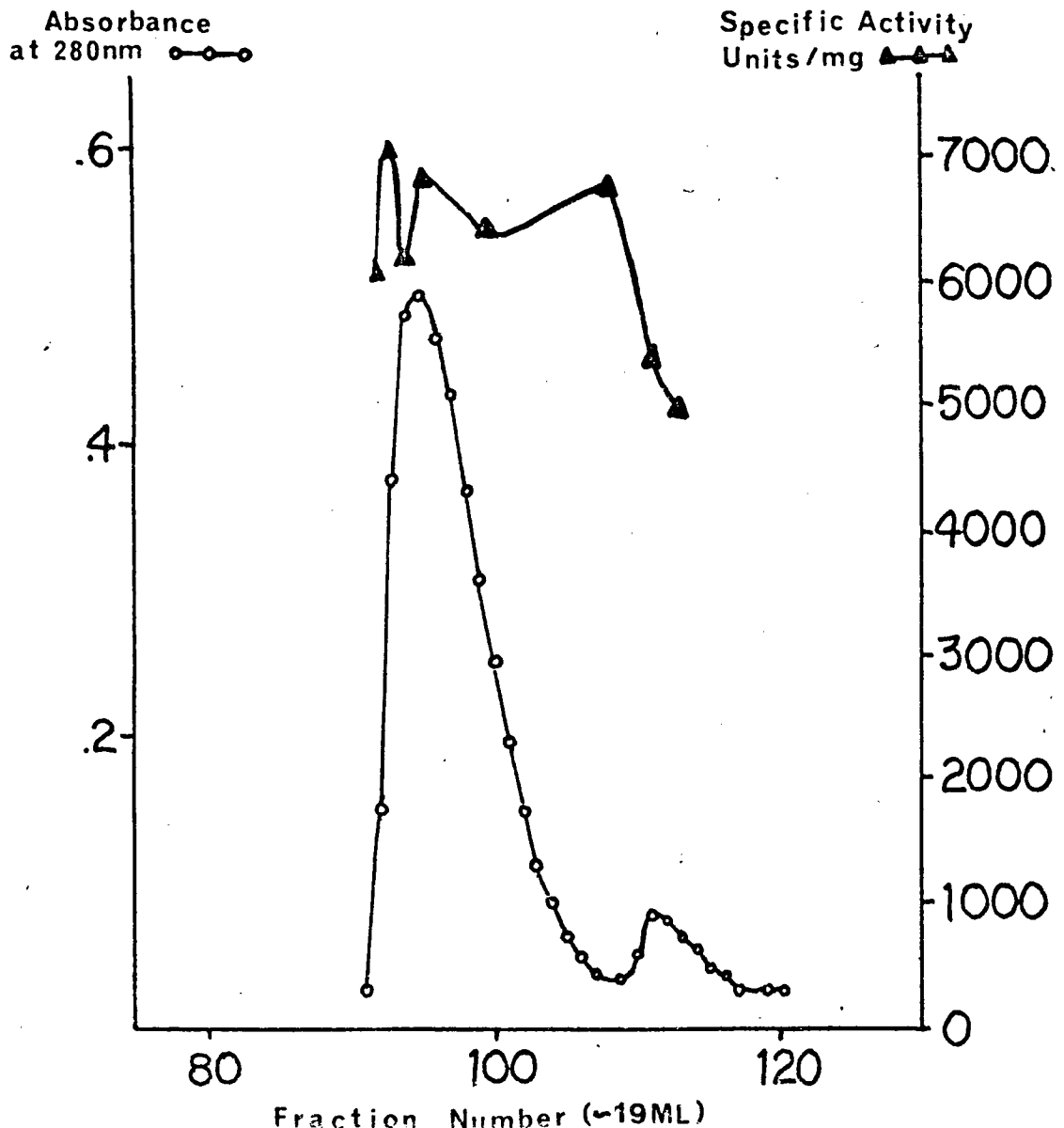


FIGURE 6: Rechromatography of Peak A and Peak B TIM Fractions on DE52 Cellulose



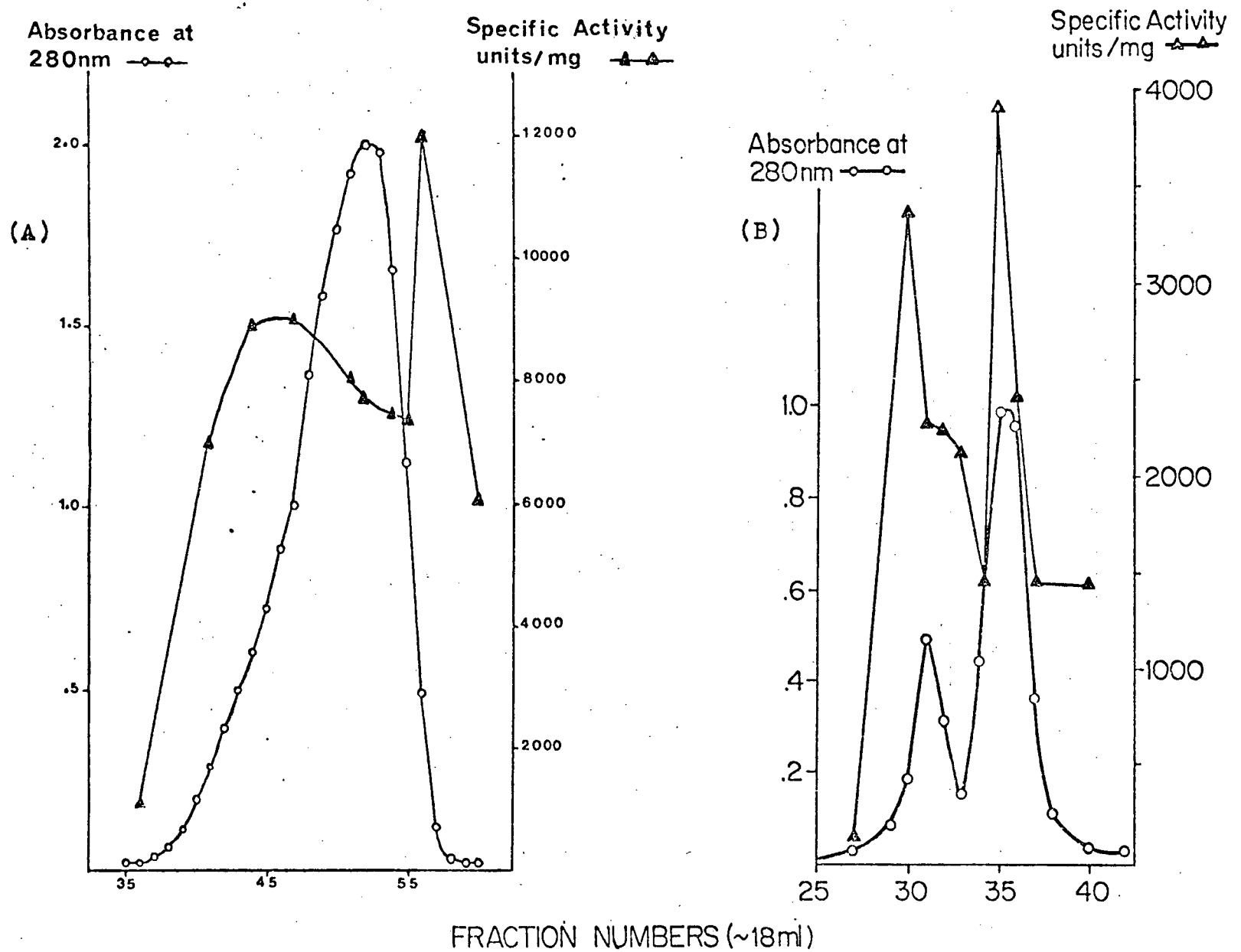
Lastly, an agarose based ion exchange resin, Biogel A DEAE, was employed in the rechromatography step. The rechromatography of peak A fractions (fig. 7A) resulted in elution of a single protein peak (well into the gradient) with the characteristic double peak specific activity profile. The slightly assymmetrical nature of the eluted peak is a feature of the agarose based DEAE resin used. The fact that this resin is capable of as good a separation of the A and B protein peaks as that shown in the DEAE Sephadex A50 and cellulose DE52 chromatographies of recombined A and B (fig. 5, fig. 6), is illustrated in figure 7B. A good separation of residual A protein and the B protein was observed. The B peak showed a single specific activity peak while the A protein had two (the second being present as a well defined shoulder of the first). The second specific activity peak was lower than the first as was discussed earlier.

In conclusion, it might be mentioned that the Biogel A DEAE resin appears to be the best choice for the anion exchanger to be used in the rechromatography step. It combines high capacity, no shrinkage of the resin, and the ability to be used at a fairly low pH, with as good a separation of TIM as has ever been observed chromatographically.

FIGURE 7: Rechromatography of TIM Fractions on Biogel A DEAE

(A) Peak A

(B) Peak B



CHAPTER III

CHARACTERIZATION OF PEAK A AND PEAK B

3.1 Methods

A. SDS Gel Electrophoresis

A slightly modified procedure of Fairbanks et al (57) was used. The procedure was carried out in a vertical electrophoresis apparatus which contained 800 mls of buffer solution in each electrode compartment and no more than 12 gel tubes (fig. 8). The gels contained 1% SDS and 5.6% acrylamide.

To facilitate the preparation of buffers, gels, etc., concentrated stock solutions were first made up and subsequently used to prepare the solutions which were used in the electrophoresis experiment. These are listed in Table II.

Gels were made by combining in a small vaccum filter flask, 1.4 mls concentrated acrylamide and NN'-Methylenebisacrylamide (Ac bis) (see Table II), 1.0 ml 10X buffer and 5.6 mls water. The solution was degassed for approximately 15 minutes and then to it was added 0.5 ml 20% SDS, 1 ml of ammonium persulfate (15 mg/ml), and 0.5 ml of 0.5% TEMED (N,N,N',N'-tetramethylenediamine), This mixture was used to fill four 0.5X11 cm pyrex tubes (previously cleaned in a concentrated HCl bath, coated with Photoflo solution, and left to dry) to within 1 cm of the top of the tube. The top of each gel was carefully covered with an overlay solution which prevents drying out of the gel. They were then left to stand for at least 12 hours to ensure complete polymerization.

Figure 8: Electrophoresis Apparatus

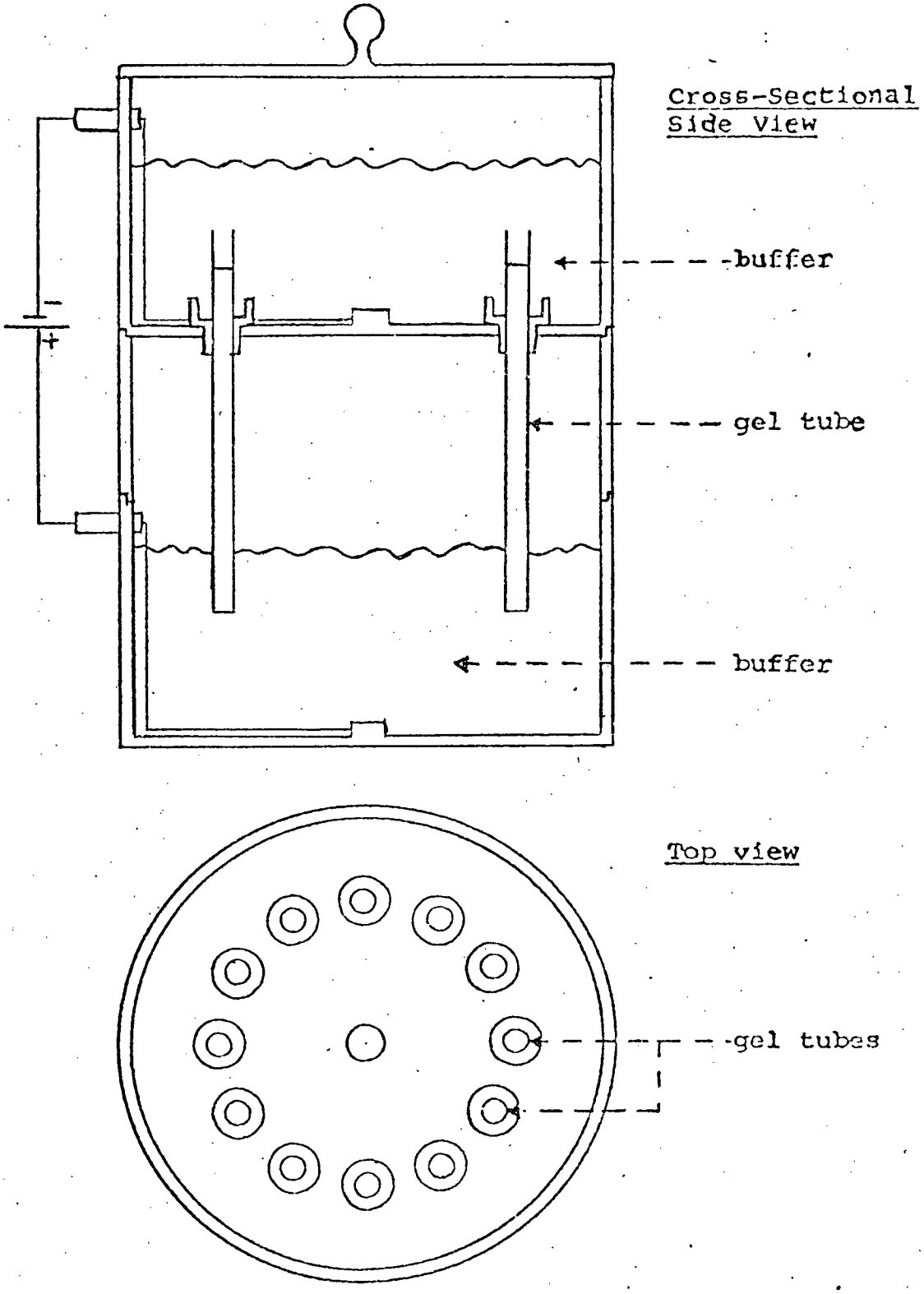


TABLE II: Stock solutions and buffers for SDS gel electrophoresis

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A. Stock solutions

1) Concentrated AcBis

40 gms acrylamide
1.5 gm N,N'-methylenebisacrylamide
H_2O to 100 mls

2) 10X Buffer

0.4 M Tris
0.2 M sodium acetate
0.02 M EDTA
pH = 7.4 with acetic acid

3) 20% SDS (W/W)

B. Electrophoresis buffer (per litre)

100 mls of 10X buffer
50 mls 20% SDS
H_2O to 1 litre

C. Denaturing Solution

2 gms SDS
10 gms sucrose
74.5 mgs EDTA
2 mgs pyronin y
0.242 gm Tris
H_2O to 100 mls, pH 8 with HCl

D. Overlay solution

0.1% SDS
0.15% Ammonium persulphate
0.05% TEMED

The proteins were prepared as follows. A small amount of the denaturing solution (Table II) was made 80mM in dithiothreitol, combined with an equal volume of protein solution and heated for 15-20 minutes at 37°C to completely denature the protein. The molecular weight standards were prepared in the same way but denatured at 60°C. Upon cooling, up to 100 µls of protein sample was carefully applied with a micropipet to the top of the gel (which was now contained in the electrophoresis apparatus and covered with buffer solution). A maximum of 100 µg of protein was loaded onto each gel which avoided excessively broad bands. Samples were run in duplicate.

Electrophoresis was carried out at a current of 5 mamp/gel and required approximately 3.5 hours under these conditions. In all cases, gels were prerun for one hour prior to sample application which ensured the removal of excess ammonium persulfate.

The molecular weight markers were denatured in separate test tubes, then combined together in one test tube and applied on the gel together. The marker proteins were run in gels separate from the sample proteins. The following proteins were used as standards: bovine serum albumin (68,600), ovalbumin (45,000), trypsin (23,800), and myoglobin (16,900).

After removal of the gels from the tubes, the position of the tracking dye was marked by notching the gels with a needle dipped in India ink. The gels were then transferred to stoppered glass tubes and agitated sequentially with the following protein staining and destaining solution: (57)

1) 25% isopropanol, 10% acetic acid, 0.025% Coomassie

blue (overnight)

2) 10% isopropanol, 10% acetic acid, 0.025% coomassie blue (6-9 hours)

3) 10% acetic acid, 0.0013% coomassie blue (overnight)

4) 10% acetic acid (overnight)

At this point, the proteins were apparent as dark blue bands. The subunit molecular weight of TIM was estimated by first plotting log molecular weight against the R_f values measured relative to the tracking dye. The points for the standard proteins fell on a straight line from which any unknown subunit molecular weight could be obtained by interpolation. (fig. 9)

B. Disc Gels

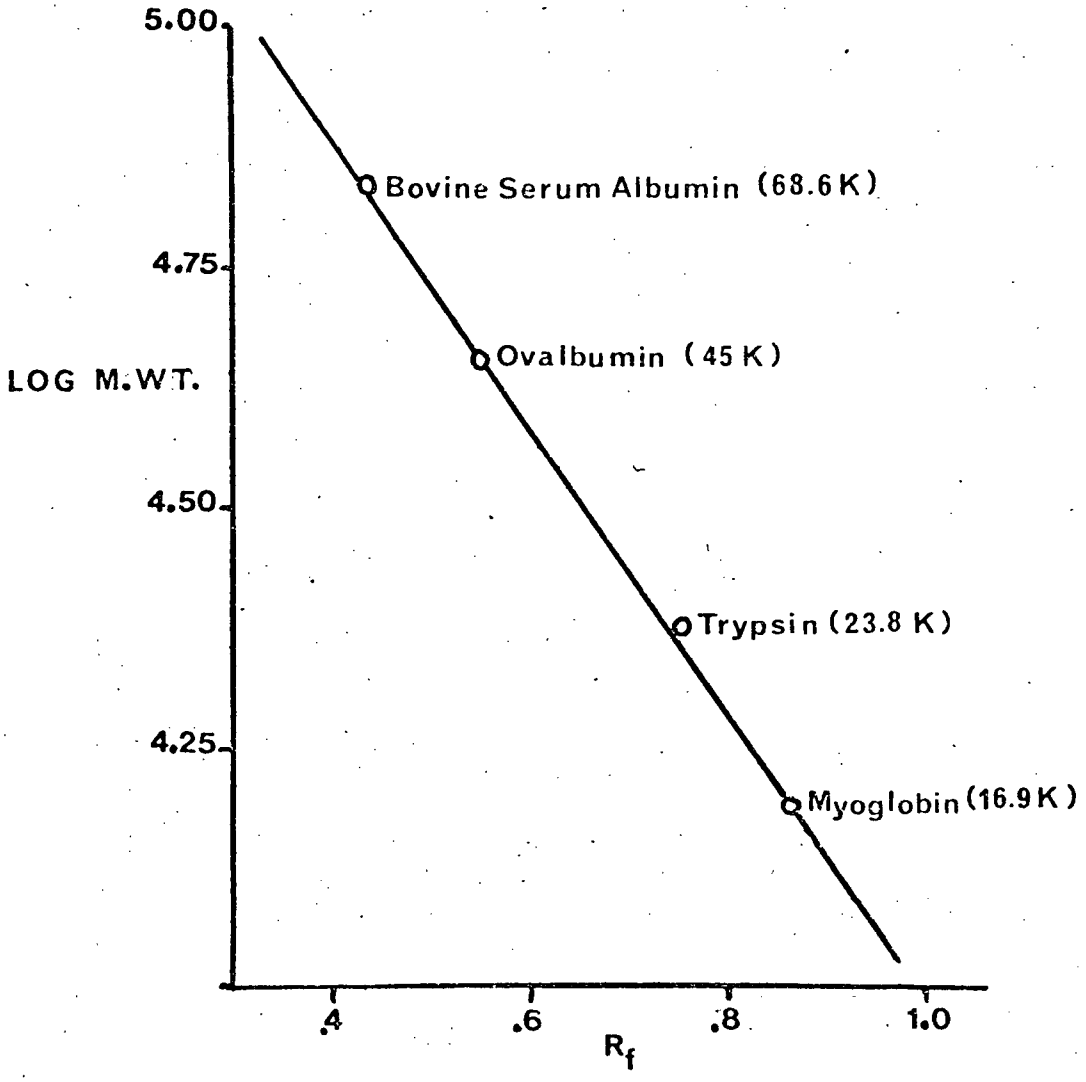
The apparatus was as for SDS gel electrophoresis. A modified method of Dietz and Lubrano was used (58). The following stock solutions were made up to facilitate preparation of the gels. Solutions A and B were stored in the cold, in the dark for up to one month. Solution C was freshly made each time.

A. 36.3 gms Tris
0.23 mls TEMED
Addition HCl to pH 8.5
H_2O to 100 mls

B. 6 gms acrylamide
160 mgs methylene-bis-acrylamide
H_2O to 20 mls

C. 140 mgs ammonium persulfate
H_2O to 100 mls

FIGURE 9: SDS Gel Electrophoresis of Protein Standards



Gel Mixture:

Four 7.5% gels were made by mixing 1.0 ml A, 2.0 mls B, 1.0 mls water, and 4.0 mls C. The solution was poured into 0.5X10 cm tubes which had been previously coated with Photoflo solution. The gels were carefully overlaid with about 50 µls of H_2O and then left to polymerize at least 8 hours or overnight.

Dialyzed protein was made 20% in sucrose and a couple of milligrams of bromophenol blue was added. Electrophoresis buffer for the cathode and anode reservoirs was made by a 1:9 dilution of a stock 10X buffer which had been previously made:

10X buffer - 6 gms Tris
28.8 gms glycine
to pH 8.3
H_2O to 1 liter

The bottom reservoir was filled with 800 mls of buffer and the gels placed into the apparatus. The various protein solutions to be run were carefully pipetted onto the top of the gels (usually about 20 µg protein in a volume less than 100 µls were laid on each gel) and then the tubes were filled with buffer. The top reservoir was filled with buffer, the electrodes connected and the electrophoresis begun. The experiment was performed at 4°C. The protein was concentrated down into a thin layer on top of the gel at 1 mamp/tube and then the voltage was increased to give 2 mamp/tube until the end of the experiment.

After electrophoresis, the tracking dye was notched with India ink and the protein was fixed by agitation of the gels for 30 minutes in stoppered tubes containing 10% TCA (trichloroacetic

acid). Staining of the protein was accomplished by agitation in 12.5% TCA with 0.05% Coomassie Blue for 1 hour. Finally, the color intensity was developed over a 48 hour period by agitation in 10% TCA. The gels were scanned at 550nm using a Gilson gel scanner.

C. Isoelectric Focusing

Column Isoelectric Focusing:

A diagram of the 110 ml capacity LKB 8101 electrofocusing column used in these experiments is shown in figure 10. Detailed descriptions of the experimental procedure can be found in several references (59,60) with the procedure used as follows:

The valve 12 was opened and cooling water passed through the compartments (18 and 16) until the apparatus was equilibrated at 4°C. The first solution to be added was the cathode solution (0.4 mls ethylene diamine, 12.0 gms sucrose and 14.0 mls H_2O). It was pumped in through nipple (1) with a peristaltic pump and filled up the bottom of the column. A dense solution, containing 3/4 of the Ampholytes diluted to 42 mls with water (and 28 gms sucrose dissolved in it) as well as a light solution containing ¼ of the Ampholytes and the dialyzed protein diluted to 60 mls were prepared.

The range of Ampholytes availabe from LKB include:

pH 3.5-10	pH 5-8
2.5-4	6-8
3.5-5	7-10
4-6	8-8.5
5-7	9-11

FIGURE 10: A Sketch of the Isoelectric Focussing Column

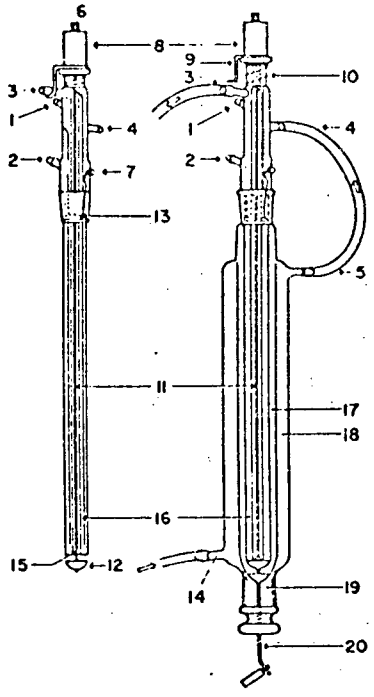


Fig. 10: Electrofocussing column of 110 ml capacity. The outer cooling jacket (18) has an inlet at 14, and an outlet at 5. From the outer jacket the water flows through a tube into the central cooling jacket at 4 and leaves the column at 3. Two platinum electrodes are used. One electrode 13, is in contact with the plug 7, is in the upper part of the column. The gas formed at this electrode escapes at 2. The other is wound on a Teflon bar 11, and gas escapes at 1. Before draining the column the central tube is closed by lifting the plug 12 which has a rubber gasket on the upper surface and seals at 15. Isoelectric focussing takes place in compartment 16, which is filled through nipple 2. At the bottom of the column there is a plug 18, with an attachment for a capilliary tube to enable the column to be fractionated.

Ampholytes were used in quantities to yield either a 1% (2.5 mls of 40%) or a 5% (12.5 mls of 40%) concentration. In addition to these ampnolytes, a small quantity of pH 3.5-10 range ampholytes was added to the 1% (0.2 ml of 40%) and the 5% (1.0 ml of 40%) columns in order to 'protect' the ends of the pH gradient from the cathode and anode solutions. From 2-20 mgs of protein may be in each protein zone depending upon the range of ampholytes used and the differences in the pI's of the proteins. For the experiments described here, about 5 mgs of protein were added.

The dense and light solutions were added to the column by means of a LKB density gradient mixer through nipple 2 with the aid of the peristaltic pump. The final solution to be added was the anode solution which contained 100 μls concentrated H_2SO_4 and 9.9 mls H_2O. The experiment was started with a voltage yielding about 2 watts of power.

The focusing of the carrier ampholytes and proteins was accompanied by a decrease in the current passing through the solution. The current was checked periodically and the voltage increased (power always kept at or under 2 watts) until it had decreased to a constant value (at constant voltage). At this point, most of the carrier ampholytes should be focused at or near their isoelectric points. To ensure complete focusing of the slower travelling protein, the experiment was continued for a further 12 hours. The entire focusing experiment takes from 24-72 hours depending upon the pH range and concentration used. More concentrated and narrower range ampholyte solutions require

the longer focusing times.

Upon completion of the procedure, the power is turned off, and valve 12 is closed to prevent the central electrode solution from mixing with the effluent. The clamp on the capillary tube (20), is opened and the column pumped out at a flow rate of about 1 ml/minute. The fractions were collected on a Gilson fractionator and their pH determined at 4°C, the temperature of the experiment. Protein, if added to the ampholyte procedure initially, was then measured by its absorbance at 280nm and TIM was assayed for using the G3P/α-glycerolphosphatedehydrogenase procedure.

The first isoelectric focusing experiment performed used pH 7-10 and pH 5-8 range ampholytes (added in 1:1 ratio with a total range of pH 5-10 obtained) at a final concentration of 1%. Resolution of the Peak A and of the Peak B protein was inadequate so 5% runs of pH 7-10 or pH 5-8 (without protein) were performed and the tubes containing carrier ampholytes in the pH 7-8 range were pooled. An appropriate amount of the pooled ampholytes (about 22 mls usually) was then used in an electrofocusing experiment which included protein. The amount of sucrose added to the dense solution was decreased to compensate for the sucrose present in the pooled ampholyte mixture. The best resolution resulted when no more than 3 absorbance units of Peak A or Peak B was used.

Polyacrylamide gel isoelectric focusing:

The apparatus for running this experiment was the same as that used for disc or SDS gel electrophoresis. The polyacryl-

amide matrix of the gel served as a substitute for the sucrose gradient of the column. The gels were either photopolymerized or chemically polymerized. Photopolymerization was the preferred method in regards to time: protein seldom needed to be concentrated before use and the experiment was started 1 hour after the pouring of the gels. However, the procedure is suitable only when the average pH of the ampholyte range is no greater than 7.0-7.5 (61). Therefore the pH 5-8 or 3.5-10 ranges were suitable for use but not the pH 7-10 range. The main disadvantage of the chemically polymerized gels is the production of artifacts due to the presence of persulfate. This has been commented on by several authors (61,62,63)

Electrofocusing in gels is fast, requires relatively small amounts of protein and uses much less ampholytes than the column technique. The disadvantage is that it is limited in determining an accurate pI for a protein since the gel is short. Standards (ie. duplicate gels without protein) are co-run with the gels containing protein. The pH profile of the standard gels is determined by slicing the gel rod, soaking the pieces in water in order to elute the ampholytes, and determination of the pH of the eluent. The protein in the other gels is stained, their position measured, and the pH at that point determined by comparison to the plot (of pH vs. distance) of the standard gel. Therefore, the accuracy of the pI depends upon an indirect determination of the pH at that point where the protein is focused and upon the accuracy of slicing.

The method used for the preparation of the photopolymerized

gels is given in Table III. Upon polymerization, the gels were placed in the electrophoresis apparatus with the anode (bottom) reservoir containing 800 mls of 0.2% sulfuric acid and the cathode (top) reservoir containing 800 mls 0.4% diethanolamine. The experiment was run at 4°C and an initial current of 1 mamp/tube. This was maintained by increasing the voltage until 350 volts had been reached. The protein and ampholytes were considered focused when a constant amperage (usually 0.5-1 mamp) at 350 volts was observed for 1-2 hours (the IEF experimental running time took on average 5 to 7 hours). At this point, the reference gels were sliced at 5 mm intervals and placed in individual test tubes with 1 ml of degassed water and then they were capped. The ampholytes were allowed to elute from the gel at 4°C, the temperature of the experiment. The pH of the gel slices could be determined after approximately one hour. If the elution was allowed to proceed overnight, nitrogen gas was bubbled into the tubes and then the tube was firmly sealed with Parafilm.

The gels to be stained (Vesterburg's quick staining method) (64) were marked with India ink at the anode end and placed in stoppered tubes. The staining procedure included:

a) incubation of the gels in a 60°C water bath (in a fume hood) in a staining solution (of methanol 75 mls, distilled water 186 mls, trichloroacetic acid 30 gms, sulfosalicylic acid 9 gms, and Coomassie Blue, 0.1%) for 15 minutes

b) replacing of the staining solution with destaining solution (of ethanol 250 mls, water 650 mls, glacial acetic acid 80 mls) and agitation of the tubes

TABLE III: Isoelectric focusing solutions for gels

Photopolymerization— Stock solutions were kept at 4°C in the dark for about one month.

A. Catalyst

1.0 ml TEMED
14 mgs riboflavin
H_2O to 100 mls

B. Acrylamide

30 gms acrylamide
0.8 gm methylene-bis-acrylamide
H_2O to 100 mls

Gel Mixture

Mix 3.0 mls of B and 0.3 ml ampholytes (40%). For each set of duplicate gels, take 1.1 ml of the above and add to it 2.75 mls H_2O (containing dialized sample if the gels are to include protein). The mixture is poured into the 0.5X10 cm tubes (to approximately 8 cms height) and exposed to bright light for approximately 1 hour to complete polymerization. The tubes had been previously coated with Photoflo solution.

Chemical polymerization— Stock solutions were kept at 4°C in the dark for about one month. The catalyst solution was made fresh each time.

A. Acrylamide

3.05 gms acrylamide
H_2O to 10 mls

B. Bis-acryl

100 mgs methylene-bis-acrylamide
H_2O to 10 mls

C. Catalyst

150 mgs ammonium persulfate
H_2O to 10 mls

Gel Mixture

Mix 1.25 mls A, 1.25 mls B, 3.30 mls H_2O, and 2.0 mls pooled ampholyte mixe. Add to this solution 25 µls TEMED and 200 µls of C. The four gels (of approximately 8 cm height) are poured immediately into the 0.5X10 cm tubes which had been previously coated with Photoflo solution, and allowed to polymerize for at least 8 hours before use.

The destaining solution was usually replaced every 30 minutes for the first 2 hours and then left in a fresh solution overnight to complete the destaining process. The gels were scanned at 550nm when destaining was complete.

The pH range 5-8 was found to be inadequate for resolution of the protein bands, since the pI's of the proteins in Peak A and B appear to be very similar. Therefore, a 5% column isoelectric focusing experiment was performed in the usual way but with a pooling of those ampholytes in the pH 7.3-7.9 fractions. The concentration of the pooled ampholytes was such that 0.5 ml was required for each 1% gel. Polymerization in this range of ampholytes was found to occur with less ease than the pH 7-10 range ampholytes (and not at all by photopolymerization) so that 50% more persulfate than the usual 1% solution (ie. a 1.5% catalyst used) was required to complete polymerization. The presence of sucrose from the pooled ampholytes did not interfere adversely with the experiment. It has been observed (66) that sucrose stabilizes the pH gradient and enables the proteins to be successfully focused in a lower percent acrylamide gel, with a lower voltage and longer focusing time. Because of the polymerization problems experienced, a lower percent acrylamide gel was not used but the gels were prepared as described in Table III. Since TIM is not excessively large or assymmetrically shaped, it experiences no problems in passing through the pores of the higher percent acrylamide gels. One of the main advantages of the 3.5% gels described by Doerr and Chramback (66) would be that large or assymmetrically shaped pro-

teins such as immunoglobulins could be focused.

Upon polymerization, the gels were placed in the electrophoresis apparatus with the anode (bottom) buffer in place. The presence of sucrose in the gels makes them less ridgid and less able to adhere to the sides of the wall of the glass tubes when placed in a vertical position. Therefore, small pieces of dialysis tubing, held in place by rubber bands around the bottom of the tube, prevented the gels from slipping out into the lower reservoir. Protein (in a 25% sucrose solution in a volume under 100 μls) was carefully laid on the top of the gel with a micropipet. Next, 100 μls of 20% sucrose was laid on top of the protein, followed by 100 μls of 10% sucrose. The cathode buffer was added to fill the rest of the tube and then the top reservoir was carefully filled with buffer. The buffers, experimental running conditions, temperature, elution of ampholytes, and staining of protein were as described for the photo-polymerization method. The gels were allowed to focus for 13 hours rather than the 5-7 hours used for the photopolymerized gels. The narrower pH range of the ampholytes used in the chemically polymerized gels take longer to travel to their isoelectric point. The gels were scanned at 550nm upon completion of destaining.

D. Amino Acid Analysis

The amino acid data was obtained from duplicate 24 hour hydrolyses of approximately 0.05 μmoles 2X chromatographed Peak A. Tryptophan was assayed for using Witkop's procedure (66) of reaction of N-bromosuccinimide (NBS) with the indole ring of trypto-

phane to yield oxindole.

The assay procedure was carried out in 8M urea (adjusted to pH 4 at 22°C with acetic acid) to ensure complete titration of all tryptophan residues. The titration proceeds by stepwise additions of 5 μl of 10mM NBS to 2.0 mls of protein solution (OD_{280}=1.5-2) contained in a 3.0 ml cuvette. The titration proceeded with a decrease in the absorbance at 280nm. 5 μl additions continued until a minimum absorbtion was reached with corrections for volume increase being made. The absorbance decrease at 280nm may be related to the OD of tryptophan in the protein sample by the empirical factor of 1.31 which allows for the oxidation product of oxindole. The E_{280} for tryptophan is $5500M^{-1}$.

3.2 Results

A. Subunit Molecular Weight

An SDS gel electrophoresis molecular weight determination was performed using protein from the two active TIM peaks of the initial DEAE Sephadex A50 chromatograph. The following gels were run (in addition to the molecular weight markers):

A. 20 μgs of Peak A

B. 20 μgs of Peak B

C. 20 μgs of Peak A plus 20 μgs of Peak B

Protein was taken from the center fractions of the two active TIM peaks. In all cases, single bands were observed upon completion of the electrophoresis experiment (with exception of gels containing marker proteins). A subunit molecular weight of 24,500 was observed for Peak A, 24,700 for Peak B, and 24,500 when center fractions of Peak A and B were co-run in the same gel. The presence of single bands was an indication of protein homogeneity. It would appear that the TIM active fractions from both peaks have the same, or nearly the same molecular weight. The values of 49,000-49,400 obtained for the dimeric molecular weight are within a reasonable deviation from the molecular weight of 48,500 which McVittie calculated from partial specific volume and sedimentation equilibrium measurements (11). SDS gel molecular weight determinations are considered accurate to within 5-10% of the true molecular weight.

SDS gel electrophoresis performed on twice chromatographed material gave results which were within 5% of the subunit molecular weight determined for the once chromatographed protein.

These second values were 23,300 for Peak A, 23,100 for Peak B, and 23,000 for when Peak A and Peak B were co-run in the same gel. Once again, single bands were obtained. The protein was found to be homogeneous by SDS gel electrophoresis.

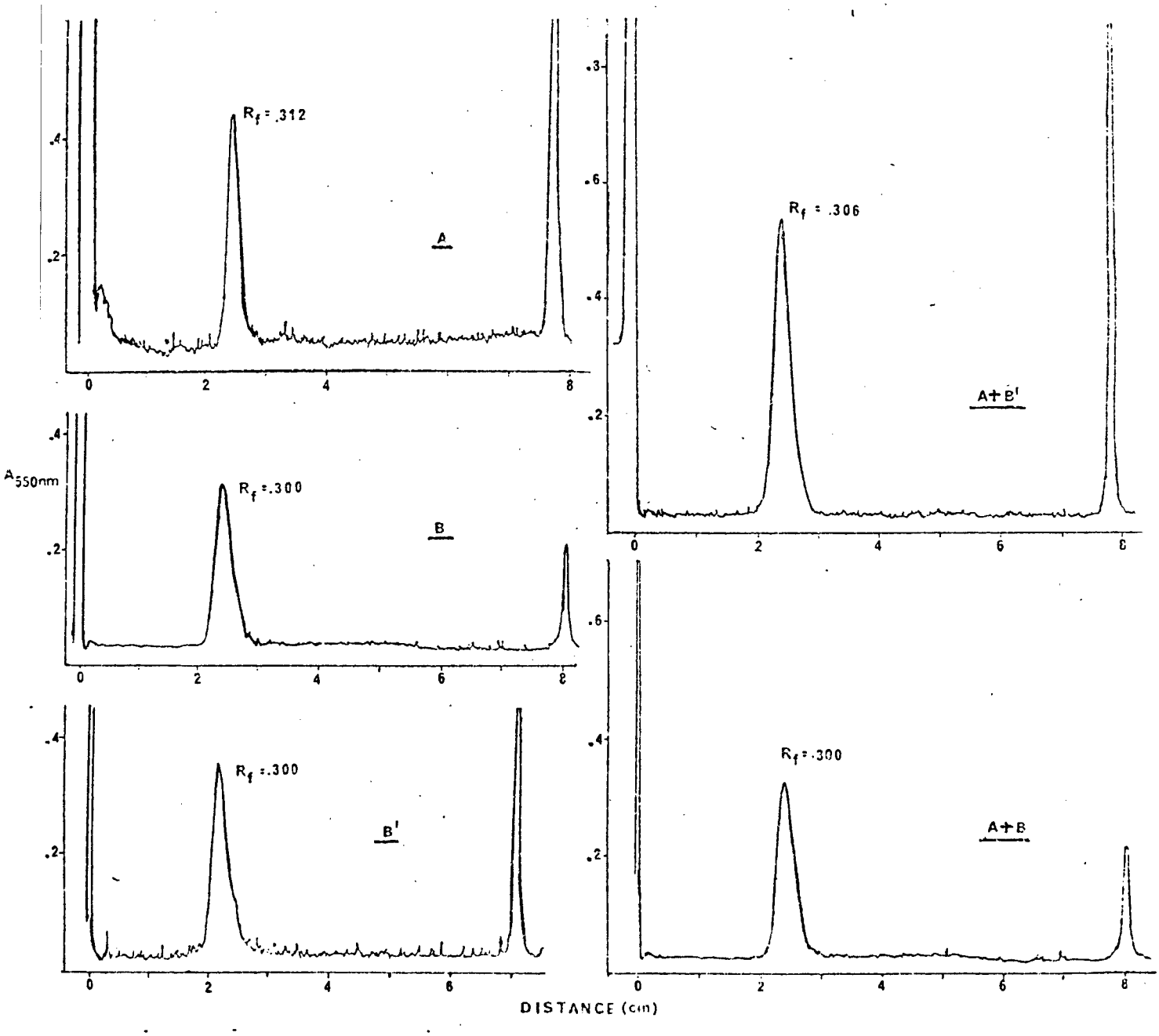
The conclusion which may be drawn from the SDS gel electrophoresis results is that the heterogeneity of the TIM active protein (as observed in the chromatographic protein and activity profiles as well as the isoelectric focusing data which follows) is not based on molecular weight differences. Even the IX chromatographed material (with the exception of the FEM modified protein) was homogeneous by SDS gel electrophoresis.

B. Disc Gel Electrophoresis

The results of the pH 8.5 disc gel electrophoresis experiment is shown in figure 11. The R_f values, reported for the proteins, were obtained from measurements taken from the gels. There is little significant difference in the electrophoretic mobility of the various samples (4% greatest difference). Scopes (20) reported only one band for disc gel electrophoresis of chicken muscle TIM at pH 8.5, but he also indicated that starch gel electrophoresis had demonstrated the presence of a minor component. The minor component could correspond to the Peak B protein which has been shown in this thesis to be separable chromatographically.

The results shown here, support Scope's observation of one band by disc gel electrophoresis. The mixing of the protein from the two chromatographic (TIM active) peaks still results in one protein zone. Even the peak B protein which was one year

FIGURE 11: Disc Gel Electrophoresis Using Fresh Protein from Peak A (A) and Peak B (B), and Old Protein from Peak B (B^{v})



old was homogeneous (by this method). The homogeneity of TIM by this method and the very minor contaminent observed in the starch gel electrophoresis has encouraged the belief in the literature that chicken muscle TIM is relatively free from isozymes.

However, the isoelectric focusing data to be described in the following section as well as the chromatographic separation observed, indicates the presence of isozymes. The protein in peak B comprises almost 8% of the protein found to possess triose phosphate isomerase activity.

The fairly large degree of isozymic contamination could affect the validity of crystallographic and amino acid sequence data which has been recently published (18). It appears probable that both the x-ray crystal structure at 2.5Å resolution and the amino acid sequence were performed on protein which was not pure. The implication of this (in terms of the correctness of the published results) could be important if the isozymic structural differences are significant. In the interpretation of the electron density map, there were 23 side chains which were a poor fit with the map in regions where the electron density was relatively strong (including 6 pairs of equivalent residues from the two subunits) and there was one short section of polypeptide chain, residues 168-176 in one subunit, which could not be followed easily in the electron density map. These problems were partially resolved by the authors of the crystal structure by considering that they were looking at two independent images of essentially identical structure (ie. 2 identical subunits). However, if there was substantial isozymic impurity

present in the protein crystals used, the assumption would be no longer valid and the possibility of incorrect assignments of amino acids exists.

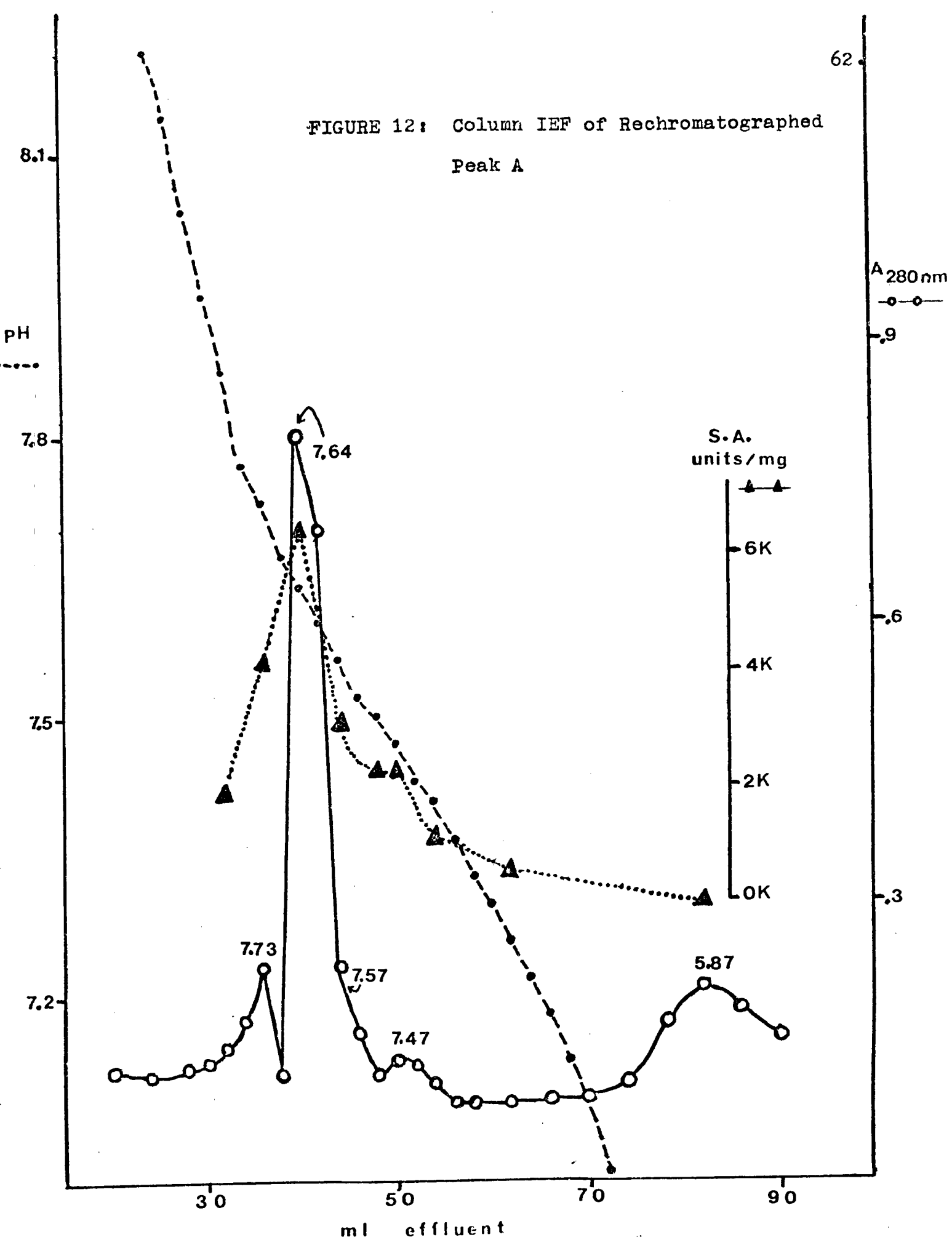
C. Isoelectric Focusing

Isoelectric focusing (IEF) is a sensitive method of separating ampholytes, especially proteins, according to their isoelectric point. Therefore, the IEF technique, which is characterized by very high analytical resolution and by simplicity of apparatus and method, can be used for both preparative separations of proteins as well as isoelectric point (pI) characterization of proteins. As little as a 0.02 pI difference in proteins may be observed. As will be demonstrated in this section, it is possible to observe separations which cannot be seen by disc gel electrophoresis.

Probably the IEF method which has the most potential for sensitivity and accuracy is the column method, as discussed in the methods section. It has allowed, because of its high resolution and reproducibility of pI value, a characterization of the chromatographically separate Peaks A and B of TIM.

The first experiment to be reported here is the column isoelectric focusing of 6 absorbance units of rechromatographed Peak A using the narrow range ampholytes (pH 7-8) which had been prepared from a 5% ampholyte column run. The results shown in figure 12 indicate the presence of a major protein and a major specific activity peak with a pI of 7.64. The A_{280} plot showed a shoulder with pI=7.57 as well as a minor peak at 7.47 which

FIGURE 12: Column IEF of Rechromatographed Peak A



might have some significance when compared to other runs, which contain Peak B protein, which will be described shortly. There was also a protein with pI=7.73 which has some TIM activity and one with a pI=5.87 which has none. The latter was probably enzyme which had been aggregated by the pH conditions of the experiment.

In the electrofocusing of 4.3 absorbance units of rechromatographed Peak B(see figure 13) the minor peak at pH 7.72 reappeared as did the nonactive 280nm absorbing peak at pH 5.92. However the major features were a protein peak at 7.57 and a well defined shoulder at 7.44. Resolution of the 7.57 and 7.44 pI peaks could probably be improved by a narrower gradient or less protein. The specific activity profile showed two peaks which appear to correspond to the proteins with isoelectric points of 7.57 and 7.44. The presence of the two small peaks at pH 7.57 and 7.47 in the protein profile of the isoelectric focusing of Peak A (fig.12) can now be ascribed (with some degree of certainty) to the presence of Peak B contaminant.

Better resolution of the two proteins in Peak B is visible in figure 14 which illustrates a column IEF experiment of rechromatographed Peak A and Peak B. Three major protein peaks were eluted from the IEF column with pI's of 7.66, 7.55 and 7.46. Once again there is a minor amount of protein possessing some TIM activity at about pH 7.7. In addition, there was a small peak of some activity at pH 7.38. Less Peak A protein than Peak B was used in this experiment. The specific activity profile showed three peaks but they only roughly corresponded

FIGURE 13: Column IEF of Rechromatographed Peak B

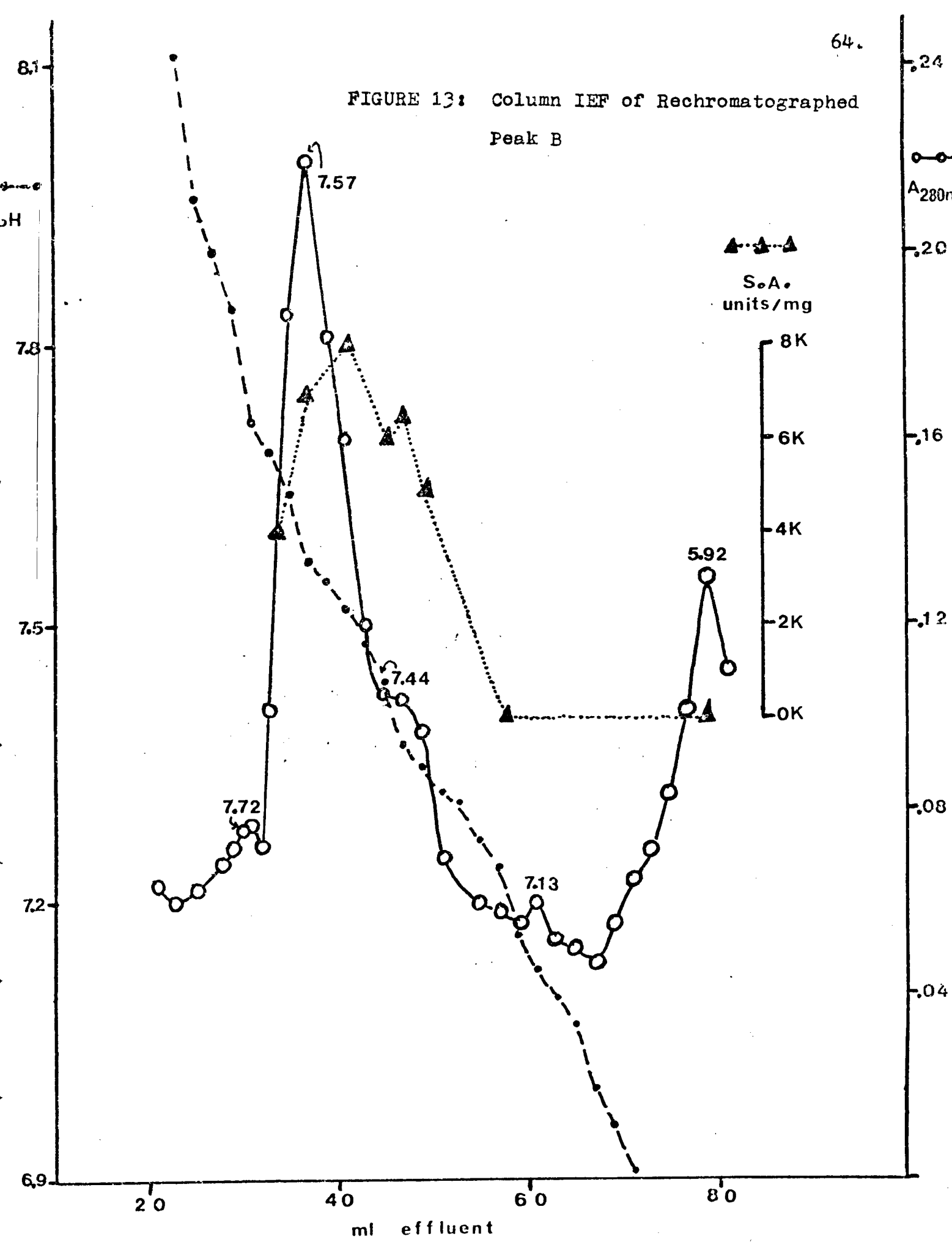
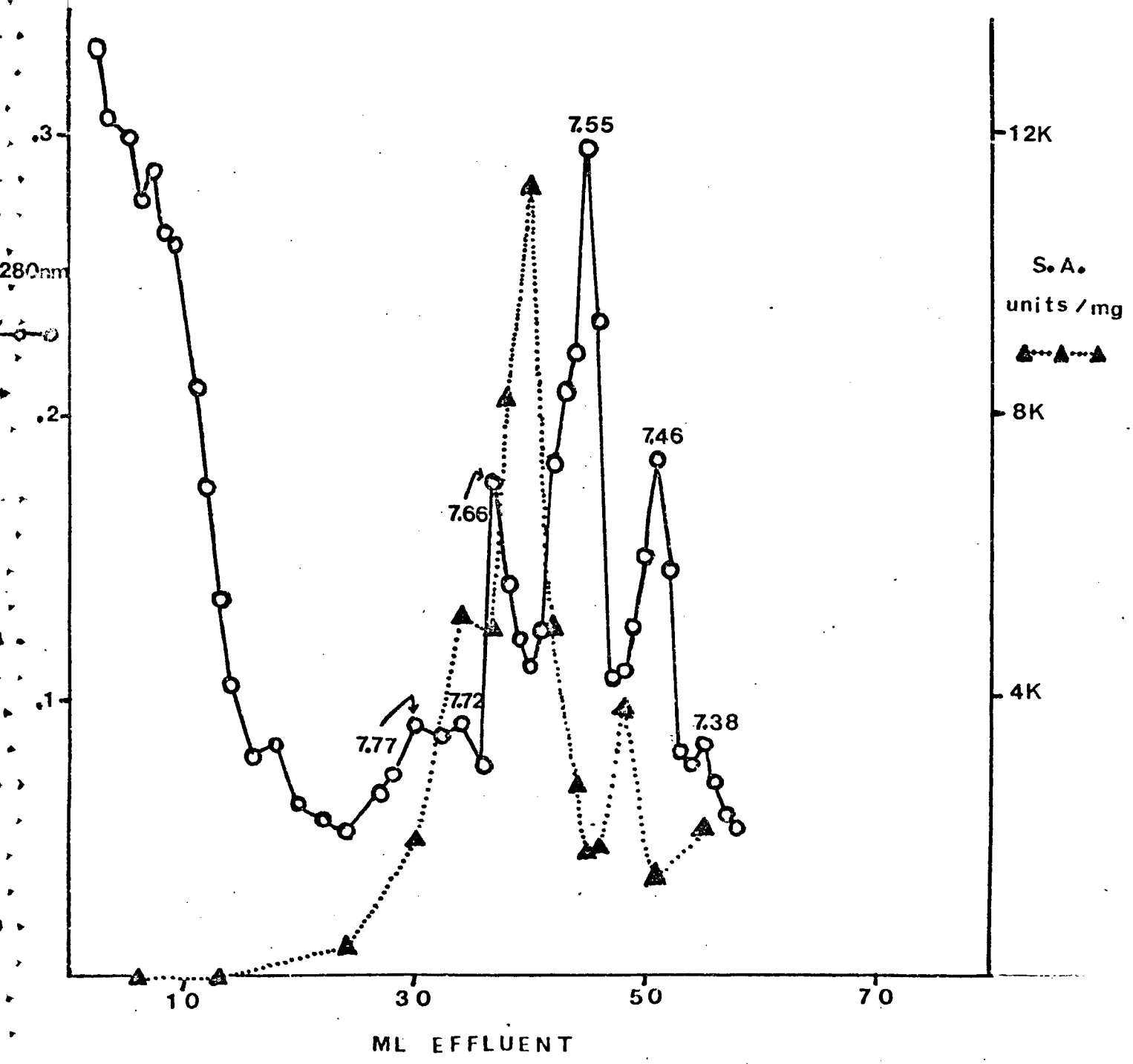


FIGURE 14: Column IEF of Rechromatographed Peak A and Peak B



to the three major peaks visible by determining the absorbance of fractions at 280nm.

The results of the column IEF indicated the presence of three proteins with triose phosphate isomerase activity: the first with a pI of about 7.65 (separable chromatographically into Peak A) and the second and third with pI's of 7.56 and 7.45 respectively isolated chromatographically together in Peak B. There is approximately 0.1 pH units difference between protein I (pI=7.65) and protein II (pI=7.56) and between protein II and protein III (pI=7.48).

The separation of the three proteins was further demonstrated in the gel IEF experiment which was performed using the narrow range ampholytes, specially prepared by 5% column IEF runs. The results illustrated in the gel scans of figure 15 indicates the focusing of twice chromatographed Peak A into a single component with pI=7.64, twice chromatographed Peak B into three components of pI's equal to 7.66, 7.56 and 7.49, and finally the focusing of Peak A and Peak B into three components with pI's of 7.64, 7.56,7.46. The photograph of the gels (fig. 16) demonstrates the clarity of the separation of the three proteins. Minute traces of bands other than the major three proteins are slightly visible in the photograph but are not visible at all in the gel scans which indicates that contamination is not present to any significant extent.

The presence of the three triose phosphate isomerase active proteins as observed by the IEF experiments, suggests very strongly three TIM isozymes.

FIGURE 15: Gel Isoelectric Focussing of Peak A and Peak B

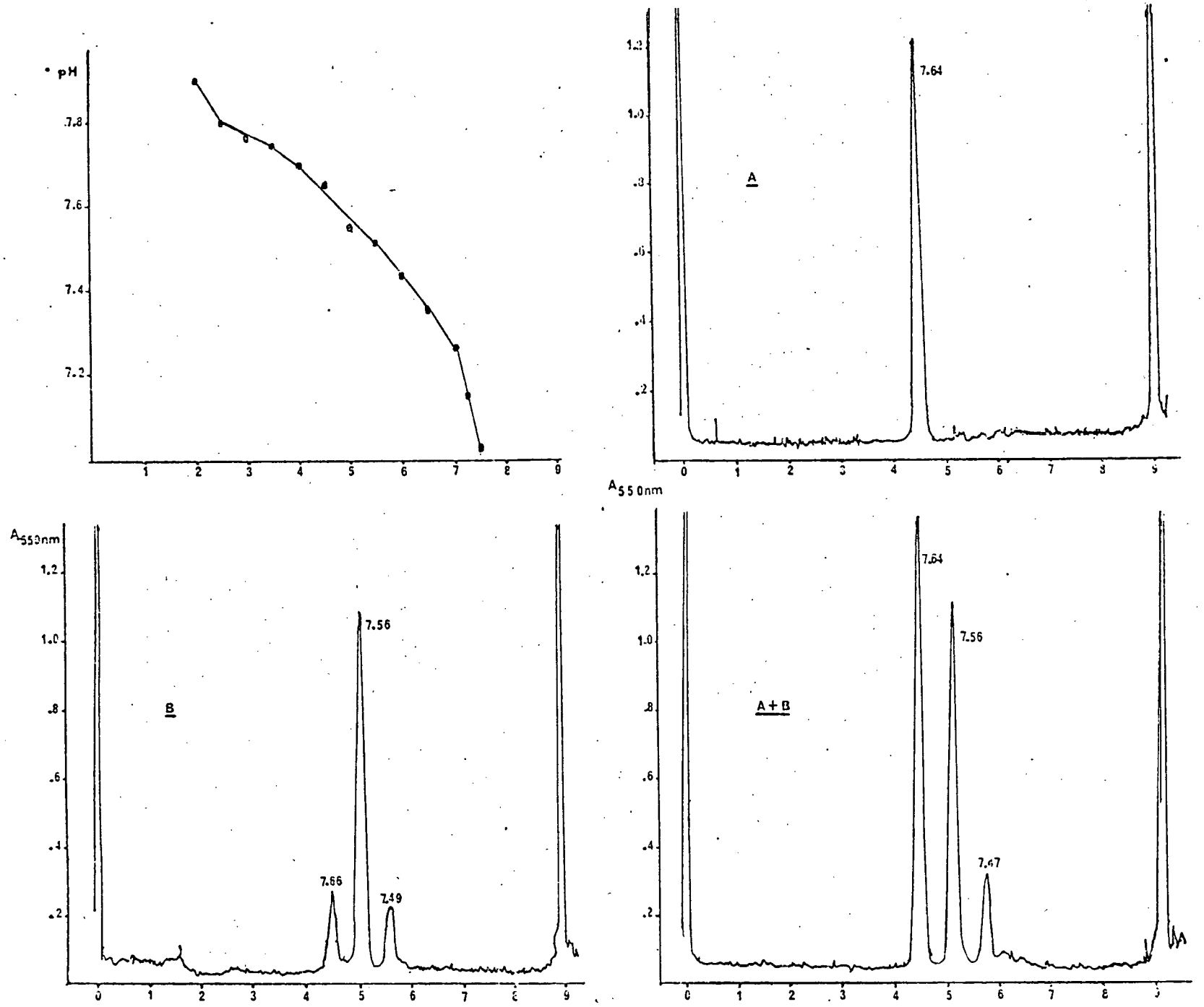
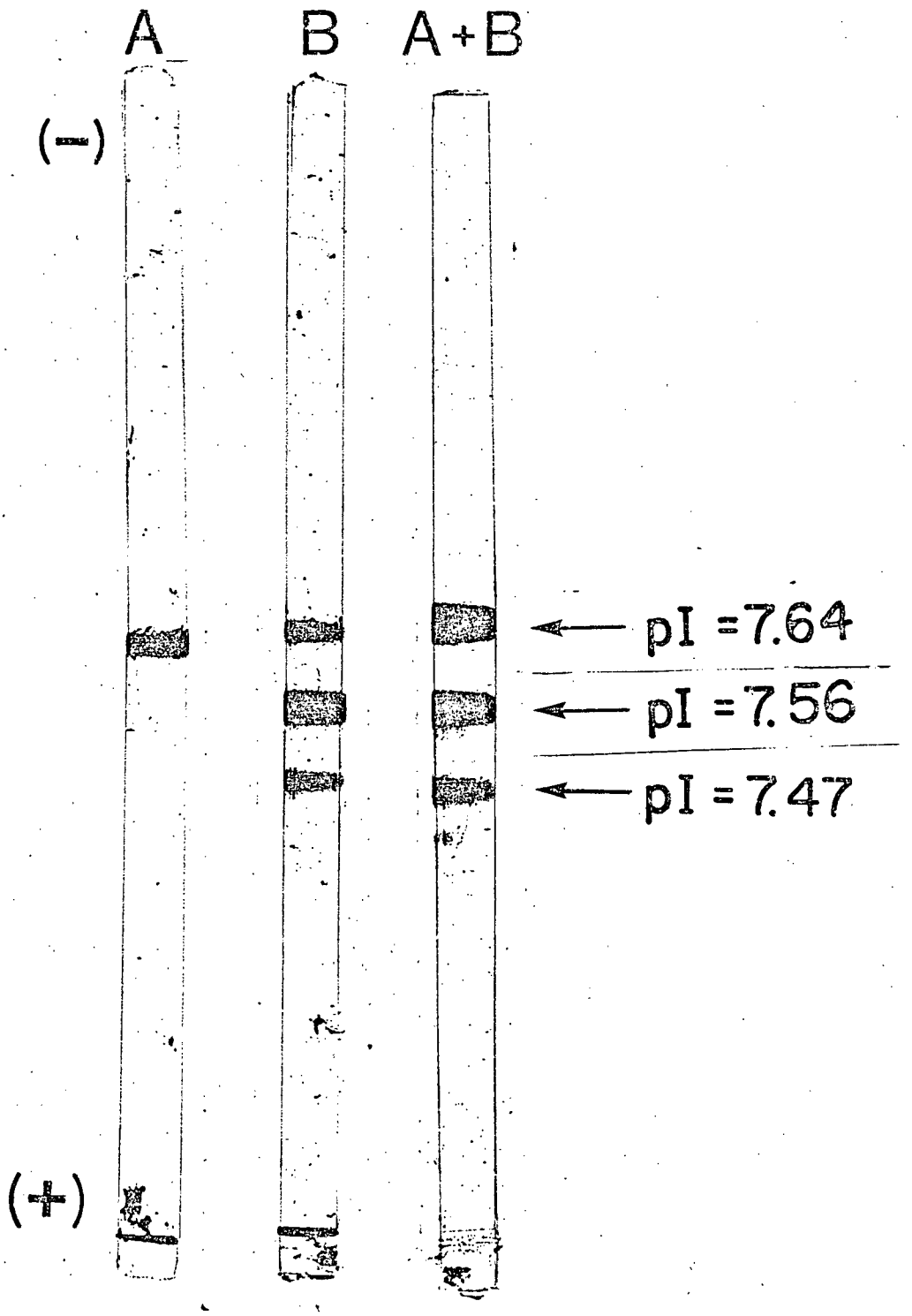


FIGURE 16: Sample Gels of Gel Isoelectric Focussing of Peak A and Peak B



The presence of more than one enzyme form for chicken breast muscle TIM has an evolutionary basis. Gracy et al has has published results giving the electrophoretic molulity from starch gel electrophoresis of triose phosphate isomerases from various tissues. He also indicated the number of isozymes present. All vertebrates reported possess three isozymes. It was necessary to go to the evolutionary level of a crab to observe two isozymes and of a beetle to observe one. It would therefore be inconsistent if only one molecular form of TIM was found in chicken breast muscle.

On the basis of the isozymic forms of other vertebrate triose phosphate isomerase (eg: human and rabbit), as well as the pI results, it is possible to tentatively suggest the basis of the chicken isozymes to be a result of two protein chains α and β. The presence of 2 distinct chains α and β would give the possibility of 3 isozymes: α_2, $\alpha\beta$, and β_2. Heterogeneity of subunits has been observed in other triose phosphate isomerases (6,24). The relative stabilities of the three forms as well as the actual relative quantities of α and β chains would determine the ratios of α_2 enzyme to $\alpha\beta$, and to β_2. With the data available at this point a ratio of about 80:6:1 for α_2:$\alpha\beta$:β_2 may be calculated if the major enzyme form present in Peak A (pI= 7.64) is assigned the α_2 designation and the two minor isozymes in the Peak B are assigned $\alpha\beta$(pI=7.57) and β_2 (pI=7.49) designations respectively.

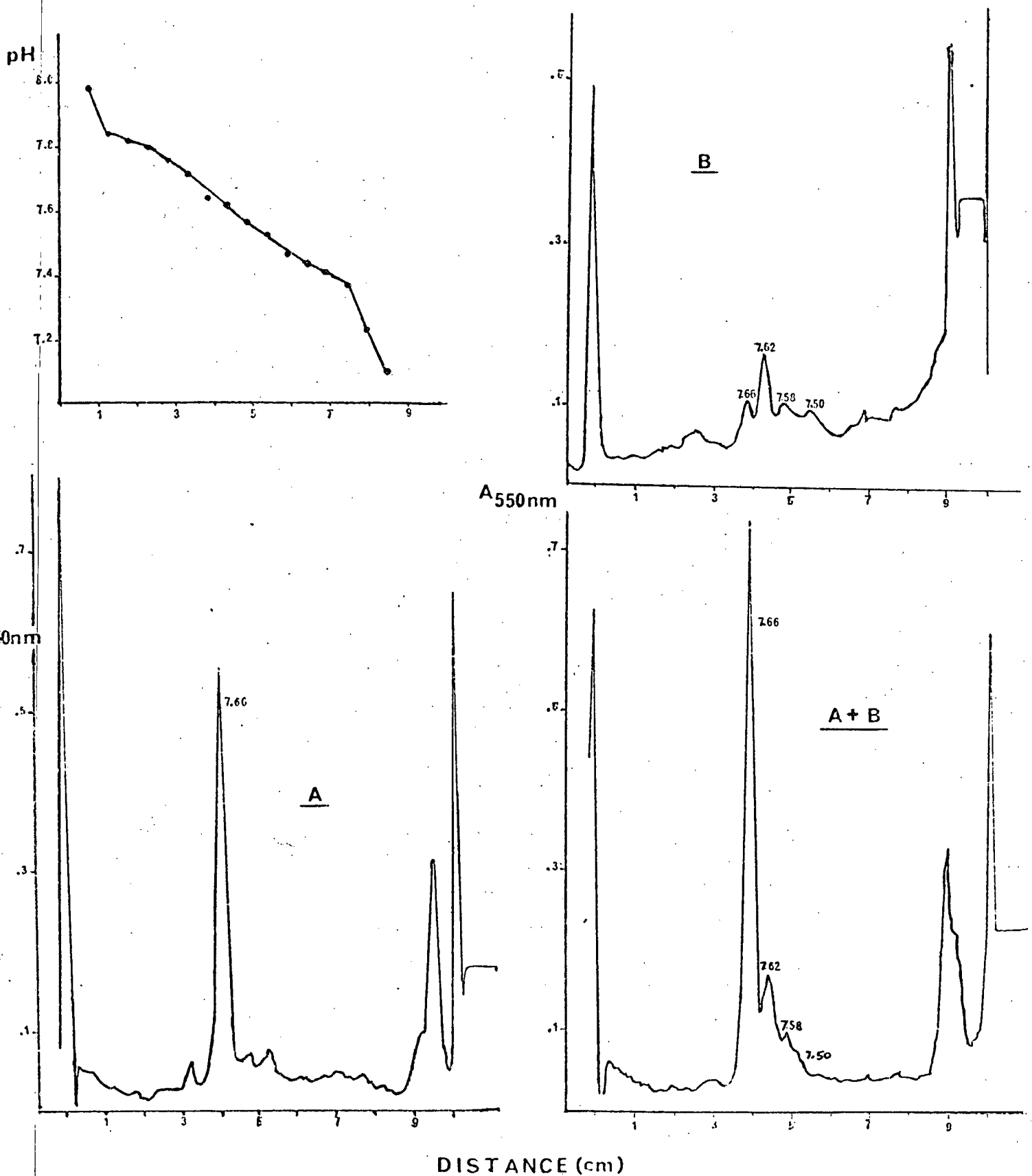
The two protein chains could have some genetic origin

(ie. two separate genes) or it is possible that they arise from some epigenetic process (ie. post translational changes in the actual protein itself which may occur in the 'in vivo' situation). The first possibility would result from the presence of two distinct genes (possibly arising from mutation at one or more sites) which code for protein chains of differing amino acid sequence. The second possibility of epigenetic processes would involve some sort of chemical modification of the protein occurring in the cell itself. A well known example of this is the formation of proteolytic enzymes from pro-enzymes.

The presence of a fourth enzyme form which has no 'in vivo' basis was observed when another gel isoelectric focusing experiment was performed which involved the focusing of fresh twice chromatographed Peak A with older (about 1 year) twice chromatographed Peak B (fig. 17). The B protein used, still possessed at least 50% of its initial specific activity. The focusing of the A protein was similar to that obtained in other IEF experiments (fig. 15) with a pI=7.66 found for the major protein zone. However the B protein was now found to contain 4 proteins of different pI: 7.66, 7.62 (not observed previously with fresh B protein), 7.58 and 7.50. Three peaks were accounted for by comparison to the gel IEF runs of fresh A and fresh B protein: protein I (pI=7.66), protein II (pI=7.58) and protein III (pI= 7.50). The presence of a peak with pI=7.66 in the B protein probably indicates contamination with A protein.

The protein with pI=7.62 (from Peak B protein) was a new occurrence. There was no trace of it when fresh B protein was

FIGURE 17: Gel Isoelectric Focussing of Fresh Protein from Peak A and Old Protein from Peak B



used (fig. 15). While the pI=7.62 protein has taken over as the dominant protein in the B peak, the protein with pI=7.58 has diminished in quantity relative to the other B peak proteins. It would seem likely that, with age, there is a change in the pI=7.58 protein to give an altered protein with pI=7.62.

The appearance of the pI=7.62 protein after a considerable length of time and not in the original fresh preparation of B, makes it clear that the 7.62 form has no genetic origin but rather arises from a modification of existing protein. Modification of reactive carboxyl, amino, or hydroxyl groups in the 'in vitro' as well as 'in vivo' situation may occur with a concomittant change in pI. Some of the most likely changes would be loss of NH_3 from asparagine or glutamine as well as SH oxidation (of cysteine) to -SOH, $-SO_2H$, or $-SO_3H$. Replacement of even a single carboxyl group in hemoglobin has been known to cause distinct changes in its mobility (67). There is also the possibility that with time, the conformation of the protein has changed, with a resultant alteration in the exposure of charged amino acid side chains and hence a change in its movement in an electric field and in its pI.

D. Amino Acid Analysis

The amino acid data for 1 subunit is given below, along with the published values (17).

TABLE IV: Amino Acid Analysis of Peak A TIM

Amino acid	2X chromatographed TIM-Peak A	Literature value
cys	4.01	4.0
arg	7.40	7.5
meth	1.98	2.0
tyr	3.80	3.9
his	7.32	7.6
asp	16.52	20.0
val	17.29	24.4
ileu	14.42	16.6
lev	16.00	17.0
phe	7.00	7.8
pro	7.95	8.9
lys	21.28	23.1
ala	25.18	28.2
gly	23.90	27.0
glu	24.90	25.8
ser	10.94	13.5
thr	10.26	10.2
trp	5.02	5.0
TOTAL (nearest whole integer)	225	253

A molecular weight of 48,064 was calculated for the Peak A, (α_2 isozyme) triose phosphate isomerase. This is significantly lower than the published literature value of 54,400 obtained from the above (literature) amino acid data. There are significant differences in aspartic acid and valine amino acid numbers with much smaller deviations apparant in isoleucine, leucine, phenylalanine, proline, lysine, alanine, glycine, glutamic and serine residues. The leucine, phenylalanine, proline, and glutamic acid residues deviate by only 1 residue which could be within the error of the experiments.

Amino acid data from the other two isozymes will be necessary before any assumptions can be made concerning the amino acid differences between the three protein forms.

CHAPTER IV

PROTEIN MODIFICATIONS

4.1 Introduction

Protein modification is a strategy used by the biological protein chemist to probe the structure of a protein. In the case of enzymes, the effect of modification upon the active site, and hence the catalytic capabilities of the system, can give valuable information concerning the enzymatic mechanism. For example, in the case of triose phosphate isomerase, considerable interest has been focused on the modification of an enzymatically essential glutamic acid residue. This is covered in some detail in the introduction to this thesis.

Possible sites of modification in any protein include the sulfhydryl group of cysteine, the imidazole group of histidine, hydroxyl group of serine, the ϵ-amino group of lysine, the ω-carboxyl group of aspartic and glutamic acids and the phenolic group of tyrosine.

The sulfhydryl group of cysteine has attracted attention due to its high nucleophilic and redox reactivity and its ability to enter into characteristic and selective reactions. The specificity of the modification is important for both the analytical determination of numbers of sulfhydryl residues present as well as a structural probe of the protein. Therefore it is fortunate that the protein sulfhydryl group of cysteine has been found to be very reactive to many reagents.

The high nucleophilicity of mercaptide ions is given by the

particular electron structure of the sulfur atom with its high polarizability. The thiolate anion is considered to be one of the strongest biological nucleophiles; in addition to the polarizability of the sulfur electrons, there are empty d-orbitals, permitting d-orbital overlap and thereby increasing nucleophilicity . For these reasons, cysteine is a good choice for modification of proteins.

The SH group of cysteine takes part in most reactions in the form of the mercaptide anion (RS^-). It was calculated by Benesch and Benesch (68) that at pH 7.4, (physiological pH) 6% of the SH's of free cysteine were ionized. In the active sites of enzymes, it has been found that the pK of cysteine may vary from 7 to 9. Examples include the active site SH of phosphoenolpyruvate carboxykinase with a pH of 7.3 (69) and ficin which has been found to include a cysteine residue with pK of 8.55 (70) in its active site. Microscopic environments which cause this variation include proximity to positive charges (pK_{SH} decreases) and negative charges (pK_{SH} increases). Ionization of sulfhydryls are particularily depressed when the cysteine is in a hydrophobic microenvironment, buried within the protein. The pK's in this case are commonly found to be above 9.

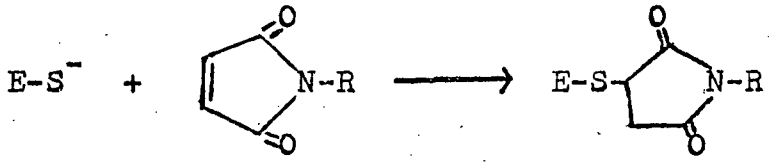
The protein chemist's interest in cysteine is a result of the significance of the SH group for specific functions of a number of enzymes, hormones and other biologically active proteins which play a central role in the normal course of many physiological processes, as well as their exceptional reactivity.

As a biologically active functional group, the sulfhydryl has been known to be responsible for noncovalent binding of substrates and cofactors, direct covalent participation in the catalytic act and maintenance of the native catalytically active conformation of an enzyme.

With the biological importance of cysteine in mind as well as the potential role of chemical sulfhydryl modifications in elucidating protein structure and function, it is well to note the high reactivity and diversity of chemicals reactions which distinguishes the SH group: alkylation, acylation, oxidation, thiol-disulfide exchange, reactions with sulfenyl halides, and the formation of mercaptides, hemimercaptols and mercaptols. The following is a short listing of some of the reaction classes along with examples of reagents.

Reaction Class	Reagent	Comment
1. Transition Metals	CoII, NiII, CuII, HgII	mercury complexes among the most stable
2. Oxidation	H_2O_2	in absence of metals, fairly specific for cysteine and methionine
3. Nucleophilic Addition	N-ethyl maleimide	vary from reversible condensations with aldehydes and ketones to fairly irreversible reactions with maleimide
4. Displacement	a) haloacetol phosphates b) DTNB	highly specific, resulting in stable products

The alkylating labels include some of the more reactive reagents although the problem does exist of the resulting thiol product being quite unstable in the aqueous environment and hydrolyzing. However, one class of labels, maleimides, usually have considerable success in forming a stable covalent linkage with cysteine. The reaction itself involves addition of the sulfhydryl to an activated double bond:

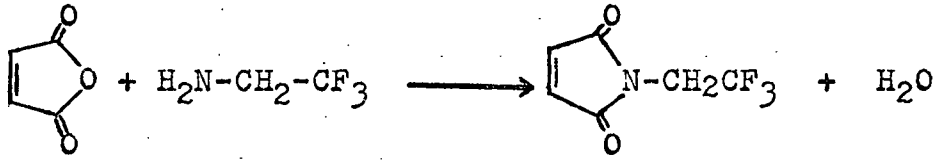


This Michael-type conjugate addition is irreversible and goes rapidly in alkaline media. There is an enhancement of rate as pH increases since the mercaptide anion of cysteine is the most reactive species.

An important competing reaction could be the addition of the ϵ-amino groups of lysine or the imidazole group of histidine to a maleimide via an analogous mechanism. The specificity of the reagent for cysteine is maintained by keeping the pH at or below pH 7.0 at which point the reaction of ϵ-amino groups (or imidazole) is insignificant in the time period required for titration of the SH groups which is usually under an hour. At pH 7.0, the rate of reaction for simple thiols is on the order of 1000 times faster than for simple amines (71). Therefore, maleimides can be highly specific reagents although there is always the possibility that the maleimide may react with some residue other than cysteine which possesses increased reactivity as a result of its particular environment in the protein.

A widely used maleimide is N-ethyl maleimide (NEM) which is employed as a sulfhydryl reagent. NEM absorbs strongly at around 300nm (ε=620$M^{-1}cm^{-1}$) which allows one to follow the reaction of the label by observing the decrease in absorbance at 300nm as the reaction proceeds. This reagent was found to be suitable for chemical modification of triose phosphate isomerase, the results of which are reported in this section.

A fluorine containing analogue of NEM was synthesized by D.G. Clark for this work:

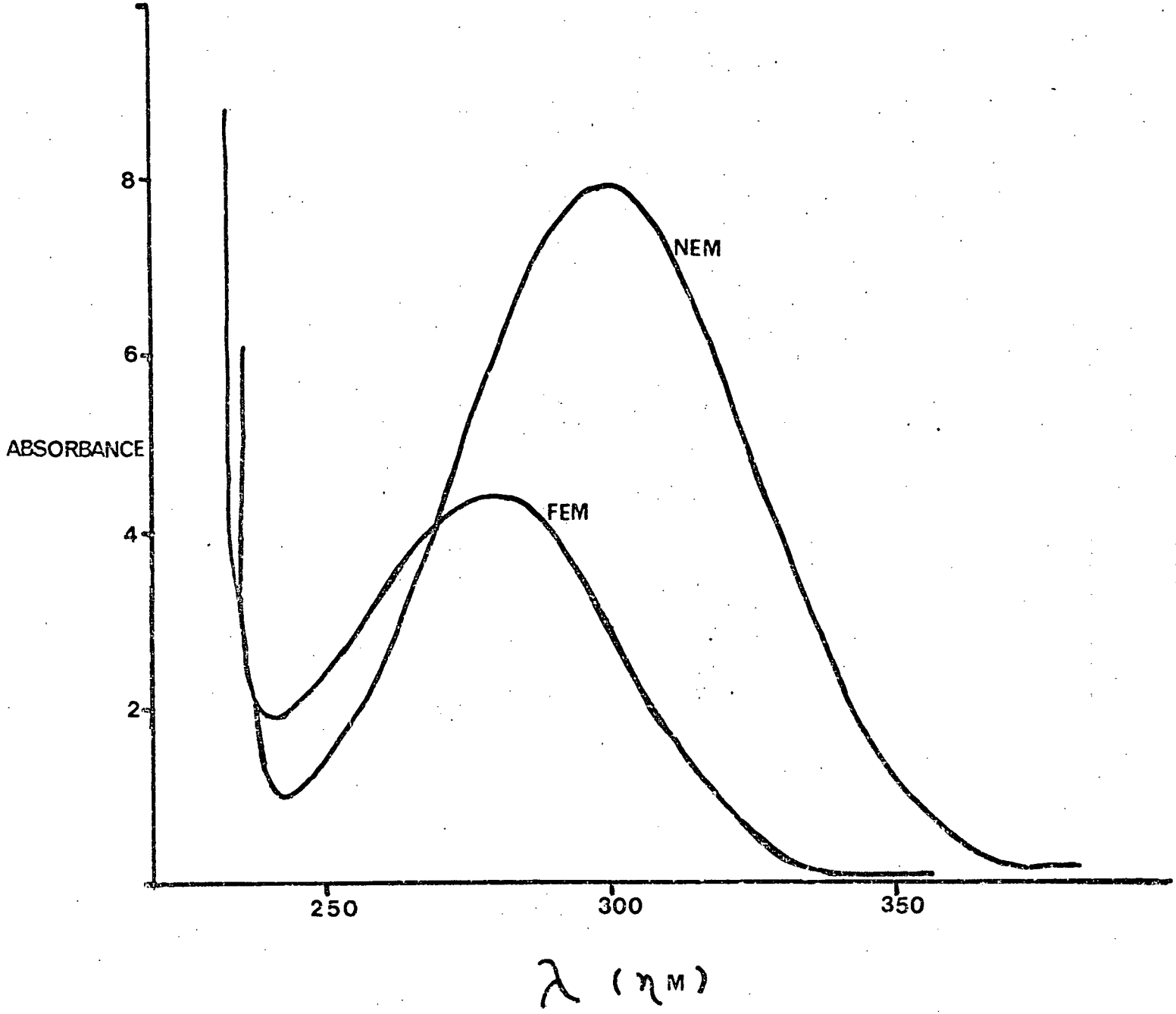


The resulting trifluro-N-ethyl maleimide (FEM) has a broad maximal absorbance centered at about 280nm (ε=390). This 280nm λ_{max} makes direct spectrophotometric observation of SH modification by FEM difficult since there is a strong interference with protein absorbtion (and hence problems with blanking). The values obtainable by the direct spectrophotometric observation have a possibility of about 10% error.

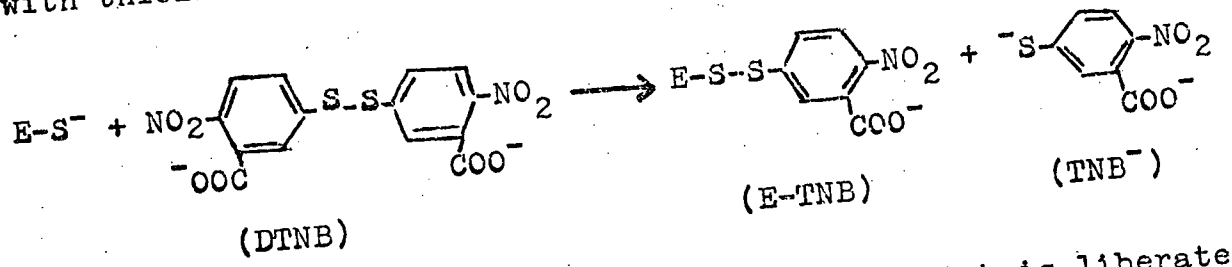
Figure 18 illustrates the ultraviolet spectra of both NEM and FEM. The electron withdrawing effect of the fluorine atoms appears to activate the malieimide double bond even more strongly resulting in a more facile addition to cysteine than the hydrogen analogue, NEM.

The FEM modifications reported here have a special interest in light of the reagent's potential as a NMR label.

FIGURE 18: Ultraviolet Spectra of NEM and FEM



Modification of triose phosphate isomerase was also carried out using SH-disulphide interchange methods based on reactions with disulfides which are considered to be among the most specific reagents for protein SH groups. A particularily effective reagent which has been used in this work is Ellman's (72) reagent, 5,5'-dithiobis (2-nitrobenzoic acid) which reacts with thiols as follows:



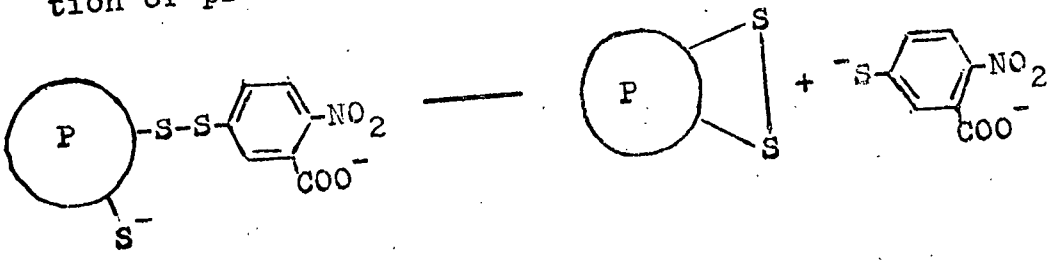
(DTNB) (E-TNB) (TNB^-)

The strongly colored thionitrobenzoate anion which is liberated may be quantated by its absorbance at 412nm ($\varepsilon = 1.36 \times 10^4 M^{-1} cm^{-1}$ at pH 8.0).

However, it must be realized that with some proteins (73, 74, 75), two reactions are possible after some SH's have reacted:

(1) the normal intermolecular reaction of the first SH's (as above)

(2) an intramolecular reaction of SH with the mixed disulfide product which results from the reaction of protein -SH with DTNB.



Whether or not the intra molecular reaction succeeds in competing successfully with the initial intermolecular reaction, the end result is the same: release of one equivalent of nitrothiopholate anion (TNB^-) for each SH group that reacts.

The absorbance of the reaction products at 412nm is highly dependent upon pH. Consequently, there is a limit to the range of pH's which may be used with DTNB (usually pH 7.5-8 only). In addition, there is a problem with autoxidation of the nitrophenolate anion as well as difficulties in using the reagent with colored proteins (eg. heme containing proteins) which absorb strongly at 412nm.

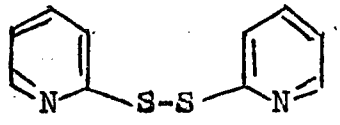
Some of the difficulties may be gotten around by following Butterworth's procedural changes (76): the protein is first modified with DTNB, then isolated and finally reacted with dithiothreitol (DTT). The DTT very rapidly liberates TNB^- from the protein and allows determination of the number of SH's by observing the increase in absorbance at 412nm. This method may not be used for those proteins which are able to undergo the intramolecular disulfide formation and resultant elimination of TNB^-. However, when this method is used, it is possible to obtain values as correct as by the method initially described although it is not possible to observe the kinetics of the modification of the protein with DTNB.

Another method of liberating TNB^- is by displacement with cyanide:

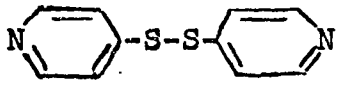
$$E\text{-}S\text{-}(TNB) \xrightarrow{(CN^-)} E\text{-}S\text{-}CN + TNB^-$$

This reaction also has the advantage of allowing the protein to become labelled with C^{13} via $C^{13}N^-$ or C^{14} via $C^{14}N^-$. The $C^{13}N^-$ protein could be used in CMR studies while the $C^{14}N$ protein would make the modified protein radioactive.

A second aromatic disulfide was used in the modification studies to be described. There are two analogues of the dithiodipyridine disulfide described by Grassetti and Murray (77) possible:



(1)



(2)

The 4,4'dithiopyridine(2), is the most sensitive reagent since upon reaction with a SH group it releases a 4-thiopyridone which has as extinction coefficient of 19,800 at 324nm which is considerably larger than the maximum molor extinction coefficient of 7060 at 343 for the 2-thiopyridone. The 4,4' reagent was used exclusively:

(4-PDS) (4-TP)

The pyridine disulfides have the advantage of being able to be used over a much wider pH range than DTNB. It is particularily useful for the lower pH's, where DTNB cannot be employed, since the diminishing level of $E\text{-}S^-$ is compensated for by the increasing reactivity of the reagent. The electron withdrawing properties of the pyridine ring (and hence the reactivity of the disulfide) becomes stronger with protonation of the nitrogen.

4.2 Methods

A. Modification with Maleimides

(a) N-ethylmaleimide (NEM)

Approximately 20 mgs of twice chromatographed triose phosphate isomerase from Peak A in a 100mM pH 6.5 phosphate buffer was gently stirred overnight in the cold with 25mM dithiothreitol (DTT) to ensure complete reduction of cysteine residues. The protein was desalted by passing it down a Sephadex G25 column (1.5X20cm) equilibrated and eluted with the 100mM pH 6.5 phosphate buffer. Approximately 2 ml fractions were collected on a Gilson microfractionator and the absorbance at 280nm for each fraction was determined. A fraction with A_{280} equal to 8.02 was selected for the modification and kinetic study.

A Zeiss PMQ II visible-UV spectrophotometer was set up for constant temperature runs at 20°C. The reaction was initiated by placing 1 ml of protein into a 3 ml cuvette containing 2 mls of a N-ethyl maleimide solution in the pH 6.5 buffer. The maleimide solution was of such a concentration as to give 20X molar excess of maleimide over protein; final concentrations were 41.8 μM in protein and 850 μM in NEM. A reaction blank consisted of 2 mls of the NEM solution and 1 ml of buffer. The modification of the protein was monitored by following the change in absorbance at 300nm as a function of time. (fig. 21) A decrease was observed until a constant value was obtained in less than 1 hour.

(b) Trifluoro-N-ethyl maleimide (FEM)

Peak A TIM was prepared for chemical modification as was

the protein in the NEM experiments. However, either pH 6.0 or pH 6.5 100mM phosphate buffer was used for the modification reaction conditions. When pH 6.0 buffer was to be used in the modification, the protein was stirred overnight in pH 7.0 buffer so as to ensure the stability of the protein. In all other cases, the protein was reduced at the same pH as the modification was carried out.

In the first series of experiments (pH 6.0), 70 mgs enzyme was allowed to react in the cold with 20X excess FEM. The modified protein was desalted on a Sephadex G25 column, eluted with pH 6.0 100mM phosphate buffer and those fractions with an absorbance at 280nm over 1.0 were kept for sulfhydryl determination with DTNB. The DTNB reaction was initiated by adding 1 ml of enzyme containing about 1 absorbance unit protein to a 3 ml cuvette containing 2.0 ml of the reagent stock solution containing 100mM phosphate pH 8.0, 10mM EDTA and 5.25mM DTNB. The blank consisted of 2.0 mls of stock reagent and 1.0 ml of pH 8.0 100mM phosphate buffer. The reaction was followed by observing the increase in absorbance at 412nm until a constant value was observed after 2 hours.

In the same experimental series, an aliquot of the FEM modified protein was stirred in DTT overnight (25mM) and then reacted with DTNB.

A similar series of experiments was performed but with the FEM modification at pH 6.5. Both the reduction of the unmodified protein and its desalting on the Sephadex G25 column were performed at pH 6.5. The protein was modified under 20X molar

excess reagent conditions and then the modified protein was desalted on a pH 8.0 100mM phosphate conlumn. The DTNB determination proceeded as before but made use of a reagent stock solution containing 1% sodium dodecylsulfate (SDS) in addition to the 100mM phosphate pH 8.0, 10mM EDTA and 5.25mM DTNB.

An attempt was made to follow the kinetics of the FEM modification by observing the decrease in the absorbance at 280nm as the protein reacts with trifluoromaleimide at pH 6.5. Thus, 0.123 μmoles of TIM was allowed to react with 2.065 μmoles FEM, complete reaction taking place in under 30 sec. Thus the modification was too rapid (and would necessitate use of a stop-flow spectrophotometer) to follow using normal spectrophotometric techniques. However, it did allow a direct determination of the number of SH's which had reacted with FEM.

The specific activity of the NEM and FEM modified protein was determined using the TIM assay procedure described earlier. Protein which had been modified by NEM as above at pH 6.5 was desalted on a Sephadex G25 column which was eluted with 100mM pH 7.5 triethanolamine buffer, the appropriate dilution was made, and the activity was determined. The same procedure followed the FEM modification experiment. Activity measurements were also performed on a control of unmodified Peak A protein after DTT reduction.

B. Modifications with Disulfides

(a) 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)

Twice chromatographed Peak A protein was reduced overnight in the cold with 25mM dithioerythretol (DTE) or DTT in pH 7.0 100mM phosphate, and then desalted on a Sephadex G25 column eluted with 100mM phosphate pH 8.0 buffer. Eluted fractions (~2mls) of A_{280} greater than 0.8 were used for the SH determination. The reaction was initiated by addition of 1.0 ml enzyme solution to 2.0 mls of stock reagent (100mM phosphate pH 8.0, 10mM EDTA, 5.25mM DTNB) in a 3 ml cuvette. The reaction cell was blanked against a cuvette containing 1.0 ml buffer and 2.0 mls reagent. The reaction was followed via an increase in the absorbance at 412nm until a constant value was obtained after approximately 2 hours. Upon completion of titration of the 'exposed' SH's, a small quantity (~10 mgs) of solid SDS was added to the reaction cell. Within one minute, the protein was completely denatured, thus facilitating the reaction of the 'buried' SH's. The final absorbance at 412 was determined giving a value for total protein sulfhydryl. The above procedure was repeated for twice chromatographed Peak B protein.

Experiments involving the displacement of TNB^- were performed on twice chromatographed Peak A protein which had been modified with DTNB as before except that reaction was carried out in a 37°C water bath instead of room temperature in order to decrease the reaction time. The modified protein was desalted on a Sephadex G25 column (1.5X20 cm) eluted with a pH 7.5 triethanolamine buffer. Approximately 4 mls of A_{280}=0.181 protein sample was

obtained. One half of the protein (ie. 2 mls) was placed in a cuvette and 1 mg of dithioerythreitol was added. Liberation of TNB^- (as observed by an increase in absorbance at 412nm) was almost instantaneous. The second half of the protein sample (ie. 2mls) was also placed in a cuvette and a crystal of KCN (1 mg) was added. The reaction cell holder in the spectrophotometer was thermostated at 20°C. Liberation of TNB^- was again observed by following the increase in absorbance at 412nm. The dispacement by CN^- was slower than that by DTE and data for a kinetic analysis was collected.

(b) 4,4' dithiopyridine

The modifications of TIM with the second disulfide, 4,4'-dithiopyridine were performed in a similar manner to the DTNB reaction except that pH 6.5 was chosen for the reaction condition. As before, the protein was reduced overnight (in the cold) in 25mM DTE or DTT in preparation for the chemical modifications. Desalting of the reduced, unmodified protein was performed on a Sephadex G25 column (1.5X20 cm) eluted with pH 6.5 100mM phosphate buffer. Fractions containing protein with A_{280}=1.0 were employed in the experiment. The reaction was initiated by placing 50 µls of stock reagent (26 mgs of 4,4'dithiopyridine per ml) delivered via a Lang-Levy pipet into a 3 ml cuvette containing 1.95 mls of pH 6.5 100mM phosphate buffer and 1.0 ml enzyme. The modification was observed by following the increase in absorbance at 324nm until a constant value was obtained, usually less that 2 hours. A determination of total sulfhydrals was

accomplished by adding a small amount of solid SDS at this point and observing the increase in A_{324}.

A 4,4'dithiopyridine titration at pH 6.5 was also performed on protein which had been modified by DTNB at pH 8.0. The unmodified Peak A protein was reduced in the usual way, desalted at pH 8.0, reacted with DTNB (in the cold for several hours), desalted at pH 6.5 and finally allowed to react with the pyridine disulfide. Upon completion of the titration of reactive groups, SDS was added and an additional increase in the absorbance at 324nm was observed.

A series of specific activity measurements of the various modified proteins was performed. In all cases, the proteins were modified under the conditions described previously. The protein samples included:

(1) Peak A TIM modified with DTNB

(2) DTNB modified protein which had been allowed to react with CN^-

(3) DTNB modified protein which had been allowed to react with DTE

(4) 'control' sample of unmodified protein

4.3 Results

A. Maleimides

(a) N-ethylmaleimide

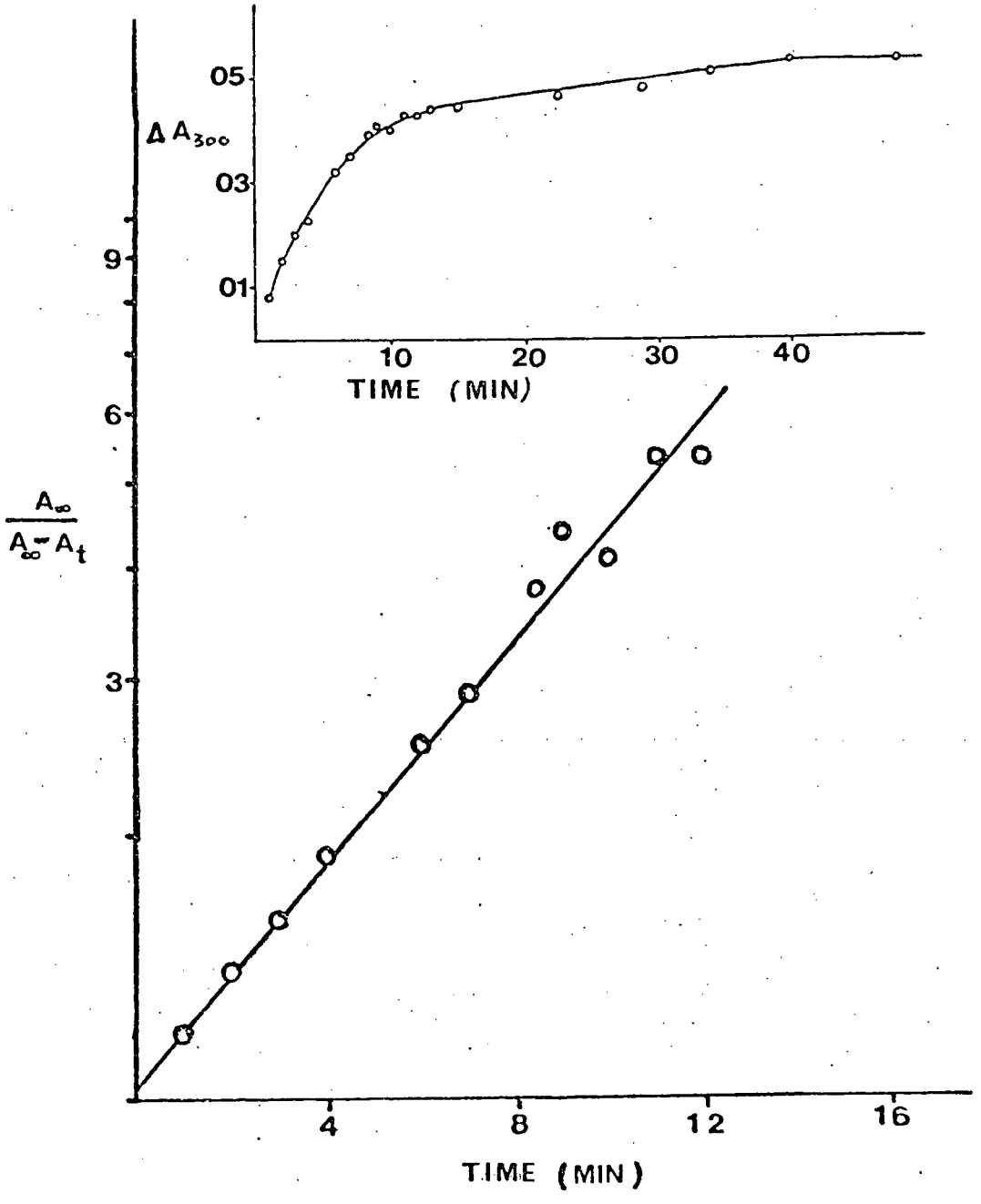
Figure 19 illustrates the pseudo first order modification of triose phosphate isomerase with N-ethylmaleimide. Data points were plotted to the third half life and a least squares technique was employed to fit the best straight line to the data. A pseudo first order rate constant of 2.39×10^{-3} sec^{-1} was calculated (20 C).

A value of 2.05 was calculated for the number of sulfhydryls modified. At the pH of the experiment (6.5) it is improbable that any residues other than cysteine was being modified. Since TIM is a dimer, the near integral value of 2 suggests that one cysteine per monomer is exposed to attack by NEM when the protein is in a native state. However, it should be realized that there is a possibility that the conformation of the protein is such that 2 cysteine residues are exposed on one monomer but none on the other. In view of the fact that recent x-ray work on chicken muscle TIM demonstrated a symmetrical molecule (18) this appears unlikely.

The kinetic analysis demonstrates that both SH's appear to be reacting with NEM at the same rate. This is in contrast to results which will be reported in the last chapter with kinetics of disulfide modification. With the data available from these experiments, it cannot be decided with any certainty which cysteines are being modified by NEM.

FIGURE 19: Pseudo First Order Analysis of the Modification of TIM with NEM

Insert: Increase in the Absorbance as the Reaction Proceeds



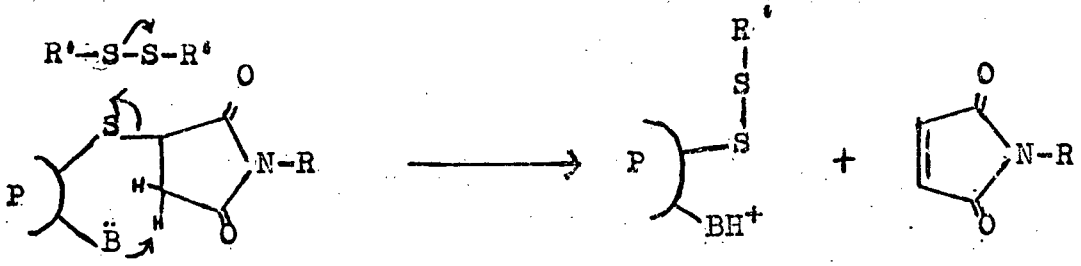
(b) Trifluoro N-ethylmaleimide (FEM)

Peak A triose phosphate isomerase, when modified at pH 6.0 or 6.5 by FEM, was found to also be able to react with DTNB. It will be shown in later segments of this section that both FEM and DTNB are able to modify 2 cysteine residues of TIM. Therefore, if one assumes that the same residues are being modified by a FEM modification or a DTNB modification, it is necessary to account for the type of reaction occurring if DTNB is shown to react with protein which is already modified by FEM. In this case protein which had been modified by FEM at pH 6.0, and hence had 2 residues react was observed to have 1.10 residues exposed to reaction with DTNB under nondenaturing conditions and a total of 7.28 exposed to reaction under denaturing conditions. The difference, 6.28, probably denotes the number of residues which are buried and not exposed to reaction with either DTNB or FEM. This value is similar to the value of 6 'hidden' SH's usually obtained by DTNB titrations alone (see disulfide modification segment of this section).

When the FEM modified protein was allowed to stir overnight in DTT and was then assayed with DTNB, similar results were obtained : 1.01 residues reacted under nondenaturing conditions. The difference in this case is 5.67 which is still quite close to the integral value of 6 usually obtained for a titration of hidden SH's by DTNB. If the FEM modified protein is denatured by SDS, 5.77 residues were found to react with DTNB, which again denotes a value close to 6 for the number of hidden residues.

If it is again assumed that FEM and DTNB attack the same residue, a mechanism for displacement of the FEM by DTNB must be given. Under normal circumstances, maleimide modifications are considered to be irreversible.

However, a mechanism such as the following could explain the results obtained in this particular modification of TIM:



The general base (B:) could well be a basic amino acid side chain in the vicinity of the FEM modified site which is uniquely set up to accept a proton from the maleimide.

The basic pH (8.0) of the DTNB modification makes the possibility of basic residues being in the unprotonated form more likely.

When the protein which had been modified by FEM was allowed to react with DTNB in nondenaturing solution first and then in denaturing solution, the total number of equivalents of DTNB averaged out to about 7 (one residue reacts in solution without SDS, the remaining six react when SDS is added). However, when the modified (FEM) protein was denatured before addition of DTNB, only 5.77 residues were found to react. This gives support to a maleimide displacement which is assisted by a strategically placed basic amino acid side chain. The displacement of maleimide appears to not be able to occure if the protein is denatured.

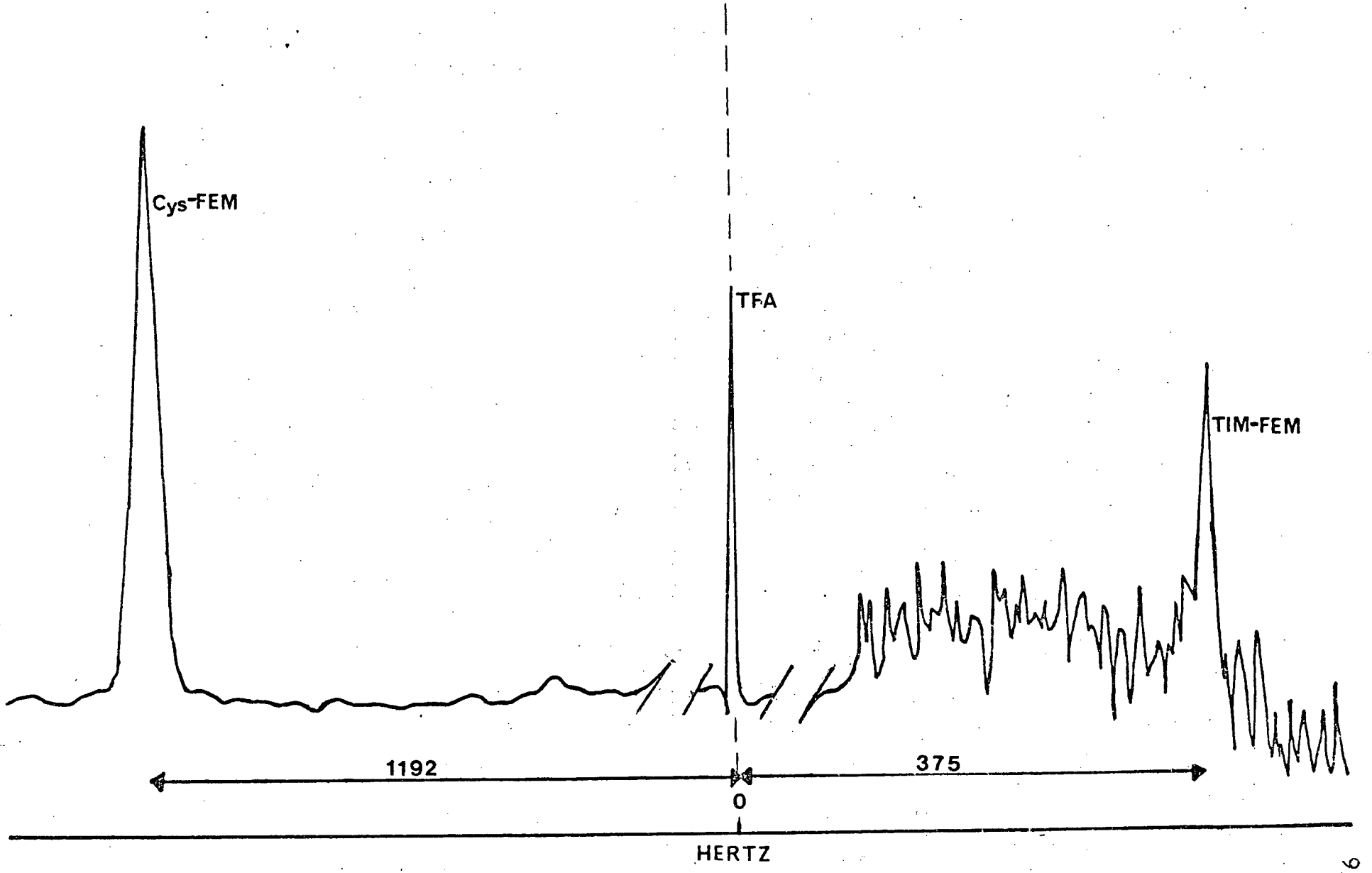
The fact that only one equivalent of maleimide is liberated suggests that there is a nonequivalence of the two modified sites which is due to some conformational difference. If the displacement is indeed dependent upon some basic amino acid side chain, even small differences could affect its ability to catalyze the reaction.

The FEM modification of TIM has a practical application in fluorine NMR of the protein. Figure 20 gives the spectra obtained from 200 mgs of modified TIM along with a reference and a model compound (all at pH 6.5).

The reference in this case is trifluroacetic acid (TFA) which has been arbitrarily set at zero. The model compound, which was formed by combining equimolar portions of N-acetyl cysteine and FEM (pH 6.5), was found to absorb 1192 hertz downfield from TFA. However the protein sample gave a signal at 375 hertz upfield from TFA which is a total of 1567 hertz from the model compound. All spectra were performed at 100MHz on the XL100. It was necessary to accumulate 100 transients for the model compound (.02 hours) and 100,000 (5.57 hours) for the protein sample.

The large chemical shift difference between the model compound and the FEM labelled protein is indicative of both the sensitivity of the fluorine nucleus to changes in environment and also the considerable alteration of the chemical environment of the FEM label. The single preliminary experiment illustrated in figure 22 can only give limited information but it does indicate the feasibility of further investigation with this system.

FIGURE 20: ^{19}F NMR of FEM Modified Triose Phosphate Isomerase



Further studies at various pH's and both in the presence and in the absence of substrate should give valuable information concerning the ionization of groups in the vicinity of the fluorine nucleus as well as changes in conformation of protein upon the binding of substrate.

(c) Specific Activity Measurements on Modified TIM

A similar kinetic analysis as that done for the NEM modification of TIM could not be performed for FEM (without stop flow techniques) because of the rapidity of the modification reaction. As discussed in the introduction to this section, this is probably due to activation of the double bond due to the electron withdrawing properties of the $-CF_3$ group. In addition, there is the possibility that the fluoroanalogue is able to more readily 'reach' the reaction sites in the protein than the NEM is, due to the changed electronic character of FEM.

However, the experiment which was performed allowed a direct determination that 1.96 SH's had been modified. Thus, the extent of modification is the same as that occurring with NEM. The two exposed SH's found in the FEM titration are especially interesting in light of the ability of DTNB to titrate one SH equivalent per mole of FEM modified protein. There appears to be a nonequivalence of the two FEM modified groups to modification by DTNB. As will be demonstrated in the kinetics section to follow, there is also a nonequivalence of the unmodified protein to DTNB modification. The nonequivalence of the two cysteine residues could be pre-existing in the native unmodified protein but also might be induced by the modification of either one or both of the SH's.

Similarities between the NEM and FEM modified proteins are demonstrated by the data on the specific activity of the modified species, as summarized below:

Protein	Specific Activity (units)	% Activity	#SH Modified
1. Control	5233	100.0	0
2. NEM modified	1204	23.0	2.0
3. FEM modified	1307	25.0	2.0

The similarity of the specific activities of the modified proteins suggests that the effect of the respective maleimides on the triose phosphate isomerase molecule is also similar. Even though the rates of modification of the two maleimides were quite different, it is likely that the actual modification (ie. the site) is the same. At this point, it would be premature to assume that since the specific activity is decreased by the modification, that the site of modification is at or near the active site. However, the recent 2.5Å resolution x-ray structure places the cysteine residue 126 in the catalytic active site of the enzyme. At the same time, it is possible that modification remote from the active site could induce a conformational change that would alter TIM's catalytic ability. Thus, if a change in conformation did result in actual changes in the structure of the active site which made catalysis more difficult, a decrease in the specific activity would be observed. This possibility has a special significance upon examination of the triose phosphate isomerases prepared for the 2.5Å resolution x-ray structure which indicated that the most reactive cysteine was at a site remote from the catalytic center. It was

found that the SH of residue 217 was the most reactive to mercury compounds such as 2-chloromercuri-4-nitrophenol. However, a smaller and more hydrophobic mercurial, ethyl-mercury phosphate, reacted with a less readily accessible pair of sulphydryl groups at residue 41.

B. Disulfides

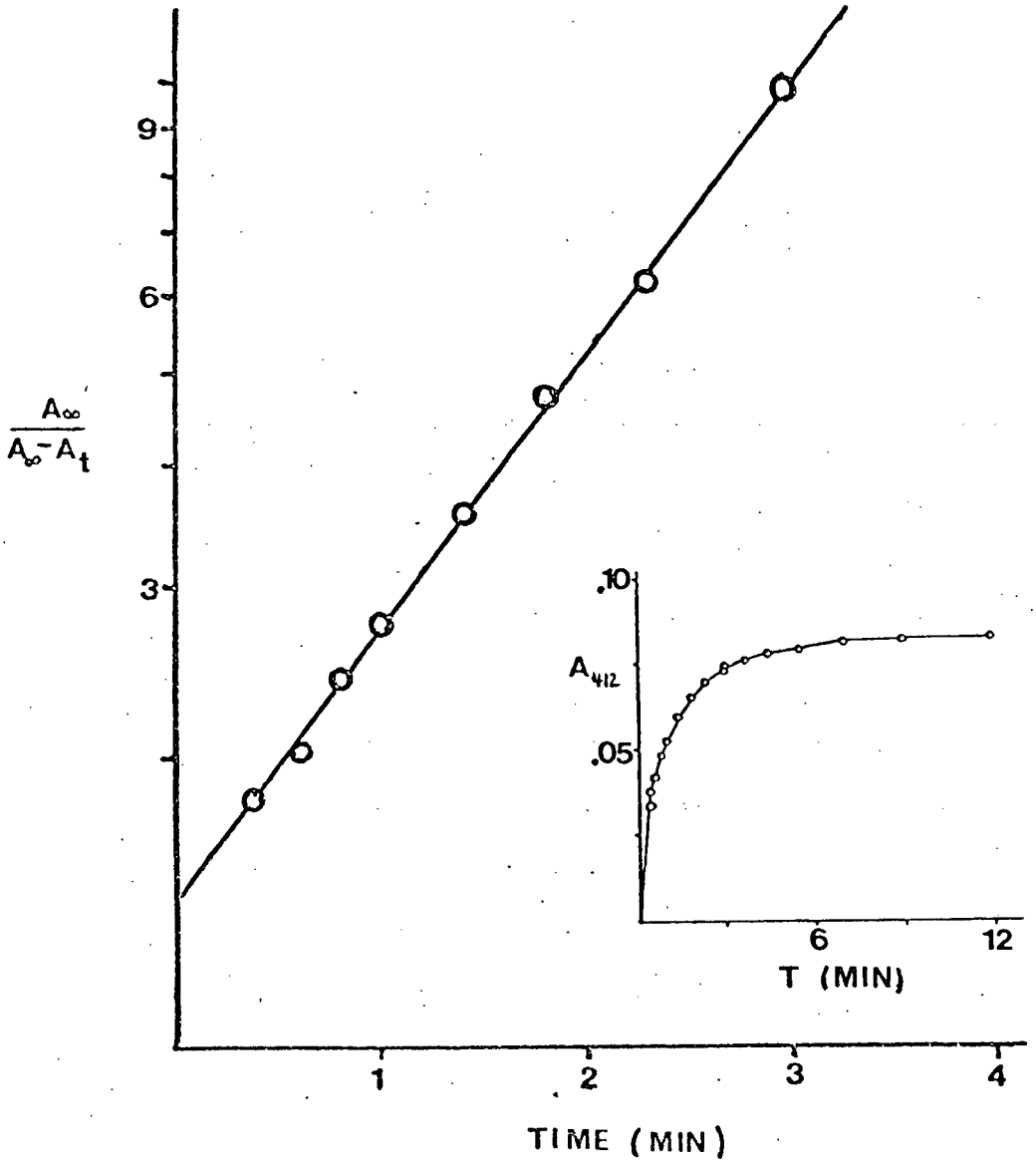
In addition to the aforementioned maleimides, it was found that TIM reacted readily with disulfides. This is in contrast to reaction with 3-bromo-1,1,1-trifluropropanone or bromoacetic acid which did not occur to any noticeable extent (Andrew M. Goetze, BSc thesis, UBC, 1973).

However, when DTNB is allowed to react with Peak A TIM, the results indicate that there are 2 SH groups which are exposed for ready modification by the disulfide: upon denaturation 6 more are able to react to give a total of 8 cysteines present. This agrees well with the 8 cysteine residues per dimer found in the amino acid analysis. Repeated experiments usually gave results which varied only one or two percent from the integer values of 2.0 and 8.0 for the respective 'exposed' and total SH titration. The results for Peak B protein were similar with 1.7 SH's being titrated initially by DTNB, and an average of 8.0 being obtained upon addition of SDS.

The cyanide displacement of TNB^- from the DTNB modified protein occurred quite readily. The pseudo first order kinetic analysis with data given to the third half life is illustrated in figure 21. A least squares treatment yielded the best straight

FIGURE 21: Pseudo First Order Analysis of the Cyanide Displacement of TNB from the DTNB Modified Peak A Protein

Insert: Increase in the Absorbance as the Reaction Proceeds



line for the data and the slope thus calculated gave a value of 11.16×10^{-3} sec^{-1} for the pseudo first order rate constant for the displacement of TNB by CN^- at pH 7.5 and 20°C.

It was observed that CN^- is able to displace 0.85 equivalents of TNB^- (average of 3 runs). Thus, once again there appears to be a nonequivalence in the ability of modified cysteine residues to react with reagents. The same explanations of nonequivalence due to conformational difference may be invoked here also. A schematic summary of the reactions includes:

$$E \xrightarrow[pH\ 8]{DTNB} E(TNB)_2 \xrightarrow[pH\ 7.5]{CN^-} E(TNB)CN$$

The nonequivalence of the DTNB modified protein was not observed upon action of dithioerythreitol on the $E(TNB)_2$ form of the enzyme. Upon addition of DTE 2.08 equivalents of TNB^- per mole of TIM were liberated. The difference between the results of the CN^- and DTE experiments might be explained by different abilities of the reagents to enter the site of DTNB modification on the enzyme.

Results from the specific activity measurements performed upon the various modified proteins are summarized below:

Protein	Specific Activity (units)	Specific Activity (%)
(1) Control	8368	100
(2) $E(TNB)_2$	2059	24.6
(3) E(TNB)CN	2410	28.8
(4) $E(TNB)_2$ + DTE	8477	101.3

The total activity of the enzyme was regained upon addition of DTE. This along with the observation of 2.08 equivalents of TNB^- liberated indicates that the TIM molecule has resumed a native unmodified state.

It is interesting to note that the specific activity of the $E(TNB)_2$ form of the enzyme (24.6%) is essentially identical to that of the maleimide modified species (25.0%). This lends support to our earlier assumption that DTNB and maleimides (ie. NEM and FEM) modify the same sites on TIM. The specific activity of the E(TNB)CN form of TIM (28.8%) is also very similar.

The reaction of TIM with the second disulfide 4,4-dithiopyridine (4-PDS) followed along the same lines as DTNB to yield values of 2.07 exposed SH titrated under nondenaturing conditions and 8.02 total SH's titrated under denaturing conditions to yield a total of 5.95 SH's 'hidden'. The specificity of DTNB appears to apply also to 4-PDS when titrating TIM. However, the greater working range of pH could make it more useful for further modification studies of TIM (or indeed any other cysteine containing protein).

A rather interesting example of the nonequivalence of the DTNB modified sites of TIM was observed when 4-PDS was allowed to react with the $E(TNB)_2$ enzyme form. The experiment illustrates that 1.01 equivalents of 4-PDS were able to react with the enzyme under non-denaturing contitions. When SDS was added (after titration of the 1.01 residues) 5.53 additional groups reacted to yield a total of 6.61 reactive groups. Again it appears that there is one of the two DTNB modified sites which is able to re-

act further. The 6 'hidden' cysteines are only able to react when the protein is unfolded.

In Torchinskii's excellent text dealing with sulfhydryl and disulfide groups of proteins, he gives a possible account of the reactions occurring in a disulfide exchange reaction (78).

$$R'\text{-}S\text{-}S\text{-}R' + R''\text{-}S\text{-}S\text{-}R'' \rightleftharpoons 2R'\text{-}S\text{-}S\text{-}R''$$

Three mechanisms for the exchange are given: one for neutral and basic solutions, one for acidic and finally a free radical mechanism. At the pH of the experiment (6.5) there should be a possibility of more than one occurring. They are given below:

(a) Basic or neutral solution

The reaction is catalyzed by thiols (catalytic quantities can arise by hydrolytic cleavage of disulfides) which carry out nucleophilic attack on a sulfur atom of the disulfide as in a thiol-disulfide exchange.

(1) $R'S^- + R''S\text{-}SR'' \rightleftharpoons R'S\text{-}SR'' + R''S^-$

(2) $R''S^- + R'S\text{-}SR' \rightleftharpoons R''S\text{-}SR' + R'S^-$

(b) Acid Media

The exchange takes place through a sulfenium cation which is formed through attack of a proton on the S-S bond.

(1) $R'S\text{-}S\text{-}R' + H^+ \rightleftharpoons R\text{-}\overset{+}{\underset{\displaystyle H}{S}}\text{-}S\text{-}R \rightleftharpoons R'SH + R'S^+$

(2) $R'S^+ + R''S\text{-}SR'' \rightleftharpoons R'S\text{-}SR'' + R''S^+$

(3) $R''S^+ + R'S\text{-}SR' \rightleftharpoons R''S\text{-}SR' + R'S^+$

The reaction(s) would be inhibited by thiols:

$$RSH + R'S^+ \rightleftharpoons RS\text{-}SR' + H^+$$

c) Free radical

The reaction occurs with participation of free radicals which can arise either from high temperatures (≥100°C) or ultra-violet radiation. Since the reaction is followed at 324nm, there is the possibility of some photolysis occurring. Radicals arising from thermal cleavage can be ruled out.

(1) $R'S\text{-}SR' \rightleftharpoons 2R'S\cdot$

(2) $R'S\cdot + R''\text{-}S\text{-}SR'' \rightleftharpoons R''S\text{-}SR' + R''S\cdot$

(3) $R''S\cdot + R'S\text{-}SR' \rightleftharpoons R''S\text{-}SR' + R'S\cdot$

Assuming that one, two or all mechanisms are operative to some extent in the case of $E(TNB)_2$ reacting with 4-PDS, the following scheme can be written:

$$E \xrightarrow[pH\ 8]{DTNB} E(TNB)_2 \xrightarrow[pH\ 6.5]{4\text{-}PDS} E(TNB)\ (4\text{-}TP)$$

CHAPTER V

KINETICS OF DISULFIDE MODIFICATION OF TIM

5.1 Methods

Twice chromatographed Peak A protein was prepared for kinetic runs by gentle stirring overnight at 4°C in 25mM dithiothreitol or dithioerthyreitol. The protein was desalted on a 1.5X20 cm Sephadex G25 column eluted with 200mM phosphate buffer, pH 6.5 in the case of 4,4'dithiopyridine runs and pH 8.0 for dithiobis-(2-nitrobenzoate) kinetics. Approximately 2 ml fractions were collected by a Gilson Microfractionator, and their absorbance at 280nm determined on a Zeiss PMQII UV-Visible spectrophotometer. Fractions with $A_{280} \geq 0.8$ were kept in ice until ready for use.

The most extensive series of kinetics involved modification of triose phosphate isomerase with 4,4'dithiopyridine. Kinetics of the modification of TIM were performed at 37°,31°,27°,25°,22°, and 15°C. The kinetics were followed both in the presence and in the absence of substrate (G3P) for each temperature cited. The progress of the reaction was followed by observing the liberation of the thiopyridone via an increase in the absorbance at 324nm. The runs were done in either duplicate or triplicate; the calculated rate constants being an average of the individual values obtained from each individual experiment. All constituents of the reaction mixture as well as the blank were equilibrated at the temperature of the experiment. The temperature

was held constant throughout the experiment by means of a circulating water bath which passed water of a constant temperature through the cell holder of the spectrophotometer.

Individual kinetic runs were initiated by rapid delivery of 50 μls of 4,4'dithiopyridine, 26 mgs/ml, via a Lang-Levy pipet into a 3 ml cuvette which contained 1 ml of protein sample ($A_{280} \cong 1$) and 1.95 mls of 200mM pH 6.5 phosphate buffer. The reaction mixture was blanked against a 3 ml cuvette containing 50 μls of reagent and 2.95 mls buffer. For those runs which were to include substrate, 100 μls of glyceraldehyde 3-phosphate (0.57mM) was added to both reaction cell and blank in place of 100 μls of buffer. At the completion of each experiment, a small amount ($\sim$10 mgs) of sodium dodecyl sulfate (SDS) was added to the reaction cell and the final A_{324} resulting from titration of all cysteines, both hidden and exposed was determined.

The kinetic experiments involving modification of TIM by DTNB were performed similarily. Runs at 35.5° and 22°C were done, both in the presence and in the absence of G3P. Preparation of the protein was at pH 8.0 as described previously; reagents, buffer, and protein were pre-equilibrated at the temperature of the experiment. The kinetic runs were performed at constant temperature.

The experiments were initiated by the pipetting of 1.0 ml of protein ($A_{280} \cong 1.0$) into a 3 ml cuvette containing 2.0 mls reagent stock solution consisting of 100mM phosphate pH 8.0, 10mM EDTA, and 5.25mM DTNB. For those kinetic runs which were

to contain substrate, 100μl of G3P (.57mM) was added along with 1.90 mls reagent, and 1.0 ml protein solution. The modification of TIM by DTNB was followed by observing the liberation of TNB^- as shown by the increase in absorbance at 412nm. The absorbance at 412nm was determined at various time intervals until a constant value was obtained. The total thiol content of the protein was then determined upon addition of solid SDS; with a further increase in A_{412} resulting from denaturation of the protein and exposure of hidden cysteine residues to modification by DTNB.

Data Treatment

The pseudo first order rate equation may be written as follows with 'a' equal to initial concetration of reactant (ie. reactive thiols), (a-x) meaning concentration of reactant at time 't' and k being the pseudo first order rate constant.

$$\ln \frac{a}{a-x} = kt$$

Since a is proportional to the final absorbance at 412nm (for DTNB kinetics) or 324nm (for 4-PDS kinetics) the substitution may be made whereby

$$\ln \frac{A_\infty}{A_\infty - A_t} = kt$$

Therefore a plot of $\ln \frac{A_\infty}{A_\infty - A_t}$ versus time theoretically gives a straight line going through the origin with slope k. All data was plotted to the third half-life.

However, the pseudo first order plots were not always observed to be linear since there were two (or more) reactive thiols and each SH not necessarily reacting at the same rate. When

the kinetic plot was bi-phasic, it was necessary to first determine the slope of that linear portion of the curve, at later time (t) where reaction of the more reactive thiol(s) was complete and the slope represents the rate of reaction for the slower reacting thiol. This slope was then subtracted from the earlier non-linear portion and a replot of the new values against time was performed. In the case of bi-phasic kinetics, the replot should now be linear and the slope would represent the rate constant for the faster reacting thiol.

A typical example of mono-phasic kinetics is illustrated in figure 22 which shows the pseudo first order plot for 4,4'-dithiopyridine (4-PDS) modification of TIM at 22°C in the absence of substrate (G3P). A typical bi-phasic plot, along with its replot, is shown in figure 23 which demonstrates the 4-PDS modification of TIM at 25°C in the absence of substrate (G3P). The results of these plus the other temperature runs are given in the section following. In all cases, a least squares analysis was performed on the linear plots.

A useful quantity to know is the 'half-life' or 'half-period' of a reaction which is the time it takes for half the original substance to disappear (ie. react). There is a simple relationship between the half-life of the reaction and the first order rate constant 'k':

$$T_{\frac{1}{2}} = \frac{0.693}{k}$$ for T in minutes when k is in min^{-1}.

The half-life, as a useful concept for semi-quantitative discussions, was calculated for all rates determined.

FIGURE 22: A Typical Example of Monophasic Kinetics-4-PDS Modification of TIM at 22°C in the Absence of Glyceraldehyde 3-Phosphate

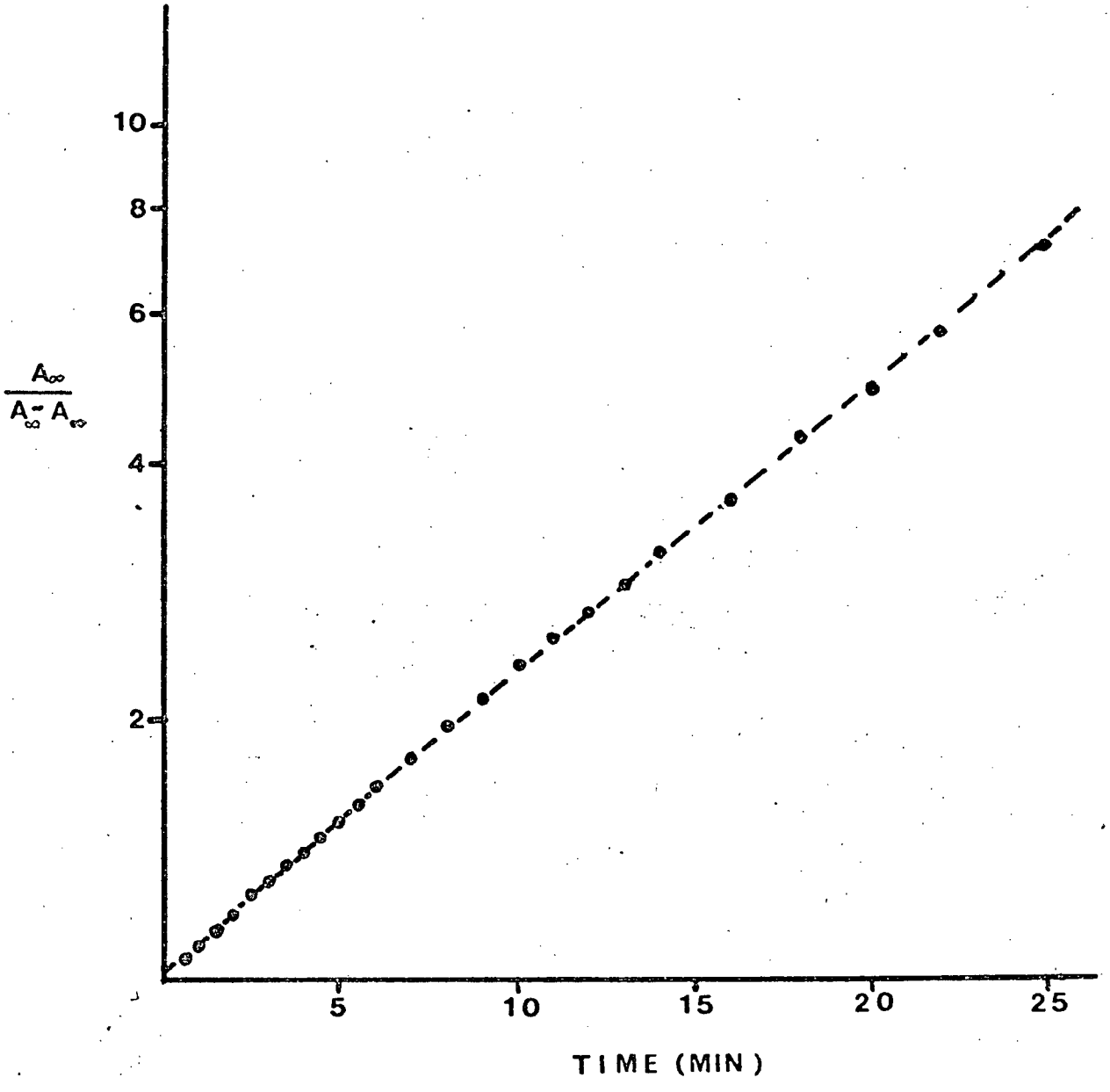
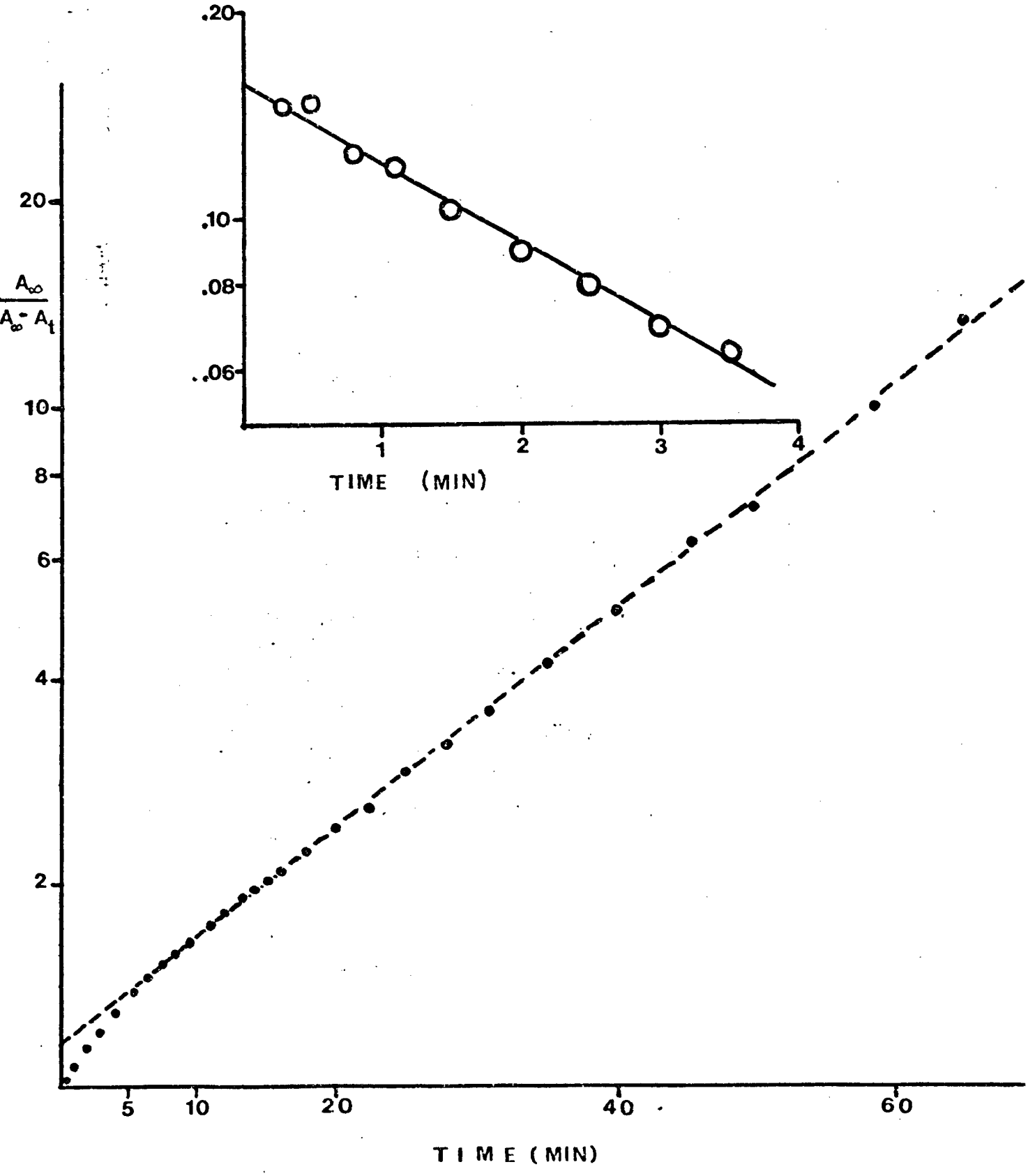


FIGURE 23: A Typical Example of Bi-phasic Kinetics-4-PDS Modification of TIM at 25°C in the Absence of Glyceraldehyde 3-Phosphate

Insert: Replot of Bi-phasic Area



An important relationship in kinetics which provides much information concerning mechanism, is one that connects the rate constant of a reaction with the temperature: the Arrhenius Law. The law may be expressed as follows:

$$k = Ae^{-Ea/RT}$$

with 'k' the rate constant, 'A' the frequency factor of the reaction, 'E_a' the activation energy, 'R' the universal gas constant and 'T' as temperature (°Kelvin). Therfore a plot of log k(sec^{-1}) versus 1/T K yields a slope of -Ea/2.303R or -Ea/4.57 with intercept A. This energy of activation represents the energy that the reactants must acquire in order to undergo reaction and as such is one measure of the ease of reaction.

5.2 Results

A. Modification of TIM with 4-PDS

The results obtained from the pseudo first order kinetic plots of the modification of TIM with 4-PDS are shown below:

TABLE V: Modification with 4-PDS

(1) In presence of Glyceraldehyde 3-Phosphate

T°C	Specific Rate Constant (min^{-1})	$T_{\frac{1}{2}}$ (min)	#SH's modified *
37	k_1 = .3950	1.75	2.0
31	k_1 = .3259 k_2 = .1681	2.13 4.12	2.0
27	k_1 = .2804	2.47	2.0
25	k_1 = .2413 k_2 = .0607	2.87 11.42	2.5
22	k_1 = .1297 k_2 = .0637	5.34 10.88	2.4
15	k_1 = .0149	46.51	2.6

* In all cases, 8.0 SH's were modified under denaturing conditions.

(2) In absence of Glyceraldehyde 3-Phosphate

37	k_1 = .3421	2.03	2.0
31	k_1 = .2880	2.41	2.0
27	k_1 = .2760 k_2 = .1432	2.51 4.84	2.0
25	k_1 = .2731 k_2 = .0372	2.54 18.63	2.0
22	k_1 = .0788	8.79	2.0
15	k_1 = .0289	23.98	2.0

From results presented in Table V-1,2, there does not seem to be any special dependence upon temperature or presence or absence of substrate when observing mono- or bi-phasic behaviour in the kinetic plots. However, the presence of bi-phasic behavior does indicate that under certain conditions (of temperature or availability of substrate) there was a non-equivalence in the potential sites of modification such that the reaction rates were able to differ by a factor of as much as eight (eg: 25°C, absence of substrate). As previously discussed in the chapter concerning protein modifications, there is the possibility of reaction of a single thiol inducing a conformational change in the protein such that the second thiol is now in a less favorable position to react.

It is evident from the listing of the number of SH's which were modified in the various experiments that the binding of substrate at temperatures at or below 25°C is able to influence the titration of a portion of a third thiol. It is known that the substrate G3P or DHAP is able to change the conformation of TIM to a significant degree (38) which might account, in part, for the exposure of the additional thiol. However, an additional temperature dependent mechanism appears to be at work since modification of more than 2.0 sulfhydryls does not occur at temperatures above 25°C. At the same time, the complete absence of a titration of a third thiol in those experiments which were performed in the absence of substrate, seems to indicate some dependence upon the conformational changes which the binding of

substrate can induce. The observation that only a portion of the third thiol (0.4 to 0.6) was titratable could indicate the existence of an equilibrium between some 'reactive' and 'non-reactive' state of the third thiol and hence in its partial modification by 4,4'dithiopyridine.

The rate constants of the most rapidly reacting thiol (k_1) were employed in an Arrhenius plot of log k versus 1/T K. The results for those experiments which contained G3P is shown in figure 24 and for those which lacked G3P is illustrated in figure 25.

A very prominent bi-phasic characteristic is apparent in both plots. For those experiments performed with substrate, the break comes at 24.6°C with the activation energy of the 4-PDS modification being 7.2 kcal/mole above this temperature and 50.0 kcal/mole below it. However, the second plot (without G3P) exhibited a break at 25.7°C with the upper limb (T>25.7°C) giving an activation energy of 4.4 kcal/mole and the lower limb (T<25.7°C) 39.9 kcal/mole.

The presence of this type of Arrhenius plot is not unknown in the literature. Copious examples are available which illustrate temperature dependent activation energies of the catalytic process of an enzyme, which is analogous to the temperature dependent reactivity of a specific group, that is the thiol of cysteine, which has been illustrated in the Arrhenius plots of figure 24 and 25.

Some insight into the temperature dependent mechanism in TIM

FIGURE 24: Arrhenius Plot for the 4-PDS Modification of TIM in the Presence of Glyceraldehyde-3-Phosphate

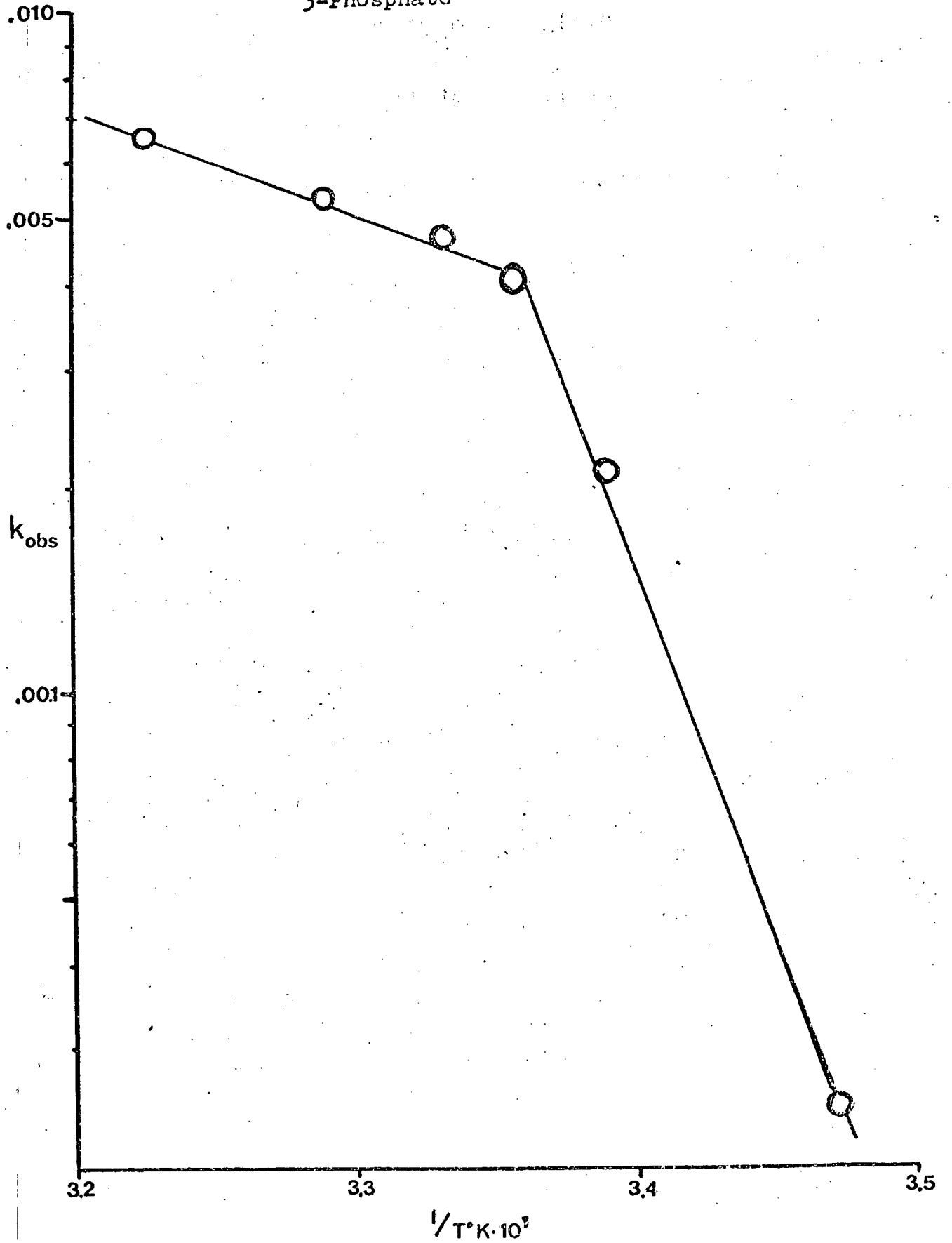
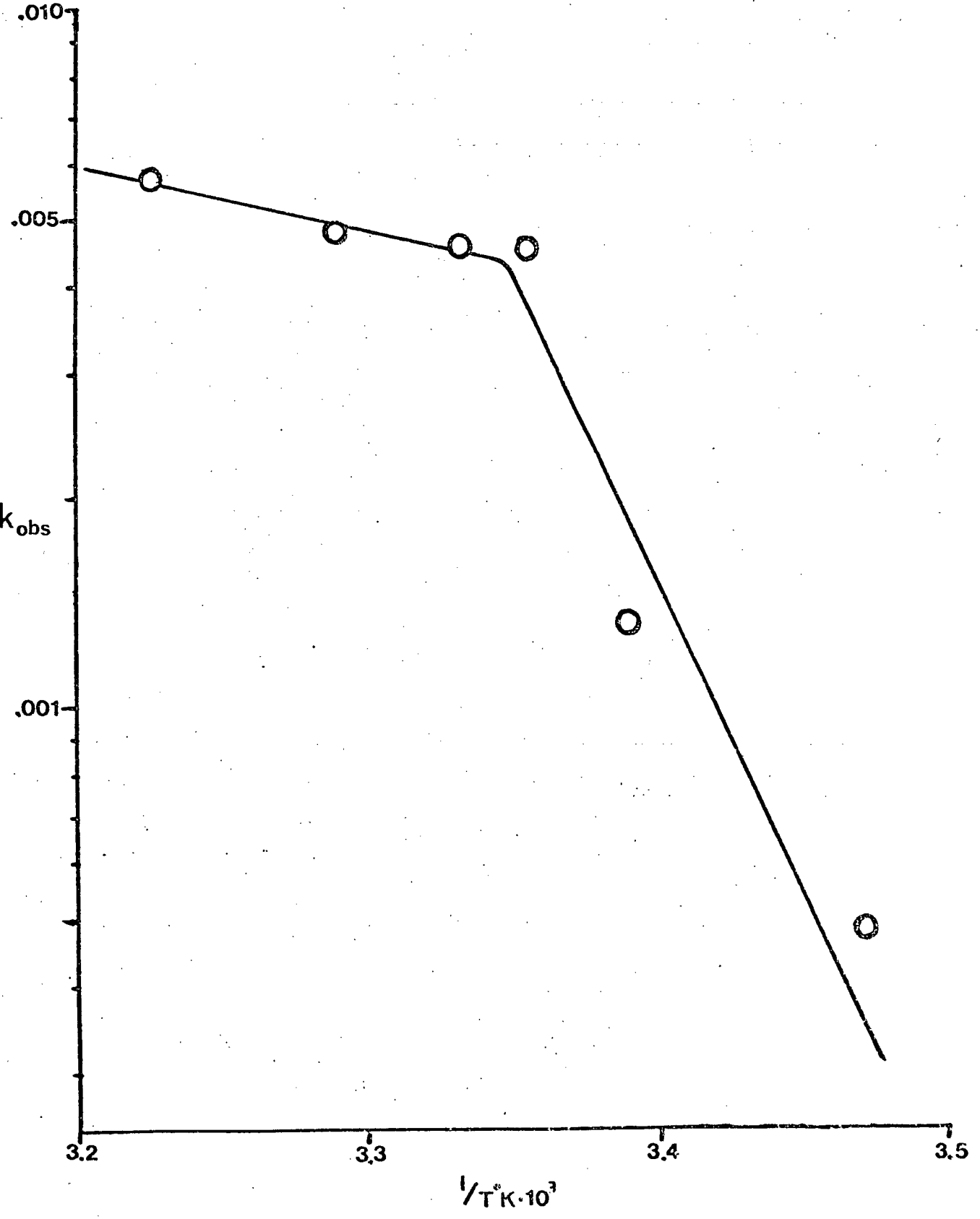


FIGURE 25: Arrhenius Plot for the 4-PDS Modification of TIM in the Absence of Glyceraldehyde-3-Phosphate



might be gained by looking more closely at some of the examples in the literature of temperature dependent processes occuring in enzymes.

Glutathione reductase from P. chrysogenum has been shown to have a catalytic activation energy of 14.3 kcal/mole between 20° and 30°C and 11.8 kcal/mole between 30°and 40°C (79). The argument of conformational change was invoked to explain the results. Massey et al (80) also obtained nonlinear Arrhenius plots for amino acid oxidase which was found to undergo a temperature dependent conformational change as observed by changes in the sedimentation constant, difference spectra, and fluorescence. The critical temperature for these phenomena coincided with the break in the Arrhenius plot. An even more radical example of a biphasic Arrhenius plot arising from temperature dependent conformational change was observed in the CMII isozyme of chorismate mutase (81). The plot illustrated an activation energy of 9.180 kcal/mole above 25°C and 99.000 kcal/mole below 25°C. No further temperature, pH, or substrate influenced cooperative interaction was observed. However, the large (11X) activation energy difference displayed by the mutase is an indication of the significant changes in conformation which may take place as a function of temperature. The reactivity of the most easily modified thiol of TIM (by 4-PDS) changes by a factor of about 7 in the activation energy in the presence of substrate and approximately 9 when substrate is not present. The modification results in the preceding chapter do suggest that Peak A TIM is sensitive to conformational change. However, an interesting continuation of

the discovery of bi-phasic Arrhenius plots for the Peak A protein, would be investigation into whether the phenomenon occurs in the other two isozymes. This could be an indication of essential differences between the different enzyme forms of chicken muscle TIM.

An interesting example of a break in an Arrhenius plot was found in muscle phosphorylase kinase (82) in which the phenomenon was entirely absent at 7 μgs/ml protein but present at 28 μgs/ml. The marked discontinuity was centered at 15°C with an activation energy of 18.200 kcal/mole at higher temperatures and 2.600 kcal/mole at lower temperatures. This unusual example of an enzyme with a higher activation energy at the higher temperature was explained by its catalytic activity being controlled by a combination of substrate binding and association-dissociation of the enzyme. This example has some interest since it has been reported that TIM's catalytic ability is affected by its concentration (83).

The kinetic titrations of TIM SH's in this thesis were carried out at protein concentrations of about 0.3 mg/ml. In the study of Poole et al (83) crystalline TIM isolated from rabbit muscle was diluted to concentrations between 1 mg/ml and 5 mgs/ml. Progressive increase of enzymatic activity with time was observed with rate of catalysis by TIM increasing up to twice the initial value observed immediately after dilution. In addition, it was found that the concentration of mercurial inhibitors required to reduce TIM activity by half, as well as the rate of reaction of thiols to iodoacetate, was dependent upon concentration of protein.

It has been suggested that the observed effects are due to conformational differences of the enzyme at different protein concentrations which could be due to the protein: protein interactions present in aggregation. The concentration of protein used in the kinetic experiments reported in this thesis (0.3 mg/ml) are high enough to show these conformation and aggregation effects.

The possibility of temperature dependent mechanisms other than conformational change was further expanded by Hipps and Nelson's reports on four esterases (84). They found that the esterases, as purified from the American cockroach were characterized by double sloped Arrhenius plots with activation energies between 6.3 and 8.5 kcal/mole at higher temperatures and between 11.6 and 14.5 kcal/mole at lower temperatures. The data was linked to association dissociation phenomenon (ie. formation of enzyme aggregates) which indicated that the cockroach gut esterases probably exist as thermally dependent molecular aggregates with different rates of hydrolytic activity in the associated and dissociated forms. Thus, lowering the temperature dissociates the aggregates into less active subunits (84). As mentioned earlier TIM is known to form concentration dependent aggregates. In addition, it has been well substantiated that there is some association of glycolytic proteins (including TIM) into large units in the '<u>in vivo</u>' situation (85,86,87,88). Therefore, the possibility of some sort of association-dissociation mechanism for the biphasic Arrhenius plot behavior of TIM cannot be ruled out.

In conclusion, it is difficult to suggest with any certainty,

the operative temperature dependent mechanism in TIM with the data available at this point. However, the mechanism is not obviously sensitized by substrate binding although the (-) substrate case does have somewhat lower activation energies. The break in the plots is about 1°C apart (24.6°C versus 25.7°C) which is within the experimental error. There is a great degree of difficulty in drawing accurate Arrhenius plots with the few temperatures which were investigated.

B. Modification of TIM with DTNB

The kinetics of the DTNB modifications of Peak A protein was investigated at two temperatures, 22°C and 35.5°C. The results are shown below:

(1) (+) substrate

T°	Specific Rate Constant	#SH modified
35.5	k_1 = .3625 k_2 = .1885	2.0
22	k_1 = .0425	2.0

(2) (-) substrate

T°	Specific Rate Constant	#SH modified
35.5	k_1 = .3555 k_2 = .2311	2.0
22	k_1 = .0355	2.0

The phenomenon of titration of more than 2.0 thiols is absent in the DTNB modification of TIM. However, biphasic behavior was still observed in the case of those runs performed at 35.5°C which indicates some similarity in enzyme changes occuring. However, the biphasic behavior was not nearly as marked as that obtained with 4-PDS and so, with the time limitations which were present for this segment of the thesis, the modifications with DTNB were not pursued beyond two temperatures. However, the close chemical reactivity of DTNB and 4,4'dithiopyridine indicates the potential interest of more extended temperature variation.

With the limited data available, it would seem inappropriate to report activation energies of the (+) substrate and (-) substrate systems. Only two points would be available for each Arrhenius plot and thus a low degree of confidence would be placed in the values obtained. In addition, caution must be exercised in deriving any interpretation from results obtained from such Arrhenius plots since the temperature interval (22-35.5°C) cuts across the breaking point (about 25°C) observed in the Arrhenius plots of 4-PDS modification.

CONCLUSIONS

In conclusion, it might be advantageous to briefly discuss the significance of the results reported in this thesis in relation to the existing knowledge of triose phosphate isomerase.

An indication of the purity of the isolated chicken muscle enzyme is that it was found to possess specific activity up to 12,000 units/mg which is at least as high or higher than the optimum of 10,000 units/mg which has been reported. Also, the chromatographic separation of TIM clearly demonstrated the first separation of chicken muscle isozymes. The first eluted peak, designated as 'A' was shown to consist of one electrophoretic moiety while the second 'B' peak was observed to contain two. The presence of the double activity peak in the specific activity profile of the DEAE-Sephadex chromatography of Peak A is difficult to account for. The possibility of protein aggregation has been discussed in this thesis and might provide some explanation for the observed activity profile. It has been well established that TIM forms part of an *in vivo* glycolytic aggregate which consists of glyceraldehyde 3-phosphate dehydrogenase, aldolase, pyruvate kinase, and lactate dehydrogenase (85,86,87) Therefore it would not be too surprising if the isolated protein aggregated as well. This particular type of protein:protein interaction could have some effect upon the enzyme's catalytic ability and hence on its specific activity. Ultracentrifugation sedimentation measurements could lead to information concerning the possibility that these aggregates do, in fact form.

There is very little possibility that the observed three isozymes are pseudo-isozymes which originate from various extents of sulfhydryl oxidation as was found for rabbit muscle phosphoglucose isomerase (90). The careful reduction of all protein with dithiolthreitol before any experimental manipulations, have ensured that all thiols are in a reduced state as shown by DTNB assay. However, this does suggest a possibility for the double specific activity profile observed for the Peak A isozyme. The chromatographies were not carried out in the presence of a reducing agent and if the thiols of the Peak A isozyme are particularily labile, it could result in pseudo-isozyme formation. These pseudo-isozymes would not be detected in the electrofocusing of the protein since the enzyme is reduced immediately before-hand. The type of facile oxidation which thiols are particularily prone to is usually reversible.

The implications of the isozymic separation on the structural determination already performed has already been discussed in section B of the characterization chapter. Extreme caution will have to be exercised in interpreting the published amino acid sequence and x-ray structure, expecially in light of the fact that there were significant differences in many amino acid residues in the amino acid analyses reported in this thesis, for the Peak A protein. In addition, the calculated molecular weight was different from the literature value by at least 5,000 gms/mole.

The chemical modifications reported were indicative of significant temperature induced conformational changes. Information

concerning thiol modification has, to date, been scanty. The foundations laid with these observations will be important not only for general understanding of the protein, but also for such spectroscopic techniques as NMR. There is a promising beginning with the ^{19}F NMR spectra observed for the FEM modified TIM. The large chemical shift difference of the labeled protein complex and the labeled model compound gives some indication of the sensitivity with which this technique will be able to detect changes in environment and hence the substrate-induced protein conformational changes.

The kinetics chapter was able to expand upon the modifications performed and led to some exciting discoveries of temperature dependent mechanisms. Little information in this area is available but the lower activation energy of the substrate enzyme complex correlates with some information available in the literature (16) concerning reaction of rabbit muscle TIM with DTNB. In one paper, Krietsch et al reported a second order rate constant for the DTNB modification in presence of substrate DHAP which was only a third of that observed in the absence of substrate. Further investigation in this area shows promise of yielding results which allow fruitful studies of the chemical and physical properties of chicken muscle triose phosphate isomerase.

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