ISOZYMES AND IN VIVO ACTIVITY OF TRIOSEPHOSPHATE ISOMERASE

THESIS

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By

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RWS

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The distribution of isozymes of triosephosphate isomerase was normal in all human tissues examined. This finding argues against the existence of tissue-specific isozymes. Normal distributions of isozymes were also found in patients with cri-du-chat syndrome. Thus it is unlikely that a gene for triosephosphate isomerase is located on the short arm of chromosome five in man.

When triosephosphate isomerases from a wide range of species were examined by starch gel electrophoresis, definite evolutionary patterns were found.

Kinetic studies were conducted on human triosephosphate isomerase under conditions simulating the intracellular environment of the erythrocyte. Calculations using the kinetic parameters obtained indicate that even in triosephosphate isomerase deficiency disease, enough enzyme activity remains that the rate of glycolysis should not become inhibited.

INTRODUCTION

Structure of the Enzyme

Triosephosphate isomerase (EC 5.3.1.1) catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (G-3-P) and is an integral step of the glycolytic pathway. Although the enzyme was discovered in the mid 1930's (Meyerhof and Lohmann, 1934; Meyerhof and Kessling, 1935), it was not until 1953 that it was isolated in crystalline form from calf muscle (Meyer-Arendt et al., 1953).

Human triosephosphate isomerase was first isolated in crystalline form from erythrocytes (Rozacky et al., 1971). It has also been isolated or partially purified from rabbit muscle (Özok and Bücher, 1960; Norton et al., 1970), rabbit liver (Krietsch et al., 1970), calf skeletal muscle (Beisenherz, 1955), bovine lens (Burton and Waley, 1968), yeast (Krietsch et al., 1970), housefly and mosquito (Chiang, 1972), coelacanth (Klob and Harris, 1972), horse and human liver (Lee et al., 1971), pea seedlings (Turner et al., 1965), algae (Meeks et al., 1968), thermophilic bacteria (Fahey et al., 1971) and chicken muscle (Trentham et al., 1969).

Since rabbit muscle triosephosphate isomerase has been the most extensively studied of the mammalian triosephosphate isomerases, it will be used here for comparison with the human enzyme. Specific activities of the rabbit enzyme (glyceraldehyde 3-phosphate as substrate) of 6,400 units per milligram of protein (at 25°C) (Krietsch et al., 1970) and 7,500 units per milligram (at 24°C) (Norton et al., 1970) are in agreement with the value of 8,000 units per milligram (at 25°C) for the human enzyme (Rozacky et al., 1971).

Recent determinations of the molecular weight of the rabbit enzyme gave values of 53,000 (Norton et al., 1970) and 57,000 (Krietsch et al., 1970). These numbers agree closely with those of 56,100 and 55,000 obtained for the human enzyme (Rozacky et al., 1971).

The amino acid composition of human triosephosphate isomerase is very similar to that of the rabbit enzyme with respect to a number of amino acids. However, some differences are apparent, particularly in serine, proline, and valine. Although the degree of amidation in either enzyme is unknown, the ratio of acidic to basic amino acid residues in the human enzyme is 1.75 and that of the rabbit enzyme is 1.59. This is in agreement with the lower isoelectric points for the human enzyme, as compared to the rabbit (Rozacky et al., 1971).

Triosephosphate isomerase from rabbit skeletal muscle has been shown to be composed of two identical subunits of approximately one half the molecular weight of the native enzyme (Krietsch et al., 1970; Norton et al., 1970; Johnson and Waley, 1970). This same dimeric structure has been shown in the case of the human enzyme (Sawyer et al., 1972).

Rabbit muscle triosephosphate isomerase has one major and two minor bands of activity when electrophoresed on starch gel (Burton and Waley, 1968) or on polyacrylamide gels (Lee and Snyder, 1971). Under isoelectric focusing conditions, the rabbit muscle enzyme has been resolved into four (Norton et al., 1970) and five peaks of activity (Rozacky et al., 1971). The human enzyme has been shown to be present as three isozymes after electrophoresis on starch gel (Kaplan et al., 1968), polyacrylamide gel (Lee and Snyder, 1971), and isoelectric focusing (Rozacky et al., 1971; Sawyer et al., 1972). Rubinson has reported a tissue specific isozyme of triosephosphate isomerase in cultured human fibroblasts (Rubinson et al., 1971). With the exception of this report, all other investigators studying the isozymes of human triosephosphate isomerase have found the same number, three, in the tissues that they have examined (Lee and Snyder, 1971; Kaplan et al., 1968). An important point in this thesis will involve a study of the isozymes of human triosephosphate isomerase, and their distribution in various tissues.

There are several possible explanations for such observed electrophoretic multiplicity. It may represent true isozymes arising from various combinations of structurally different subunits, or it may represent different states of oxidation of sulfhydryl groups, partial denaturation, different states of conformation, aggregation, or amidation.

The individual isozymes of human triosephosphate isomerase have been separated and extensively studied (Sawyer et al., 1972). By equilibrium ultracentrifugation, each isozyme was found to have the same molecular weight as the native enzyme (56,000), and each could be dissociated into subunits of 28,000.

The weight of evidence suggests that the subunits of human triosephosphate isomerase are of two different types A and B and that these two different subunits can combine to form three dimeric isozymes AA, AB, and BB. The two different types of subunits would each be coded for by different genes. These genes could be the result of a gene duplication, followed by divergent evolution. A similar basis for the isozymes of rabbit triosephosphate has been proposed (Krietsch et al., 1971).

If the isozymes of triosephosphate isomerase do indeed have a genetic basis, a study of a large number of different species could substantiate this by showing differences in isozyme patterns corresponding to evolutionary distance. Such a study will be included in this thesis.

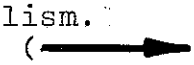
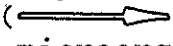
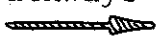
Metabolic Role of the Enzyme

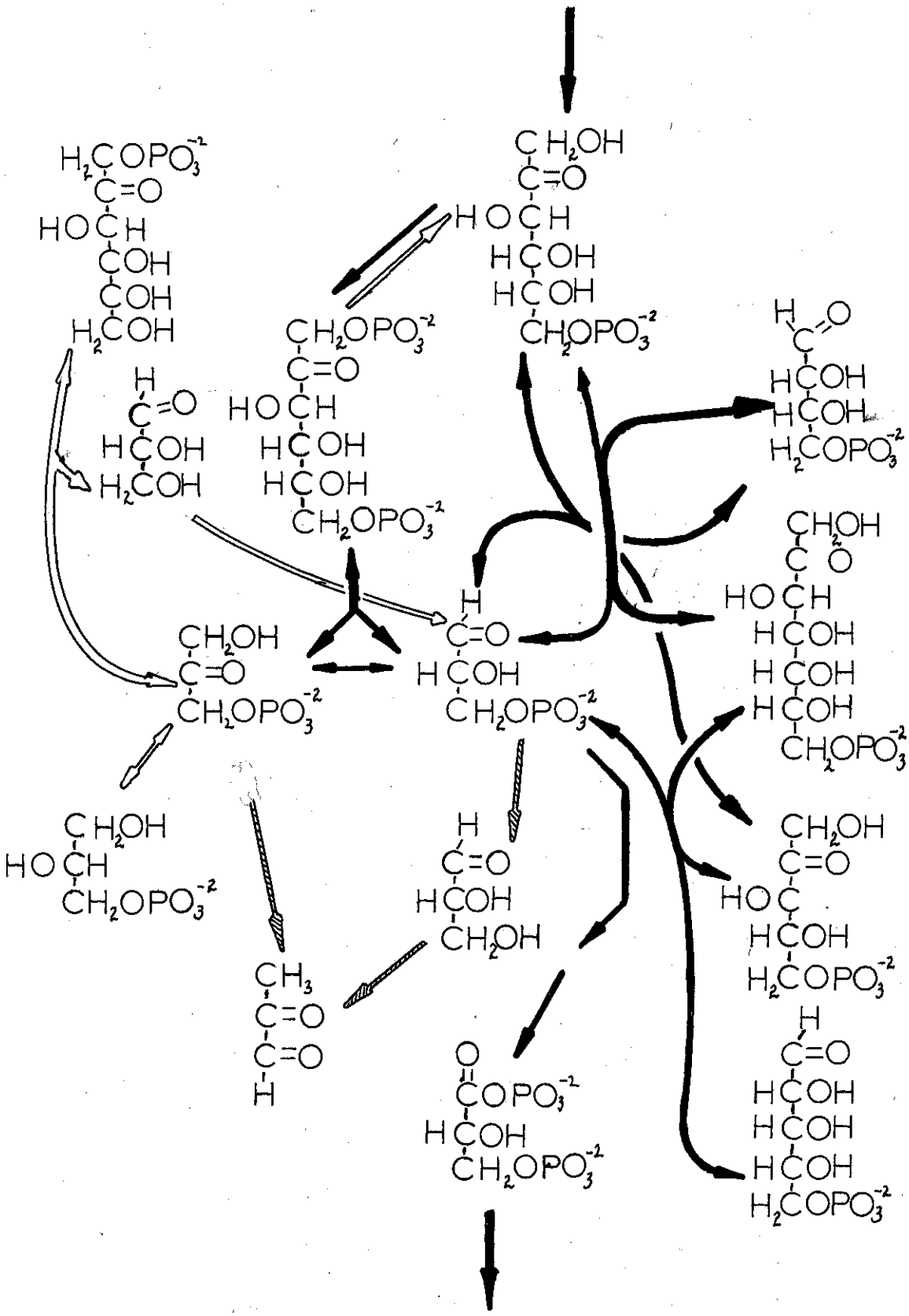
The triosephosphates also serve as intermediates in other metabolic pathways, a factor which may be important in metabolic control or interaction between these pathways. DHAP can be converted to α-glycerol phosphate by cytoplasmic α-glycerol phosphate dehydrogenase (EC 1.1.1.8). The glycerol phosphate is then channeled into triglyceride or phosphoglyceride synthesis. Another enzyme acting on DHAP is fructose 1-phosphate aldolase which functions in gluconeogenesis and is localized in the liver and kidneys. In this reaction fructose 1-phosphate is formed from the aldol condensation of DHAP and D-glyceraldehyde. The enzyme is also capable of catalyzing the condensation of the two triosephosphates to form fructose 1,6-diphosphate. DHAP is also involved in the glycerol phosphate shuttle which unidirectionally transports reducing equivalents into the mitochondria. Furthermore, it has recently been shown that certain microorganisms have a constitutive methylglyoxal synthetase which catalyzes the conversion of DHAP to methylglyoxal (Hooper and Cooper, 1971).

Glyceraldehyde 3-phosphate is also a common intermediate in several pathways. It is involved in both the transketolase and the transaldolase reactions of the pentose phosphate shunt. It can be formed from glyceraldehyde by liver glycerol kinase and is also involved in the

synthesis of glyceryl ethers. Glyceraldehyde 3-phosphate has also been shown to be converted to methylglyoxal by a phosphatase and a dehydrogenase in extracts of Pseudomonas saccharophila (Rizza and Hu, 1973). Figure 1 summarizes the various pathways which may be involved in the metabolism of the triosephosphates.

The roles played by these pathways and their intermediates vary with the type of tissue and cell in which they are found. The erythrocyte, however, provides a particularly interesting system. Since the mature red blood cell lacks mitochondria, glycolysis stops at the level of lactate and no glycerol phosphate shuttle exists. Red cells are also lacking in a number of other enzymes which normally act at the triosephosphate level. No α-glycerolphosphate dehydrogenase has been detected in these cells (Schneider et al., 1968), and the erythrocyte apparently has no other means of metabolizing DHAP except through glycolysis (Foranini and Bossu, 1969). Thus, it would seem that this very restricted metabolism in the erythrocyte simplifies the role of triosephosphate isomerase in this cell to one of allowing DHAP to be catabolyzed to lactate. Yet even in this simplified case, our understanding is apparently incomplete since investigations of metabolism in triosephosphate isomerase-deficient erythrocytes have yielded unexpected and poorly understood results (Schneider et al., 1968).

Fig. 1 --Pathways of triosephosphate metabolism. Pathways known to exist in the human erythrocyte (). Pathways found in other mammalian tissues (). Pathways presently known only in plants or microorganisms ().



The Enzyme Deficiency Disease

The genetic disease, triosephosphate isomerase deficiency, has been studied by Schneider and co-workers (Schneider et al., 1968; Valentine et al., 1966). The disorder appears to be due to a single gene mutation and is transmitted as an autosomal recessive. It is characterized by a nonspherocytic hemolytic anemia with erythrocytes very low in triosephosphate isomerase activity. There are slightly elevated levels of several other glycolytic and pentose phosphate pathway enzymes. This elevation, however, is believed to be due to a younger mean red cell age (Schneider et al., 1968). Marked neuromuscular disease, including mental and developmental retardation and general spasticity, implying muscle and central nervous system involvement, has led to the suggestion of a multiple-tissue or whole-body disease (Schneider et al., 1968). Triosephosphate isomerase levels in erythrocytes, leucocytes, skeletal muscle, spinal fluid and blood serum of afflicted individuals were all markedly lower than levels of the enzyme in unaffected individuals. In addition, the increased susceptibility to infection was interpreted as functional impairment of leucocytes. Only homozygous individuals showed these symptoms and these same individuals showed as little as five per cent of normal triosephosphate isomerase activity. The heterozygotes, on the average,

exhibited half normal levels of the enzyme but no clinical symptoms.

The metabolic basis of the disease is poorly understood. Schneider and co-workers studied erythrocytes from individuals homozygous for the deficiency in an attempt to trace the molecular chain of events leading from a deficiency of triosephosphate isomerase to the hemolysis (Schneider et al., 1968). Mature erythrocytes are entirely dependent on anaerobic glycolysis for production of high energy phosphate compounds. They have no other known pathway for metabolizing DHAP. Therefore, it was expected that a block at the triosephosphate isomerase reaction would result in a decrease in the catabolism of the three carbon compounds formed from glucose. This would in turn cause a deficiency in ATP synthesis and a decrease in the rate of glycolysis. In addition, an accumulation of DHAP and possibly of fructose 1,6-diphosphate was expected. Schneider's findings, however, were quite unexpected. There was a three-fold increase in the rate of glucose utilization. There was also no deficit in the amount of lactate formed from glucose. The ATP concentration was normal in one patient and only moderately lower in the other, and this concentration did not change during incubation. The DHAP concentrations of 1.77 and 2.19 μ mole per milliliter of packed cells were elevated approximately

twenty-fold. Fructose 1,6-diphosphate showed only a very slight increase in concentration but other intermediates were not significantly different from normal controls. These findings led Schneider and co-workers to suggest that a great proportion of glucose was metabolized by the pentose phosphate shunt thereby bypassing the triosephosphate isomerase step and achieving a slight net gain in ATP synthesis. Methylene blue caused only a slight increase in glucose utilization in triosephosphate isomerase deficient erythrocytes suggesting that the shunt was operating near maximum rates. Other possibilities advanced (Beutler, 1968) were that alternate routes for metabolism of DHAP might exist, that excess DHAP might cause abnormalities in lipid synthesis by way of α-glycerolphosphate dehydrogenase, and that DHAP or a condensate of DHAP with some other biomolecule might be toxic.

In other studies (Valentine et al., 1966), it was found that triosephosphate isomerase deficient erythrocytes subjected to oxidative stresses such as incubation with acetylphenyl-hydrazine showed vastly increased Heinz body formation. These data suggested an inability to maintain hemoglobin in a reduced state.

Foranini and Bossu (1969) have pointed out that normal triosephosphate isomerase has a turnover of about 4,300 to 5,160 μmoles of substrate per hour per milliliter

of erythrocytes (in hemolysates), but that within the erythrocyte, the rate of glycolysis is only 3 to 4 μ moles per hour per milliliter of cells. The hemolysates of homozygous triosephosphate isomerase deficient patients have about five per cent of normal activity which would still exhibit a turnover of approximately 250 μ mole per hour per milliliter of cells. Foranini and Bossu (1969) have suggested that the actual activity may be reduced an additional twenty times in whole cells due to pH, inhibitor concentrations, etc. This would still result in 12.5 μ moles per hour per milliliter of cells, which is still three to four times the normal metabolic rate observed. Thus, even with the homozygous condition, enough triosephosphate isomerase activity would be expected to remain to prevent impairment of glycolysis. However, the suggested twenty-fold reduction in activity due to intracellular conditions (Foranini and Bossu, 1969) was an assumption and was not based on any data concerning intracellular inhibitors or their concentrations. Therefore, it was of considerable interest to determine to what extent the enzyme is normally inhibited *in vivo*, and thus the assaying of human triosephosphate isomerase under conditions simulating those of erythrocyte cytoplasm as closely as possible forms a third portion of this thesis.

Triosephosphate Isomerase and Chromosome Five

Sparkes and co-workers (1969) have reported a case of the cri-du-chat syndrome involving a deletion of approximately half of the short arm of chromosome five in which the level of triosephosphate isomerase in the blood was about one-half of the normal value. They concluded that the patient was hemizygous for the triosephosphate isomerase gene and postulated that this gene was localized on the short arm of chromosome five in man.

Rüdiger and co-workers (1970) and Brock and Singer (1970a; 1970b) examined several patients with cri-du-chat syndrome but failed to observe abnormally low levels of triosephosphate isomerase. Sparkes subsequently defended his original conclusions and pointed out specific differences in assay methods of the two studies (Sparkes, 1970). Furthermore, the wide range of triosephosphate isomerase levels found in normal controls (Brock and Singer, 1970) also suggested the need for more complete investigations. Additional problems in interpretation of the cri-du-chat studies resulted from the demonstration of three isozymes of human triosephosphate isomerase (Rozacky et al., 1971; Sawyer et al., 1972; Kaplan et al., 1968). Investigations described in this thesis also attempt to resolve this controversy by detailed studies on the isozymes of

triosephosphate isomerase from patients showing the cri-du-chat syndrome.

MATERIALS AND METHODS

Materials

Electrophoresis

Triethanolamine (TEA), and imidazole were obtained from Sigma Chemical Company as the free bases, and buffers were prepared with HCl. DL-glyceraldehyde-3-phosphate (G3P) was obtained as the barium salt of the diethylacetal and converted to the free aldehyde according to the supplier's (Sigma) instructions. Reduced diphosphopyridine nucleotide (DPNH), 2-mercaptoethanol, α-glycerophosphate dehydrogenase (α-GPDH), phenazine methosulfate, MTT tetrazolium 3(4,5-dimethylthiazolyl-2-)-2-5diphenyl tetrazolium bromide, and ethylenediaminetetraacetic acid (EDTA) were also obtained from Sigma Chemical Company. Ampholines and other materials for isoelectric focusing were obtained from LKB-Produkter. Celagram 78 x 150 mm cellulose acetate sheets were purchased from Shandon Instruments. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Nutritional Biochemicals, while hydrolyzed starch was from Fisher Scientific Company. All other chemicals were reagent grade, and all solutions were prepared in distilled water.

Inhibitors

2-Phosphoenolpyruvate, adenosine triphosphate, adenosine monophosphate, adenosine 3'-5'cyclic monophosphate, adenosine 5'-diphosphate, fructose-1,6-diphosphate, glucose-1-phosphate, ribose-5-phosphate, creatine phosphate, oxalocetic acid, 2,3-diphosphoglycerate, 3-phospho-D-glycerate, and D-erythrose-4-phosphate were obtained from Calbiochem. Phosphoglycolic acid, DL-glyceraldehyde, D-fructose-1-phosphate, D-glucose-6-phosphate, pyruvic acid, fumaric acid, creatinine, creatine, ∝-ketoglutaric acid, D-galactose-6-phosphate, seduhepulose-7-phosphate, D-ribulose-1,5-diphosphoric acid, 6-phosphogluconic acid, D-ribulose-5-phosphate, fructose-6-phosphate, pyridoxal-5'-phosphate, pyridoxine, and 2-phosphoglycerate were purchased from Sigma Chemical Company. Pyridoxal and reduced glutathione were supplied by General Biochemicals. Sodium acetate, magnesium chloride, sodium bicarbonate, and sodium phosphate were from J. J. Baker Company. In each experiment the stock inhibitor solutions were converted to the sodium salts and dissolved in the buffer system being used for the assay.

Methods

Starch Gel Electrophoresis and Activity Stains

Starch gel electrophoresis was carried out by the method of Kaplan *et al.* (1968). Fourteen-centimeter gels

were prepared and run at 140 volts in a cold room. A modification of the activity stain of Kaplan et al. (1968) was used to locate the triosephosphate isomerase activity. After horizontal sectioning of the gel, the staining solution consisting of two milliliters of 50 mM TEA buffer, pH 7.8, 0.35 mM DPNH, 0.4 mM G3P, and 25 units/ml ∝-GPDH was allowed to soak into a 78 x 150 mm cellulose acetate sheet which was lightly blotted, then applied to the starch gel surface. The defluorescence of DPNH was then viewed in an ultraviolet light box.

Isoelectric Focusing

Isoelectric focusing was carried out in a sucrose density gradient in a 110-ml LKB column according to the method of Vesterberg and Svensson (1966), and the LKB instrument manual. Ampholines of the 5-7 pH range were used in all cases. The electrofocusing experiments were carried out at 600 volts for 72 hours at 4°C, and two ml fractions were collected. pH measurements were made with a glass electrode immediately after elution of the electrofocusing column.

Enzyme Assay

The TPI assay mixture consisted of 0.15 mM DPNH, 10 µg of ∝-glycerophosphate dehydrogenase, 1.5 mM DL-glyceraldehyde-3-phosphate, 1 mM EDTA, and 50 mM triethanolamine

buffer, pH 7.8, in a final volume of 1.0 ml. The change in absorbance at 340 nm resulting from the oxidation of DPNH was followed in a recording spectrophotometer with the sample chamber thermostatically controlled at 30.0°C.

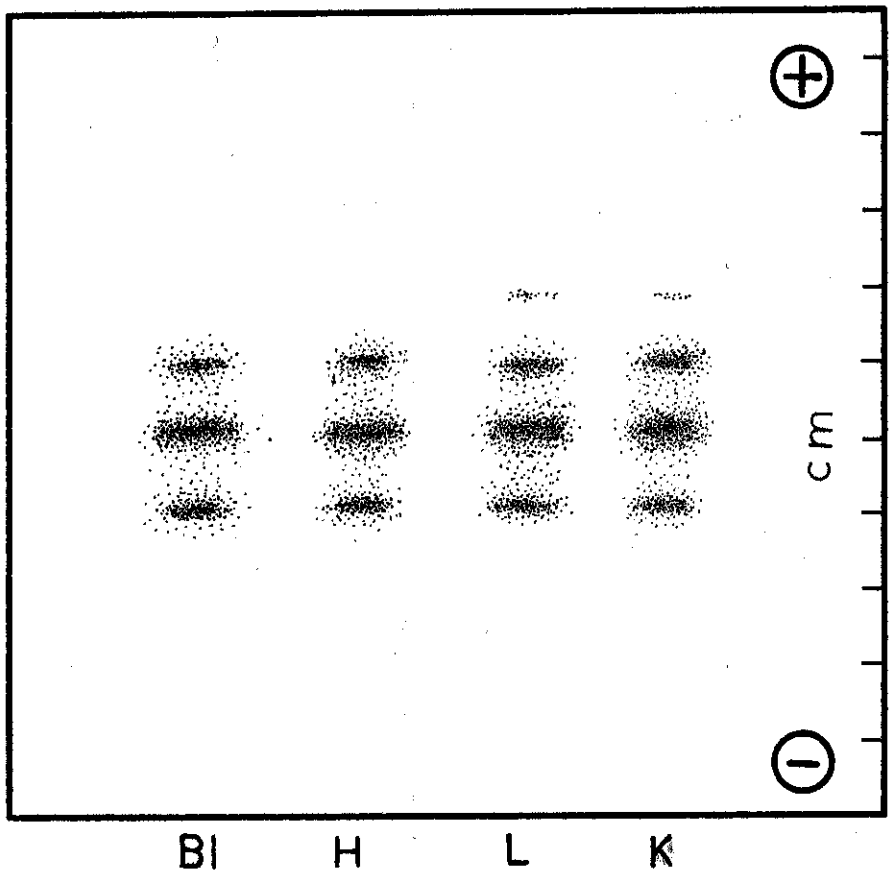
RESULTS

Comparison of Triosephosphate Isomerase Isozymes in Human Tissues

Starch gel electrophoresis of extracts of human blood, heart, liver, kidney, skeletal muscle, brain, spleen and lung each resulted in three bands of triosephosphate isomerase activity (Fig. 2). A weak, slowly developing fourth band of activity occasionally appeared just anodal to the other three, but its occurrance was reduced when the concentration of mercaptoethanol in the gel was increased. When blood lysate was allowed to age without mercaptoethanol, then electrophoresed, five bands of activity were seen (Fig. 3). When substrate, coupling enzymes, or cofactors were omitted from the stain, no defluorescence of the gel was observed.

When the separated isozymes of human triosephosphate isomerase, obtained by isoelectric focusing of the crystalline enzyme (Sawyer *et al.*, 1972), were run on starch gel electrophoresis, the correspondence of the three activity bands seen on starch gel with the three isozymes from focusing was established (Fig. 4). The relative migration of each isozyme was consistent with the isoelectric points of each form.

Fig. 2 --Starch gel electrophoresis of triosephosphate isomerase from various human tissues. Frozen tissues obtained from autopsy were thawed, homogenized in three volumes of 10 mM Tris, 0.45 mM EDTA, pH 9.3, centrifuged (25,000 x g, 1 hour), and dialized overnight against the same buffer. The abbreviations are as follows: blood (Bl); heart (H); liver (L); kidney (K); skeletal muscle (SM); brain (B); spleen (S); and lung (Lu).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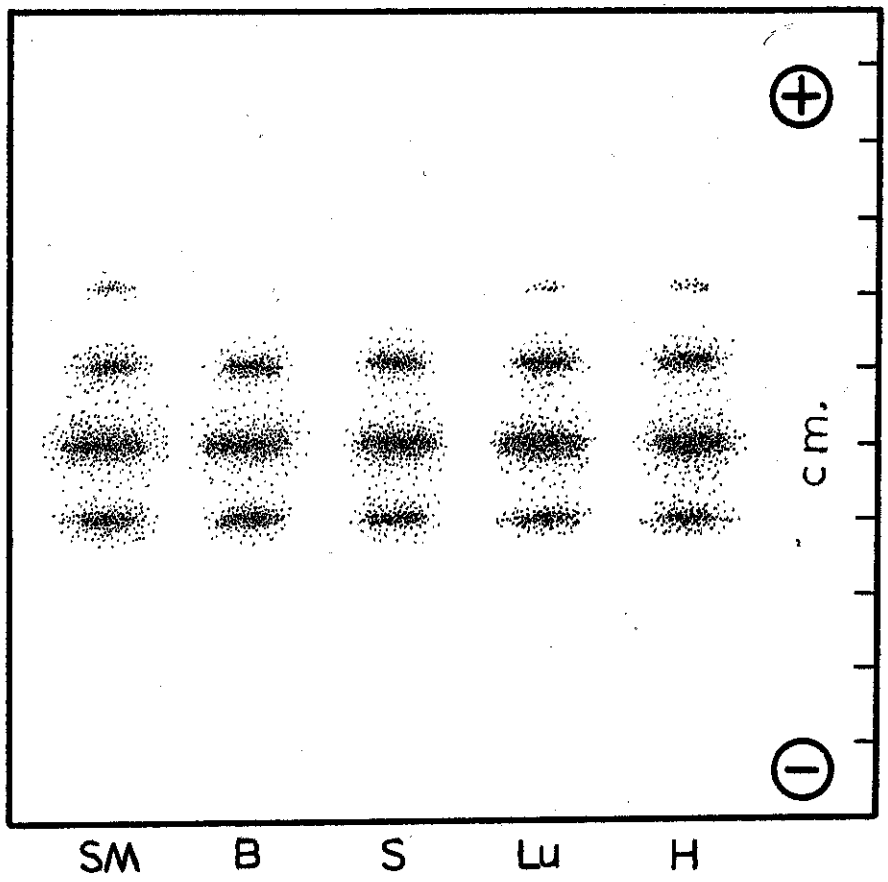
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Lu
H

Fig. 3 --Minor triosephosphate isomerase activity bands of high relative mobility appearing on a starch gel as oxidative artifacts. This sample of human erythrocyte lysate was allowed to age for one week at four degrees centigrate without mercaptoethanol. Mercaptoethanol was also deleted from the gel buffer.

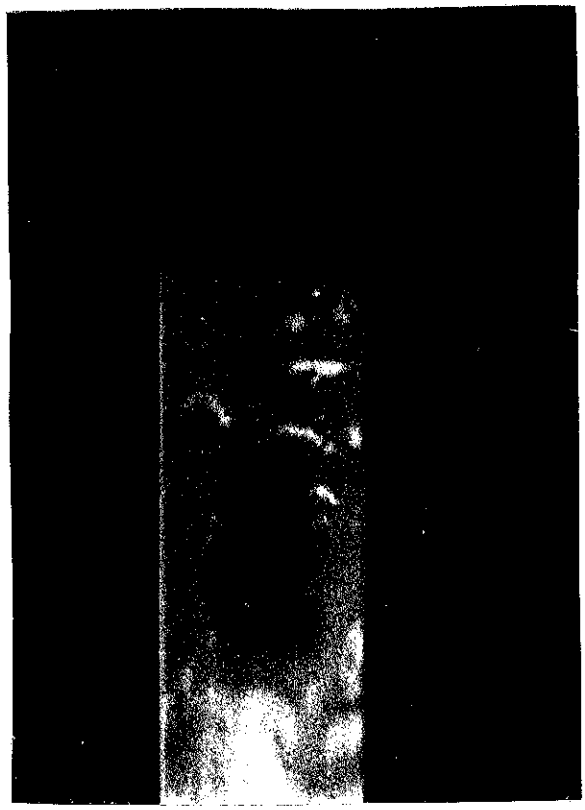
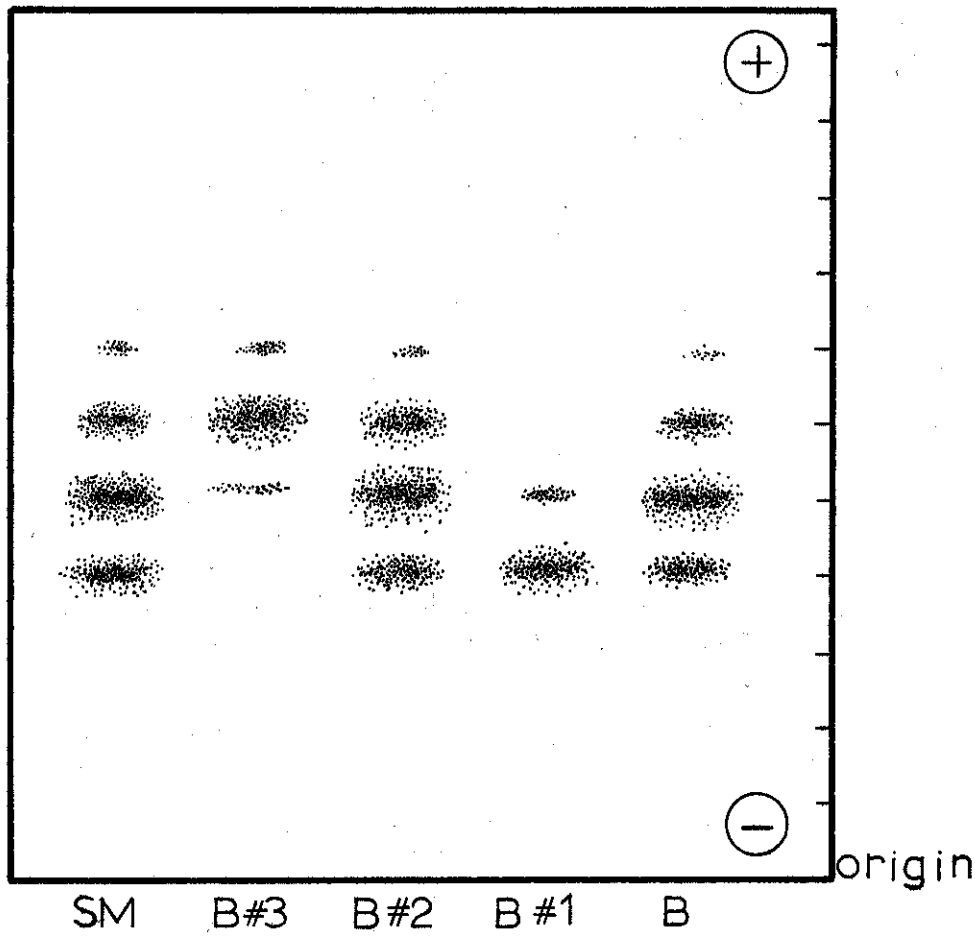


Fig. 4 --Starch gel electrophoresis of isozymes of purified human triosephosphate isomerase resolved by isoelectric focusing (Sawyer et al., 1972). The designation of each sample is as follows: skeletal muscle (SM); Component I from blood (B#1); Component II from blood (B#2); Component III from blood (B#3), and unresolved crystalline triosephosphate isomerase isolated from human blood (B).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B

Comparison of Triosephosphate Isomerase Isozymes in Other Species

In a survey of a wide range of species by starch gel electrophoresis all vertebrates gave three bands of triosephosphate isomerase activity (Table I). In some cases, however, two of the bands were of low intensity. Several crustaceans exhibited two isozymes and the lower invertebrates and microorganisms exhibited only one isozyme. The exception to this was Euglena gracilis which exhibited two isozymes.

Of all the species studied, only Rhesus monkey showed identical electrophoretic migration with the human isozymes (Fig. 5). When a 1:1 mixture of human and Rhesus muscle triosephosphate isomerase was electrofocused, only three bands of activity were found with isoelectric pH values of 6.3, 6.0, and 5.6 (Fig. 6). The only difference between the human and Rhesus enzymes was one of the relative distribution of the three isozymes. In human tissues, the central isozyme (Component II) was the most rapidly developing and the most intense in staining, followed by the anodal band (Component III), then the cathodal band (Component I). In the case of the Rhesus enzyme, however, the cathodal band appeared first and was most intense, followed by the central, and then the anodal isozyme.

TABLE I

ELECTROPHORETIC PROPERTIES OF TRIOSEPHOSPHATE ISOMERASE FROM VARIOUS SPECIES

Species[a]	Number of Isoenzymes[b]	Relative Mobility[c]
Human	3	2·8, 2·2, 1·6
Rhesus	3	2·8, 2·2, 1·6
Rabbit	3	1·0, 0·8, 0·5
Bovine	3	1·6, 1·4, 1·2
Porcine	3	1·6, 1·2, 1·0
Chicken	1 (+ 2M)	(2·5), (1·8), 1·3
Dove -- *Zenoidura macroura*	3	2·8, 2·2, 1·5
Turtle -- *Pseudemys scripta elegans*	3	2·0, 1·5, 1·0
Leopard frog -- *Rana pipiens*	3	2·0, 1·5, 1·0
Turbot -- *Scophthalmus maximus*	1 (+ 2M)	(4·2), 1·4, (0·0)
Salt water catfish -- *Galeichthys felis*	3	1·5, +0·8, -0·8

TABLE I --Continued

Species[a]	Number of Isoenzymes[b]	Relative Mobility[c]
Fresh water bass -- *Roccus chrysops*	1 (+ 2M)	(4·6), 2·0, (0·0)
Crab -- *Callinectes sapidus*	2	6·8, 6·0
Lobster -- *Homarus vulgaris*	2	6·8, 6·0
Shrimp -- *Harpiosquilla rhapidea*	2	6·5, 6·0
Shrimp -- *Palaemonetes vulgaris*	1	8·0
Beetle -- *Calosoma scrutator*	1	11·3
Cricket -- Subfamily Eneopterinae	1	12·0
Grasshopper -- *Melanoplus*	1	14·6
Clam -- *Venus mercenaria*	1	0·9
Sea snail -- *Busycon canaliculatum*	1	2·3
Squid -- *Loligo brevis*	1	2·5
Ascaris suum	1	0·9
Sea anemone -- *Astrangia danae*	1 (+ 1M)	3·8, (2·0)

TABLE I --Continued

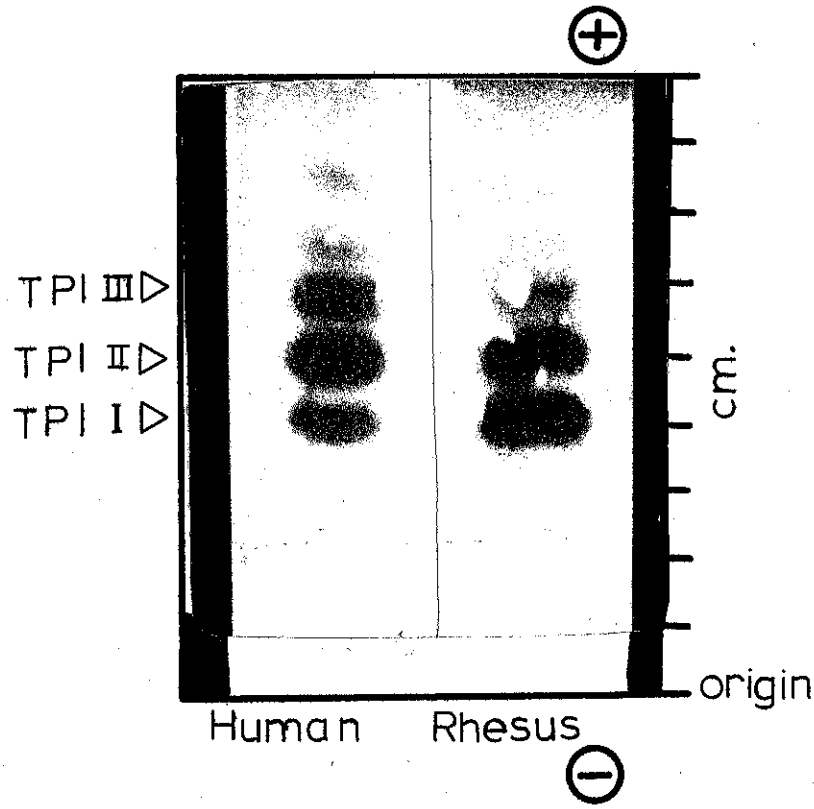
Species[a]	Number of Isoenzymes[b]	Relative Mobility[c]
Euglena -- dark-grown	1	9·6
light-grown	2	11·2, 9·5
Escherichia coli	1	12·5
Bacillus subtilis	1	15·0
Pseudomonas aeruginosa	1	10·0
Staphylococcus aureus	1	14·0

[a]Skeletal muscle tissue used in all larger animals.

[b]Number of completely resolved electrophoretic bands. Cases where minor bands were seen are also indicated (e.g. + 2M indicates two additional minor bands). The mobilities of the minor bands are indicated in parentheses.

[c]All electrophoresis was carried out at pH 8·9 in 13 per cent starch gel slabs. Electrophoresis was as in Methods, but for various time periods. Relative mobilities are expressed as mm/hr. All mobilities are given toward the anode.

Fig. 5 --Starch gel electrophoresis of human and Rhesus monkey triosephosphate isomerases. Skeletal muscle was used for both samples. Conditions were those described in Methods.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TPI III
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cm.
origin
Human
Rhesus
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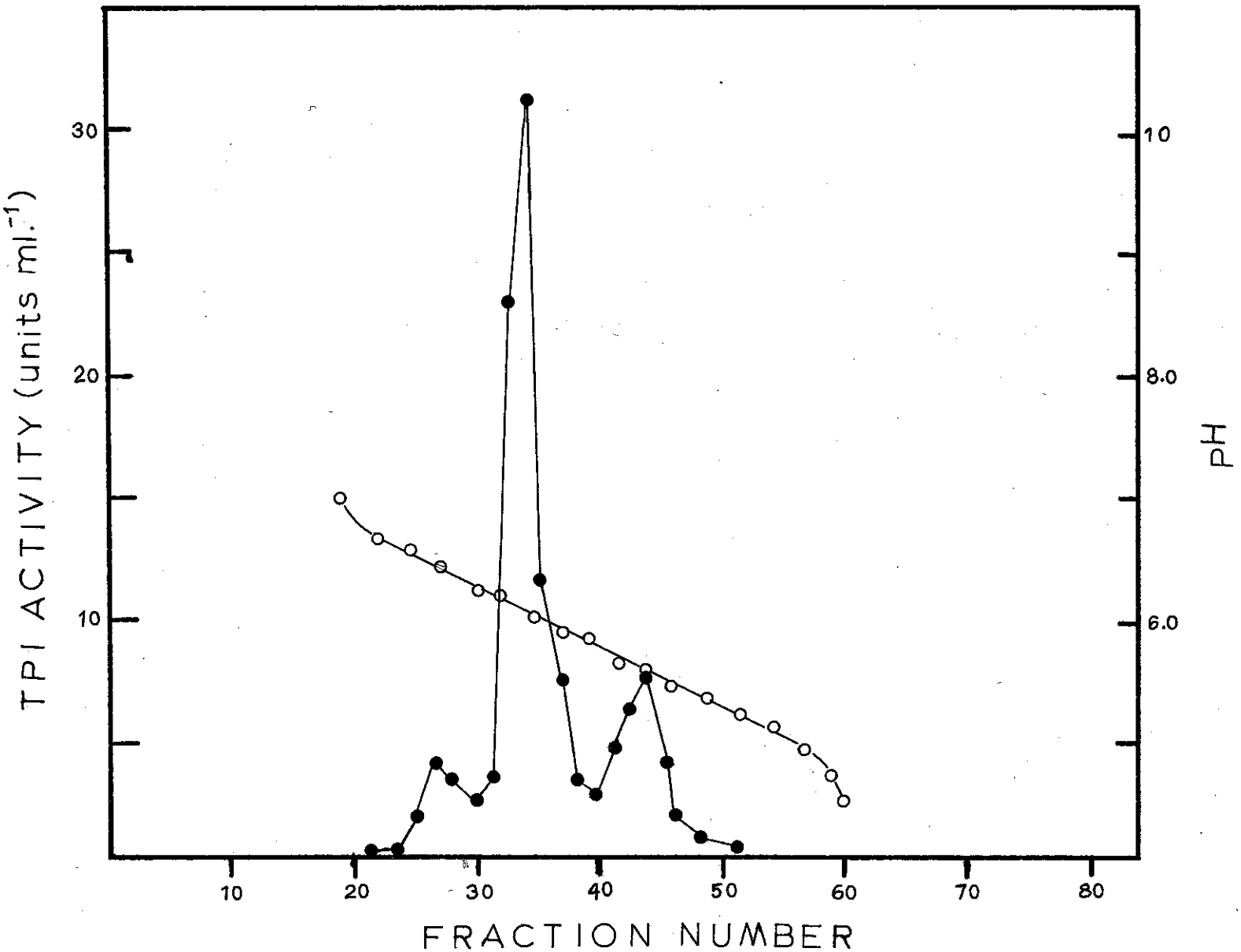
Fig. 6 --Coelectrofocusing of human and Rhesus monkey triosephosphate isomerase. Both samples were taken from skeletal muscle and mixed in a one to one activity ratio. The symbols are (●——●——●) activity and (o——o——o) pH. Electrofocusing was carried out as described in Methods.


TPI ACTIVITY (units ml.$^{-1}$)
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10
10
8.0
6.0
pH
10
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70
80
FRACTION NUMBER

Triosephosphate Isomerase in the Cri-Du-Chat Syndrome

When samples of hemolysate from patients exhibiting the three cytogenetic forms of cri-du-chat syndrome were examined by starch gel electrophoresis, no difference in the distribution of triosephosphate isomerase isozymes as compared to normal controls could be detected (Fig. 7). Subject L.B. showed no apparent deletion of the B group chromosomes yet exhibited typical cri-du-chat symptoms. This situation is rare but not without precedent (McGavin et al., 1967). Subjects R.F., L.H., and D.N. each exhibited the typical short arm deletion of chromosome five. Subject L.R. also had the short arm deletion as well as a break near the tip of the long arm with subsequent formation of a ring chromosome. Rebreaking of some of these metaphase chromosomes at the point of fusion in some preparations has revealed a virtually complete deletion of the short arm. This deletion is considerably larger than that reported by Sparkes (Sparkes et al., 1969), yet this subject and the others all appeared to have a normal complement of triosephosphate isomerase isozymes by starch gel electrophoresis.

As further corroboration of this, a hemolysate from L.R. was subjected to electrofocusing. A normal focusing profile was obtained (Fig. 8).

Fig. 7 --Starch gel electrophoresis of erythrocyte lysates from patients with cri-du-chat syndrome. Initials are those of the patients. Normal control is indicated by (c).

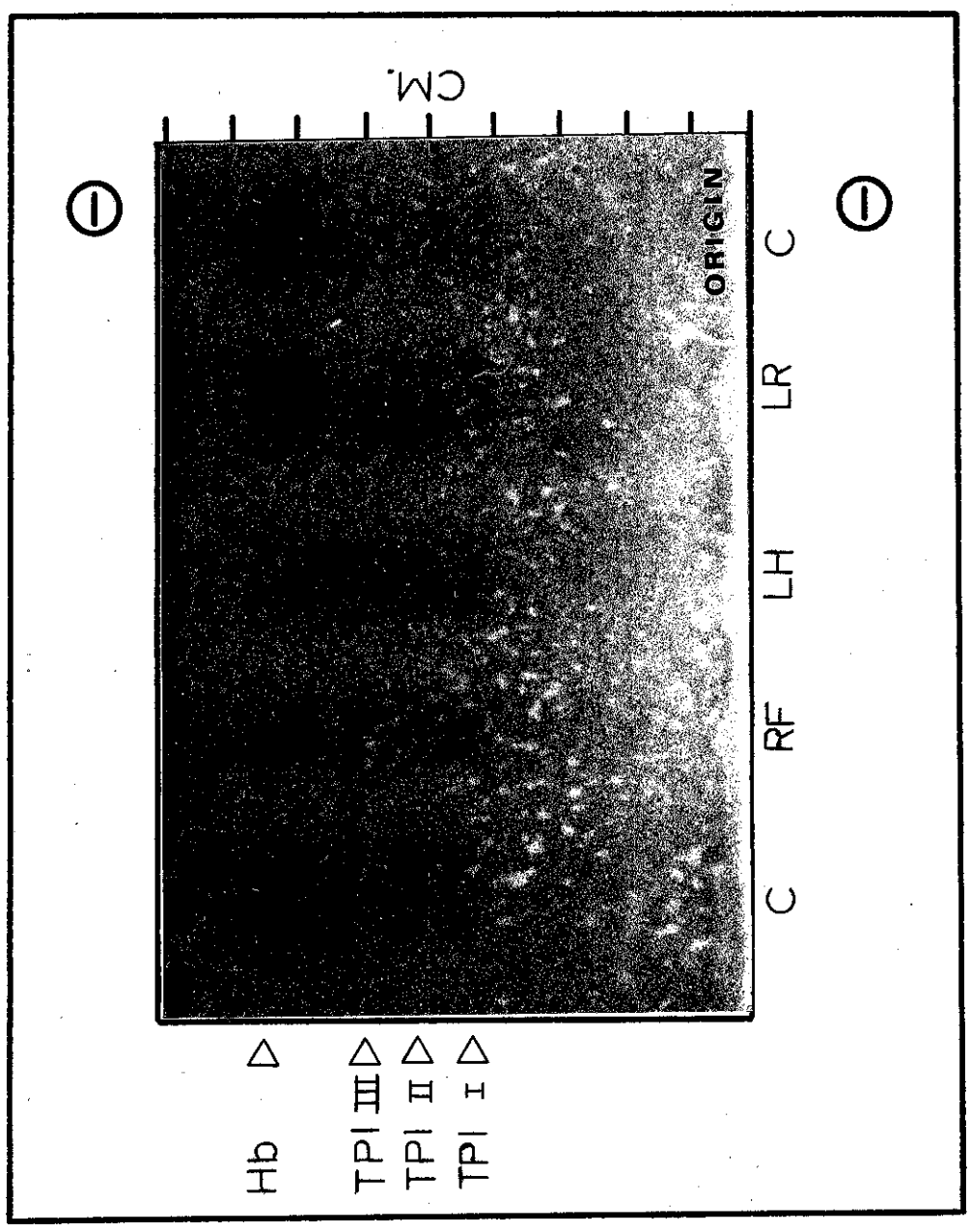
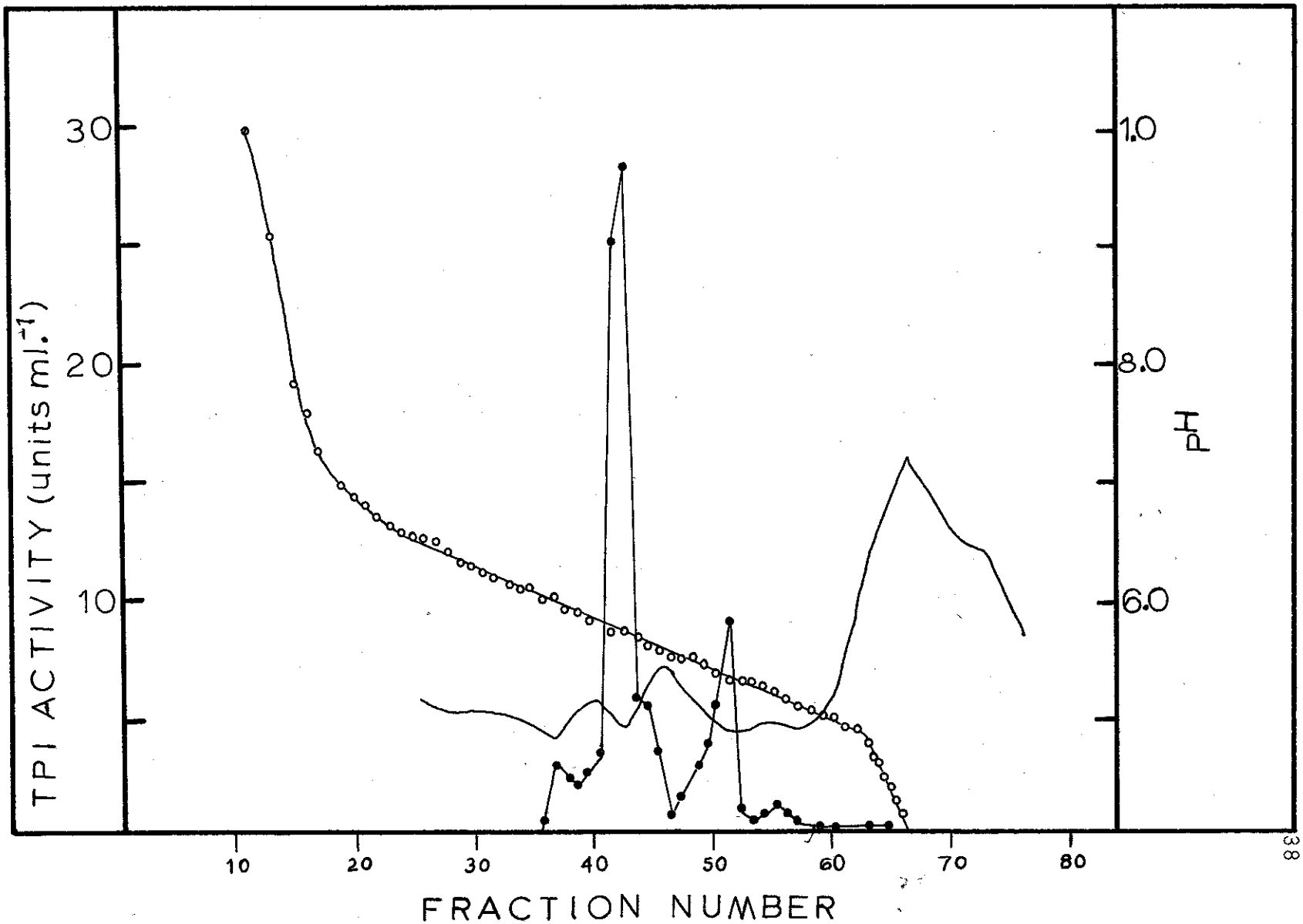

CM.
ORIGIN
C
RF
LH
LR
C
TPI I
TPI II
TPI III
Hb

Fig. 8 --Isoelectric focusing of an erythrocyte lysate from patient L.R. who exhibited a ring form of chromosome five with an essentially complete deletion of the short arm. Electrofocusing was carried out as described in Methods. The symbols are: (—●—●—)triosephosphate isomerase activity, (-oooooo-) pH, and (———)protein.


TPI ACTIVITY (units ml.$^{-1}$)
30
20
10
1.0
8.0
6.0
pH
10
20
30
40
50
60
70
80
FRACTION NUMBER

Simulated In Vivo Kinetics of Triosephosphate Isomerase

An assay system was designed to simulate properties of the erythrocyte cytoplasm likely to affect enzyme kinetics. The assay buffer was composed of fifty-millimolar triethanolamine buffer adjusted to pH 7.20 and with an ionic strength of 0.165. Assays with this system were carried out at 37°C. A large number of metabolic intermediates and potential inhibitors of triosephosphate isomerase were tested by determining their inhibition constants (Ki values) under these conditions (Table II). The strongest inhibitor among these was phosphoenolpyruvate with a Ki of 1.15 mM. However, at a normal intracellular concentration of 0.017 mM, phosphoenolpyruvate gives only a slight inhibition.

When kinetic studies were made under standard assay conditions of 25.0°C and pH 7.8 (Rozacky et al., 1971) and plotted in a Lineweaver-Burk fashion (Fig. 9) a K_m for glyceraldehyde 3-phosphate of 0.48 mM and a V_{max} of 0.0102 units per milliliter were found (one milliliter of assay mix contained 0.97 µg of human triosephosphate isomerase). Using these values, an activity of 1.30×10^{-4} units per microgram of triosephosphate isomerase was calculated at the normal erythrocyte glyceraldehyde 3-phosphate concentration of 0.006 millimolar (Yoshida, 1973).

By comparison, the Lineweaver-Burk plot for the assay carried out under simulated in vivo conditions of pH 7.2,

TABLE II

ESTIMATED INHIBITION OF TRIOSEPHOSPHATE ISOMERASE BY ERYTHROCYTE METABOLITES

Compound	Ki (mM)	Erythrocyte Concentration (mM)	Per Cent Inhibition In Vivo
GSH	70.0	3.00	0.50
ATP	24.5	1.50	0.49
Fructose 1,6-diphosphate	22.7	0.007	0.52
Cyclic AMP	24.3	*	
Glucose 1-phosphate	26.6	*	
Glucose 6-phosphate	24.9	0.04	0.52
Fructose 6-phosphate	22.4	0.015	0.52
Ribose 5-phosphate	22.5	*	
ADP	28.9	0.18	0.51
Pyrophosphate	39.3	*	

TABLE II --Continued

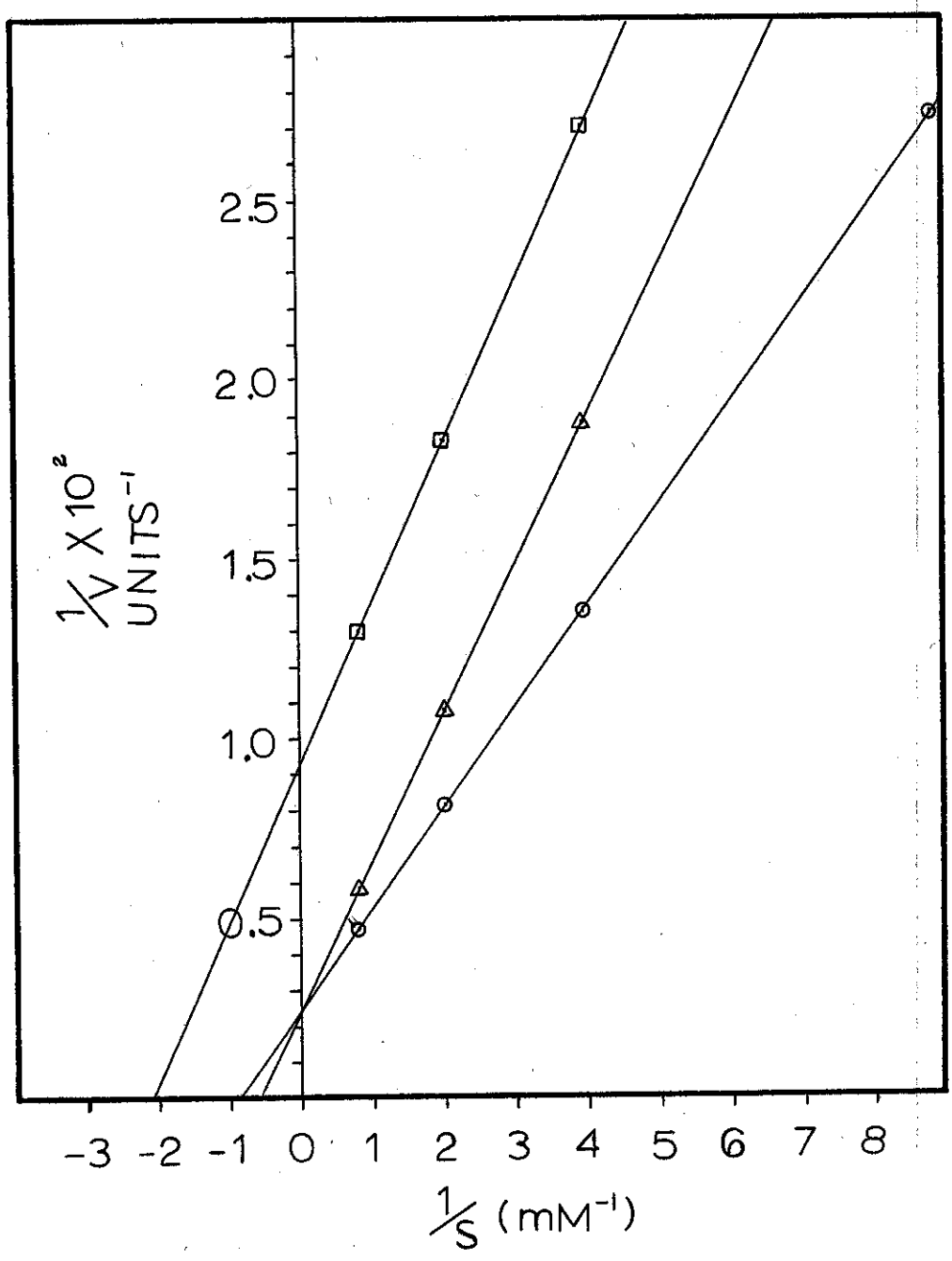
Compound	Ki (mM)	Erythrocyte Concentration (mM)	Per Cent Inhibition In Vivo
Creatine phosphate	24.0	*	
Creatine	55.8	*	
2-phosphoglycerate	21.0	0.01	0.52
3-phosphoglycerate	40.7	0.07	0.52
α-ketoglutarate	47.8	*	
Phosphoenolpyruvate	1.15	0.017	0.51
Glucose 1,6-diphosphate	25.0	0.30	0.51
Pi	> 75	0.50	< 0.52
Fumarate	> 75	0.50	< 0.52
Pyruvate	> 75	0.50	< 0.52
Creatinine	> 75	0.50	< 0.52
Glutamate	> 75	0.50	< 0.52
Bicarbonate	> 75	0.50	< 0.52

TABLE II --Continued

Compound	Ki (mM)	Erythrocyte Concentration (mM)	Per Cent Inhibition In Vivo
Acetate	> 75	0.50	< 0.52
MgCl	> 75	0.50	< 0.52
Citrate	> 75	0.50	< 0.52

*Indicates that the concentration is not known or insignificant.

Fig. 9 --Lineweaver-Burk plots. The standard assay at pH 7.8 and 25°C (⊡—⊡—⊡), the simulated *in vivo* assay at pH 7.2, ionic strength of 0.165 and 37°C (⊙—⊙—⊙) and the simulated *in vivo* assay with inhibitors at intracellular concentrations (△—△—△) are compared. All data were analyzed by the weighted least squares method as adapted to the IBM 360 computer by Cleland (1970).


2.5
2.0
1.5
1.0
0.5
1/V X 10²
UNITS⁻¹
-3 -2 -1 0 1 2 3 4 5 6 7 8
1/S (mM⁻¹)

ionic strength of 0.165 and 37.0°C gives a K_m of 1.145 mM and a V_{max} of 0.0395 units per milliliter. This represents a 2.3-fold increase in K_m and a 3.87-fold increase in V_{max}. With these values, a triosephosphate isomerase activity of 2.123×10^{-4} millimoles per minute per microgram of enzyme can be calculated at a glyceraldehyde 3-phosphate concentration of 0.006 millimolar.

The per cent of maximal velocity at this substrate concentration can be calculated using the Michaelis-Menten equation:

$$v = \frac{100\ (S)}{K_m + (S)}$$

where v is the per cent initial velocity at a given substrate concentration (S), one hundred is the maximal velocity, and K_m is the Michaelis constant.

$$v = \frac{(100)\ (0.006\ mM)}{(1.145\ mM) + (0.006\ mM)} \equiv 0.52\%\ V_{max}$$

The per cent of inhibition due to phosphoenolpyruvate under these conditions can also be calculated using standard Michaelis-Menten kinetics for linear competetive inhibition:

$$v = \frac{100\ (S)}{K_m(1 + \frac{(I)}{Ki}) + (S)}$$

where (I) is the concentration of the inhibitor and Ki is the inhibition constant.

$$v = \frac{(100)\ (0.006\ mM)}{(1.145\ mM)\ (1 + \frac{0.017\ mM}{1.15\ mM}) + (0.006\ mM)} = 0.51\%\ V_{max}$$

the per cent of inhibition, then is:

$$100 - (\frac{vi}{v} \times 100) = 100 - (\frac{0.51}{0.52} \times 100) = 2\%$$

For a fifty per cent inhibition, a phosphoenolpyruvate concentration of 1.16 mM would be required.

As an approximation of total inhibition under intracellular conditions, the following metabolites, chosen for their relatively low estimated Ki values or relatively high known concentrations in the erythrocyte, were added to the assay mix at the same concentrations at which they are found in the erythrocyte (Yoshida, 1973): adenosine triphosphate (1.5 mM), adenosine diphosphate (0.18 mM), glucose 1,6-diphosphate (0.3 mM), glucose 6-phosphate (0.04 mM), 3-phosphoglycerate (0.07 mM), 2,3-diphosphoglycerate (1.5 mM), phosphoenolpyruvate (0.017 mM), inorganic phosphate (0.5 mM), reduced glutathione (3.0 mM) and magnesium ion (60 mg/l). When kinetic studies were made at intracellular pH, temperature and ionic strength with these weak inhibitors present at the given intracellular concentrations, a Lineweaver-Burk plot was obtained (Fig. 9) which showed simple competetive inhibition with respect to the plot made under the same conditions without inhibitors. The apparent K_m was 1.62 mM and the V_{max} was 0.041 units per milliliter; essentially the same as the V_{max} under

these conditions without inhibitors. Since this plot was linear, and since the inhibition appeared to be pure competetive inhibition, a calculation of a total inhibitor constant, designated Ki^{Σ} was calculated as if a single inhibitor was involved. The Ki^{Σ} was found to be 17.24 mM. If the apparent K_m and V_{max} are used to calculate the rate at 0.006 millimolar glyceraldehyde 3-phosphate, a value of 1.563×10^{-4} millimoles per minute per microgram of enzyme is obtained. The total percentage of inhibition was not significantly affected by either pH or dielectric constant within physiological ranges.

DISCUSSION

The three bands of triosephosphate isomerase activity resulting from starch gel electrophoresis of erythrocyte lysates were shown to correspond to the three isozymes obtained by isoelectric focusing of the purified enzyme (Sawyer et al., 1972). Additional bands of activity seen in aged lysates or samples electrophoresed in low concentrations of mercaptoethanol were attributed to oxidative artifacts.

The three isozymes of triosephosphate isomerase were also found in the same relative distributions, in human blood, heart, liver, kidney, skeletal muscle, brain, spleen and lung. This argues against the possibility that the low amount (5-10 per cent) of the most basic isozyme (AA) seen in isoelectric focusing experiments of the erythrocyte enzyme (Sawyer et al., 1972; Rozacky et al., 1971) might represent a tissue specific variant which might predominate in another tissue. These results further substantiate that the isozymes of human triosephosphate isomerase seen in electrofocusing experiments are not "isolation artifacts." Additional evidence to this effect has been provided by coelectrofocusing experiments with isozymes from human liver, brain, heart and erythrocytes (Snapka et al., 1974).

It has been suggested (Kaplan et al., 1968; Sawyer et al., 1972) that the most acidic subunit of human triosephosphate isomerase (Component III) and the hybrid heterodimer (Component II) may be inactive in triosephosphate isomerase deficiency disease leaving only the most basic homodimer (AA, which shows 5-10 per cent of total activity on electrofocusing) to carry out the metabolic role of the enzyme. The results described in this thesis suggest that the same degree of enzyme deficiency would exist in all tissues thus far examined. Furthermore, they can explain the severity of this disorder, which exhibits widespread tissue damage.

Starch gel electrophoresis of samples from a number of different species has revealed an evolutionary pattern in the numbers of triosephosphate isomerase isozymes. Vertebrates, as a rule, have three isozymes although in some cases two of these are minor (less intense and slower developing). Invertebrates possess only one or two isozymes with the multiple forms being found only in some arthropods. Microorganisms showed only a single isozyme with the exception of the protist, Euglena gracilis, which exhibits two distinct isozymes, a cytoplasmic and a chloroplastic form (Mo et al., 1973). Since these two isozymes are in different subcellular compartments, they do not appear to hybridize. The doublet patterns in some marine

crustaceans are especially interesting as it suggests the presence of two isozymes which do not hybridize. Whether or not this is due to subcellular compartmentalization is not known.

Of all the species studied, only the enzyme from the Rhesus monkey showed a migration of all three bands corresponding exactly with those of the human enzyme. This suggests that the Rhesus enzyme may be identical to the human. Coelectrofocusing of a 1:1 mixture of human and Rhesus muscle triosephosphate isomerases yielded three peaks of activity with isoelectric pH values of 6.3, 6.0, and 5.6, further indicating the high degree of homology, if not identity of the isozymes from the two different species. The only difference was in relative intensities of the three bands. In human tissues, the central band (AB) developed most rapidly and was the most intensely staining. This was followed by the anodal band (BB), then the cathodal band (AA). In Rhesus, the intensity of the isozymes was (AA) > (AB) > (BB). These results suggest that the similarity of the Rhesus enzyme to the human enzyme combined with the ready availability of Rhesus tissue might make it an ideal source of enzyme for physical and chemical studies. It also suggests that a comparative study of the two species might reveal the basis for the variation in the relative distribution of the isozymes.

The overall pattern in triosephosphate isomerase isozymes, as seen in a wide variety of species, argues strongly for a genetic basis of the multiplicity of this genome. It further suggests that gene duplication occurred in a primitive common ancestor of modern vertebrates. A gene duplication may also have taken place in an ancestor of some present-day invertebrates.

The finding of normal numbers and distributions of triosephosphate isomerase isozymes in hemolysates of patients representing all three cytogenetic forms of cri-du-chat syndrome indicates that in no case was one of the cistrons for triosephosphate isomerase lost along with the genetic material involved on the short arm of chromosome five. This argument is particularly strong in the case of L.R., who was shown to have a deletion of essentially all of the short arm of chromosome five. In these same patients, Michaelis constants, levels of triosephosphate isomerase, and stabilities of enzymes were also normal (Hendrickson et al., 1973). Another recent study (Güttler and Niebuhr, 1972), involving fluorescence microscopy of chromosome five in thirteen individuals with karyotype 5p -- is in agreement with our own findings and those of Brock and Singer (1970a,b) and Rüdiger and co-workers (1970). Between all of these studies, a total of thirty-nine individuals with deletions of the short arm of chromosome

five or with cri-du-chat symptoms have been found to have normal levels of triosephosphate isomerase. A number of techniques including fluorescent microscopy and high resolution electrophoretic methods have also been employed. The cumulative evidence, therefore, argues very strongly against the localization of a cistron for triosephosphate isomerase on the short arm of chromosome five in man.

Attempts to relate the rate of the triosephosphate isomerase reaction under simulated in vivo conditions to the known rate of glycolysis yielded ambiguous results due to the poorly understood disequilibrium of this reaction in vivo. This disequilibrium has also been documented in other tissues and species (Williamson et al., 1966; Veech et al., 1969). From the kinetic studies done under the standard assay at pH 7.8 and 25°C, a rate of 1.299×10^{-4} millimoles of substrate converted per minute per microgram of triosephosphate isomerase was calculated for the in vivo concentration of 6.0 micromolar for glyceraldehyde 3-phosphate. Using a value of 8,000 micromoles per minute per milligram at 25°C for the specific activity of human triosephosphate isomerase (Rozacky et al., 1971) and a value of 85 micromoles per minute per milliliter of packed human red cells at 25°C and with saturating substrate (Chapman et al., 1962), a value of 10.625 micrograms of triosephosphate isomerase per milliliter of packed cells

is obtained. This value can then be used to calculate a rate of 1.38 micromoles of substrate converted per minute per milliliter of packed cells at a glyceraldehyde 3-phosphate concentration of 6.0 micromolar. This rate is obviously higher than the known rate of glycolysis in whole cells of 0.05-0.07 micromoles per minute per milliliter of packed cells (Foranini and Bossu, 1969). If the K_m is corrected to 0.0158 millimolar for the concentration of the aldehyde form of glyceraldehyde 3-phosphate (Trentham et al., 1969) and if the free concentration of glyceraldehyde-3-phosphate measured in the red cell is taken to be one hundred per cent aldehyde form (Trentham et al., 1969) a rate of 30.8 micromoles of substrate converted per minute per milliliter of cells is obtained. If the calculations of the Results chapter are repeated using the corrected values for the Michaelis constant (K_m) and the inhibitor constant for phosphoenolpyruvate, somewhat different results are obtained. Under physiological conditions, without inhibitors, the triosephosphate isomerase reaction is about thirteen per cent of V_{max}. Under these same conditions, the physiological concentration of phosphoenolpyruvate gives an inhibition of about seventy-six per cent.

Using the kinetic data for the assay done at intracellular conditions and with normal intracellular concentrations of inhibitors, again using the corrected apparent

K_m, a rate of 43.35 micromoles of substrate converted per minute per milliliter of packed cells (about ten per cent V_{max}) is obtained at a concentration of 6.0 micromolar glyceraldehyde 3-phosphate.

The net flux through the triosephosphate isomerase reaction can also be calculated for given concentrations of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate by using the steady-state rate equation for a reversible single substrate reaction (Haldane, 1930):

$$v = \frac{\frac{V_f[G3P]}{K_m^f}\left(1 - \frac{[DHAP]/[G3P]}{K_{TPI}}\right)}{1 + \frac{[G3P]}{K_m^f} + \frac{[DHAP]}{K_m^r}}$$

where V_f is the maximum rate in the direction glyceraldehyde 3-phosphate to dihydroxyacetone phosphate; and K_m^f and K_m^r are the Michaelis constants for glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, respectively. Several assumptions and corrected values must be used. The apparent K_m for the simulated *in vivo* reaction with inhibitors, corrected for true concentrations of the substrate glyceraldehyde 3-phosphate (Trentham *et al.*, 1969) is used. The experimentally determined concentrations of free glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in human erythrocytes (Yoshida, 1973) are used, and the assumption made that these are present one hundred

per cent in the forms used by triosephosphate isomerase (Trentham et al., 1969; Reynolds et al., 1971). The corrected K_{eq} for the triosephosphate isomerase reaction (Reynolds et al., 1971) is also employed. One obtaines:

$$v = \frac{\frac{(0.041)[0.006]}{(0.0534)}\left(1 - \frac{(0.017)/(0.006)}{420}\right)}{1 + \frac{[0.006]}{(0.0534)} + \text{negligible}}$$

$v = 4.13$ μm/min/ml assay mix

$v = 4.258$ μm/min/μg TPI

$v = 45.24$ μm/min/ml packed cells

This is in very close agreement with the value of 43.35 micromoles per minute per milliliter of packed cells that was obtained by assuming a true equilibrium at the triosephosphate isomerase reaction (and therefore rate forward equal rate reverse) and glyceraldehyde 3-phosphate concentration of 6.0 micromolar. However, this is about 750 times the rate of glycolysis.

In the cases of triosephosphate isomerase deficiency disease (Schneider et al., 1968), triosephosphate isomerase activity in the erythrocyte was only about five per cent of normal, and dihydroxyacetone phosphate concentrations were about 20 to 24 times greater than normal controls. The rate of glucose utilization was increased three fold from 0.03 micromoles per minute per milliliter of packed cells in normal controls to 0.1 micromole per minute per

milliliter cells in the deficient erythrocytes. All other glycolytic intermediates including glyceraldehyde 3-phosphate were present at the same levels in both normal controls and the deficient cells.

A value for the rate of the triosephosphate isomerase reaction in triosephosphate deficient erythrocytes can be calculated by using the steady-state rate equation. The same corrected apparent K_m is used as in the previous example, the V_{max} is reduced to five per cent of its original value, and the dihydroxyacetone phosphate concentration is increased twenty-four fold. The third factor in the denominator is no longer negligible at this increased dihydroxyacetone phosphate concentration. As a conservative approximation, the corrected K_m for dihydroxyacetone phosphate at 25.0°C (Rozacky et al., 1971) is used in this term. The rate calculated is 1.03 micromoles per minute per milliliter of packed cells. This is still about seventeen times faster than the normal rate of glycolysis in erythrocytes. Even if it is assumed that Schneider's increased rate of glucose utilization is due to glycolysis, the calculated rate of the triosephosphate isomerase reaction is about ten times greater. Thus, it appears that even in the deficiency disease, with only five per cent of normal triosephosphate isomerase activity, this enzyme does not restrict the rate of glycolysis. These

calculations, of course, are based only on the best available data. If triosephosphate isomerase were associated with another protein or a membrane *in vivo*, the kinetic parameters might be altered significantly. There is also a possibility that some unknown compound, not included among those examined as inhibitors, may affect the kinetics of triosephosphate isomerase *in vivo*. Since there is no evidence for these cases at the present time, conclusions must be drawn from the available data. These findings together with Schneider's findings suggest new possibilities for the molecular basis of the disease. Schneider (1968) found normal levels of other glycolytic intermediates and elevated glucose utilization in triosephosphate isomerase deficient erythrocytes. He was also unable to find an appreciable decrease in ATP or dihydroxyacetone during incubation. Once it is assumed that glycolysis is functioning normally or even at an elevated rate in the triosephosphate isomerase deficient erythrocytes, the abnormally elevated steady-state dihydroxyacetone phosphate concentration remains as a likely cause of the problem. The equilibrium constant for the triosephosphate isomerase reaction, $K_{eq} = \frac{[DHAP]}{[G3P]}$, is 420 at 37.0°C when calculated on the basis of the percentages of the keto form of dihydroxyacetone phosphate and the aldo form of glyceraldehyde 3-phosphate in the equilibrium mixtures (Reynolds

et al., 1971). In normal human erythrocytes, however, the ratio of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate is about 2.8 (Yoshida, 1973), a great displacement from equilibrium. In the triosephosphate isomerase deficient erythrocytes, this ratio is about 68; closer to the true equilibrium constant than the ratio in normal erythrocytes. This suggests that the cause of the disequilibrium may be in the triosephosphate isomerase molecule itself rather than in an external cause such as binding of one of the substrates to other enzymes. A thorough kinetic study of the mutant enzyme might lead to an explanation of the disequilibrium phenomenon.

The high concentration of dihydroxyacetone phosphate in the deficient erythrocytes could lead to the observed hemolytic anemia by several different mechanisms. Several investigators have considered the idea that the high concentration of dihydroxyacetone phosphate could affect lipid synthesis and thus the membrane composition by way of α-glycerolphosphate dehydrogenase (Schneider et al., 1968; Foranini and Bossu, 1969). However, this dehydrogenase has not been shown to exist in erythrocytes, which apparently can only metabolize dihydroxyacetone phosphate by way of glycolysis (Schneider et al., 1968). This mechanism, of course, remains a possibility in the case of other tissues.

In order to explain the hemolytic anemia, a link must be found between the increased levels of dihydroxyacetone

phosphate and the increased lability of triosephosphateisomerase deficient erythrocytes. Under normal physiological conditions, 0.5 to 3 per cent of total hemoglobin is oxidized to methemoglobin per day. These oxidative forces are balanced by a number of reducing agents which are at least 250 times as active as the oxidative forces (Foranini and Bossu, 1969). The most important of these reductive potentials are NADPH, reduced glutathione (GSH), and NADH. In the erythrocyte, NADPH is produced exclusively by the pentose phosphate shunt. In addition to being directly involved in reduction of methemoglobin, NADPH maintains glutathione in the reduced state by way of glutathione reductase. Together NADPH and GSH account for over 20 per cent of the reducing contributions to methemoglobin (Scott et al., 1965). GSH also controls H_2O_2 breakdown through glutathione peroxidase. This enzyme, not catalase, is the main mechanism in erythrocytes for the breakdown of hydrogen peroxide and thus its removal as an oxidative threat (Foranini and Bossu, 1969). It has been shown that oxidative damage can be produced in erythrocytes only under conditions where all GSH has been oxidized or inactivated with SH reactive compounds or is reduced due to genetic defects (Foranini and Bossu, 1969). If the pentose shunt were prevented from operating at a normal rate, a shortage of GSH would be caused resulting in hemolysis due to

oxidative damage to structural and enzymatic proteins as well as hemoglobin oxidation and polymerization (Heinz body formation).

It is possible that the elevated levels of dihydroxyacetone phosphate could depress the activity of the shunt by specifically inhibiting a key enzyme or enzymes.

An even more likely link between elevated dihydroxyacetone phosphate concentrations and depression of the pentose shunt is suggested by reports of non-enzymatic conversion of trioses to methylglyoxal by solutions of polyvalent anions (Riddle and Lorenz, 1968; Riddle and Lorenz, 1972). Methylglyoxal is extremely toxic to most cells and especially toxic to mammalian cells. Enzymes for conversion of methylglyoxal to lactate are commonly believed to serve as scavengers for methylglyoxal produced non-enzymatically from glycolizing tissues (Kiessling, 1950). Although a methylglyoxal synthase has recently been shown to exist in bacteria (Cooper and Anderson, 1970; Hopper and Cooper, 1971; Freedberg et al., 1971), no such enzyme has yet been detected in mammalian cells. A number of anions present in the erythrocyte have been shown to catalyze the non-enzymatic conversion of dihydroxyacetone and glyceraldehyde to methylglyoxal at physiological conditions of pH and temperature (Riddle and Lorenz, 1968). These include phosphate, bicarbonate, α, β-glycerophosphate, glucos

glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate. Although the rate of hydrolysis of the phosphate group of dihydroxyacetone phosphate in the erythrocyte is not known, it is likely to occur to some extent even if non-enzymatically. The dihydroxyacetone formed could then be non-enzymatically converted to methylglyoxal. Damage to avian sperm cells incubated in glycerol, with impairment of respiration and motility has recently been traced to methylglyoxal formed from the glycerol by way of several steps (Riddle and Lorenz, 1973). Whether or not the glyoxalase system could effectively lower such an elevated level of methylglyoxal is not known. Even a slightly elevated steady-state concentration of methylglyoxal could result in slow cumulative damage to the cell. Methylglyoxal is an inhibitor of certain sulfhydryl enzymes and is known to damage cell membranes (Racker et al., 1954; Kun, 1950). Riddle and Lorenz (1968) have speculated that the practice of using iodoacetate or fluoride as glycolytic inhibitors would increase the levels of triosephosphate and that this would spontaneously lead to an accumulation of free trioses due to the action of ubiquitous phosphatases.

Most of these various possibilities can be easily tested. A survey for inhibition of pentose shunt enzymes by either dihydroxyacetone phosphate or methylglyoxal is a straight forward and informative experiment. The rate of

conversion of dihydroxyacetone phosphate to the free triose and formation of an elevated steady-state concentration of methylglyoxal could both be studied in cell extracts or under simulated cytoplasmic conditions of pH, ionic strength, temperature and presence of ions capable of catalyzing the dihydroxyacetone to methylglyoxal reaction.

The presence of phosphatases capable of dephosphorylating dihydroxyacetone phosphate or a low level of constitutive methylglyoxal synthase could account at least in part for the disequilibrium in the triosephosphate isomerase reaction. The possibility should also be considered that in the presence of such phosphatases and in view of the cytotoxic effects of methylglyoxal it might be an advantage to an organism if the steady-state concentration of dihydroxyacetone could be kept at the lowest possible level. Thus the disequilibrium in this system, which is so commonly observed, might be an evolutionary adaption which prevents a high steady-state concentration of methylglyoxal.

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