# ISOZYMES OF HUMAN TRIOSEPHOSPHATE ISOMERASE: ISOLATION AND CHARACTERIZATION

DISSERTATION

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By

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The isolation and purification of triosephosphate isomerase from human erythrocytes, cardiac and skeletal muscle, liver, and brain has been described. Subsequent isolation and characterization of three isozymes from these tissues was effected. The native enzyme was found to be homogeneous with respect to other proteins by ultracentrifugation, isoelectric focusing, and disc gel electrophoresis. The pure native enzyme and isozymes are dimers exhibiting molecular weights of approximately 56,000 and are composed of two subunits of molecular weight 28,000. Isoelectric pH values of the isozymes were 6.7 (Component I), 6.5 (Component II), and 6.1 (Component III). Basic kinetic and denaturation stability parameters were determined for both the human and rabbit enzyme. Rabbit muscle triosephosphate isomerase was found to be more stable toward guanidinium chloride-induced denaturation than the human enzyme. The isozymes from either source were found to differ in their stabilities; Component I denaturing fastest, with Component II denaturing at a rate intermediate to I and III.

By coupling the enzyme to an insoluble matrix, the subunits were found to be catalytically active. Two different types of immobilized enzyme were characterized by routine kinetic methods and denaturation stability studies. The enzyme linked to the matrix through a "spacer" was found to approach the native enzyme in characteristics, whereas properties of the directly-bound enzyme were drastically changed. It was found that the individual subunits of the spacer-bound immobilized enzyme had activity.

The three forms of the enzyme are best explained by an AA, AB, and BB system of dimers with each subunit being a product of individual cistrons. A hypothesis is proposed to explain the alteration of the enzyme in the genetic disease, triosephosphate isomerase deficiency.

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# INTRODUCTION

Essentially all of the inheritance in man is determined by the information carried in his forty-six chromosomes. If elongated, all of the chromosomal material in the average adult human body would reach to the sun and back sixty-five times (Lehninger, 1970; Guyton, 1971). It is no surprise then, that these twenty-three gene pairs, or loci, are subject to variation by a number of factors. These variations may affect both the quality and quantity of the specific protein. These changes are reflected both directly in tha amino acid sequence of the gene products (proteins), and indirectly, through physiological and metabolic effects. The variant protein may cause no detectable deleterious effects, may limit an individual's metabolic and physiological capacity, or be fatal.

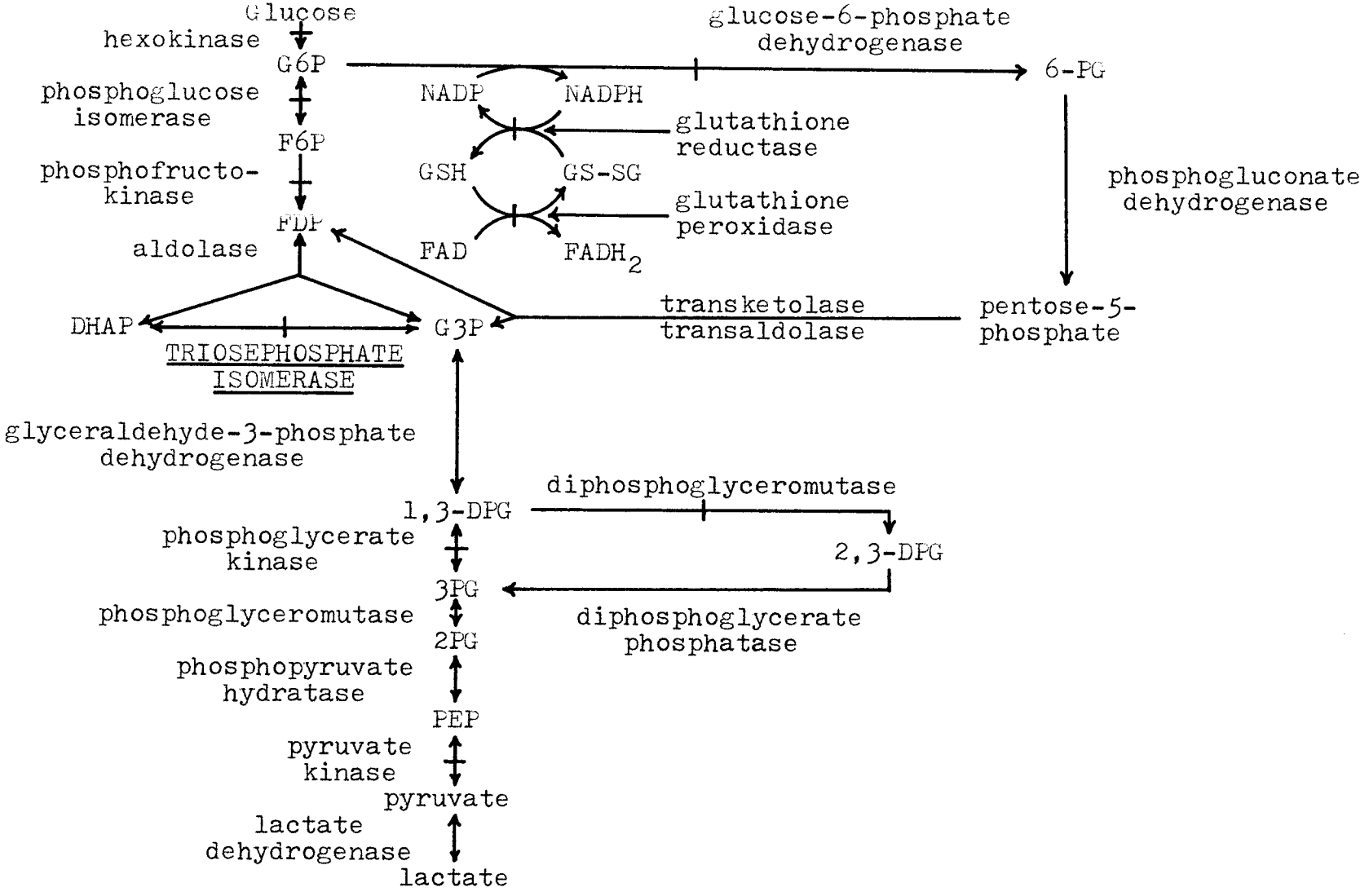
As early as 1906 Garrod (1908a,b,c,d) had recognized the relationship between metabolic deficiencies and heredity, but in only the last twenty years has progress in research related to inherited diseases soared. In 1968, McKusick published a catalogue (McKusick, 1968) of 1,545 genetically determined variations in proteins. However, only ten per cent of these variations had been linked to specific proteins (Childs, 1968). Many of these proteins have been isolated and studied, in both the normal and abnormal forms, from blood, because of its

accessibility. Indeed, because the human red blood cell has such a simple metabolic composition and because a large number of deleterious heritable diseases have been associated with it, the erythrocyte has been under great biochemical scrutiny for many years.

Although the population of mature erythrocytes comprises only approximately twenty-three per cent of the total number of cells in the average adult (Harris and Kellermeyer, 1972), without the red blood cell all mankind would cease to exist. Thus, any defect associated with the red cell is important to the whole organism. The glycolytic and pentose phosphate pathways through catabolism of glucose are the only sources of energy (in the form of ATP) and reducing equivalents in the mature erythrocyte. Of approximately eighteen enzymes involved in red cell carbohydrate metabolism, twelve have been associated with inherited genetic defects (Stanbury et al., 1972) as shown in Figure 1.

In 1965 Schneider and coworkers (Schneider et al. 1965) described a genetic disease caused by an abnormal form of triosephosphate isomerase (E.C.5.3.1.1). Although rare, this is the most severe genetic disorder associated with a glycolytic enzyme, causing death generally by the age of five, from cardiac and respiratory failure (Schneider et al., 1968a). Characterized by nonspherocytic hemolytic anemia, increased susceptibility to infection, neurological disorders,

Figure 1. Metabolic blocks in carbohydrate metabolism of the red blood cell. These blocks have been shown to be inherited.


Glucose
hexokinase
G6P
glucose-6-phosphate dehydrogenase
6-PG
phosphoglucose isomerase
NADP
NADPH
glutathione reductase
F6P
phosphofructo-kinase
GSH
GS-SG
glutathione peroxidase
phosphogluconate dehydrogenase
FDP
aldolase
FAD
FADH2
transketolase
transaldolase
pentose-5-phosphate
DHAP
G3P
TRIOSEPHOSPHATE ISOMERASE
glyceraldehyde-3-phosphate dehydrogenase
diphosphoglyceromutase
1,3-DPG
phosphoglycerate kinase
2,3-DPG
3PG
phosphoglyceromutase
diphosphoglycerate phosphatase
2PG
phosphopyruvate hydratase
PEP
pyruvate kinase
pyruvate
lactate dehydrogenase
lactate

developmental retardation, and unexplained sudden death, this metabolic disease is believed to be due to a single gene mutation which is transferred as an autosomal recessive (Schneider et al., 1965 and 1968a). The enzyme deficiency was found not only in erythrocytes, but also in spinal fluid, muscle tissue, and leucocytes. Schneider et al. (1968b) provided data suggesting an altered enzyme occurred in the genetic disease and Kaplan et al. (1968) demonstrated electrophoretic differences in the normal and abnormal enzymes. They observed three electrophoretic forms of triosephosphate isomerase in hemolysates from normal subjects. The two more acidic bands were absent in hemolysates from the enzyme-deficient patients.

Multiple forms of triosephosphate isomerase had been observed as early as 1944 (Meyerhof and Beck, 1944). However, it was not until the studies of Kaplan et al. (1968) that these were linked to a genetic disease. Since neither the normal nor mutant enzyme had been isolated, their chemical, physical, and catalytic properties were unknown. In order to determine the specific site(s) and the nature of this genetic lesion, it was necessary to compare the properties of the enzyme from normal and abnormal tissue at the molecular level.

Triosephosphate isomerase was studied as early as 1935 (Meyerhof and Kiessling, 1935) and crystallized from calf

muscle in 1953 (Meyer-Arendt et al., 1953), but only recently has this enzyme come under intensive investigation. Purification procedures have been developed from numerous tissues and species (Table I) and the enzyme has been studied in partially pure form from pea leaves (Anderson, 1971; Anderson and Advani, 1970), pea seeds (Turner et al., 1965), two chlorophyta (Meeks et al., 1968), Euglena gracilis (Mo et al., 1973), cockroaches (Chiang, 1971a), primates and human fibroblasts (Rubinson et al., 1973), a psychrophile (Shing et al., 1972), and various other species (Scopes, 1968; Snapka et al., 1974). However, the human enzyme had only been studied in crude lysates (Kaplan et al., 1968; Rubinson et al., 1971) or in partially purified form (Lee et al., 1971).

The molecular heterogeneity associated with human triosephosphate isomerase which was observed by Kaplan et al. (1968) and Rubinson et al. (1971), could have been caused by the electrophoretic system, partial denaturation, oxidation of sulfhydryl groups, dissociation or aggregation, different degrees of amidation, or covalent modification. The multiple forms could also represent conformational isomers in addition to the possible genetic basis (i.e., the existence of more than one unique polypeptide chain which could give rise to multiple combinations of oligomers). The basis for the multiple forms of the enzyme was of particular interest in view of the genetic enzyme-deficiency disease. If two nonidentical subunits exist which are the products of two genes,

TABLE I

SOURCES OF ISOLATED TRIOSEPHOSPHATE ISOMERASE

| Source of Enzyme | Final Specific Activity (units/mg) | Reference |
|---|---|---|
| Rabit muscle | 5,500 | Meyerhof and Beck (1944) |
| | 2,750 | Czok and Bucher (1960) |
| | 8,000 | Krietsch et al. (1970) |
| | 7,800 | Norton et al. (1970) |
| Bovine muscle | 4,725 | Meyer-Arendt et al. (1953) |
| | 9,450 | Beisenherz (1955) |
| Chicken muscle | 10,800 | Trentham et al. (1969) |
| | 11,200 | Putnam et al. (1972) |
| | 6,300 | McVittie et al. (1972) |
| | 10,000 | Furth et al. (1974) |
| Equine liver | 3,183 | Lee et al. (1971) |
| Human liver | 2,397 | Lee et al. (1971) |
| Mosquito | 2,000 | Chiang (1972) |
| Housefly | 2,412 | Chiang (1972) |
| Coelacanth | 7,000 | Kolb and Harris (1974) |
| Brewer's yeast | 10,000 | Krietsch et al. (1970) |
| Baker's yeast | 9,600 | Hawkinson et al. (1972) |
| Bacillus stearothermophilus | 4,500 | Fahey et al. (1971) |

an individual with the genetic disease might produce both a normal and an abnormal subunit. The basis of this enzyme multiplicity seemed worthy of detailed study also with regard to the normal physiological roles of these multiple forms. True isozymes frequently exhibit tissue- or organ-specific (Harris, 1971) distribution and distinct catalytic or regulatory properties. Multiplicity of triosephosphate isomerase had also been suspected for some time in other species and from one to eight electrophoretic forms have been reported from rabbit muscle (Krietsch et al., 1971; Coulson et al., 1970; Norton et al., 1970; Lee et al., 1971) and in a number of other species and tissues (Snapka et al., 1974; Scopes, 1968; Rubinson et al., 1974). However, Krietsch and coworkers (1971), who were the first to examine these multiple forms in detail, found essentially no differences in the chemical, physical, or catalytic properties of the isozymes. Recently Corran and Waley (1974) proposed that slight structural modifications might give rise to the isozymes from rabbit muscle.

A study of the interaction and activity of the individual subunits of triosephosphate isomerase and how these interactions affect the isozyme system in vivo became important in understanding both the molecular aspects of the normal enzyme and the clinical pathology of the genetic disease. Guanidinium chloride denaturation was chosen as the method of study of the subunit interactions because of its successful

application in detailed studies of monomeric proteins. Furthermore, the use of matrix-bound enzyme systems, whereby the individual subunits could be separated from one another, seemed ideally suited for these studies.

The present study was undertaken to purify and fully characterize triosephosphate isomerase from human tissue. The study was designed not only to reveal basic structure-function relationships of the normal enzyme but also to provide information relating to the genetic defect associated with this enzyme. A number of chemical, physical, catalytic, and denaturation stability studies were carried out on the native enzyme and the three isozymes. The activity of the individual subunits was investigated using immobilized triose-phosphate isomerase which was also characterized.

## MATERIALS AND METHODS

### Materials

Whatman phosphocellulose powder (P11) with an exchange capacity of 0.74 meq/g and diethylaminoethyl (DEAE) cellulose (DE11) with an exchange capacity of 0.9 meq/g were obtained from Reeve Angel. Sephadex and DEAE-Sephadex (Sephadex A-50, exchange capacity 3.0 meq/g) were obtained from Pharmacia Fine Chemicals, while all Ampholines and isoelectric focusing equipment were obtained from LKB Instruments. Iodoacetic acid, urea, rabbit muscle triosephosphate isomerase (3300 units/mg), α-glycerophosphate dehydrogenase (200 - 300 units/mg), Sepharose 4B, DL-glyceraldehyde-3-phosphate (diethyl acetal, monobarium salt), dihydroxyacetone phosphate (dimethylketal, cyclohexylammonium salt), phenazine methosulfate, 3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyl tetrazolium bromide, the sodium salts of NAD and NADH and enzyme grade guanidinium chloride were obtained from Sigma Chemical Company. Glyceraldehyde-3-phosphate dehydrogenase (100 units/mg) was obtained from Boehringer Mannheim Corporation. Free DL-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate were prepared according to instructions from the supplier (Sigma Chemical Company). Tris(hydroxymethyl)-aminomethane (Tris), triethanolamine, ammonium sulfate,

ethylenediamine tetraacetic acid (EDTA), N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), N,N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), and glycine amide were all enzyme grade reagents obtained from Nutritional Biochemicals. Iodoacetic acid and urea were recrystallized before use. Guanidinium chloride was heated at 60° for six hours, then stored in a vacuum desiccator. Methylene bisacrylamide, acrylamide, N,N,N',N'-tetramethylethylenediamine, and ammonium persulfate were obtained from Bio-Rad Laboratories. Metallographic plates for ultracentrifugation and plastic-backed thin layer cellulose chromatographic plates, number 6064, were obtained from Eastman Kodak. All other chemicals were analytical grade.

## Methods

### Enzyme Assay

Triosephosphate isomerase activity was monitored in both the forward and reverse directions by enzymatic methods. A modification of the method of Beisenherz (1955) was most frequently used in which triosephosphate isomerase was coupled to excess α-glycerophosphate dehydrogenase and the rate of isomerization followed by oxidation of NADH followed at 340 nm. For the free enzyme, this assay mixture consisted of 0.15 mM NADH, 2.1 mM D-glyceraldehyde-3-phosphate,[1]

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[1]The L-isomer is present but is not an inhibitor (Plaut and Knowles, 1972).

20 g of α-glycerophosphate dehydrogenase, and 50 mM triethanolamine buffer containing 1 mM EDTA and 14 mM 2-mercaptoethanol at pH 7.6, in a final volume of 0.94 ml. For assaying the immobilized enzyme, the assay mixture consisted of the same concentrations of components in a final volume of 2.95 ml. In the assay procedure in the reverse direction, triosephosphate isomerase was coupled to excess glyceraldehyde-3-phosphate dehydrogenase and the reduction of NAD was followed at 340 nm (Marquardt et al., 1968). This assay mixture consisted of 0.2 mg glyceraldehyde-3-phosphate dehydrogenase, 2.5 mM dihydroxyacetone phosphate, 0.15 mM NAD, 6 mM sodium arsenate, and 50 mM triethanolamine buffer, pH 7.6, containing 1 mM EDTA and 14 mM 2-mercaptoethanol, in a final volume of 1.0 ml. Both triosephosphate isomerase (Turner, 1965) and α-glycerophosphate dehydrogenase (Sigma Chemical Company Catalogue) are inhibited by sulfate ions. Therefore, the coupling enzymes α-glycerophosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were always dialyzed extensively against assay buffer before use to remove the ammonium sulfate in which they were stored. Since arsenate ion is a competitive inhibitor of triosephosphate isomerase, correction for this inhibition was always taken into consideration when calculating enzyme activity obtained by the reverse assay procedure.

All enzyme assays were carried out in a Beckman Kintrac VII spectrophotometer thermostated at 30° and equipped with

a cuvette stirrer. Because the solution in the cuvette could be stirred when activity assays of immobilized triosephosphate isomerase were run, there was no interference due to settling of the Sepharose beads and linear assay recordings were always obtained. One unit of enzyme activity is defined as the amount catalyzing the isomerization of 1.0 $\mu$mole of triose-phosphate per minute at 30°.

## Protein Concentration Determination

Protein concentration of crude extracts and impure fractions was determined by the Lowry method (1951). However, during chromatographic steps in enzyme purification, the absorbance at 280 nm was used. The $E^{1\%}_{1\ cm}$ at 280 nm for human triosephosphate isomerase was determined to be 12.9 by the method of Walsh and Brown (1962).

## Chromatography

Phosphocellulose, DEAE-cellulose, and DEAE-Sephadex were washed according to the method of Peterson and Sober (1962), and equilibrated with appropriate buffer by the method of Himmelhoch (1971). Ion exchange Sephadex was swollen in deionized water prior to washing. All ion exchange chromatography was carried out at 0 - 5°. Ion exchange cellulose columns were packed under nitrogen pressure as described by Himmelhoch (1971) while DEAE-Sephadex was packed under gravity. Columns were eluted by

gravity flow. When a NaCl concentration gradient was used to develop a column, the chloride concentration in the fractions was determined by the method of Schales and Schales (1941). Analytical gel filtration was performed according to the method of Andrews (1965) using Sephadex G-100 which had been swollen and equilibrated according to the instructions of the supplier.

## Subcellular Fractionation

Fractionation of human liver cells into their constituent mitochondrial, cytosolic, and microsomal fractions, was accomplished by a combination of the methods of Johnson and Lardy (1967) and Ernster and Low (1955). Cell nuclei were prepared by the method of Blobel and Potter (1966).

## Electrophoresis

Polyacrylamide gel electrophoresis was carried out in standard 7% alkaline gels (pH 8.9) according to the method of Davis (1964). The gels were stained with either 0.1% (w/v) Amido Black or 0.25% (w/v) Coomassie Blue in methanol-acetic acid-water (45:10:45) and destained by a modification of the method of Gathercole and Klein (1971). Gels were stained for triosephosphate isomerase activity by the coupled assay described by Scopes (1968, 1964). Polyacrylamide gels were quantitatively scanned on a Canalco Model E recording microdensitometer.

## Isoelectric Focusing

Density gradient isoelectric focusing was carried out using 1% LKB ampholines in a 110-ml LKB column, according to the method of Vesterberg and Svensson (1966). The cathode and basic solution [2% (v/v) ethanolamine] were at the bottom of the column, while the anode and acidic solution [1% (v/v) phosphoric acid] were at the top. 2-Mercaptoethanol at a final concentration of 14 mM was normally added to the gradient solutions to serve as a reducing agent (Ikehara, 1972). All solutions and gradients were prepared according to the LKB instruction manual (LKB Instruments, Inc.) and each experiment was carried out for the indicated time at 600 volts at 4°. Fractions (1.0 ml) were collected, their pH values determined at 4°, the triosephosphate isomerase activity measured, and absorbance at 280 nm determined. The contents of the tubes containing the enzyme were pooled and dialyzed for at least thirty-six hours against 10 mM triethanolamine buffer, pH 7.5, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 100 mM NaCl.

Polyacrylamide gel isoelectric focusing was carried out in a Metalloglass Model M-137A apparatus according to the supplier's instructions. The gels contained 7% (w/v) acrylamide and 2% (w/v) ampholines. After polymerization, current was applied to the gels for thirty minutes prior to sample application to remove the ammonium persulfate and establish

the pH gradient. Samples were applied in 50 mM triethanolamine, pH 7.6, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 10% (w/v) sucrose. Samples were focused at 1 ma per gel until a potential of 400 volts was attained, at which time electrofocusing was terminated. Gels were sliced into fifty portions using a Canalco lateral gel slicer, and each slice was crushed, vigorously agitated in assay buffer with a Vortex mixer for thirty seconds, and allowed to extract for four hours. An aliquot was then withdrawn and the enzyme assayed in the normal manner.

## Peptide Maps

Peptide maps were produced for each of the human triosephosphate isomerase isozymes by the following procedure. The enzymes were reduced with 2-mercaptoethanol and S-carboxymethylated with freshly recrystallized iodoacetic acid by a modification of the method of Crestfield *et al.* (1963). The lyophilized enzyme (1.0 mg) was dissolved and stirred in 75 µl of 0.5 M Tris buffer, pH 8.5, containing 0.25 M EDTA, and 1 µl of 2-mercaptoethanol in a specially-constructed sidearm flask. Recrystallized urea (60 mg) was added to the solution while 3 mg of freshly recrystallized iodoacetic acid, dissolved in 100 µl of buffer , was added to the sidearm. The apparatus was evacuated and flushed with nitrogen several times, and denaturation and reduction were allowed to proceed for four hours in the dark under nitrogen. The iodoacetic

acid was added, and carboxymethylation was allowed to proceed in the dark at pH 8.5 for twenty minutes. The reaction was stopped by the addition of 5 $\mu$l of 2-mercaptoethanol, and the pH was adjusted to 2.0 with 1 M formic acid. Samples were then dialyzed in the dark against 1% (v/v) formic acid. The S-carboxymethyl protein was then lyophilized, dissolved in 0.5 ml of 2% (v/v) trimethylamine, and the pH adjusted to 8.0 with 1 M formic acid. L-1-Tosylamido-2-phenylethyl-chloromethyl-ketone trypsin was added to this solution, and the digestion was carried out in a stirred vial at room temperature. The extent of reaction was monitored with a combination glass electrode and a recording pH meter. Trypsin was added in four separate aliquots at zero, thirty, sixty, and 180 minutes, and digestion was allowed to proceed for a total of four hours with the pH maintained at 7.8 - 8.0 by the addition of 2% (v/v) trimethylamine. The total trypsin to triosephosphate isomerase weight ratio was 1:50. Samples were subsequently lyophilized to dryness and dissolved in approximately 50 $\mu$l of 2% (v/v) trimethylamine. From 50 to 200 g of sample were then spotted on cellulose-coated thin-layer chromatography sheets (20 x 20 cm). Electrophoresis was conducted at pH 5.5 in pyridine-acetic acid-water (10:3:300) at 300 volts (15 ma) in a Varsol-cooled (4°) microscale mapping chamber similar to that used by Tauri et al. (1972). Ascending chromatography was carried out in

butanol-pyridine-acetic acid-water (50:33:1:40) at room temperature, and the chromatograms were air-dried. Peptides were located by spraying with ninhydrin, or ninhydrin-collidine.

## Amino Acid Analysis

Although all amino acid analyses were performed generally by standard methods, some modifications were necessary for samples obtained from polyacrylamide gels or for the enzyme covalently bound to Sepharose 4B. After exhaustive dialysis against deionized water, the enzyme was hydrolyzed for twenty-four, forty-eight, and seventy-two hours at 110° *in vacuo* with 5.7 N HCl containing 14 mM 2-mercaptoethanol with norleucine and α-amino-β-guanidinopropionic acid added as internal standards (Walsh and Brown, 1962). The hydrolysates were subsequently lyophilized and analyzed on a Beckman Model 120 C amino acid analyzer according to Spackman *et al.* (1958). Values for serine and threonine were extrapolated to "zero time" of hydrolysis. Duplicate runs were made at each time period of hydrolysis and three different triosephosphate isomerase preparations were analyzed. Tryptophan was determined by the spectrophotometric method of Edelhoch (1967), and cysteine was measured as either cysteic acid following performic acid oxidation (Hirs, 1956), by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman, 1959), or as S-carboxymethylcysteine.

When the material to be analyzed was obtained from polyacrylamide gels, the following procedure was utilized. Gels were removed from the electrophoresis chamber and immediately placed on Sepraphore III polyacetate strips (2.5 x 15 cm) which had been previously soaked in an activity stain solution (Scopes, 1964). As soon as the bands of enzyme activity were visible on the strips (approximately two to three minutes), the corresponding areas of the gels were sliced and collected. Care was taken to carry out this step rapidly to avoid diffusion. The appropriate gel slices were then mixed with 3.0 ml of 5.7 N HCl and hydrolyzed for forty-eight hours *in vacuo* at 110°. After hydrolysis, the samples were filtered through a 0.45-micron Millipore filter to remove the insoluble gel material. For the immobilized enzyme, a weighed, lyophilized aliquot was processed in the same manner.

## Ultracentrifugation

Analytical ultracentrifugation of both rabbit muscle and human triosephosphate isomerase was carried out in a Beckman Spinco Model E ultracentrifuge equipped with electronic speed control and RTIC temperature regulation. Sedimentation equilibrium experiments were performed by the meniscus depletion method (Yphantis, 1964; Van Holde, 1967) and analyzed in the standard fashion (Yphantis, 1964). Sedimentation velocity experiments were carried out according

to the method of Chervenka (1967) and analyzed using a computer program for the IBM 360-50 (Mo, 1974). Schlieren photographs and Rayleigh interference fringes were measured on a Nikon Model 6C microcomparator equipped with digital x, y encoders. Densities and viscosities of all solutions were either determined directly or calculated as described by Kawahara and Tanford (1966). A value of 0.725 ml/g was calculated as the partial specific volume from amino acid compositions for human triosephosphate isomerase.

When rabbit muscle triosephosphate isomerase was subjected to sedimentation velocity ultracentrifugation under denaturing conditions, the following procedure was used. Two $2^{\circ}$ double sector cells equipped with quartz windows and 12 mm aluminum-filled Epon centerpieces were used in each run with one cell being equipped with a $1^{\circ}$ wedge window. All runs were made at $30^{\circ}$. One sector of each cell was filled with 0.35 ml guanidinium chloride/buffer D (20 mM triethanolamine, 2 mM EDTA, 28 mM 2-mercaptoethanol, 200 mM NaCl, pH 7.5) solution, while the sample sector contained 0.35 ml of the same solution plus enzyme. For each run, two 0.25-ml aliquots, each containing buffer D and a different concentration of guanidinium chloride, were injected into two separate vials using calibrated syringes. At "time zero," a 0.25-ml aliquot of triosephosphate isomerase/buffer D solution was added to each vial simultaneously. Within two minutes after mixing, a 10 $\mu$l

aliquot of each sample had been prediluted into the same concentration of guanidinium chloride/buffer D solution and assayed for enzymatic activity. Within thirty minutes after mixing, acceleration of the rotor had begun and was carried out smoothly at 10 amps in order to minimize heat convection in the cells. All runs were made at 60,000 rpm and photographs were taken at five minute intervals. The part of the sample that was not used in ultracentrifugation was incubated at 30° and assayed during the run while photographs were being taken, in order to correlate changes in enzymic activity directly with changes in the sedimentation coefficient. The change in the partial specific volume for the rabbit muscle enzyme in going from the native to the denatured state was estimated to be from 0.741 ml/g to 0.744 ml/g (Lee and Timasheff, 1974).

## Standardization of Solutions

The concentration of the substrate D-glyceraldehyde-3-phosphate was determined using the coupled enzymatic assay described in "Enzyme Assay" except that excess triosephosphate isomerase was used with a limiting amount of substrate. By omitting the triosephosphate isomerase from the assay mixture, the concentration of dihydroxyacetone phosphate was similarly determined. Substrates were always prepared fresh

daily and maintained at 0°.[2] Solutions of ligands used in inhibition and denaturation rate studies were prepared immediately before use by dissolving the proper amount of anhydrous crystals in assay buffer.

Guanidinium chloride concentrations were determined by refractometry in a Bausch-Lomb refractometer thermostated at 30° ± .05° (Gordon, 1972). All guanidinium chloride solutions were prepared by quantitative volumetric dilution of a standardized solution.

## Kinetics

All kinetic parameters were determined at 30°. Michaelis constants ($K_m$) and maximum velocity values ($V_{max}$) were determined from data analyzed by the weighted least squares method of Wilkinson (1961) as adapted to the IBM 360-50 by Cleland (1967). Since all the inhibitors used in this study were competitive inhibitors, inhibition constants ($K_i$) were evaluated using the following formula:

$$K_m' = K_m \left(1 + (I)/K_i\right)$$

where $K_m'$ is the apparent Michaelis constant determined in the presence of inhibitor, $K_m$ is the value determined in the absence of inhibitor, (I) is the concentration of inhibitor, and $K_i$ is the dissociation constant for the enzyme-inhibitor complex.

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[2]The concentration of D-glyceraldehyde-3-phosphate decreased as much as thirty-three per cent in three hours if incubated at 30°.

In pH optimum and pH stability experiments, the hydrogen ion concentration of each buffer was recorded immediately before and after each assay to insure that no substantial change in pH had occurred. In pH stability experiments, assays were run at "time zero" and after a thirty-minute incubation period at 30°. In these experiments, all buffers consisted of 100 mM "controlling buffer" containing 1 mM EDTA and 14 mM 2-mercaptoethanol, and were adjusted to the desired pH with either HCl or NaOH. All pH values were determined at 30°. There was always an excess of the coupling enzyme in the assay mixture over the entire pH range.

In the denaturation titrations of human and rabbit muscle triosephosphate isomerase, where enzyme concentration was not the experimental variable, the concentration of triosephosphate isomerase in the denaturant solution was 150 ng/ml. In a normal experiment, 10 or 20 $\mu$l of a prediluted enzyme stock solution was added to 1.0 ml of guanidinium chloride/assay buffer solution. Assays were run at "time zero" and after a thirty-minute incubation period at 30°. All assay recordings were linear and no renaturation was detectable during the time span of the assay. In all cases, the guanidinium chloride in the aliquot to be assayed did not affect the coupling enzyme which always remained in excess. When the concentration of triosephosphate isomerase was excessive in the denaturant solution, the aliquot to be assayed was

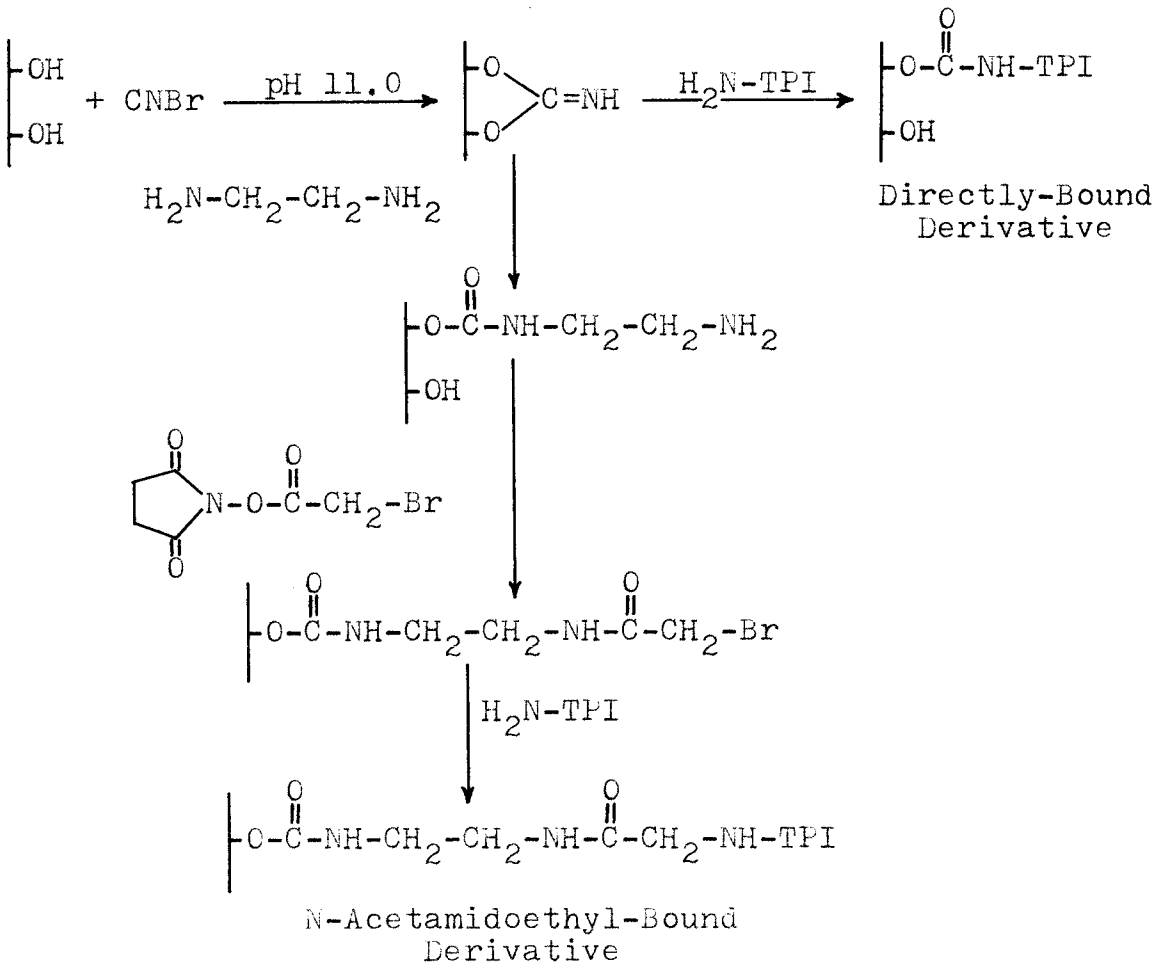
prediluted into the same concentration of guanidinium chloride as in the denaturant solution. In order to obtain a representative sample when an aliquot of matrix-bound triosephosphate isomerase was to be assayed, the solution was shaken as the sample was removed with an Eppendorf micropipet equipped with a tip from which the lower 3 mm had been removed.

Denaturation rates were measured and calculated by the method of Srere (1966) and Chervenka (1960). An aliquot containing 150 $\mu$g of native triosephosphate isomerase was added at "time zero" to 1.0 ml of the appropriate concentration of guanidinium chloride/assay buffer mixture. Assays were then run at "time zero" and at designated times during a 120-minute period. The per cent of the remaining activity was plotted against time in a semilogarithmic fashion to yield the first order rate constant for denaturation ($k_L'$ or $k_o'$).

## Coupling of Triosephosphate Isomerase to Sepharose 4B

Two methods (Cuatracasas and Anfinsen, 1971) of covalently binding rabbit muscle triosephosphate isomerase to Sepharose 4B were employed (Figure 2). In the first method, approximately 10 ml of Sepharose 4B were washed extensively with deionized water and filtered to form a moist cake. Twenty-five mg of cyanogen bromide was dissolved in 10 ml of deionized water and added to a 1:1 dilution of the filtered Sepharose 4B and deionized water. The pH of this solution

Figure 2. Coupling of rabbit muscle triosephosphate isomerase to a matrix. The enzyme was covalently bound to Sepharose 4B following the procedure as described in "Methods".


+ CNBr
pH 11.0
C=NH
$H_2N$-TPI
Directly-Bound Derivative
$H_2N-CH_2-CH_2-NH_2$
$N-O-C-CH_2-Br$
$H_2N$-TPI
N-Acetamidoethyl-Bound Derivative

was immediately adjusted to pH 11.0 and maintained with 0.1 N NaOH. Small quantities of ice were added to maintain the temperature at approximately 20°. After the reaction was complete (about twenty minutes), 10 ml of crushed ice were added and the mixture was filtered and washed with 120 ml of cold 10 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA. This solution was stirred gently at 4° for twenty-four hours then ethanolamine, pH 8.0, was added to a final concentration of 0.1 M. This mixture was stirred gently for another twenty-four hours at 4°. After the reaction period, all triosephosphate isomerase which was not covalently bound was removed by filtering and washing on a Buchner funnel with 10 mM sodium phosphate buffer, pH 8.0, containing 1.0 M NaCl and 1 mM EDTA.

In the second method, the enzyme was bound to Sepharose 4B through a spacer. Aminoethyl-Sepharose 4B was produced by adding cyanogen bromide-activated Sepharose to an equal volume of cold ethylenediamine at pH 10.0. After the reaction had proceeded for sixteen hours at 4°, the aminoethyl-Sepharose was washed with four liters of cold deionized water and stored at 4°. One millimole of bromoacetic acid and 1.2 millimoles of N-hydroxysuccinimide were dissolved in 8 ml of dioxane. To this solution was added 1.1 millimoles of dicyclohexylcarbodiimide with stirring. After seventy minutes, the dicyclohexylurea was removed by filtration on

a Buchner funnel and the entire filtrate (consisting primarily of dissolved N-bromoacetoxysuccinimide) was added directly to a 20-ml suspension of aminoethyl-Sepharose at 4° in 0.1 M sodium phosphate, 1 mM EDTA, pH 7.5, in a total volume of about 50 ml. This mixture was stirred gently for thirty minutes, then filtered and washed with two liters of 0.1 M NaCl. The enzyme was covalently bound to this bromoacetaminoethyl-Sepharose derivative by reacting 5 mg of enzyme in 0.1 M $NaHCO_3$ with 10 ml of a suspension of the "spacer-Sepharose" derivative at pH 9.0 for two days at room temperature. After the incubation period, the triosephosphate isomerase-acetamidoethyl-Sepharose 4B derivative was washed with 10 mM sodium phosphate buffer, pH 7.5, containing 1.0 M NaCl and 1 mM EDTA, until no enzyme activity was detected in the eluate. The two triosephosphate isomerase-Sepharose 4B derivatives were stored at 4° and there was no detectable loss in enzymatic activity after six months storage.

## Composition Coefficients

Composition coefficients were calculated by the method of Dedman *et al.* (1974) using a Fortran IV computer program adapted to the IBM 360-50 computer.

# RESULTS

## Isolation of Triosephosphate Isomerase from Human Erythrocytes

### Fraction I - Hemolysate

One unit (450 ml) of human blood was drawn into 67.5 ml of standard acid-citrate-dextrose anticoagulant solution. All subsequent purification steps were performed in an ice bath or cold room at 0 - 5° unless otherwise specified. Plasma was removed after centrifugation at 8,000g for sixty minutes and the buffy coat and leucocytes were removed with a pasteur pipet by mild vacuum. The erythrocytes were washed four times by resuspending them in 500 ml of cold 0.145 N NaCl followed by centrifugation as above. After washing, the erythrocytes were suspended in cold deionized water, frozen and thawed, and the lysate centrifuged at 8,000g for ninety minutes to remove the cell debris. The pH of the lysate was adjusted to 7.0 with cold 1.0 M sodium phosphate buffer.

### Fraction II - Phosphocellulose

A 5 cm pad of phosphocellulose, which had been previously cleaned and equilibrated in 10 mM sodium phosphate buffer, pH 7.0, was packed into a large plastic-fritted vacuum funnel (25 cm diameter). The hemolysate was applied slowly and evenly to the ion exchange cellulose followed by elution under

a mild vacuum with cold 10 mM sodium phosphate buffer, pH 7.0. Fractions of 200 ml each were collected and monitored for triosephosphate isomerase activity and total protein (Figure 3). Under these conditions, the triosephosphate isomerase activity was eluted in the void volume, while most of the other proteins, including hemoglobin, remained bound to the ion exchanger.

Fractions 2 - 10 which contained the triosephosphate isomerase activity were collected, concentrated, and dialyzed against 10 mM triethanolamine buffer, pH 8.0, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. This concentrate (Fraction II) routinely exhibited a 10 - 15-fold purification over Fraction I with 70 - 80% recovery of enzyme activity.

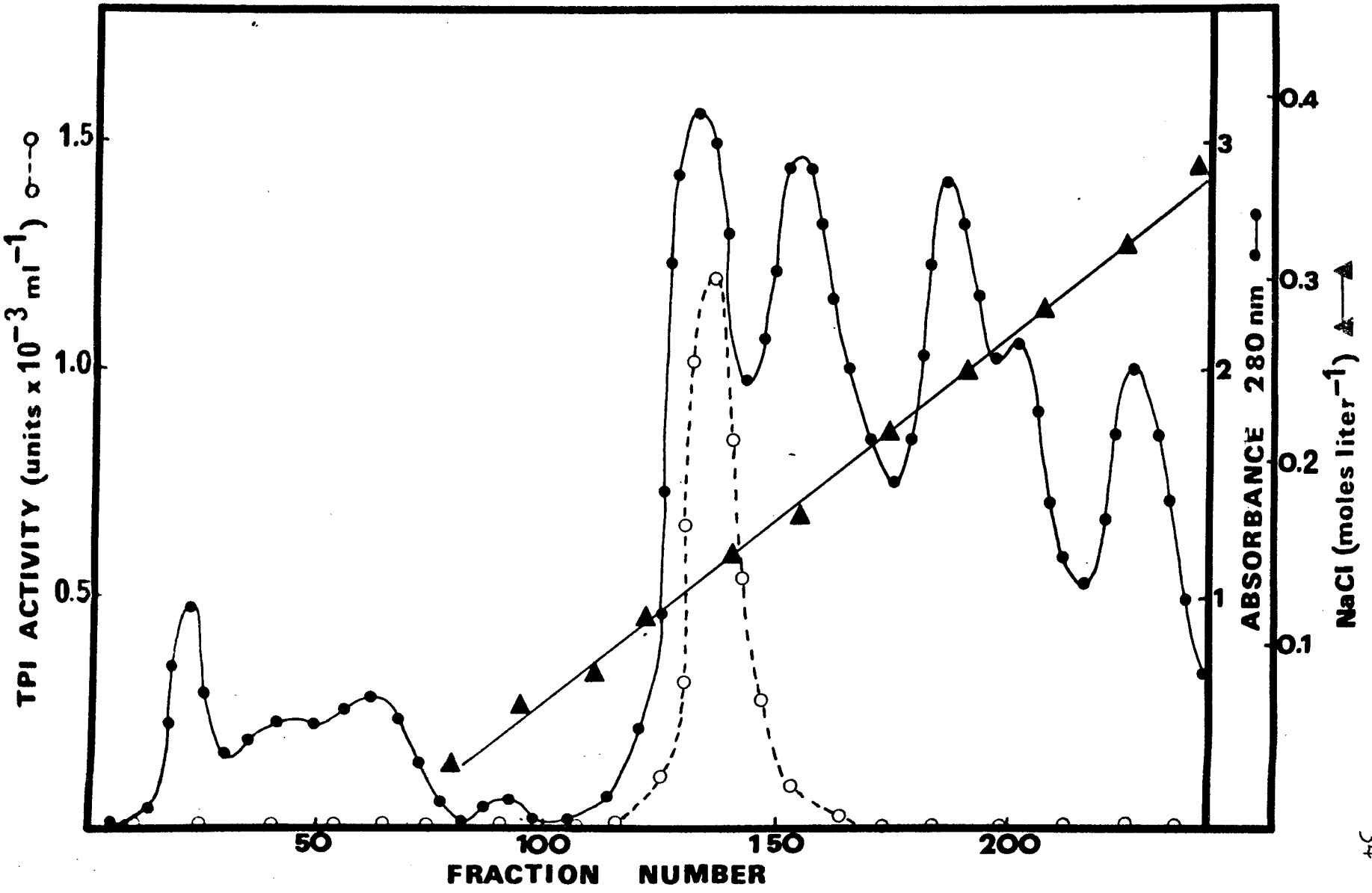
## Fraction III - First DEAE

Fraction II was applied to a 2.5 x 75 cm column of DEAE-cellulose which had been cleaned and equilibrated in 10 mM triethanolamine buffer, pH 8.0, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. A volume of 600 ml of this buffer was passed through the column at a flow rate of 30 ml per hour after which a linear salt gradient was applied. The gradient was composed of 1.0 liter of the column buffer in the mixing chamber and 1.0 liter of the same buffer, but containing 0.4 M NaCl, in the reservoir. The elution profile shown in Figure 4 indicates that under these conditions, triosephosphate isomerase was eluted with approximately

Figure 3. Fractionation of triosephosphate isomerase on phosphocellulose by batch process. The hemolysate (663 ml of Fraction I, 88.6 mg of protein per ml) was added to a plastic-fritted vacuum funnel (30 x 25 cm) packed to a height of 5 cm with phosphocellulose which had been washed and equilibrated with 10 mM sodium phosphate, pH 7.0, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. The column was developed as described in "Results" and after the triosephosphate isomerase activity was eluted from the column, the protein remaining on the exchanger was washed from the column with 1 M NaCl. All fractions were monitored for triosephosphate isomerase activity (○) and protein concentration (●).

TPI ACTIVITY (units x $10^{-2}$ $ml^{-1}$) o--o
ABSORBANCE 280 nm ●—●
VOLUME (liters)
1
2
3
4
5
2
4
6
8
10

Figure 4. Column chromatography of triosephosphate isomerase on DEAE-cellulose. Fraction II (934 ml, 3.3 mg of protein per ml) was applied to a DEAE-cellulose column which was equilibrated with 10 mM triethanolamine, pH 8.0, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. This same buffer was pumped through the column at a flow rate of 30 ml per hour and a linear salt gradient was established as described in "Results." Fractions (10 ml each) were assayed for triosephosphate isomerase activity (○); protein concentrations (●); and NaCl concentration (▲).

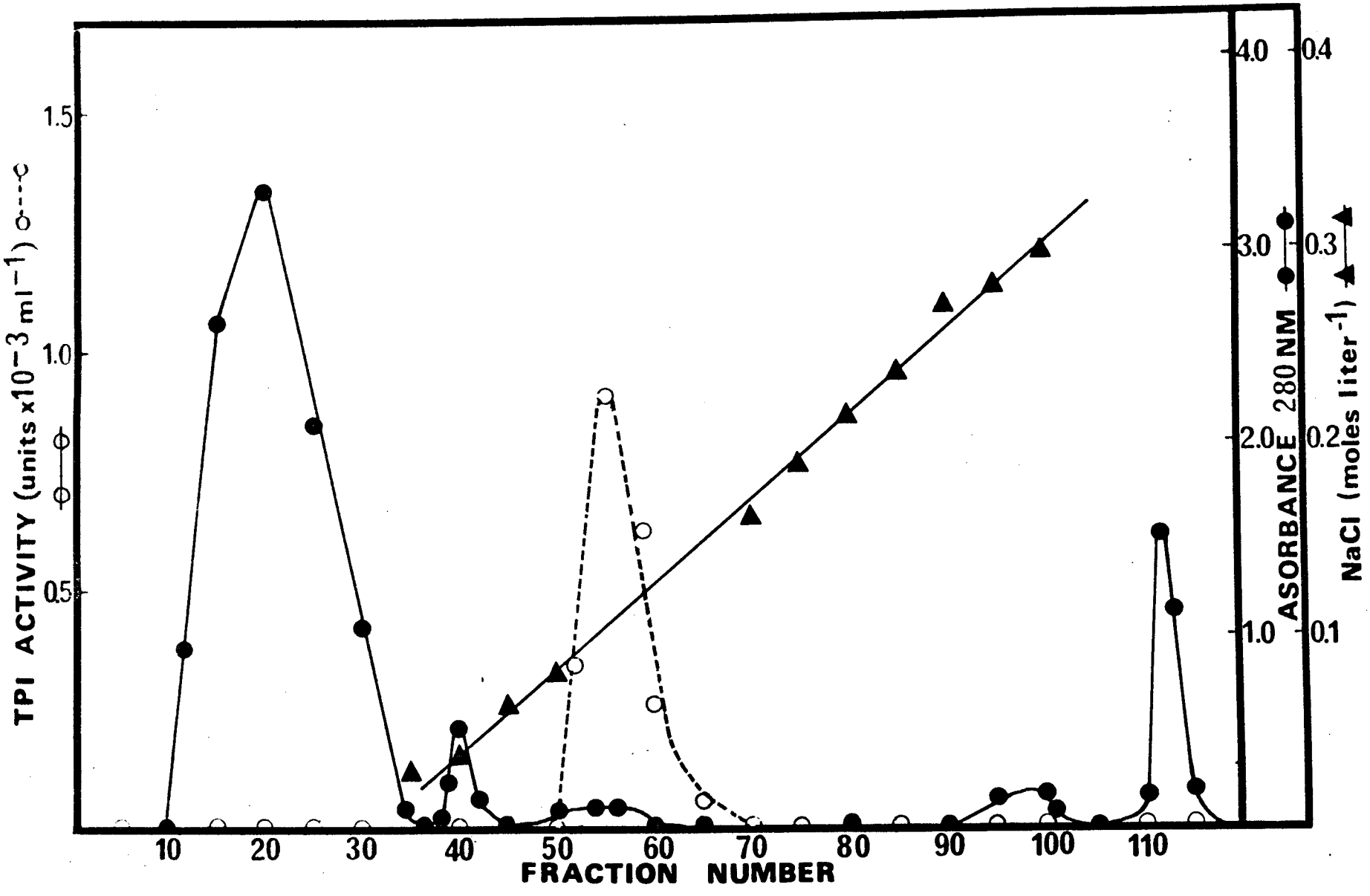

TPI ACTIVITY (units x $10^{-3}$ $ml^{-1}$) o---o
1.5
1.0
0.5
50
100
150
200
FRACTION NUMBER
3
2
1
ABSORBANCE 280 nm
0.4
0.3
0.2
0.1
NaCl (moles $liter^{-1}$)

0.15 M NaCl. Fractions containing triosephosphate isomerase activity (123 - 150) were collected and concentrated and dialyzed by ultrafiltration against 10 mM triethanolamine buffer, pH 8.5, containing 1 mM EDTA and 14 mM 2-mercapto-ethanol. This concentrate (75 ml) was designated as Fraction III.

## Fraction IV - Second DEAE

Fraction III was applied to a 2.1 x 22 cm column of DEAE cellulose which had been previously cleaned and equilibrated at pH 8.5 with 10 mM triethanolamine containing 1 mM EDTA and 14 mM 2-mercaptoethanol. After all of the sample had been applied, 300 ml of column buffer was passed through the column which eluted a large quantity of protein (Figure 5). After this initial elution, a shallow linear salt gradient consisting of 500 ml of column buffer in the mixing chamber, and 500 ml of column buffer containing 0.1 M NaCl as the limit buffer, was applied. The column was eluted at 20 ml per hour and the peak of triosephosphate isomerase activity was found in the third protein peak at approximately 0.025 M NaCl. Fractions 51 - 60 were pooled, concentrated and dialyzed by ultrafiltration against 50 mM triethanolamine, pH 7.6, containing 1 mM EDTA and 14 mM 2-merceptoethanol. The volume was reduced to 4 ml and was designated as Fraction IV.

Figure 5. Rechromatography of triosephosphate isomerase on DEAE-cellulose. Fraction III (75 ml, 4.2 mg protein per ml) was added to a DEAE-cellulose column (2.1 x 22 cm) which was equilibrated with 10 mM triethanolamine, pH 8.5, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. The column was developed as described in "Results" and assayed for triosephosphate isomerase (○); protein concentration (●); and NaCl concentration (▲).


TPI ACTIVITY (units x10$^{-3}$ ml$^{-1}$) o---o
1.5
1.0
0.5
10
20
30
40
50
60
70
80
90
100
110
FRACTION NUMBER
4.0
3.0
2.0
1.0
ASORBANCE 280 NM
0.4
0.3
0.2
0.1
NaCl (moles liter$^{-1}$)

## Fraction V - Crystallization

The enzyme was crystallized by dialysis of Fraction IV against a 0.40 saturated solution of ammonium sulfate. An amorphous precipitate formed during the early stages of crystallization which was removed by centrifugation. The enzyme suspension was returned to the dialysis bag and the ammonium sulfate concentration increased by the addition of saturated ammonium sulfate until crystallization of triosephosphate isomerase began at 0.76 saturation. After forty-eight hours, the needle-like crystals (Figure 6) were collected by centrifugation. Upon storage, the enzyme showed no loss of activity for at least six months.

A summary of the purification is shown in Table II. The enzyme was routinely purified 4,000 - 5,000-fold with an overall recovery of 50 - 60% and a specific activity of 8,000 moles of D-glyceraldehyde-3-phosphate isomerized per minute per milligram of protein at $30^{\circ}$. This corresponds to a molecular activity of $4.44 \times 10^{5}$ moles of D-glyceraldehyde-3-phosphate isomerized per minute per mole of enzyme. The specific activity of crystalline human triosephosphate isomerase was not increased by rechromatography on DEAE cellulose, phosphocellulose or Sephadex G-100. The overall recovery was limited by the approximate thirty per cent loss during the batch step on phosphocellulose (Fraction II). Attempts to achieve this initial fractionation by column chromatography

Figure 6. Photomicrograph of crystals of triosephosphate isomerase isolated from human erythrocytes. Crystals in 0.76 saturated ammonium sulfate were photographed at room temperature under oil immersion and with phase contrast. The total magnification is 1000-fold.

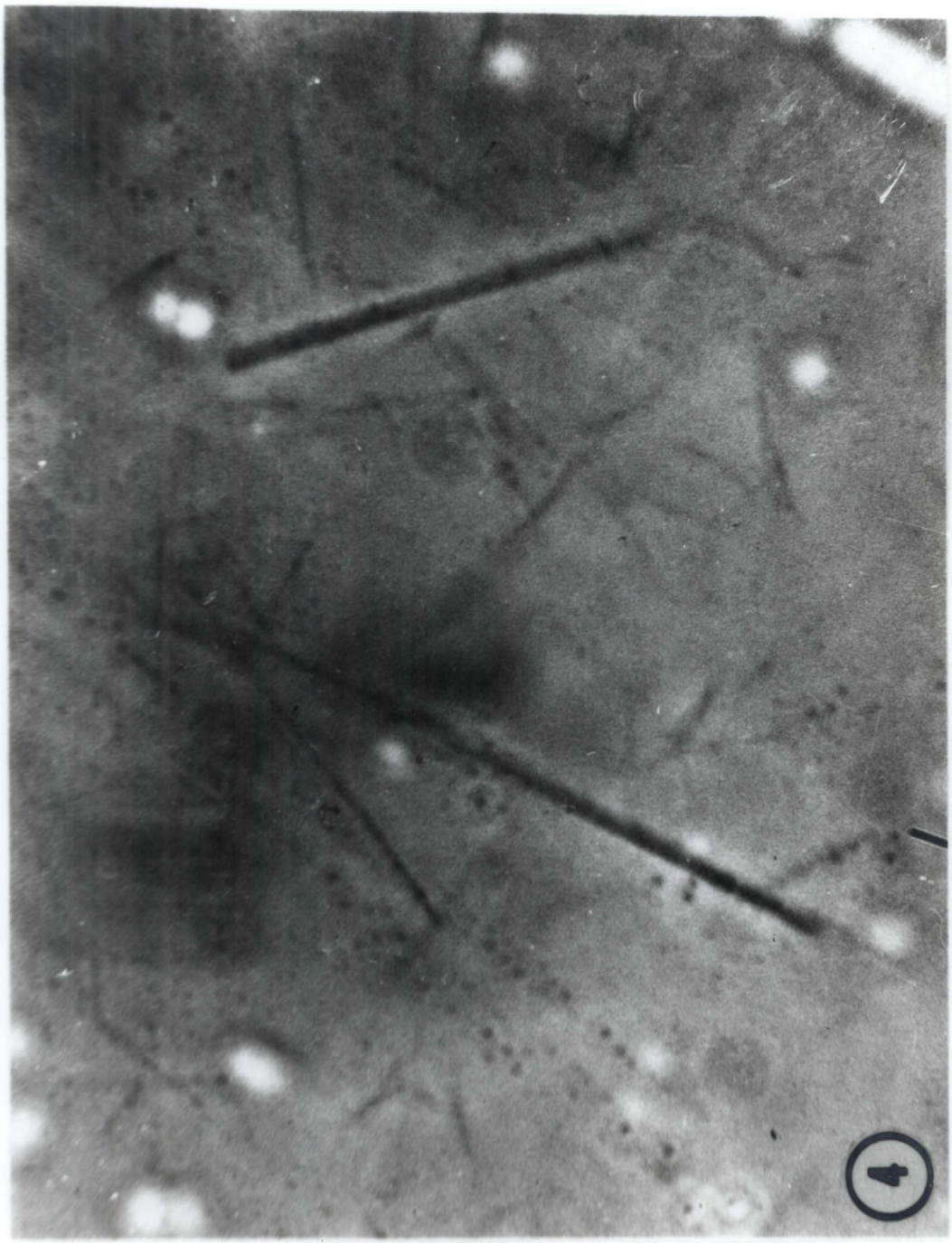


TABLE II

PURIFICATION OF ERYTHROCYTE TRIOSEPHOSPHATE ISOMERASE

| | Fraction | Total Activity (units) | Total Protein (mg) | Specific Activity (units/mg) | Purification | Per Cent Recovery |
|---|---|---|---|---|---|---|
| I. | Hemolysate* | 104,200 | 58,742 | 1.8 | (1) | (100) |
| II. | Phosphocellulose | 73,900 | 3,082 | 24.2 | 13.4 | 71 |
| III. | First DEAE | 73,000 | 315 | 232 | 121 | 70 |
| IV. | Second DEAE | 69,000 | 8.6 | 8,020 | 4,460 | 66 |
| V. | Crystals | 54,000 | 6.7 | 8,060 | 4,500 | 52 |

*Volume of the hemolysate was 663 ml.

were unsuccessful due primarily to overloading and flow stoppage due to the large amount of hemoglobin. Attempts to precipitate the hemoglobin by solvent extraction were also unsuccessful because the triosephosphate isomerase activity was lost due to denaturation. The complete purification of the enzyme from erythrocytes requires approximately two weeks if all buffers and ion exchange celluloses have been preliminarily prepared.

## Isolation of Triosephosphate Isomerase from Other Human Tissues

The enzyme was also successfully isolated from human cardiac and skeletal muscle, liver, and brain tissue by a modification of the procedure developed for isolation of the erythrocyte enzyme. Described below is a general method for the isolation of the enzyme from either of these other tissues.

### Fraction I - Homogenate

A portion of tissue was sliced, passed through a meat grinder, then blended twice for sixty seconds each, in a Waring blender. Homogenization was effected in two volumes of cold 10 mM triethanolamine buffer, pH 7.2, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. This homogenate was centrifuged at 8,000g for forty minutes, the supernatant solution collected, and the pellet resuspended and washed three times as described. The supernatant solutions were

combined, passed through glass wool to remove lipid material and designated as Fraction I (740 ml). The pH of Fraction I was adjusted to 7.0 with 1.0 M triethanolamine, pH 7.0.

## Fraction II - Phosphocellulose

Fraction I was applied to a 2.5 x 125 cm column of phosphocellulose which had been cleaned and equilibrated with 10 mM triethanolamine buffer, pH 7.2, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. The column was eluted at thirty ml per hour and the triosephosphate isomerase activity eluted in the void volume. The fractions containing the triose-phosphate isomerase activity were collected, concentrated and dialyzed by ultrafiltration against 10 mM triethanolamine, pH 8.0, containing 1 mM EDTA and 14 mM 2-mercaptoethanol.

## DEAE-Cellulose and Sephadex Fractionations

The fraction from phosphocellulose chromatography was fractionated on two successive DEAE-cellulose columns at pH 8.0 and pH 8.5 as outlined in the isolation procedure from erythrocytes. However, after the last DEAE fractionation, a high molecular weight impurity remained which was removed on a 2.5 x 95 cm Sephadex G-200 column to yield the pure enzyme.

A summary of the purification from cardiac muscle is shown in Table III. The purification procedure for the enzyme from the other human tissues was essentially identical. A comparison of Tables II and III illustrates two significant

TABLE III

PURIFICATION OF CARDIAC MUSCLE TRIOSEPHOSPHATE ISOMERASE

| | Fraction | Total Activity (units) | Total Protein (mg) | Specific Activity (units/mg) | Purification | Per Cent Recovery |
|---|---|---|---|---|---|---|
| I. | Homogenate* | 207,000 | 10,200 | 20.3 | (1) | (100) |
| II. | Phosphocellulose | 189,000 | 1,950 | 97.0 | 4.8 | 91 |
| III. | First DEAE | 168,000 | 56 | 3,000 | 148 | 81 |
| IV. | Second DEAE | 159,000 | 22.7 | 7,000 | 345 | 77 |
| V. | Gel Filtration | 150,000 | 13.6 | 11,000 | 542 | 72 |

*Original sample consisted of 160 grams wet weight of cardiac tissue.

differences in the purification from these tissues and that from erythrocytes. First, a much smaller extent of purification (e.g. 540-fold for cardiac muscle) is needed to yield a homogeneous protein with a specific activity of 11,000 units per mg. Second, the overall recovery from these other tissues is approximately twenty per cent higher than that from erythrocytes.

## Homogeneity Studies of Human Triosephosphate Isomerase

### Ultracentrifugation

In order to establish not only the homogeneity of purified human triosephosphate isomerase, but also to determine certain basic physical parameters of the enzyme, a series of ultracentrifugation experiments were performed. When subjected to sedimentation velocity ultracentrifugation (Figure 7), human cardiac muscle triosephosphate isomerase sedimented as a single boundary with a sedimentation coefficient ($s_{20,w}$) of 4.11S (5.0 mg per ml). When human erythrocyte triosephosphate isomerase was subjected to meniscus depletion sedimentation equilibrium centrifugation as outlined in "Methods," a linear distribution of ln C *vs.* $r^2$ (Figure 8) was highly indicative of a homogeneous protein and yielded a weight average molecular weight, $\bar{M}_w$, of 56,000 daltons. The human cardiac muscle enzyme yielded an identical molecular weight when subjected to sedimentation equilibrium

Figure 7. Sedimentation velocity ultracentrifugation of human cardiac muscle triosephosphate isomerase. Sedimentation velocity ultracentrifugation was carried out at 20° at 60,000 rpm in an An-D rotor equipped with a 12 mm double sector cell with sapphire windows. The sample sector contained 0.35 ml of enzyme (5.0 mg per ml) in a buffer consisting of 10 mM triethanolamine, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1 M NaCl, pH 7.5. The other sector contained buffer only. This photograph was taken 72 minutes after reaching two-thirds speed. Migration is from left to right.

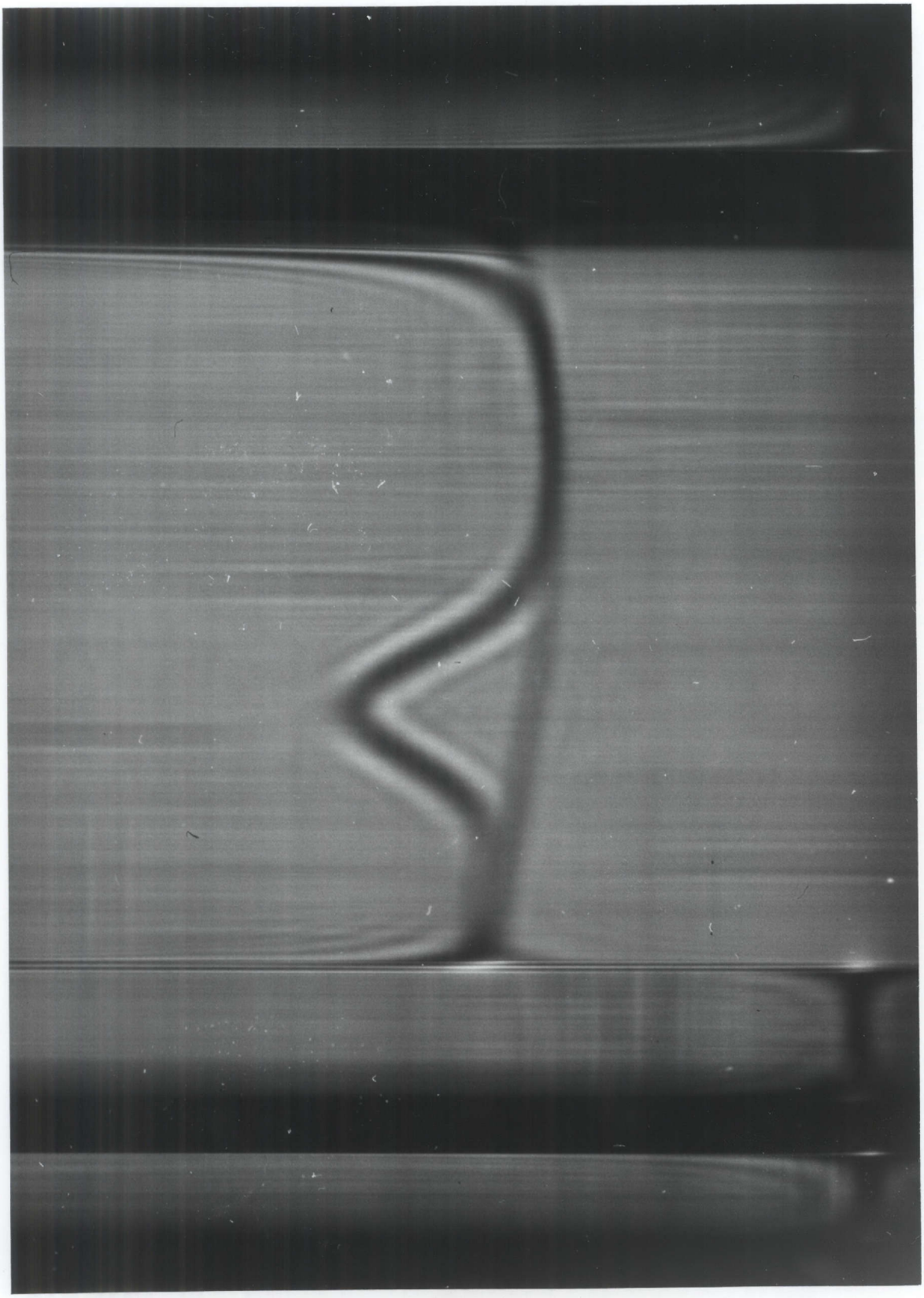
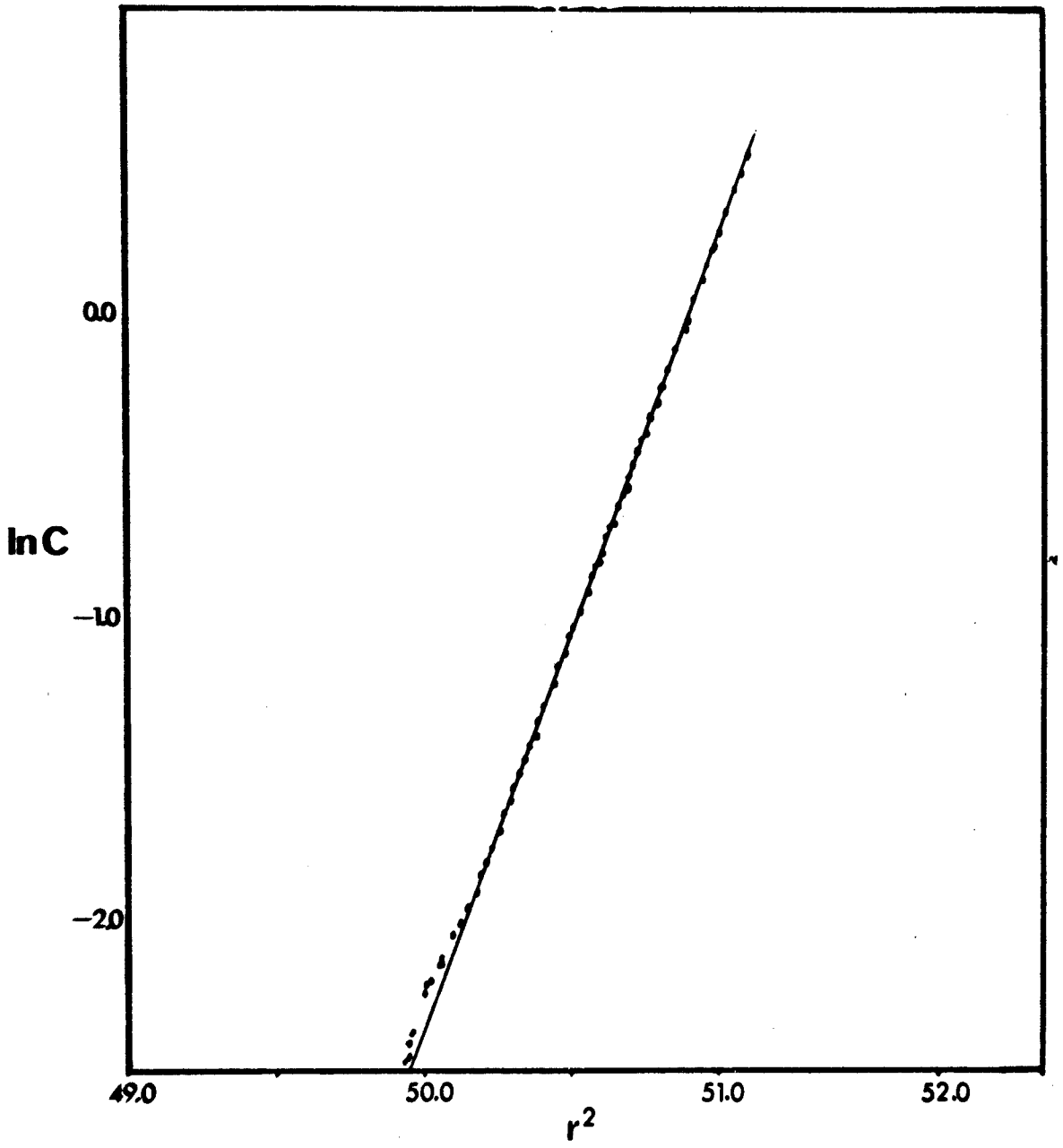


Figure 8. Sedimentation equilibrium ultracentrifugation of human erythrocyte triosephosphate isomerase. Ultracentrifugation was carried out at 20° at 27,690 rpm in the An-D rotor in 12 mm double sector cells with sapphire windows. The sample sector contained 100 microliters of enzyme (0.48 mg per ml) in a buffer consisting of 10 mM tris-(hydroxymethyl)aminomethane, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1 M NaCl, pH 7.5. The other sector contained only the buffer. Photographs of the Raleigh interference patterns were taken after equilibrium had been established and the protein concentration, C, was calculated from the fringe displacement in millimeters as measured on the microcomparator. The abscissa represents the square of the distance (cm) from the center of rotation.


0.0
ln C
−1.0
−2.0
49.0
50.0
51.0
52.0
r²

ultracentrifugation. The enzyme isolated from skeletal muscle, liver and brain sedimented as single boundaries with sedimentation coefficients ($s_{20,w}$) of 4.12S (5.0 mg per ml). This sedimentation coefficient corresponds to a molecular weight of approximately 56,000 (Chervenka, 1967) for a globular protein. When human cardiac muscle or erythrocyte triosephosphate isomerase was subjected to analytical gel filtration on a calibrated Sephadex G-100 column, a molecular weight of approximately 55,000 was obtained. Thus, with respect to molecular weight, human triosephosphate isomerase from all tissues examined is identical. When the sedimentation coefficient and the molecular weight are substituted into the Svedberg equation, a diffusion coefficient of $6.70 \times 10^{-7}$ $cm^2$ $sec^{-1}$ is calculated, from which a frictional ratio of 1.27 is obtained and thus a Stoke's radius, r, of 32 Å can be calculated (Van Holde, 1971). Although these ultracentrifugation studies strongly suggested a homogeneous protein, as clearly illustrated recently by Feramisco et al. (1974), this technique in itself should not be regarded as a rigorous criterion of purity. Therefore, electrophoretic studies were also employed to assess the homogeneity of this protein.

## Electrophoresis

When purified human erythrocyte triosephosphate isomerase was subjected to polyacrylamide gel electrophoresis and stained

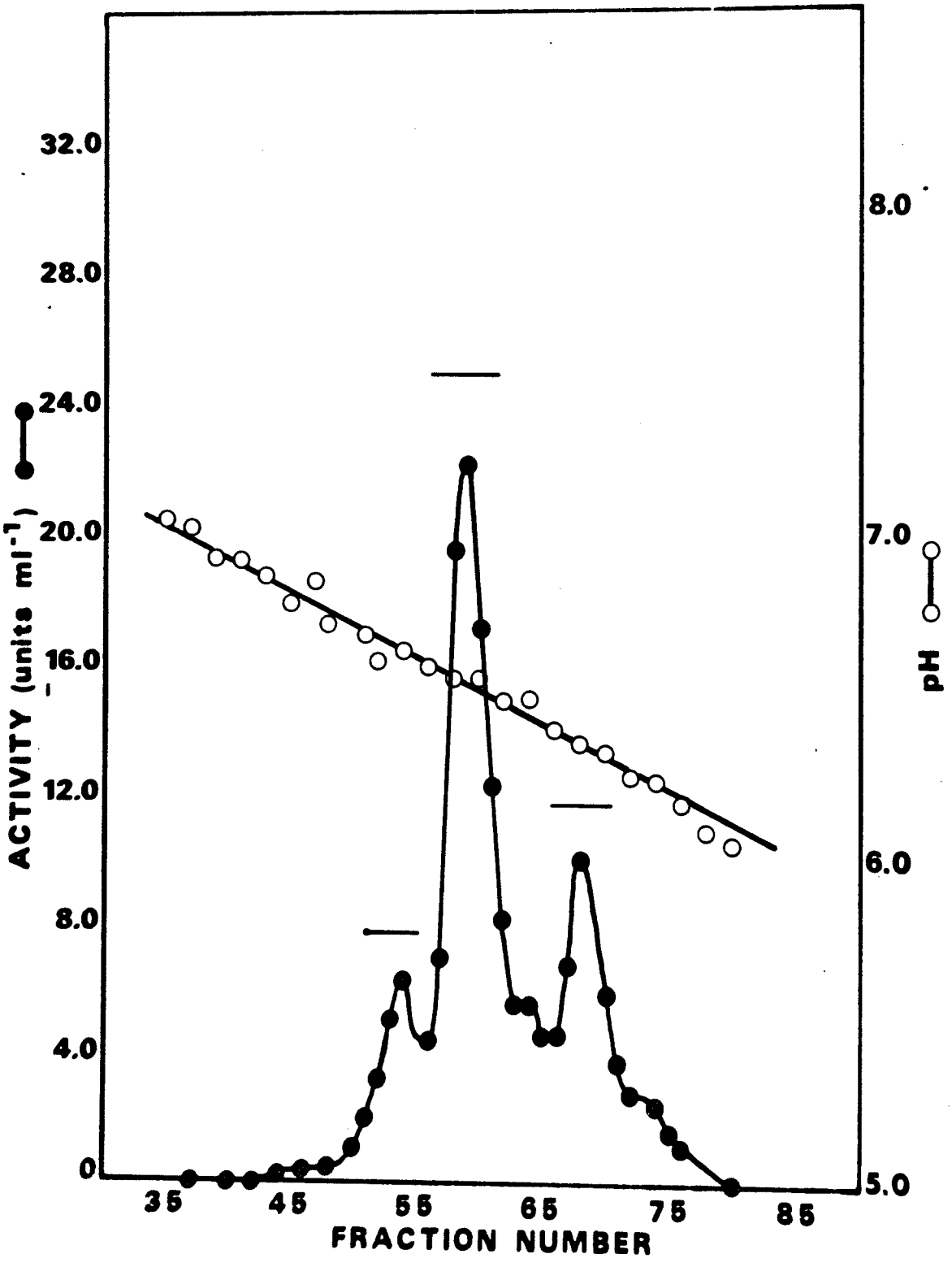
for protein as described in "Methods," three bands of protein were observed. Upon staining gels with either of two different types of triosephosphate isomerase activity stains, (Scopes et al., 1968; Kaplan et al., 1968), three bands of triosephosphate isomerase activity were clearly visible, corresponding to the three bands stained for protein.

## Establishment of Isozymes of Human Triosephosphate Isomerase

### Isoelectric Focusing

When crystalline human erythrocyte triosephosphate isomerase was subjected to isoelectric focusing, three catalytically active components were resolved (Figure 9). Component I accounted for only 5 - 10% of the total activity and focused with an apparent isoelectric pH of 6.7. The major component (II) focused at pH 6.5 and comprised approximately 70 - 75% of the total enzyme activity, while a third component (III) was observed at pH 6.1 and accounted for 20 - 25% of the total activity. This pattern was unaltered under a variety of conditions including extending the focusing time to seven days, or reversing the electrodes. The same patterns and activity ratios were observed on seven different preparations of the erythrocyte enzyme, each from different blood donors. The specific activities for the three forms (average of five isoelectric focusing experiments) were found to be 4,000 - 4,800 for Component I, 9,000 - 11,000 for Component II, and 5,000 - 5,800 for Component III.

Figure 9. Isoelectric focusing of crystalline human erythrocyte triosephosphate isomerase. Crystalline triosephosphate isomerase (0.05 mg) (specific activity 7200 units/mg) was electrofocused for 92 hours at 4° in 1% narrow range ampholines (pH 5 - 7) as described in "Methods." Fractions of 1 ml each were collected and assayed for pH (○) and triosephosphate isomerase activity (●). The fractions indicated by the horizontal bar were pooled for refocusing experiments.


32.0
28.0
24.0
20.0
16.0
12.0
8.0
4.0
0
ACTIVITY (units ml⁻¹)
8.0
7.0
6.0
5.0
pH
35
45
55
65
75
85
FRACTION NUMBER

## Isozyme Refocusing

When Component III was collected (as indicated in Figure 9) and refocused under identical conditions, most of the enzymatic activity was found at pH 6.1 (Figure 10A). However, some activity was observed which corresponded to Component II. This appeared to be due to contamination of Component III, rather than to an equilibrium process, since a second refocusing of Component III (Figure 10B) yielded only triosephosphate isomerase with an isoelectric pH value of 6.1. Component I, when refocused the first time (Figure 11A), showed contamination from Components II and III, but the relative amount of pH 6.7 material was markedly increased. Upon a second refocusing of Component I, only the pH 6.7 isozyme was obtained. Component II yielded a single species (pI = 6.5) on the first refocusing (Figure 11B). Thus, the three electrophoretic forms of human triosephosphate isomerase refocused as distinct species and did not seem to be artifacts produced by the electrofocusing system. Furthermore, each of the forms was a stable, isolable species.

Recently, multiple electrophoretic forms of phosphoglucose isomerase were demonstrated to be the result of oxidative artifacts (Payne *et al.*, 1972; Blackburn *et al.*, 1972) which resulted in "pseudoisozymes" (Blackburn *et al.*, 1972) with catalytic activity. In this case, the electrophoretic multiplicity could be reversed by treatment with

Figure 10. Isoelectric refocusing of Component III. A. Component III from Figure 8 was refocused for 91 hours in 1% ampholines (pH 5 - 8). Otherwise, conditions were as in Figure 9. The material indicated by the horizontal bar was pooled and 55 units were refocused (B) under identical conditions in pH 5 - 7 ampholines.

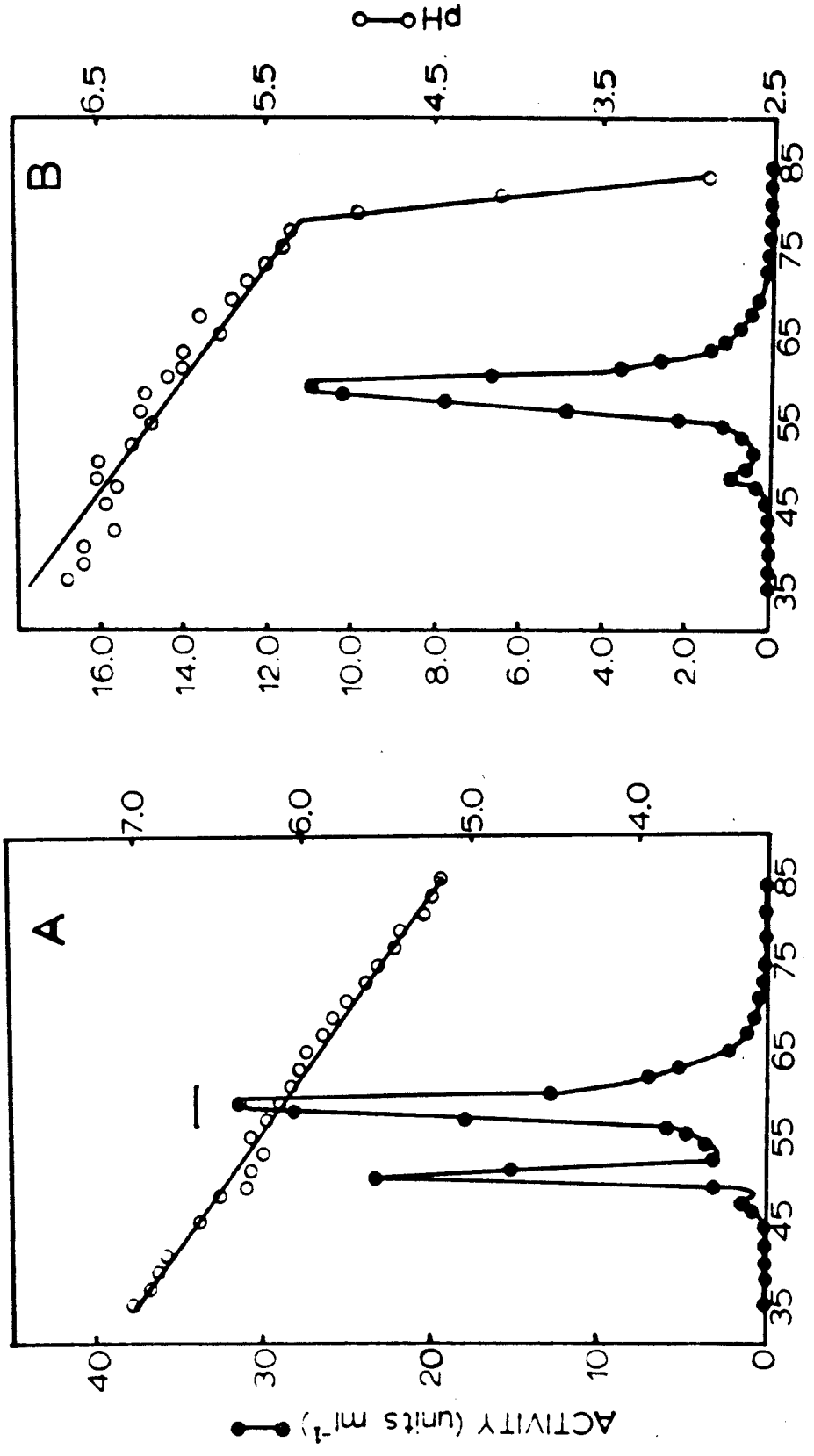
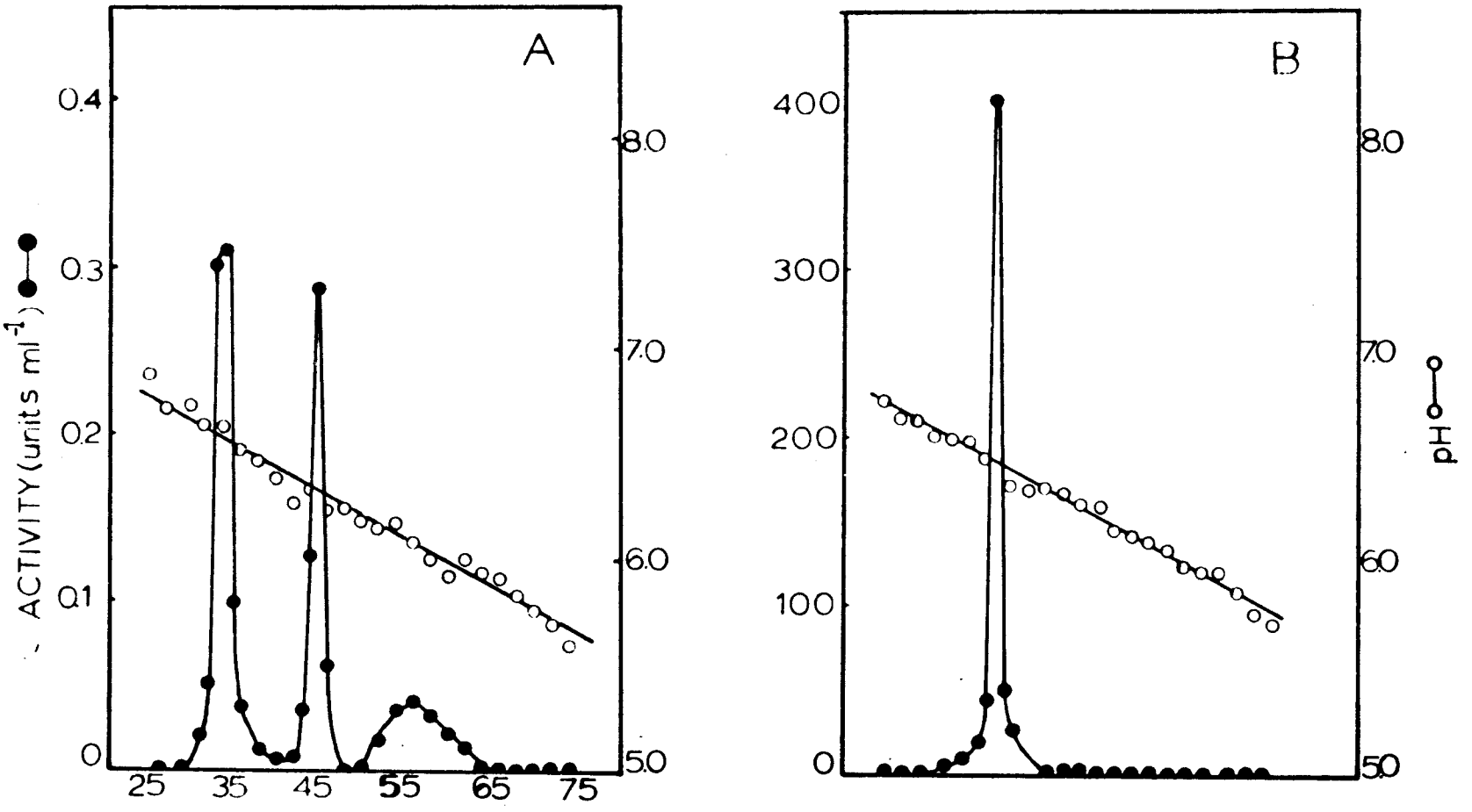

A
B
ACTIVITY (units ml⁻¹)
pH
FRACTION NUMBER

Figure 11. Isoelectric refocusing of Components I and II. Three units of Component I (A) and 500 units of Component II (B) were refocused in pH 5-7 ampholines as in Figure 10.


A
B
0.4
0.3
0.2
0.1
0
8.0
7.0
6.0
5.0
400
300
200
100
0
8.0
7.0
6.0
5.0
25
35
45
55
65
75
ACTIVITY (units ml$^{-1}$)
pH
FRACTION NUMBER

reducing agents (Payne et al., 1972; Blackburn et al., 1972; Tilley and Gracy, 1974). However, treatment of tissue extracts or crystalline triosephosphate isomerase with 2-mercaptoethanol or dithioerythritol caused no alteration in the electrofocusing patterns. Furthermore, the three isozymes of human triosephosphate isomerase were observed in fresh or aged (24 hours at 25°) erythrocytes when the hemolysates were electrofocused under identical conditions. The addition of the proteolytic inhibitor phenylmethane sulfonyl fluoride likewise had no effect on the electrofocusing profile. Thus, the multiplicity did not seem to be the result of oxidative artifacts, the isolation procedure, or proteolysis.

## Isozyme Molecular Weights

When human triosephosphate isomerase was subjected to sedimentation equilibrium ultracentrifugation in 6 M guanidinium chloride, again a linear ln C vs. $r^2$ analysis indicated homogeneity and provided a weight average molecular weight of 28,000. These data indicated that the enzyme was a dimer composed of two subunits of essentially identical molecular weights.

Table IV presents the molecular weights of the native isozymes of human cardiac muscle triosephosphate isomerase and of the subunits of the isozymes after dissociation in guanidinium chloride. Essentially identical values for the molecular weights of the three were obtained, demonstrating

TABLE IV

MOLECULAR WEIGHTS OF NATIVE AND DISSOCIATED HUMAN CARDIAC MUSCLE TRIOSEPHOSPHATE ISOMERASE ISOZYMES

| Component | Molecular Weight* | |
|---|---|---|
| | Native** | Subunit*** |
| I (pI - 6.7) | 56,400 ± 2,200 | 28,700 ± 1,100 |
| II (pI - 6.5) | 56,200 ± 2,100 | 29,100 ± 1,000 |
| III (pI - 6.1) | 57,000 ± 2,700 | 28,900 ± 900 |

*Results of two to four high-speed meniscus depletion sedimentation equilibrium ultracentrifugation experiments on each sample.

**Samples from isoelectric focusing were collected, concentrated, and dialyzed against buffer consisting of 10 mM triethanolamine, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 M NaCl, pH 7.5. Weight average molecular weights were calculated in the standard manner (Yphantis, 1964).

***Samples from isoelectric focusing were dialyzed, lyophilized to dryness, and dissolved in buffer consisting of 6 M guanidine hydrochloride, 1.0 M NaCl, 10 mM 2-mercaptoethanol, and dialyzed for two days against the same solution. Sedimentation equilibrium ultracentrifugation experiments were carried out as described in "Methods."

their close structural similarity. Sedimentation velocity ultracentrifugation of Components II and III corroborate these data ($s_{20,w}$ = 4.09S and 4.14S for Components II and III, respectively) which are essentially identical to those for the unresolved enzyme ($s_{20,w}$ = 4.12S). This shows that the isozymes are not the result of different degrees of aggregation or dissociation, and that all three isozymes are dimers of molecular weight approximately 56,000.

Identical isoelectric focusing patterns and activity distributions (Table V) were observed for the enzyme purified from human cardiac and skeletal muscle, liver, and brain. Starch gel electrophoresis of these purified enzymes and crude extracts from other human tissues including lung, spleen, and kidney also corroborated these data (Snapka et al., 1974). Thus, these data failed to demonstrate a tissue-specific localization of the three forms of this enzyme since the relative distribution of the three isozymes was essentially identical in these tissues.

Some difficulty was encountered in establishing the precise isoelectric pH values for the three isozymes from the different tissues. The separation of the three forms was reproducible from experiment to experiment (i.e. Components I and II differed by 0.21 $\pm$ 0.05 pH and Components II and III by 0.40 $\pm$ 0.05 pH), but the absolute values of the isoelectric pH of each component varied as much as 0.5 pH unit

TABLE V

ISOELECTRIC FOCUSING OF TRIOSEPHOSPHATE ISOMERASE IN HUMAN TISSUE EXTRACTS

| Tissue | Isozyme | Isoelectric pH | Relative Per Cent of Total Activity |
|---|---|---|---|
| Erythrocyte (6)* | I | 6.75 | 6 |
| | II | 6.54 | 70 |
| | III | 6.17 | 25 |
| Cardiac Muscle (3) | I | 6.71 | 5 |
| | II | 5.50 | 70 |
| | III | 6.11 | 25 |
| Liver (1) | I | 6.68 | 10 |
| | II | 6.51 | 65 |
| | III | 6.08 | 25 |
| Brain (2) | I | 6.75 | 3 |
| | II | 6.51 | 70 |
| | III | 6.01 | 27 |

*The number in parentheses represents the number of isoelectric focusing experiments on that tissue. The isoelectric pH values and relative percentages represent averages of the experiments on that tissue.

from one electrofocusing experiment to another. The pH gradient from electrofocusing is known to be influenced by several factors including absorption of $CO_2$, temperature, concentration of reducing agents and the sucrose gradient (Anderson, 1971; Haglund, 1971; Ikehara et al., 1972). In view of this difficulty, it was necessary to carry out co-electrofocusing experiments to conclusively establish the identical nature of the three components from different tissues.

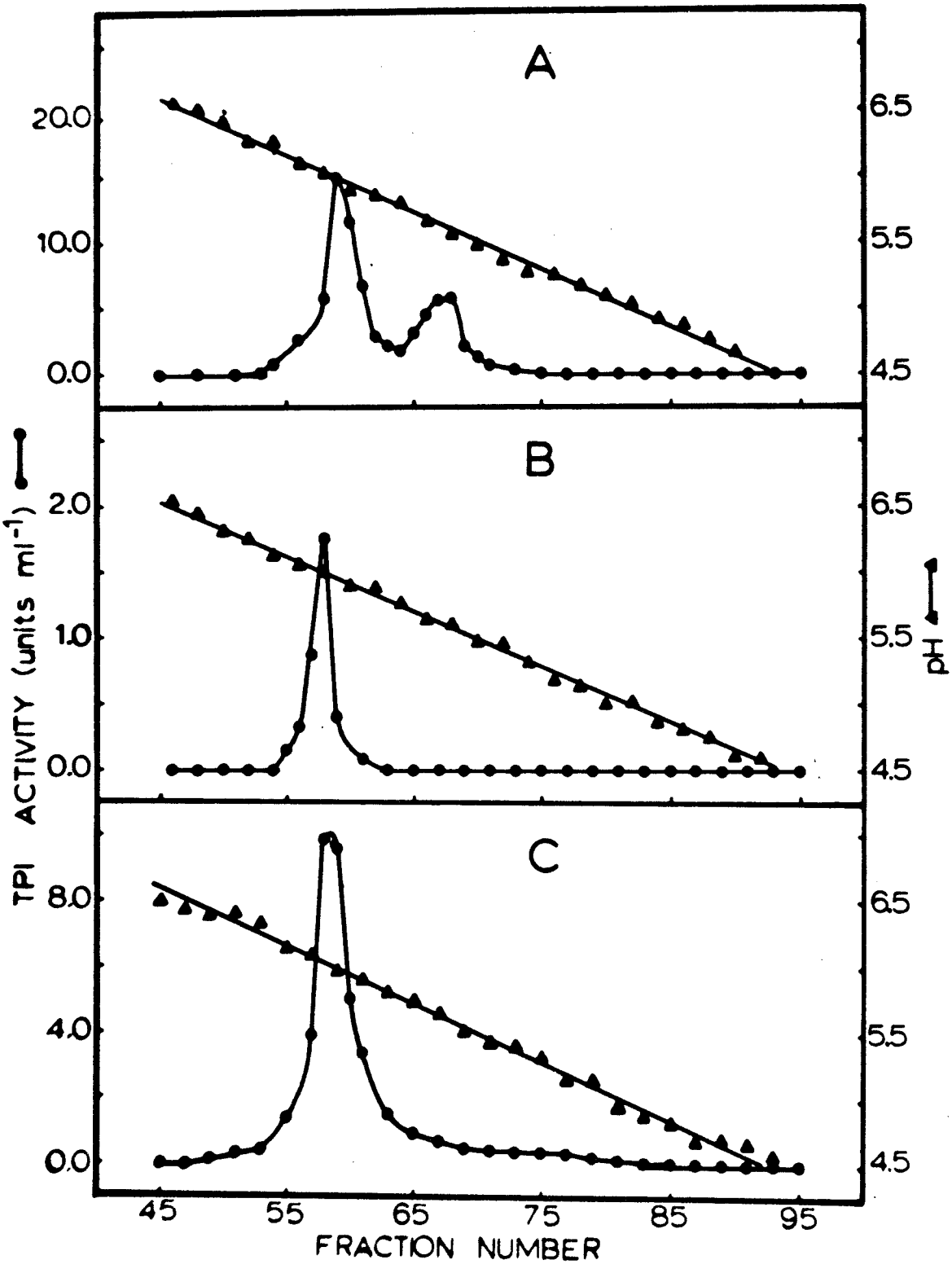
## Isozyme Cofocusing

When equal amounts of erythrocyte Components II and III were cofocused with heart Component II, the pattern shown in Figure 12A was obtained. Component II, with an isoelectric pH of 6.5, contained twice the amount of enzyme activity as the peak which focused at pH 6.1. Thus, it appears that within the resolving power of this technique, the Component II isozymes of heart and erythrocyte are identical. When erythrocyte Component II and brain (Figure 12B) or liver (Figure 12C) Component II were cofocused, a single enzymatically active peak resulted, further testifying to the identical electrophoretic nature of the triosephosphate isomerase isozymes from various tissues.

## Subcellular Fractionation of Human Liver Cells

Because organelle-specific isozymes of triosephosphate isomerase had been documented in the unicellular protist,

Figure 12. Isoelectric cofocusing of human heart, brain, and liver triosephosphate isomerases with the erythrocyte enzyme. A. Crystalline triosephosphate isomerase from erythrocytes (27.9 units of Component II and 27.9 units of Component III) was mixed with 27.8 units of Component II isolated from human cardiac muscle. The mixture was subjected to isoelectric focusing at 4° in 1% ampholines (pH 5 - 7) at 600 v for 79 hours. Fractions were eluted and assayed for pH (▲) and enzyme activity (●). B. Erythrocyte triosephosphate isomerase Component II (4.3 units) was mixed with Component II from brain (4.2 units). Electrofocusing and analysis were described in (A). C. Erythrocyte Component II (17 units) was mixed with 16 units of Component II from liver. Electrofocusing and analysis were as described in (A).


A
B
C
20.0
10.0
0.0
2.0
1.0
0.0
8.0
4.0
0.0
6.5
5.5
4.5
TPI ACTIVITY (units ml⁻¹)
pH
45
55
65
75
85
95
FRACTION NUMBER

Euglena gracilis (Mo et al., 1973) and in pea leaves (Anderson, 1971), human liver was fractionated into its subcellular constituents. As shown in Table VI, essentially all of the triosephosphate isomerase activity was found in the cytosolic fraction. The small amount of activity detected in the other fractions was probably due to cross contamination from the cytosolic fraction.

## Isozyme Hybridization

Since it appeared that the three forms of human triosephosphate isomerase might be the result of an AA, AB, and BB distribution of dimers, subunit hybridization experiments were carried out. After human triosephosphate isomerase had been dissociated, essentially 100% of the activity was recovered by removal of the denaturant by dialysis. Reassociation and reactivation were favored by a high salt concentration and the presence of reducing agents.

Components I, II, and III from either human erythrocytes or cardiac muscle were dissociated and reassociated, subjected to polyacrylamide gel electrophoresis, and stained for total protein (densitometer tracings are presented in Figure 13). Figure 13A indicates that native Component II yielded only a single band on polyacrylamide gels. After dissociation and reassociation of Component II (Figure 13B), three bands of protein were observed which corresponded to the three parent types, I, II, III. Dissociation and reassociation of

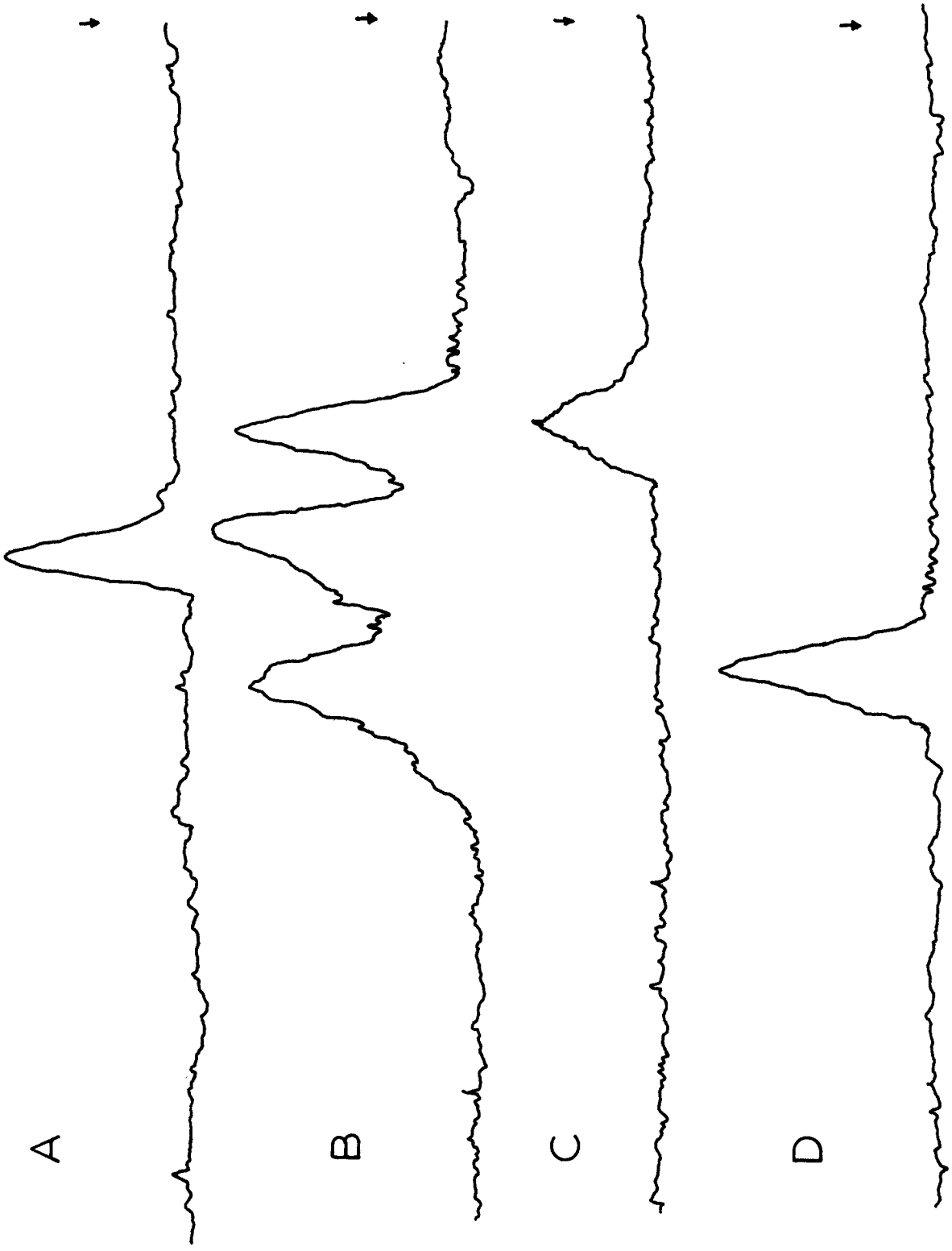
TABLE VI

TRIOSEPHOSPHATE ISOMERASE ACTIVITY CONTENT OF ORGANELLES FROM HUMAN LIVER CELLS

| Fraction | Total Enzyme Activity (units)* | Per Cent Activity in Homogenate |
|---|---|---|
| Homogenate | 11,280.0 | 100.0 |
| Cytosolic | 14,440.0 | 128.0 |
| Nuclear | 773.8 | 6.9 |
| Mitochondrial | 147.3 | 1.3 |
| Microsomal | 69.0 | 0.6 |

*Original sample was 10.0 g wet weight of tissue.

Figure 13. Polyacrylamide gel electrophoresis of dissociated and reassociated triosephosphate isomerase isozymes. One hundred micrograms of crystalline human erythrocyte triosephosphate isomerase were added to 2.0 ml of 10 mM triethanolamine buffer, pH 7.5, containing 1 mM EDTA, 14 mM 2-mercaptoethanol 0.1 M NaCl, and the solution was assayed for activity. The sample was then dialyzed at 4° against the same buffer but without guanidinium chloride. Samples of 30 - 50 μg each were run for 4 hours at 2 ma per gel in 7% polyacrylamide gels containing 10 mM 2-mercaptoethanol. Gels were subsequently stained for 12 hours in Amido Black as described in "Methods." After destaining, gels were scanned on a Canalco recording microdensitometer, and the recording was traced. The arrow indicates the top of the gel, with the anode to the left and the cathode to the right. A, native Component II; B, Component II after dissociation and reassociation; C, Component I after dissociation and reassociation; and D, Component III after dissociation and reassociation.


A
B
C
D

Component I (Figure 13C) or III (Figure 13D) resulted in only single bands which corresponded to the native isozymes I and III. When Components I and III were dissociated and reassociated within the same mixture, the isozyme distribution pattern was essentially identical with that shown in Figure 13B. Similar studies were carried out by electrofocusing in polyacrylamide gels with identical results.

## Amino Acid Compositions

Table VII contains the amino acid compositions of the three human triosephosphate isomerase isozymes. Significant differences (more than one residue change per subunit) were found in the content of histidine, serine, glycine, valine, and leucine. The greater histidine content of Component I is consistent with its more basic isoelectric point. Coincidental with the greater histidine content of Component I is its lower ratio of total acidic to basic amino acids (1.76) as compared to those of Component II (1.98) and III (2.07), assuming that the degree of amidation is the same for all components. For those amino acids which showed significant differences, the values obtained for Component II were intermediate to the values for Components I and III and are consistent with its designation as a heterodimer.

A comparison of the compositions of the human erythrocyte isozymes and the rabbit muscle isozymes (Krietsch *et al.*, 1971) revealed significant differences in the compositions

TABLE VII

AMINO ACID ANALYSIS OF TRIOSEPHOSPHATE ISOMERASE ISOZYMES FROM HUMAN ERYTHROCYTES

| Amino Acid | Residues per 56,000 g | | |
|---|---|---|---|
| | Component I | Component II | Component III |
| Lysine | 31.7 | 30.2 | 29.8 |
| Histidine | 14.8 | 10.1 | 7.9 |
| Arginine | 16.5 | 17.1 | 18.4 |
| Aspartic Acid | 60.4 | 61.2 | 61.5 |
| Threonine | 19.9 | 20.1 | 19.3 |
| Serine | 48.2 | 38.6 | 32.4 |
| Glutamic Acid | 51.1 | 52.3 | 52.1 |
| Proline | 32.0 | 31.8 | 32.4 |
| Glycine | 42.7 | 45.7 | 54.2 |
| Alanine | 41.7 | 42.1 | 41.6 |
| Half-cystine** | 9.4 | 10.1 | 9.7 |
| Valine | 37.5 | 32.7 | 30.1 |
| Methionine | 4.2 | 4.2 | 4.0 |
| Isoleucine | 17.1 | 15.9 | 15.1 |
| Leucine | 41.5 | 45.0 | 48.3 |
| Tyrosine | 13.3 | 13.9 | 14.2 |
| Phenylalanine | 18.6 | 20.2 | 20.9 |
| Tryptophan*** | 9.2 | 8.6 | 8.6 |

*A total of five analyses (two from samples recovered from polyacrylamide gels and three from isoelectric focusing) were run on each component. The values given represent the average. In no case was the deviation from the mean greater than four per cent. Total recovery of amino acids on a protein basis yielded average weight recoveries of 102.1, 98.9, and 101.6 per cent for Components I, II, and III, respectively.

**Determined spectrophotometrically with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

***Determined spectrophotometrically by the method of Edelhoch(1967).

from the two sources. The most significant difference was in the greater content of basic amino acids of the rabbit muscle isozymes which is consistent with their more basic isoelectric pH values.

Based on the methionine content, minimum molecular weights of the isozymes of 12,700 were calculated. Each isozyme can be cleaved into a minimal number of peptides by reacting the methionines with cyanogen bromide. The rabbit muscle and human erythrocyte isozymes contain identical quantities of methionine, cysteine, and tryptophan.

When the amino acid compositions of human erythrocyte triosephosphate isomerase were statistically compared to those of the rabbit muscle isozymes, and the unresolved enzyme from rabbit liver, chicken, coelacanth and yeast, the composition coefficients exhibited in Table VIII were obtained. Composition coefficients were derived by Dedman et al. (1974) to measure the degree of sequence homology between homologous enzymes of different species from their amino acid analysis data. These values vary between zero and one, those closer to unity indicating greater sequence homology between the compared proteins. The data show (Table VIII) that the human isozymes correlate best with their counterparts from rabbit muscle. In general, as the evolutionary age of the species increases, the composition coefficient should decrease. However, if this trend was strictly followed, the coelacanth and chicken would be in reverse order in the table since

TABLE VIII

COMPOSITION COEFFICIENTS OF TRIOSEPHOSPHATE ISOMERASE

| Species | Human Isozymes | | |
|---|---|---|---|
| | I | II | III |
| Rabbit muscle[b] | | | |
| I | .810 | .814 | .800 |
| II | .804 | .811 | .798 |
| III | .809 | .822 | .808 |
| Rabbit liver[c] | .792 | .829 | .822 |
| Coelacnath[d] | .772 | .809 | .805 |
| Chicken muscle[e] | .678 | .711 | .716 |
| Brewer's yeast | .646 | .680 | .669 |

[a]Calculated by the method of Dedman et al. (1974).

[b]Krietsch et al. (1971).

[c]Krietsch et al. (1970).

[d]Kolb et al. (1974).

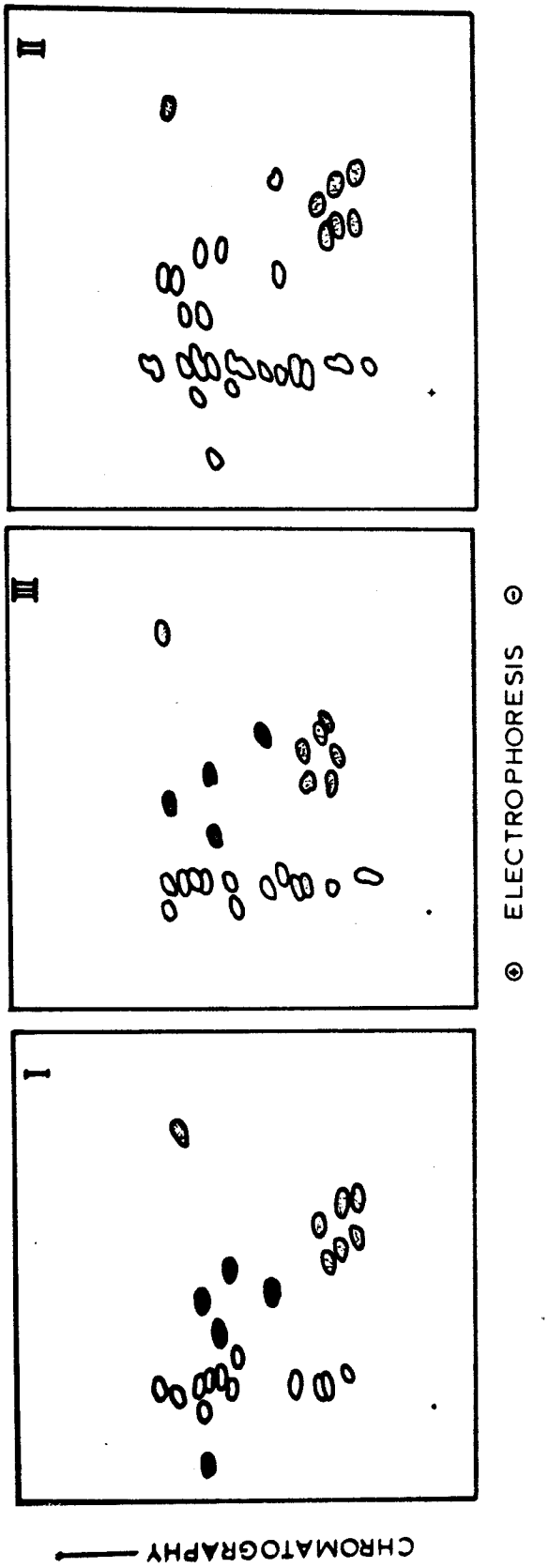
[e]Furth et al. (1974).

paleontological data (Dickerson and Geis, 1969) indicate that the coelacanth evolved approximately 400 million years before the chicken. It is interesting that the composition coefficient of human Component III and the chicken muscle enzyme is higher than with Component I and II. This similarity is in agreement with alkaline starch gel electrophoresis data which show the chicken muscle enzyme to have approximately the same electrophoretic mobility as Component III of the human enzyme (Snapka et al., 1974).

## Peptide Maps of the Triosephosphate Isomerase Isozymes

Tryptic peptide maps of the three isozymes from crystalline human cardiac muscle triosephosphate isomerase are illustrated in Figure 14. Tryptic digestion was complete; no insoluble precipitate was encountered and no ninhydrin-positive core material was observed at the origin. The prints are from direct tracings from thin layer plates. The number of peptides observed for Components I and III is consistent (plus or minus one peptide) with the content of lysine and arginine obtained from amino acid analysis and assuming a dimeric quaternary structure. The two types of subunits seem to be composed of largely identical, yet distinctly different, polypeptides. Particularly noteworthy is the cluster of six peptides in the lower right quadrant of the maps, and the lone peptide near the upper right quadrant,

Figure 14. Tryptic peptide maps of human erythrocyte triosephosphate isomerase isozymes. The isolated components were reduced, S-carboxymethylated and digested with trypsin as described in "Methods." Samples were applied at the small dot indicated near the lower left corner. Electrophoresis was carried out first, followed by chromatography in the second dimension. Unique peptides are shown in shading.


III
II
I
⊕ ELECTROPHORESIS ⊖
CHROMATOGRAPHY

which have identical migrations in the three isozymes. Many of the peptides which displayed little electrophoretic migration suggest homologous peptides of the three variants. There were however, several distinct differences in the tryptic maps of Components I and III. The dark shaded spots represent peptides which are unique to the two types of homodimers. The three lightly shaded peptides on the maps of the homodimers appeared to be similar but were not identical. These differences were clearly apparent in the map of the heterodimer (II) where all of the unique peptides appeared. These unique peptides were clearly separated, and therefore, should be amenable to further structural studies by extracting them from the thin layer plates by the recent procedure of Tilley et al. (1974).

## Triosephosphate Isomerase and Chromosome Number Five

When triosephosphate isomerase from patients exhibiting the cri du chat syndrome was assayed under optimal conditions, the activity per unit volume of red cells was found to be normal (Table IX). Similarly, no significant differences were seen in the kinetic constants for the forward or reverse reaction between the cri du chat and normal individuals. Because these cri du chat patients each had different types and sizes of chromosome 5 deletions (Hendrickson et al., 1973), the results suggested that there was no triosephosphate

TABLE IX

TRIOSEPHOSPHATE ISOMERASE IN INDIVIDUALS WITH CRI DU CHAT SYNDROME

| Sample | TPI Activity* | Michaelis Constants** | |
|---|---|---|---|
| | | D-Glyceraldehyde-3-phosphate (mM) | Dihydroxyacetone Phosphate (mM) |
| Normal | 441 ± 135 | 0.015 ± 0.005 | 0.48 ± 0.10 |
| D. N. | 482 | 0.016 | 0.47 |
| L. R. | 411 | 0.016 | 0.46 |
| L. B. | 385 | 0.016 | 0.45 |
| R. F. | 412 | 0.017 | 0.49 |
| L. H. | 438 | 0.018 | 0.48 |

*Micromoles of D-glyceraldehyde-3-phosphate converted to dihydroxyacetone phosphate per minute per $10^{10}$ red cells at $30^{\circ}$, pH 7.6. The normal represents a total of 22 individuals.

**Corrected to actual concentrations of free aldehyde and ketone in solution required for one-half maximum velocity.

isomerase deficiency associated with the cri du chat syndrome. When hemolysates from cri du chat syndrome patients were subjected to starch gel electrophoresis and isoelectric focusing, each of the three isozymes was observed with the same isoelectric pH values and activity distribution as in normal individuals (Hendrickson et al., 1973). Thus, in contrast to previous suggestions of Sparks et al. (1969, 1970), it appears that the genes for triosephosphate isomerase are not located on chromosome five and that triosephosphate isomerase deficiency is in no way related to the cri du chat syndrome.

## Kinetics of the Isozymes

The basic kinetic parameters of crystalline human erythrocyte triosephosphate isomerase are shown in Table X. The values for D-glyceraldehyde-3-phosphate were very similar to those values determined by Krietsch et al. (1971) for the rabbit muscle isozymes. The values for both substrates were quite similar to those for the unresolved rabbit muscle enzyme (Reynolds, 1971) and the chicken muscle enzyme (Plaut and Knowles, 1972). Neither allosteric interactions nor substrate inhibition were observed with either substrate, in contrast to the studies of Chiang with the mosquito and housefly (1972) and the rabbit muscle enzymes (1971b). Although the $K_m$ values for the substrates are only slightly different for the three isozymes, the values for Component II

TABLE X

KINETIC PROPERTIES OF HUMAN ERYTHROCYTE TRIOSEPHOSPHATE ISOMERASE ISOZYMES*

| Property | | I | II | III |
|---|---|---|---|---|
| $K_m$ | D-Glyceraldehyde-3-phosphate | $9.9 \times 10^{-6}$ | $13.2 \times 10^{-6}$ | $19.8 \times 10^{-6}$ |
| | Dihydroxyacetone Phosphate | $5.3 \times 10^{-4}$ | $3.4 \times 10^{-4}$ | $2.9 \times 10^{-4}$ |
| pH Optima** | | 7.8 | 7.8 | 7.8 |
| pH Stability Optima** | | 7.0 - 8.5 | 6.5 - 9.5 | 6.0 - 8.2 |

*The Michaelis constants, $K_m$, for D-glyceraldehyde-3-phosphate were corrected for the free aldehyde concentration and the $K_m$ values for dihydroxyacetone phosphate were corrected for inhibition by arsenate and for concentration of free ketone in solution.

**Buffers and pH ranges: 4.0 - 5.5, acetate; 5.5 - 7.05, imidazole; 7.25 - 9.0, triethanolamine; 9.4 - 11.6, β-alanine. All of these buffers also contained 14 mM 2-mercaptoethanol.

are intermediate to those for I and III, and again suggest differences in the subunits. The pH optima were found to be essentially identical for the three isozymes. However, Component II appeared to have been afforded a greater range of stability by the hybridization of the two different subunits.

## Denaturation and Dissociation Studies

When the isozymes of human triosephosphate isomerase were denatured in 0.72 M guanidinium chloride, the denaturation rate patterns revealed that Component II denatured in two stages, both of which were pseudo-first order (Figure 15), and that the rate of each stage was essentially identical to that of the two homodimers (Table XI). A ratio of 1:0.6 for the denaturation rates of Components I and III, respectively, was determined not only for the human isozymes but also for those from rabbit muscle.

To further study subunit interactions in triosephosphate isomerase, attempts were made to determine if the dissociated monomeric subunit had catalytic activity. Hsu and Neet (1973) were able to convert native rabbit muscle aldolase into a rapidly associating-dissociating system in 1.2 M $MgCl_2$. However, when rabbit muscle triosephosphate isomerase was dialyzed against 1.0 M $MgCl_2$ buffer, no change was observed in the sedimentation coefficient ($s_{20,w}$). Although the activity of the enzyme was inhibited 1,000-fold by the $MgCl_2$, as measured by discontinuous assays, this inhibition was

Figure 15. Denaturation rate determination of human muscle triosephosphate isomerase isozymes. The isozymes (0.63 ug/ml) were incubated at 30° in 50 mM triethanolamine buffer, pH 7.6, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 0.8 M guanidinium chloride. Aliquots of Component I (○), Component II (□), and Component III (△) were removed and assayed at the indicated times as described in "Methods."

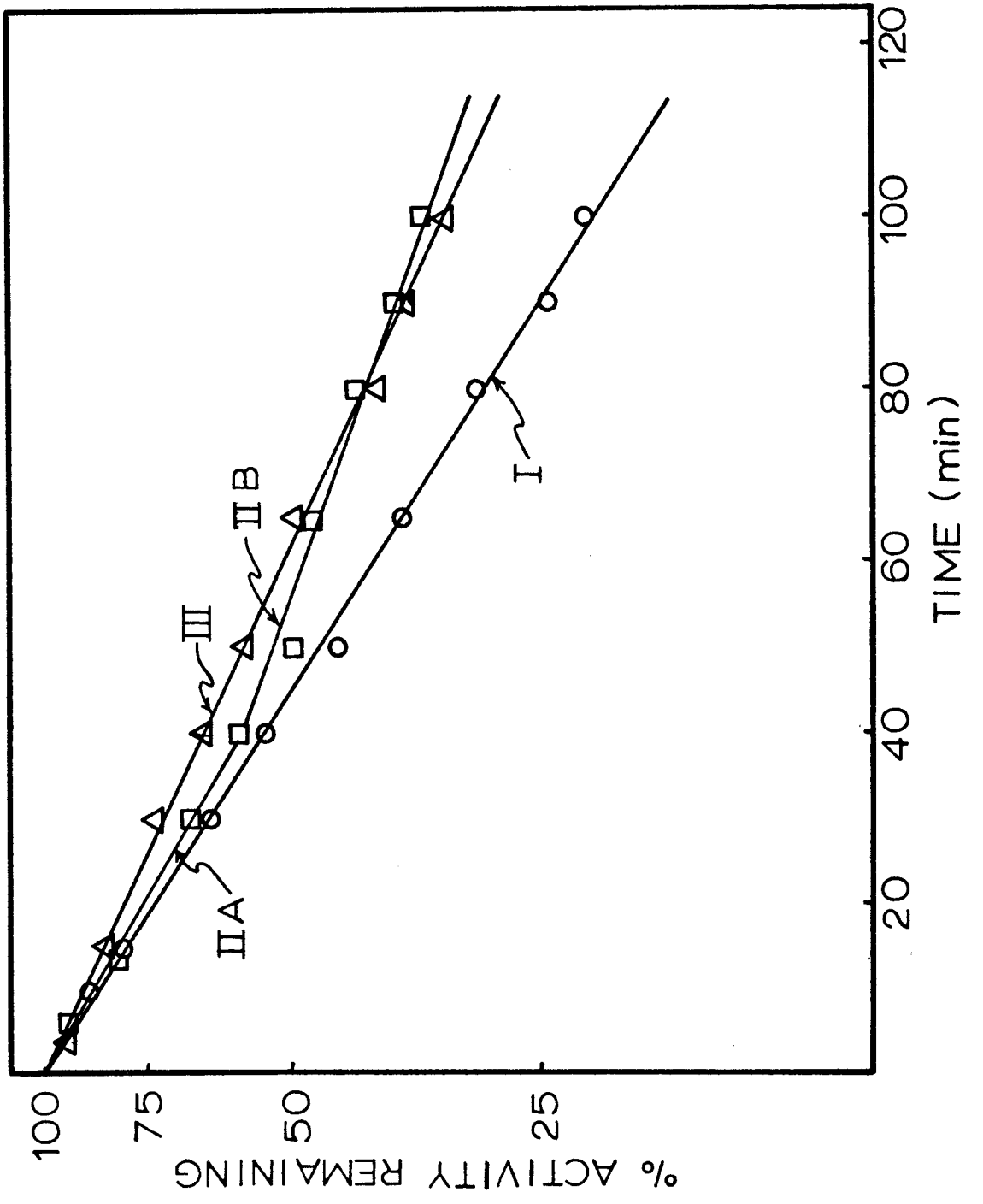

% ACTIVITY REMAINING
100
75
50
25
TIME (min)
20
40
60
80
100
120
I
IIA
IIB
III

TABLE XI

DENATURATION RATES OF TRIOSEPHOSPHATE ISOMERASE ISOZYMES[a]

| Isozyme | Human Erythrocytes ($sec^{-1}$)[b] | Rabbit Muscle ($sec^{-1}$)[c] |
|---|---|---|
| I | $2.43 \times 10^{-4}$ | $3.07 \times 10^{-4}$ |
| IIA[d] | $2.55 \times 10^{-4}$ | $3.54 \times 10^{-4}$ |
| IIB[d] | $1.69 \times 10^{-4}$ | $1.94 \times 10^{-4}$ |
| III | $1.5 \times 10^{-4}$ | $1.86 \times 10^{-4}$ |

[a]Values represent the average of 3 - 5 experiments for each isozyme.

[b]Rates were determined in 0.72 M guanidinium chloride.

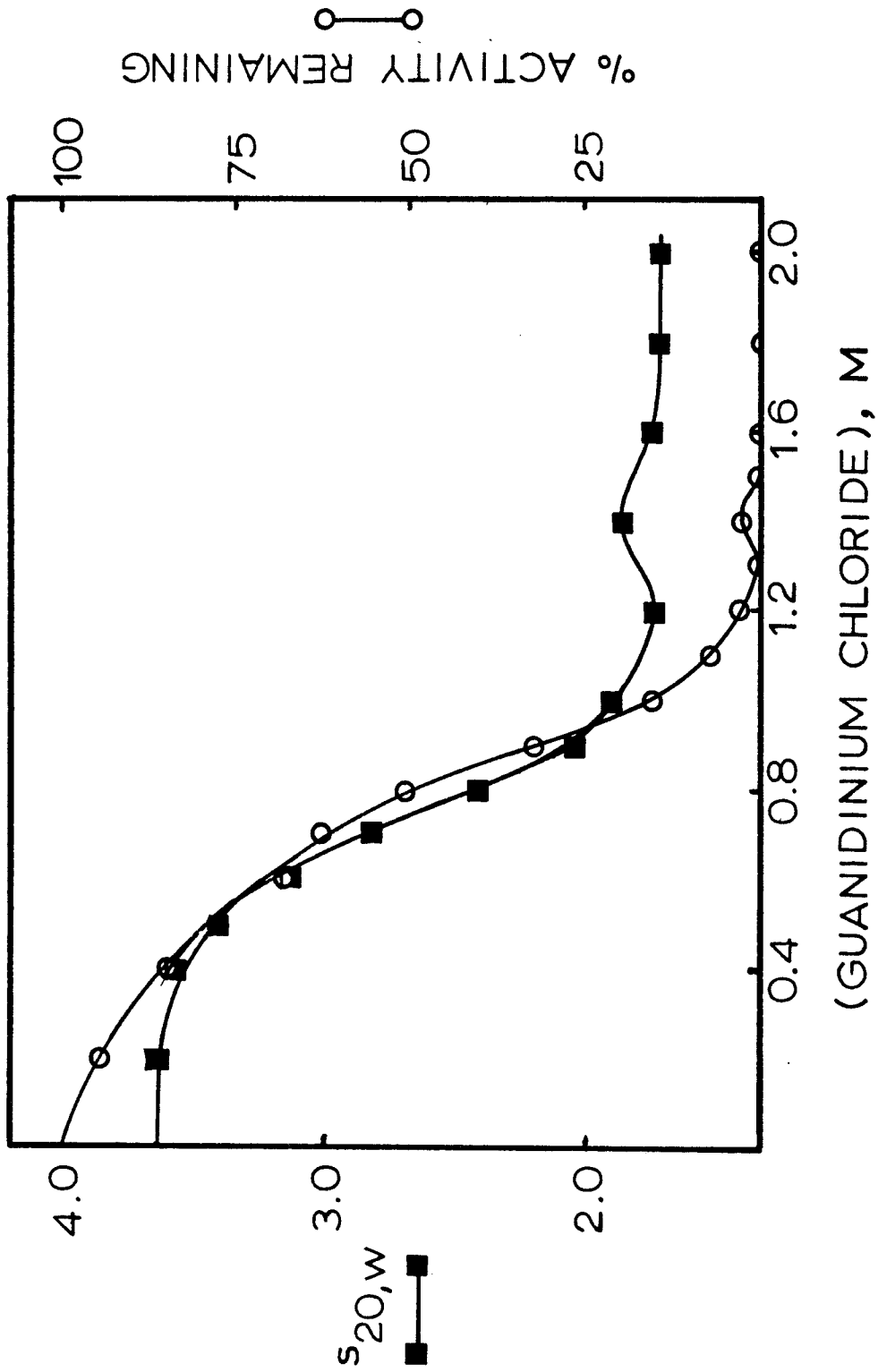
[c]Rates were determined in 0.80 M guanidinium chloride.

[d]Rate for IIA is first stage of denaturation, while IIB is the second stage.

likely due to complexation of the substrate, D-glyceraldehyde-3-phosphate, with $Mg^{2+}$. Triosephosphate isomerase was also dialyzed against NaCl in an attempt to dissociate the enzyme, but neither 1.0 M nor 3.1 M NaCl resulted in dissociation of the enzyme. Therefore, other approaches were sought to dissociate the enzyme.

Because it had been shown previously (vide supra) that human erythrocyte and rabbit muscle triosephosphate isomerase were reversibly denatured in 6 M guanidinium chloride, and because the properties of this denaturant are well documented (Tanford, 1968 and 1970; Gordon, 1972; Lee and Timasheff, 1974), guanidinium chloride was chosen as the dissociating-denaturing agent to be used in these studies. When rabbit muscle triosephosphate isomerase was incubated for thirty minutes in various concentrations of guanidinium chloride, it was found that a sharp decrease in activity occurred between 0.7 M and 0.9 M with fifty per cent inactivation occurring at 0.8 M guanidinium chloride (Figure 16). In contrast to the 0.8 M guanidinium chloride required to inactivate and denature the rabbit muscle enzyme, only 0.72 M guanidinium chloride was required to inactivate the human cardiac muscle enzyme to the same extent. The $s_{20,w}$ values of the enzyme were determined under "activity-assay conditions" (see "Methods") and exhibited denaturation titration curves with transitions in the sedimentation

Figure 16. Denaturation titration of rabbit muscle triosephosphate isomerase. The denaturation titration curve for the enzymes were followed by activity assays (○) and $s_{20,w}$ values (■). Sedimentation velocity ultracentrifugation and enzyme activity assays were performed as described in "Methods" at a protein concentration of 6.79 mg/ml and were both determined at 30°.


% ACTIVITY REMAINING
100
75
50
25
4.0
3.0
2.0
$s_{20,w}$
0.4
0.8
1.2
1.6
2.0
(GUANIDINIUM CHLORIDE), M

coefficient coincident with the loss in catalytic activity (Figure 16). The decrease in sedimentation coefficient from 3.65 to 1.7S is indicative of a transition from the native dimer to an unfolded monomer. When the titration was carried out over 116,000-fold range of protein concentration (0.012 $\mu$g per ml to 1.4 mg per ml), fifty per cent inactivation always occurred at 0.8 M and 0.72 M guanidinium chloride for the rabbit muscle and human enzymes, respectively.

By monitoring the rate of denaturation in the presence and absence of various ligands, insight into both the relationship of ligand binding to enzyme stability and the mechanism of denaturation was studied. Substrates, substrate analogs and a variety of metabolic intermediates were included in the denaturation medium. When rate constants were determined in the absence ($k_o'$) and in the presence ($k_L'$) of these ligands, substrate, $\alpha$-glycerolphosphate and 2-phosphoglycollate, protected the enzyme against denaturation most effectively as shown in Table XII. The fact that the most effective ligand in the protection against denaturation was 2-phosphoglycollate, supports its classification as a transitionstate analog (Wolfenden, 1969).

The dissociation constants ($K_{diss}$) of substrate, $\alpha$-glycerolphosphate, and 2-phosphoglycollate and the number of these molecules bound per subunit (n) were determined, using the method of Chervenka (1960), in which the denaturation rates are determined in the presence of various

TABLE XII

EFFECT OF LIGANDS ON DENATURATION RATE RATIOS

| Ligand | $k'_L/k'_o$ | |
|---|---|---|
| | Human TPI | Rabbit TPI |
| 2-Phosphoglycollate | .73 | .67 |
| D-Glyceraldehyde-3-phosphate | .80 | .76 |
| α-Glycerolphosphate | .89 | .84 |
| Ribose-5-phosphate | ... | .95 |
| 2,3-Diphosphoglycerate | ... | .94 |
| Phosphoglycerate | ... | .98 |
| Glyceraldehyde | ... | .97 |
| cyclic-AMP | ... | .97 |
| AMP | ... | 1.02 |
| ADP | ... | .99 |
| ATP | ... | .99 |
| IDP | ... | .97 |
| $P_i$ | ... | 1.02 |

concentrations of these ligands. Assuming that n molecules of ligand bind per site on the enzyme molecule, then for each site,

$$EL_n \quad E + nL$$

and

$$K_{diss} = \frac{(E)\ (L)^n}{(EL_n)}$$

where (E) is the concentration of free enzyme, (L) is the concentration of free ligand, and $(EL_n)$ is the concentration of enzyme-ligand complex. In the absence of ligand, the denaturation-rate expression is

$$v_o = k_o' \ (E_o)$$

while the rate expression in the presence of ligand is

$$v_L = k_L' \ (EL_n)$$

where $E_o$ is the initial concentration of enzyme. Therefore, if $L_o$ is the initial concentration of ligand,

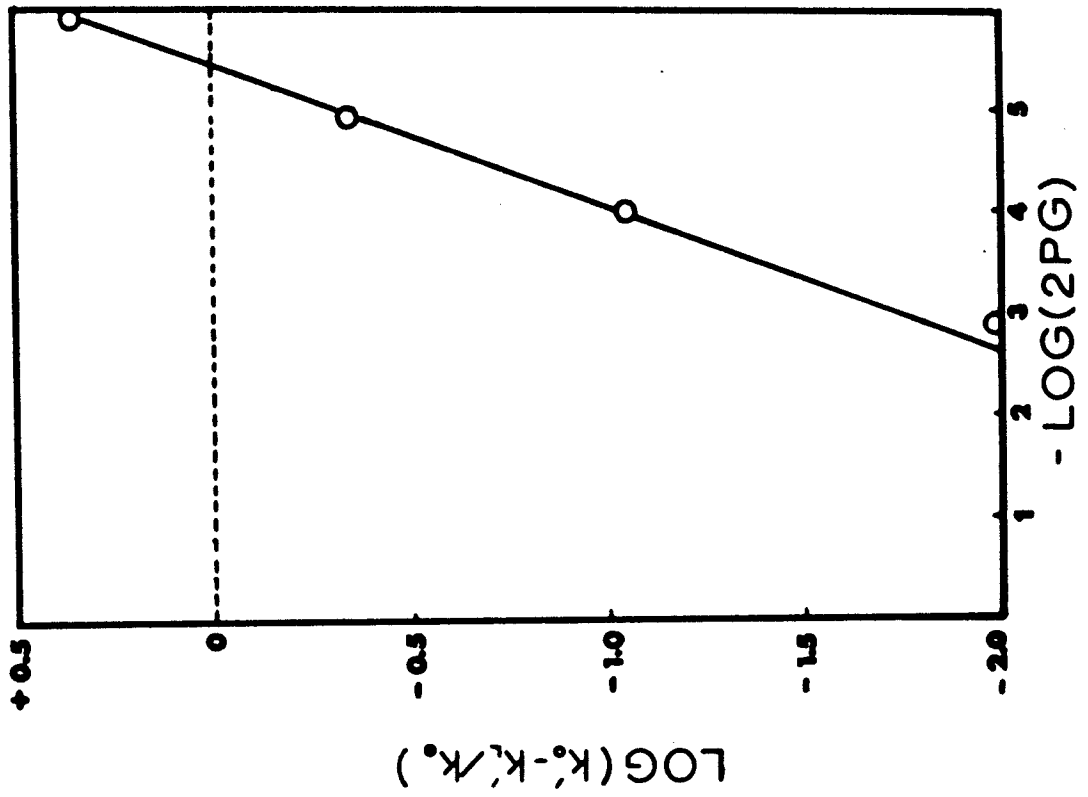
$$\log \frac{v_L' - v_o'}{v_o} = n \log (L_o) - \log K_{diss}.$$

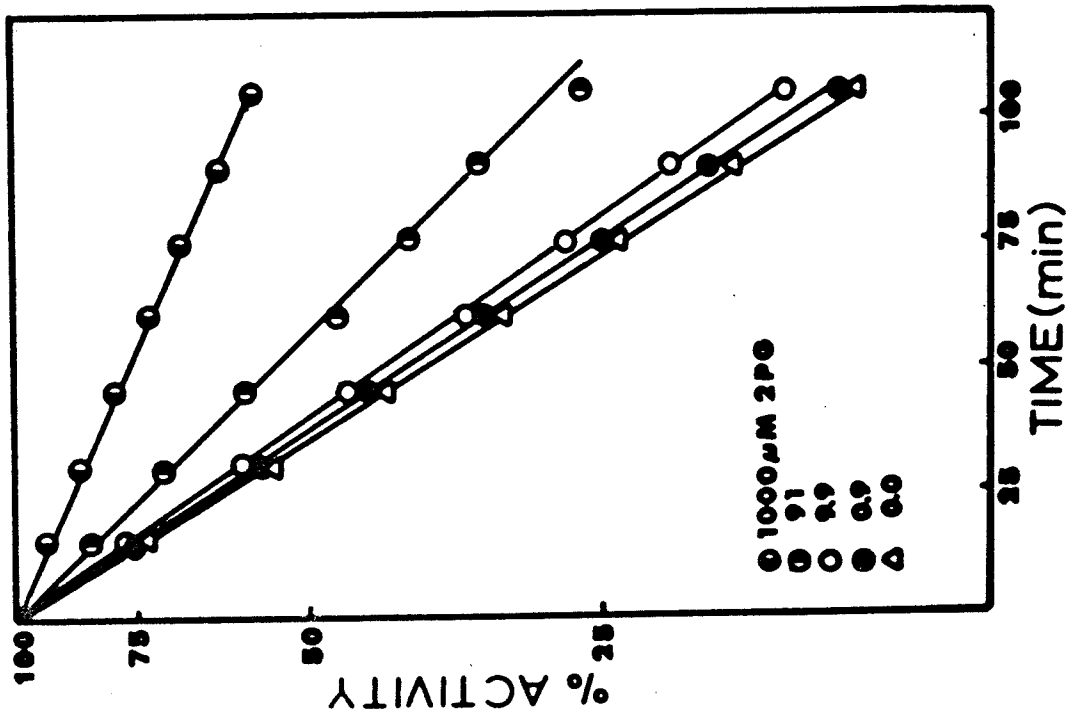
Since it is more accurate and convenient to use first-order rate constants than initial velocities,

$$\log \frac{k_o' - k_L'}{k_o'}$$

was plotted against $-\log (L_o)$. Data for denaturation in the presence of 2-phosphoglycollate are shown in Figure 17. Similar data were obtained for substrate and $\alpha$-glycerol-phosphate. This interpretation of the data is valid only if

Figure 17. Denaturation rate of human triosephosphate isomerase in the presence of 2-phosphoglycollate. Human cardiac muscle triosephosphate isomerase was incubated in 50 mM triethanolamine, pH 7.6, containing 14 mM 2-mercaptoethanol, 1 mM EDTA, 0.8 M guanidinium chloride, and various concentrations of 2-phosphoglycollate. A. Assays were run at the designated time and plotted in a semilogrithmic fashion *vs* time. The slopes of these curves were used to calculate $k_o'$ and $k_I'$. B. $k_I'$ and $k_o'$ were plotted in the manner shown to obtain *n* from the slope and $K_{diss}$ from the x-intercept as described in "Results."


LOG(k'₀-k'ᵢ/kᵢ)
+0.5
0
-0.5
-1.0
-1.5
-2.0
- LOG(2PG)
1
2
3
4
5


% ACTIVITY
100
75
50
25
TIME(min)
25
50
75
100
1000μM 2PG
0.1
0.2
0.3
0.4

$k_o'$ is independent of enzyme concentration. This criterion was found to be true over at least a 100,000-fold range of triosephosphate isomerase concentrations from 12 $\mu$g per ml to 1.85 mg per ml. Therefore, the decrease in the denaturation rate does, in fact, reflect a decrease in the equilibrium concentration of uncomplexed enxyme. As can be seen in Table XIII for 2-phosphoglycollate, a value of $3 \times 10^{-6}$ M for $K_{diss}$ and a value of 0.7 for n were obtained, which is indicative of the binding of approximately one 2-phosphoglycollate molecule per subunit. Similar values of 0.9 and 0.85 for n were obtained, respectively, for D-glyceraldehyde-3-phosphate and $\alpha$-glycerolphosphate, and dissociation constants of $7.6 \times 10^{-4}$ M and $4.0 \times 10^{-4}$ M were determined for both of these ligands, respectively. The $K_{diss}$ values for the inhibitors were in excellent agreement with the $K_i$ values determined by normal Michaelis-Menton kinetics (Table X). Because the triosephosphates are in equilibrium, the $K_{diss}$ value is in excellent agreement with the expected $K_m$ value obtained by normal kinetics, since the equilibrium favors dihydroxyacetone phosphate.

By following the denaturation rate of ribonuclease at different pH ranges in the presence of various ligands, Chervenka was able to postulate the amino acid residues and the type of interaction of these ligands involved in the stabilization of the enzyme. It was also possible to

TABLE XIII

DISSOCIATION CONSTANTS AND NUMBER OF LIGAND BINDING SITES PER SUBUNIT

| Species | $K_{diss}$ (mM) | | | n * | | |
|---|---|---|---|---|---|---|
| | G3P* | α-GP* | PPG* | G3P | α-GP | PPG |
| Rabbit muscle TPI* | 0.78** | 0.41 | 0.003 | 0.90 | 0.85 | 0.74 |
| Human erythrocyte TPI | 0.76 | 0.39 | 0.0025 | 0.91 | 0.86 | 0.74 |

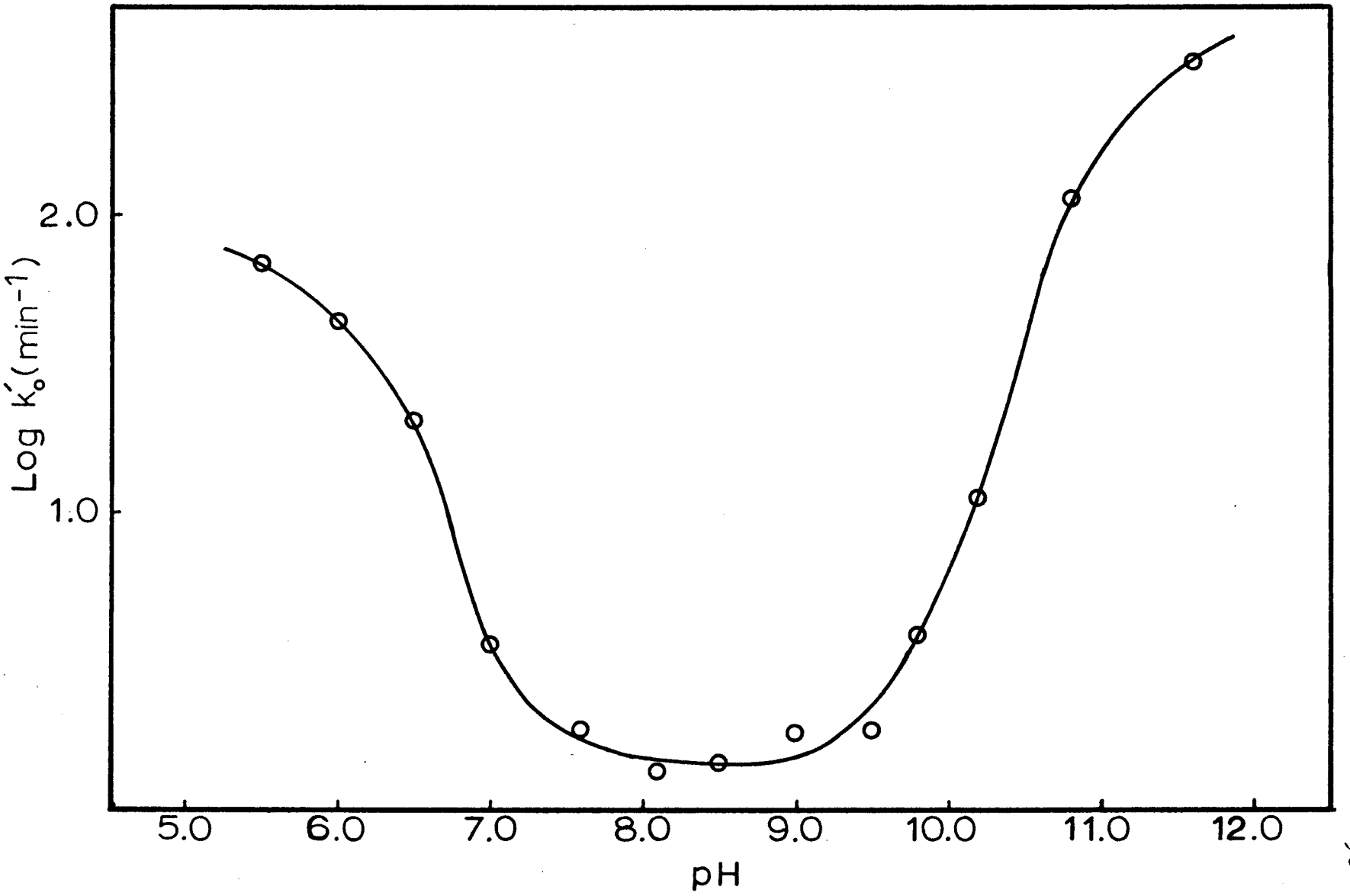
*G3P = D-glyceraldehyde-3-phosphate; α-GP = α-glycerolphosphate; PPG = phosphoglycollate; TPI = triosephosphate isomerase; n = number of ligand binding sites per subunit.

**This $K_{diss}$ value is representative of an equilibrium mixture of D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

speculate upon the amino acids directly involved in maintaining the enzyme in its native conformation (Chervenka, 1960). Therefore, the effect of pH upon the rate of denaturation of rabbit muscle triosephosphate isomerase was examined and is illustrated in Figure 18. As a control, the rate of inactivation of triosephosphate isomerase by pH alone was determined. These data suggest that the titration of amino acid residues with pKa values of 6.5 - 7.0 and 10.2 - 10.8 greatly enhance the denaturation process. Denaturation rates were first order at all pH values, suggesting that the mechanism of denaturation is similar at all pH values.

Triosephosphate isomerase has been suggested to exist as a multienzyme complex with aldolase and perhaps glyceraldehyde-3-phosphate dehydrogenase *in vivo* (Frieden, 1971). However, ultracentrifugation experiments in our laboratory have thus far failed to demonstrate such an association. Furthermore, when equimolar concentrations of rabbit muscle aldolase, and glyceraldehyde-3-phosphate dehydrogenase were included with triosephosphate isomerase, the rate and extent of denaturation was unchanged relative to the control containing only triosephosphate isomerase and guanidinium chloride.

Figure 18. Denaturation rates of rabbit muscle triose-phosphate isomerase vs pH. Denaturation rates were determined as described in "Methods" in 1.0 M sodium acetate, pH 4.0 - 5.0; imidazole, pH 5.0 - 7.0; triethanolamine, pH 7.0 - 9.0; or β-alanine, pH 9.4 - 11.6; containing 0.8 M guanidinium chloride, 1 mM EDTA, 14 mM 2-mercaptoethanol, and 150 ug of enzyme. The rate $k'_8$ represents the difference between the rate in the presence of the denaturant and the rate of a sample in an identical incubation mixture at the same pH but in the absence of the denaturant.


Log k'o(min-1)
2.0
1.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH

## Kinetic Studies of Matrix-Bound Rabbit Muscle Triosephosphate Isomerase

In an effort to evaluate the activity and properties of individual subunits of triosephosphate isomerase, the use of an immobilized enzyme appeared to be simple and well-suited for this particular study. Triosephosphate isomerase from rabbit muscle was bound directly or *via* an acetamidoethyl linkage to Sepharose 4B as outlined in "Methods." Figure 19 shows that when these immobilized derivatives were subjected to denaturation titration, the concentration of guanidinium chloride needed for fifty per cent inactivation of the directly bound derivative was 1.75 M. This is in contrast to 1.1 M and 0.8 M guanidinium chloride required to cause the same degree of inactivation in the acetamidoethly-bound and the free enzyme, respectively. This intermediate denaturation equivalency value for the spacer-bound derivative would be expected since the enzyme would be assumed to have greater conformational flexibility than the directly-bound derivative, yet less than the native enzyme. These data suggested that the acetamidoethyl-bound derivative might be more similar to the native enzyme than the directly-bound derivative. Therefore, other properties of these derivatives were investigated (Table XIV). The $K_m$ values for the substrates, D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, were increased slightly and were essentially identical for both matrix-bound derivatives. On the other

Figure 19. Denaturation titration of free and matrix-bound rabbit muscle triosephosphate isomerase. The enzyme was covalently attached either directly or *via* an acetamidoethyl spacer to CNBr-activated Sepharose 4B as described in "Methods." The free enzyme, along with the two immobilized derivatives were incubated for 30 minutes at 30° in various concentrations of guanidinium chloride and assayed for enzymatic activity at "time zero" and after the incubation period. Immobilized-enzyme assays were run in 50 mM triethanolamine, pH 7.6, containing 1 mM EDTA and 14 mM 2-mercaptoethanol in a 3 ml cuvette with continuous stirring as described in "Methods"; spacer-bound, (□); directly-bound, (△). Free enzyme (○) assays were run by the routine assay as described in "Methods."

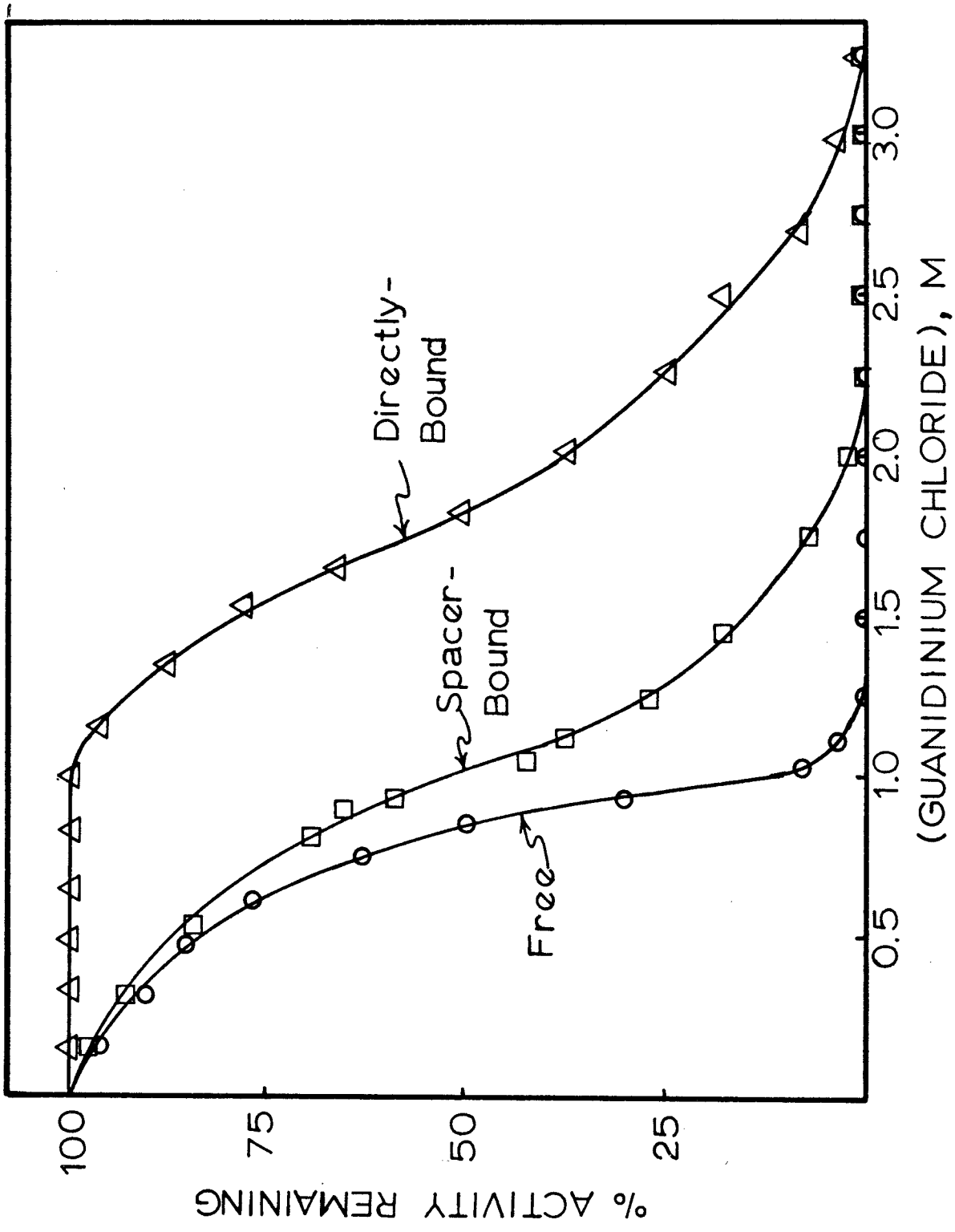

% ACTIVITY REMAINING
100
75
50
25
0.5
1.0
1.5
2.0
2.5
3.0
(GUANIDINIUM CHLORIDE), M
Directly-Bound
Spacer-Bound
Free

TABLE XIV

KINETIC CONSTANTS FOR MATRIX-BOUND AND FREE RABBIT MUSCLE TRIOSEPHOSPHATE ISOMERASE

| Ligand | Free (M)* | Directly-Bound (M)* | Spacer-Bound (M)* |
|---|---|---|---|
| D-Glyceraldehyde-3-phosphate | $3.9 \times 10^{-4}$ | $2.2 \times 10^{-3}$ | $2.0 \times 10^{-3}$ |
| Dihydroxyacetone phosphate | $9.8 \times 10^{-4}$ | $2.5 \times 10^{-3}$ | $2.1 \times 10^{-3}$ |
| α-Glycerolphosphate** | $3.6 \times 10^{-4}$ | $1.4 \times 10^{-2}$ | $1.4 \times 10^{-3}$ |
| 2-Phosphoglycollate** | $2.2 \times 10^{-6}$ | $1.4 \times 10^{-2}$ | $6.4 \times 10^{-6}$ |

*"Directly-bound" designates the enzyme which was covalently attached to CNBr-activated Sepharose 4B with no interstitial hydrocarbon chain. "Spacer-bound" designates the enzyme which was covalently linked to Sepharose 4B *via* an acetamidoethyl spacer as described in "Methods."

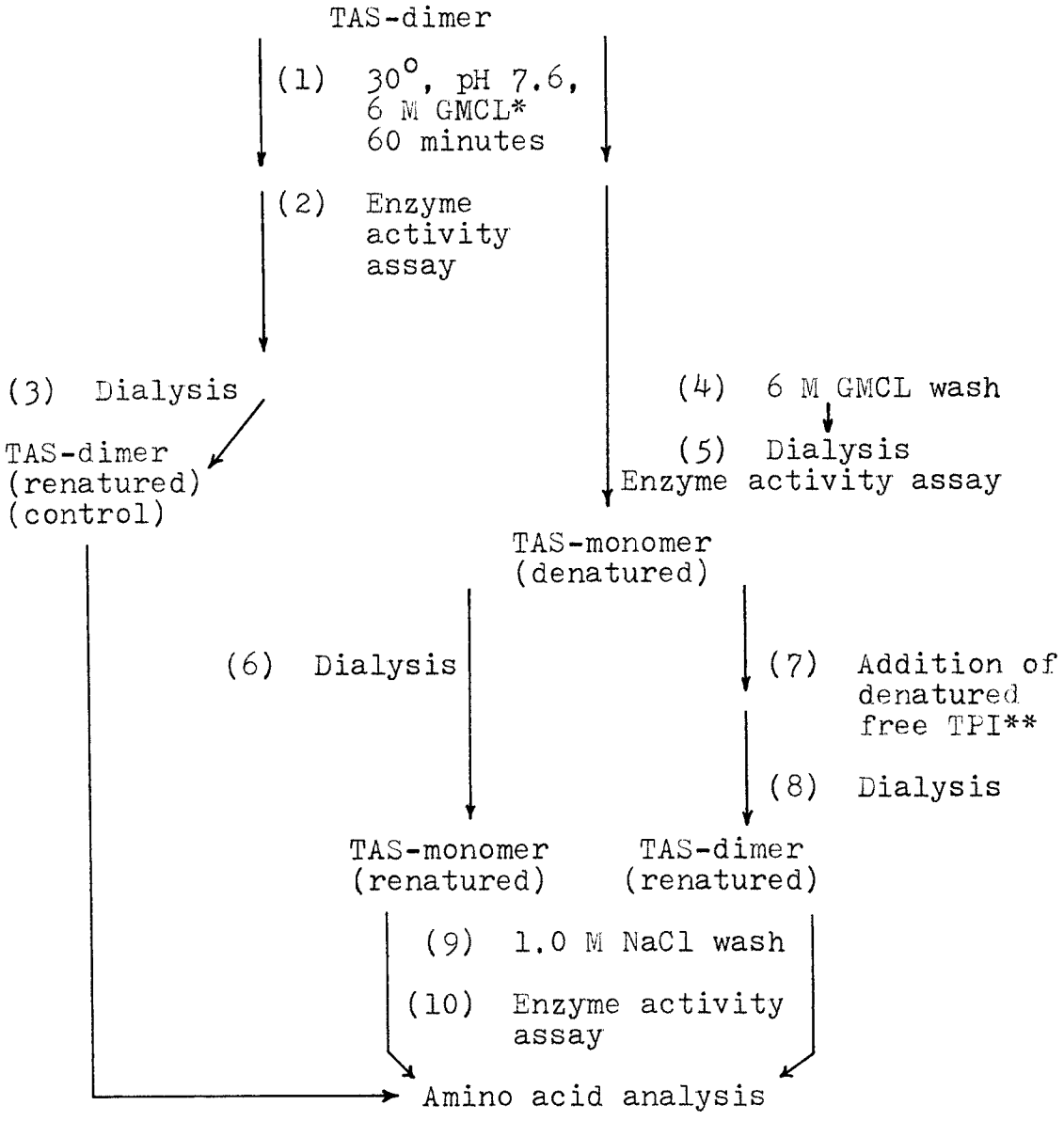
**Dissociation constants were determined using Lineweaver-Burk reciprocal plots at constant inhibitor concentration. Both inhibitors were ascertained to be competitive with respect to substrate.

hand, while the $K_i$ values for α-glycerolphosphate and 2-phosphoglycollate were increased only 3 - 4-fold in the acetamidoethyl-bound derivative, they were increased 38- and 6100-fold, respectively, in the directly-bound derivative. These data further suggested that when the enzyme was bound *via* the acetamidoethyl linkage, its kinetic properties more closely approximated those of the free enzyme. Most intriguing is the loss of the ability of 2-phosphoglycollate to act as a competitive inhibitor and as a protective agent against denaturation in the directly-bound enzyme. 2-Phosphoglycollate has been reported to cause a five per cent and a three per cent change, respectively, in the unit cell dimension of the crystals of triosephosphate isomerase from baker's yeast (Hawkinson *et al.*, 1972) and chicken muscle (Banner *et al.*, 1972). The highly restricted conformation of the directly-bound enzyme may preclude such a large conformation change.

## Individual Subunit Activity of Triosephosphate Isomerase

For the determination of the individual subunit activity, experiments on triosephosphate isomerase-acetamidoethyl-Sepharose 4B (TAS) were carried out by way of the sequence outlined in Figure 20. The native TAS (TAS-dimer) was incubated in 50 mM triethanolamine buffer, pH 7.6, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 6 M guanidinium chloride for sixty minutes at 30°. After the sample was

Figure 20. Outline of procedure for determination of individual subunit activity. A more detailed discription is provided in "Results."




*Guanidinium chloride.

**Triosephosphate isomerase.

assayed for enzymatic activity, one third of the sample was begun dailyzing immediately against renaturation buffer (vide infra). The TAS-monomer was then prepared by washing the previously denatured TAS-dimer on a glass-fritted funnel with ten volumes of the previously used buffer containing 6 M guanidinium chloride. At this point, the TAS-monomer was resuspended in two volumes of the buffered guanidinium chloride solution and half of the sample was dialyzed against renaturation buffer (10 mM triethanolamine buffer, pH 7.5, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 0.1 M NaCl). To the other half of the denatured TAS-monomer solution was added 0.5 mg of free, denatured triosephosphate isomerase monomers, in the same buffered guanidinium chloride solution as above. This solution was then dialyzed against renaturation buffer. After dialysis, each aliquot was washed with 10 mM triethanolamine buffer, pH 7.6, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 1.0 M NaCl until no more enzymatic activity appeared in the wash. The two derivatives were then assayed for enzymatic activity and centrifuged simultaneously at 1,000g for ten minutes to obtain compact slurries of each. Amino acid analysis was then carried out on each sample after hydrolysis for twenty-five hours. To quantitate the relative amount of protein bound to Sepharose, the valine content was calculated. As seen in Table XV, TAS which had been denatured, washed with

TABLE XV

INDIVIDUAL SUBUNIT ACTIVITY DETERMINATION

| Fraction | Activity | | Per Cent Recovery | Relative Per Cent Recovery | Relative Per Cent Valine Per mg Sepharose |
|---|---|---|---|---|---|
| | Before Denaturation* | After Denaturation And Renaturation | | | |
| Control | 10.4 | 3.42 | 34.0 | (100) | (100) |
| TAS--no TPI added** | 41.7 | 7.24 | 17.3 | 50 | 51 |
| TAS--TPI added*** | 41.7 | 17.85 | 42.8 | 125 | 99 |

*Enzymatic activity prior to incubation in 6 M guanidinium chloride.

**No free, denatured triosephosphate isomerase (TPI) monomers were added to this TAS sample after it had been incubated and washed with 6 M guanidinium chloride.

***Free, denatured triosephosphate isomerase (TPI) monomers were added to this sample after incubation and washing with 6 M guanidium chloride.

denaturant, and dialyzed against renaturation buffer in the absence of free denatured triosephosphate isomerase monomers, exhibited essentially fifty per cent of the activity regained by the control.

This suggested the possibility that each subunit itself might be active and that the washing with guanidinium chloride was removing one subunit, leaving a TAS-monomer. This interpretation was strengthened by the results of the readdition of free, denatured triosephosphate isomerase monomers to the TAS-monomer and the removal of denaturant. As seen in Table XV, this resulted in essentially a two-fold gain in activity upon reconversion to the TAS-dimer. Further substantiation of this interpretation is seen from the specific activities of the TAS-monomer and the TAS-dimer. The derivative to which free, denatured enzyme had not been added during the final dialysis, had only fifty per cent of the valine content as the derivative to which free, denatured monomers had been readded (Table XV). Thus, these data suggest that the individual subunits of the enzyme bound to a matrix are active and that they are capable of binding an additional subunit, under the proper conditions, to reform the matrix-bound dimer.

# DISCUSSION

In order to elucidate the molecular basis of a genetic disease involving a single protein, the normal protein must first be characterized for a comparison to the aberrant protein. For this reason, human triosephosphate isomerase has been isolated from five tissues including cardiac and skeletal muscle, brain, liver, and erythrocytes. The most desirable sources of the enzyme were skeletal and cardiac muscle for the following reasons: their high content of the enzyme (200 mg per kg of wet tissue); the low overall purification necessary to obtain pure enzyme (540-fold); and the high overall recovery (70 - 80%) using a relatively simple and mild purification procedure. Although Lee et al. (1971) attempted to isolate triosephosphate isomerase from human liver, only partial purification was achieved as evidenced by a lower specific activity (2,400 units per mg).

The types of fractionation methods employed by Lee and coworkers (which also characterize other procedures for isolation of this enzyme from other species: Norton et al., 1970; Krietsch et al., 1970; Chiang, 1972; Meeks et al., 1968, McVittie et al., 1972) could be damaging to the enzyme. Heat, solvent, and ammonium sulfate precipitation steps used in these procedures could lead not only to loss or modification of

catalytic activity, but might also give rise to conformational subforms of the enzyme (Krietsch et al., 1971). The omission of these types of fractionation methods in our procedure yielded an enzyme of equal or higher specific activity and gave a higher overall recovery than from any other mammalian source. The purification procedure described by Putnam et al. (1972) for the chicken muscle enzyme required the same number of steps to yield pure enzyme (specific activity 11,200 units per mg), but the overall recovery was only forty-one per cent. Thus, the procedure outlined for human triosephosphate isomerase appears to be quantitatively, and in many cases, qualitatively superior to other procedures (Table XVI).

The average specific activity of the human enzyme from all tissues was 8,000 units per mg which corresponds to a molecular activity of $4.44 \times 10^5$ moles of D-glyceraldehyde-3-phosphate isomerized per minute per mole of protein. Because its molecular activity far exceeds any of the other glycolytic enzymes, it is difficult to conceive of this enzyme ever becoming rate limiting in glycolysis, even in triosephosphate ismoerase deficiency disease where ten per cent of its enzymatic activity still remains (Schneider et al., 1968b). However, the effective in vivo levels of the enzyme cannot be correlated with the levels measured at maximal velocity in vitro because of the lower substrate concentration and additive effects of in vivo inhibitors (Yoshida, 1973).

TABLE XVI

RESULTS OF ISOLATION PROCEDURES FOR TRIOSEPHOSPHATE ISOMERASE

| Sources of Enzyme | Per Cent Recovery | Final Specific Activity (units/mg) | Dimer Molecular Weight (daltons) | Reference |
|---|---|---|---|---|
| Bacillus stearothermophilus | .. | 4,500 | 54,000 | Fahey et al. (1971) |
| Brewer's yeast | .. | 10,000 | 56,000 | Krietsch et al. (1970) |
| Baker' yeast | .. | 9,600 | 56,000 | Hawkinson et al. (1972) |
| Coelacanth | .. | 7,000 | 50,000 | Kolb and Harris (1972) |
| Mosquito | 86 | 2,000 | 60,000 | Chiang (1972) |
| Housefly | 21 | 2,400 | 60,000 | Chiang (1972) |
| Equine liver | 20 | 3,183 | 48,000 | Lee et al. (1971) |

TABLE XVI--Continued

| Sources of Enzyme | Per Cent Recovery | Final Specific Activity (units/mg) | Dimer Molecular Weight (daltons) | Reference |
|---|---|---|---|---|
| Bovine muscle | .. | 9,450 | ..... | Meyer-Arendt et al. (1953) |
| Rabbit liver | .. | 6,400 | 56,000 | Krietsch et al. (1970) |
| Rabbit muscle | .. | 8,000 | 56,000 | Krietsch et al. (1970) |
| | 21 | 7,800 | 53,000 | Norton et al. (1970) |
| | .. | 2,750 | ..... | Czok and Bucher (1960) |
| | .. | 9,450 | ..... | Beisenherz et al. (1955) |
| | .. | 5,500 | ..... | Meyerhof and Beck (1953) |
| Chicken | 41 | 11,200 | 48,400 | Putnam et al. (1972) |
| | .. | 10,800 | 48,000 | Trentham et al. (1969) |
| | .. | 6,300 | 48,400 | McVittie et al. (1972) |
| | .. | 10,000 | 54,400 | Furth et al. (1974) |
| Human liver | 13 | 2,397 | 48,000 | Lee et al. (1971) |

Even though it has the highest molecular activity of the glycolytic enzymes, human triosephosphate isomerase is one of the smallest oligomeric enzymes known (Klotz and Darnall, 1969). Composed of two single polypeptide chains of molecular weight 28,000, it exhibits a dimer molecular weight of approximately 56,000. These values are similar to those obtained by other workers for the enzyme from other species (Table XVI). The human enzyme does appear to have a slightly higher molecular weight (56,000) than the chicken muscle enzyme (53,000), although other properties of these enzymes are quite similar. The dimeric structure and molecular weight of triosephosphate isomerase has apparently remained essentially constant for at least 1,200 million years (brewer's yeast molecular weight = 56,000, Krietsch et al., 1970) throughout the gamut of species from Bacillus stearothermophilus (Fahey et al., 1971) to Homo sapien. This conservation of structure reflects the important position that this enzyme occupies in metabolism, forming a critical link between the pentose phosphate pathway, glycolysis, and glyceride synthesis.

A variety of electrophoretic techniques demonstrated that the three triosephosphate isomerase isozymes did not exhibit a tissue-specific distribution similar to aldolase (Penhoet, et al., 1966) and lactate dehydrogenase (Anderson and Weber, 1966). The latter two enzymes each have one

isozyme predominating in one tissue while still another functions in another tissue. Nor were the human isomerase isozymes found to be organelle-specific as were the cytoplasmic and chloroplastic triosephosphate isomerase isozymes from Euglena gracillis (Mo et al., 1973) and pea leaves (Anderson, 1971). Isozymes of triosephosphate isomerase are not unique to human tissues since multiple electrophoretic forms have been observed from numerous other species, both vertebrate and invertebrate (Burton and Waley, 1968; Coulson et al., 1970; Scopes, 1968; Krietsch et al., 1970; Norton et al., 1970; Snapka et al., 1974). In a recent screening of thirty species, Snapka et al. (1974) found three zones of triosephosphate isomerase activity in most higher vertebrates after starch gel electrophoresis.

The three forms of crystalline human triosephosphate isomerase were observed under a variety of conditions and were shown not to be artifacts produced by the oxidation of sulfhydryl groups, the electrophoretic systems, aggregation or dissociation, proteolysis, or the isolation procedure. Because the two proposed homodimers were not interconvertible by dissociation and reassociation, they did not appear to be conformers as previously suggested for the rabbit muscle enzyme by Burton and Waley (1968). Based on these results, the dissociation and reassociation experiments and the structural studies, the multiple forms were most readily

explained in terms of an AA, AB, and BB, distribution of dimers.

A genetic basis for these forms was strongly suggested by electrophoretic data of Kaplan and coworkers (1968). Using hemolysates of normal and triosephosphate isomerase-deficient individuals, their data showed that the most negatively charged component (Component III, BB) and the intermediate species (Component II, AB) were markedly decreased or absent in the patient with the genetic disease. This inferred that there was a different cistron coding for each subunit and that the mutation occurred in the cistron which coded for the B subunit such that Components II and III were affected. If the A and B subunits failed to form a heterodimer, or if they dimerized but produced an inactive heterodimer, the AA homodimer (Component I) would remain as the only enzymatically active species in the triosephosphate isomerase-deficient individual. When human erythrocyte triosephosphate isomerase was subjected to isoelectric focusing, a ratio of activity of approximately 5:70:25 (AA:AB:BB) was produced. Thus, if the AB and BB forms were inactive, the enzyme-deficient patient would be expected to retain only about five per cent of the normal level of activity. This was precisely the case. Schneider and coworkers (1968b) reported five to ten per cent of the normal level of triosephosphate isomerase activity in their patients homozygous for the genetic defect.

The presence of two distinctly different polypeptide chains was conclusively demonstrated by the amino acid compositions and tryptic peptide fingerprints. The presence of several unique tryptic peptides from Components I and III strongly argued against conformational changes, deamidation, or covalent modification as the basis of the multiplicity.

That the isozymes of triosephosphate isomerase are the result of two cistrons is further substantiated by Krietsch et al. (1970) and Snapka et al. (1974). Krietsch and coworkers found that the enzyme purified from brewer's yeast contained two subunits having different amino-terminal residues (alanine and valine). In a survey of a variety of species, Snapka and coworkers found that in bacteria and fungi, only one triosephosphate isomerase species could be detected by starch gel electrophoresis. In certain crustacea, two enzymatically active forms of the enzyme were observed (i.e., AA and BB with no AB present), while in vertebrates, three bands of enzymatic activity were detected. These data not only implicate two cistrons coding for triosephosphate isomerase, but also suggest gene duplication occurring early in evolution. This contrasts with the studies of Rubinson and coworkers (1973) who postulated that duplication of the triosephosphate isomerase gene occurred at the time of differentiation of the homonoids. It is especially interesting in studies of Snapka et al. (1974) that chicken muscle exhibited only one

major band of activity while three were observed from dove and other higher vertebrates. Exceptions involving chicken muscle triosephosphate isomerase were also observed in molecular weights (56,000 for human and rabbit; 53,000 for chicken) and composition coefficients (the chicken-human comparison was lower than the coelacanth-human comparison). These exceptions may be effects of a change in the basic structure-function relationships of the enzymes in the chicken which may be reflected by the difference in chromosome number of chicken (seventy-eight) and human (forty-six).

Despite the structural differences of the isozymes shown in the present study, they appear to be catalytically very similar to one another and to the rabbit muscle enzyme (Norton et al., 1970; Krietsch et al., 1970), the chicken muscle enzyme (Plaut and Knowles, 1972; Putnam et al., 1972), the pea leaf enzyme (Anderson, 1971), and the pea seed enzyme (Turner et al., 1965). The $K_m$ values for both substrates were slightly different for the isozymes, with Component II having a value intermediate to Components I and III. These data also supported Component II as being a hybrid of I and III.

In contrast to the similarity of the basic kinetic parameters of the isozymes, important differences were observed in their rates of denaturation in guanidinium

chloride. Kuehl and Sumsion (1970) and Johnson and Velick (1972) have suggested that steady-state concentrations of enzymes are highly regulated by their rates of synthesis and degradation. Thus, the higher lability of Component I *in vivo* could lead to its faster degradation and could explain the low concentration (i.e., five to ten per cent) of this isozyme in tissue homogenates and in the pure enzyme. If their stability *in vitro* is paralleled *in vivo*, Component I would be degraded faster than either II or III. Dölken and Pette (1974) suggest that the *in vivo* half-life of proteins is regulated in part by interactions of substrates and inhibitors with the protein. The stability properties of triosephosphate isomerase have been shown to be influenced by the substrates and certain metabolites.

A comparison of the stabilities of human and rabbit triosephosphate isomerases showed the human enzyme to be more labile toward guanidinium chloride denaturation. Based on a two-state mechanism of denaturation (Tanford, 1970), there is a difference of 525 cal per mole in the free energy of denaturation of the enzyme from the two species. This is not without precedent since Knapp and Pace (1974) found bovine cytochrome *c* to be more stable toward guanidinium chloride denaturation than the equine protein by 700 cal per mole, even though their amino acid compositions differ by only three amino acids. Because these three amino acids

occur in an apparently hypervariable region of this protein, Knapp and Pace suggested that the purpose of these changes was to change the in vivo native-denatured equilibrium such that stability towards proteolytic digestion would be altered. Thus, changes in structural stability would affect the physiological stability of the protein. Robinson (1974) has suggested that amino acid substitutions which affect the glutamine and asparagine content in a protein may also directly affect its half-life in vivo. Thus, the difference in stability in vitro of the rabbit and human triosephosphate isomerase may reflect differences in the protein degradation rates in the enzymes in vivo.

When the denaturation rates of rabbit muscle triosephosphate isomerase were determined over a wide pH range, the titration of one or more amino acids with $pK_a$ values of 6.5 - 7.0 and 10.2 - 10.8 caused marked alteration in the rates of denaturation. The susceptibility to guanidinium chloride-induced denaturation was enhanced by protonation of the former group(s) and deprotonation of the latter group(s). The denaturation rates followed first order kinetics over the entire pH range, which suggested that denaturation was perhaps occurring by the same mechanism at all pH values. Because amino acids may exhibit different $pK_a$ values in proteins than when free in solution, speculation of the groups involved is somewhat tenuous. However, it is of

interest to point out that histidine is the only amino acid which has a pKa value near the first equivalency point, while lysine ($\epsilon$-$NH_2$), tyrosine (-OH), and cysteine (-SH), are all candidates for the second equivalency point. More detailed thermodynamic investigations of denaturation and the determination of the amino acid sequence would be required to ascertain the group(s) involved.

The mechanism of the denaturation of triosephosphate isomerase could occur by a number of pathways, but the present results suggest the following possibilities. The subunits could unfold simultaneously while joined together, then separate; they could sepearate, then unfold; or they could unfold and dissociate simultaneously. In an attempt to distinguish these mechanisms, it was necessary to determine if each subunit had catalytic activity. To do this, the enzyme was covalently bound to Sepharose 4B. When triosephosphate isomerase was bound directly to Sepharose, a number of its kinetic properties changed drastically, along with its susceptibility to denaturation. Subsequently, the enzyme was found to be joined to the matrix through both subunits in this derivative, precluding its use in this study. However, the properties of the native enzyme were more closely approximated by the enzyme bound to Sepharose 4B with an acetamidoethyl linkage through only one subunit. Using the acetamidoethyl-bound enzyme, the denaturation-renaturation data

suggested that the individual subunits may be catalytically active. This is in contrast to the renaturation kinetic data of Waley (1973) which suggested that the subunits were not active.

The parallel loss of enzymatic activity and change in the sedimentation coefficient coupled with the individual subunit activity data seem to argue against a dissociation into subunits without unfolding. These data argue even more strongly against an unfolding which destroys catalytic activity without dissociation. Thus, these data are consistent with a concerted dissociation and unfolding system. Nonetheless, the subunits being active when immobilized does not necessarily imply their being active as free monomers in the presence of 0.7 to 1.0 M guanidinium chloride. In view of this, further studies remain to unequivocally distinguish the mechanisms of denaturation.

That the structural integrity of triosephosphate isomerase was intimately related to its active site conformation, was indicated by the protection against denaturation by certain ligands. Banner and coworkers (1971) found by 6Å resolution X-ray analysis that 2-phosphoglycollate produced a six per cent contraction in the crystal unit cell volume of the chicken muscle enzyme. In addition, the metabolite α-glycerolphosphate effected a unit cell contraction of three per cent, as did the substrate. Thus, the degree of

conformational contraction in the crystal state is paralleled by the degree of protection against denaturation by these ligands. These data suggest that the contractions observed in the crystalline state may also occur in solution.

If contraction of the protein actually occurs in solution, this could explain the protection against guanidinium chloride-induced denaturation. The current theory of denaturation by guanidinium chloride (Lee and Timasheff, 1974; Gordon, 1972) is that it "unzips" the protein. Apparently, an insipient solvation of the protein occurs, causing initial disruption of the tertiary structure. More denaturant then inundates the protein through a cooperative process, until the molecule is completely denatured.[3] Therefore, if by some mechanism, the protein is able to "tighten" its native conformation, more denaturant would be required to disrupt the highly ordered protein structure.

Certain ligands (e.g., 2-phosphoglycollate) protect triosephosphate isomerase in its native state against denaturation, possibly by inducing a conformational change. However, when the enzyme was bound directly to a matrix, these same ligands afforded no protection to the enzyme. Furthermore, over twice the concentration of denaturant was required to cause the same extent of denaturation in this

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[3]Each molecule of denaturant appears to interact with two peptide bonds and with aromatic amino acid residues (Lee and Timasheff, 1974).

derivative as in the native enzyme. In contrast, the derivative bound to the Sepharose 4B *via* an acetamidoethyl linkage not only had kinetic properties approximating those of the native enzyme, but was also protected against denaturation by these ligands. These data suggest that the directly-bound enzyme is restricted in its conformation, whereas, the spacer-bound derivative has a conformation that is much more flexible. Because of its similarity to the native protein, the spacer-bound derivative should be of great utility in future experiments.

The matrix-bound subunits of triosephosphate isomerase were shown to be catalytically active and capable of combining with another subunit to form matrix bound dimers. These observations coupled with data from other workers suggest the following possibilities relating to the triosephosphate isomerase-deficient system in which a defective B subunit [B] is produced.

Case 1. The [B] subunit will not dimerize with the (A) subunit.

(A) + [B] ⟶ (A)(A) + [B] or [B][B]

Result: (a) 42 - 47% of normal enzymatic activity exhibited by (A)(A) homodimer.
(b) no heterodimer catalytic activity observed upon electrophoresis.

Case 2. The B subunit dimerizes with an A subunit but does not affect its activity.

(A) + [B] ⟶ (A)(A) + (A)[B] + [B] or [B][B]

Result: (a) 42 - 47% of normal enzymatic activity exhibited by (A)(A) and (A)[B] dimers.
(b) enzymatic activity band appears for A B upon electrophoresis.

Case 3. The B subunit binds to and alters the activity of the A subunit.

(A) + [B] → (A)(A) + (A)[B] + [B] or [B][B]

Result: (a) 5 - 10% of normal activity observed.
(b) band of enzyme activity may or may not be observed for heterodimer upon electrophoresis.

Cases 1 and 2 seem unlikely because in no case has 42 - 47% of the normal activity been reported in the homozygous-deficient individuals.

When Kaplan and coworkers (1968) subjected 1:50 diluted hemolysates of a triosephosphate isomerase-deficient patient and normal individuals to starch gel electrophoresis, only the most basic component (AA) appeared in the deficient hemolysate. However, when the hemolysates were diluted only 1:6, the basic isozyme (AA) again appeared and was accompanied by a more slowly stained band. This minor band appeared in the same position as the heterodimer (AB) in the normal subjects. Thus, case 3, in which the activity of the A subunit is modified is supported by these data. X-ray data of chicken muscle triosephosphate isomerase (Banner et al., 1971) have shown that the active-site regions are near the subunit contact sites. Thus, when the normal A and defective B subunits dimerize, the conformational change imposed on

the active site of the A subunit could decrease its catalytic activity eight- to ten-fold.

Therefore, triosephosphate isomerase deficiency disease appears to be the manifestation of the production of a defective B subunit with little or no catalytic activity. Furthermore, indirect evidence could suggest that this defective B subunit is capable of dimerizing with a normal A subunit and in so doing decreases the activity of the normal subunit in the affected AB heterodimer.

A number of studies could be employed to unequivocally define the genetic defect. Immunoprecipitation studies with the abnormal enzyme would ascertain the presence of the B subunit and the AB heterodimer. Isolation and complete structural analysis of the three forms from the deficient individual would follow. These data would define the change in the primary sequence of the polypeptide chain but would not ascertain the topographical result of the lesion in the native protein. This could only be accomplished by X-ray studies. Certainly, subunit binding studies could be carried out with the normal matrix-bound subunit and the defective subunit to ascertain if the defective subunit will dimerize.

Using crosslinking agents such as dimethyl suberimidate (Davies and Stark, 1970) to covalently bind the subunits together, one might cleave this derivative with a protease and isolate the subunit binding site peptides. Antibody could

then be produced specifically for the binding site peptide and used in studying the defective subunit. If no defective B subunit was available, modification of the binding site residues in the normal subunit could ascertain which amino acids are involved in binding.

Clearly, tissue cultures of fibroblasts from the enzyme-deficient individual would be the most effective method of investigating the disease. Not only would these cells provide a continuous source of the enzyme, but various factors affecting the production and degradation of this enzyme *in vivo* could also be evaluated. The most important use of such cell cultures would be to investigate the effects of *in vivo* levels of metabolic intermediates on this enzyme. Because the stability of the isozymes could be closely associated with the severity of this genetic disease, the half-life of the isozymes should also be determined. Cells from amniotic fluid samples could also be cultured in order to effect prenatal diagnosis of this enzyme. A cell line which was deficient in triosephosphate isomerase would be most effective in studying the effects of the build up of certain metabolites, especially dihydroxyacetone phosphate, on cell function.

This project has established the foundation for the study of the enzyme and provided some insight into the molecular basis of triosephosphate isomerase deficiency disease.

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