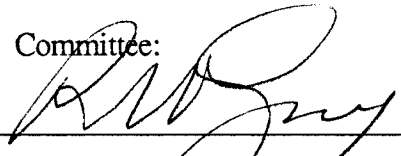
MOLECULAR AGING OF TRIOSEPHOSPHATE ISOMERASE

APPROVED :

Graduate Committee:



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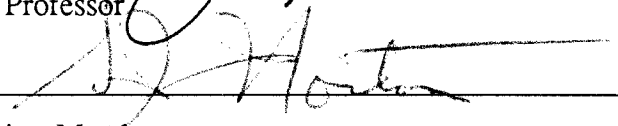

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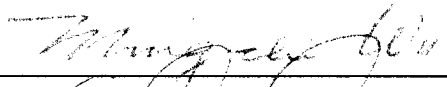

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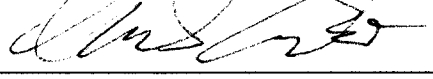

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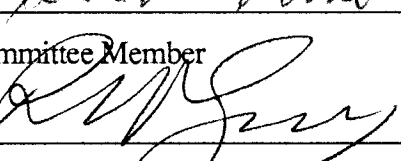

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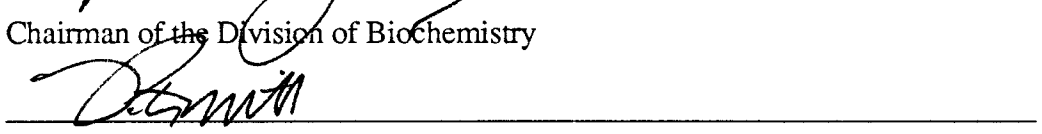

Chairman of the Division of Biochemistry

Dean of the Graduate School

MOLECULAR AGING OF TRIOSEPHOSPHATE ISOMERASE

DISSERTATION

Presented to the Graduate Council of the
North Texas State University in Partial
Fullfilment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

K. Ümit Yüksel, M.S.
Denton, Texas
May, 1987

Yüksel, K. Ümit, Molecular Aging of Triosephosphate Isomerase. Doctor of Philosophy (Biochemistry), May, 1987, 120 pp., 8 tables, 29 illustrations, bibliography, 137 titles.

The isozymes of triosephosphate isomerase (TPI, EC 5.3.1.1) in higher animals are generated from specific deamidations of asparagine-15 and asparagine-71. Deamidation destabilizes TPI by introducing four negative charges into the subunit contact region. Thus, deamidation has been proposed to be an initial step in the normal degradation of the enzyme. The deamidated TPI isozymes accumulate in aging cells.

To understand the mechanism, regulation and significance of these deamidations, factors affecting the intrinsic rates of deamidations of TPI, the effects of deamidation on the structure of the protein, and the effects of structure on the deamidation of TPI were studied both *in vitro* and *in vivo*.

Elevated temperature and pH enhanced the *in vitro* deamidation rates. The rates dependent on the specific buffer ions indicating a general base catalysis mechanism. The presence of denaturants and lower protein concentrations enhanced deamidation suggesting increased exposure of the specific asparagines to the solvent enhances nucleophillic attack of water on these particular asparagines. Substrate promoted deamidation and exhibited saturation kinetics. The enhancement of deamidation by substrate may be related to conformational changes in the catalytic center around the labile asparagines. The presence of substrate may, in part, account for the rapid rate of deamidation *in vivo*. Increasing deamidation with increasing number of catalytic turnovers suggests that this process may be the first example of the "wear and tear" theory of aging at the molecular level. Faster rates of deamidation along with impaired proteolysis explains the accumulation of deamidated forms of TPI in aging cells.

In addition to the specific studies on the "abnormal" forms of TPI, development of general methods to assist in the detection and characterization of the accumulation of "abnormal" forms of other proteins in aging were sought. Quantitation of silver stained proteins on polyacrylamide gels was examined by atomic absorbtion spectrophotometry, along with the factors effecting staining properties. This method is very sensitive and independent of the color of the stain. Analyses revealed that silver deposition correlated with protein concentration, but did not always yield visible stains.

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LIST OF ABBREVIATIONS

BICINE	N,N-bis(2-hydroxyethyl)glycine
DEAE	diethylaminoethane
DHAP	dihydroxyacetone phosphate
$EtNH_2$	ethanolamine
FBS	fetal bovine serum
G3P	glyceraldehyde 3-phosphate
G3PDH	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
HEPES	4-(2-hydroxethyl)-1-piperazine ethanesulfonic acid
MEM	Eagle's minimum essential medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
Na-EDTA	ethylenediamine tetra-acetic acid disodium salt
PAGE	polyacrylamide gel electrophoresis
PAGIEF	isoelectric focusing on polyacrylamide gels

PBS	Dulbecco's phosphate buffered saline
PMS	phenazine methosulfate
SDS	sodium dodecyl sulfate
SPS-PAGE	polyacrylamide gel elctrophoresis in presence of SDS
Ser-Xtend	artificial serum additive
TEA	triethanolamine
TPI	triosephosphate isomerase (EC 5.3.1.1)
TPI-B	undeamidated form of TPI
TPI-A_x	deamidated form(s) of TPI; $x \geq 1$, number of deamidations per dimeric TPI molecule
TRIS	tris(hydroxymethyl)aminomethane
α-GDH	α-glycerophosphate dehydrogenase (EC 1.1.1.8)
3-PGA	3-phosphoglyceric acid

CHAPTER I

INTRODUCTION

Background

Proteins and Aging

Aging can be defined as the physical and chemical changes of a living organism which occur as a function of time. Although macroscopic changes such as gray hair, wrinkled skin and loss of physical strength have long been associated with aging, their origins are not known. More recently, the aging process has been observed to correlate with many molecular changes including, isozyme shifts (Dovrat and Gershon, 1981), altered activity of enzymes (Melloni *et al.*, 1981), accumulation of inactive enzymes (Wiederanders and Oelke, 1984), reduced stability (Wulf and Cutler, 1975) and changes in coenzyme binding (Gafni, 1981). While it is difficult to elucidate all cause and effect relationships, the accumulation of unstable enzymes is clearly associated with the aging process.

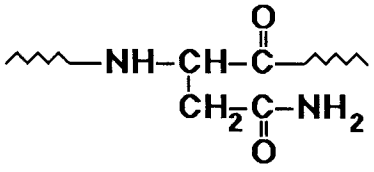
Two major theories of aging have emerged: the "error catastrophy" theory of Orgel (1963) and the "wear and tear" theory of Rubner (1908). The error catastrophy theory suggests that errors in translation and/or transcription can cause the accumulation of inactive proteins, and ultimately lead to cellular entrophy and cell death. The wear and tear theory, on the other hand, proposes that aging is the result of the overall extent of metabolism. Although both theories have survived testing over many years, current experimental data favor the wear and tear theory (Rothstein, 1985). If the protein synthetic machinery were to become defective as suggested by the error hypothesis, altered forms of all or most proteins should be observed in aging cells. The number of known altered proteins is surprisingly less than one might predict based on this hypothesis. On the other

hand, many subtle transcriptional and translational defects may go unrecognized in most of the systems designed for protein separation and thus, many more "abnormal" proteins might exist in aging cells than detected by current methods. Other lines of experimental evidence argue against faulty synthesis being the sole source of the abnormal proteins. Altered forms of aldolase (Dovrat and Gershon, 1981), glyceraldehyde 3-phosphate dehydrogenase (Jedziniak *et al.*, 1986), glucosephosphate isomerase (Cini and Gracy, 1986) and glucose 6-phosphate dehydrogenase (Dovrat *et al.*, 1986) have been found in the nucleus region of the eye lens of rat, human, bovine and rat, respectively. The nucleus of the eye lens is devoid of protein synthesis; hence, altered proteins would appear to be generated by routes other than errors in synthesis. Secondly, the lowering of metabolic rates by restricting physical activity, lowering environmental temperature or lowering rates of maturation by caloric deprivation, prolong life span well beyond that of controls (Sohal and Allen, 1985). Higher oxygen consumption rates have been correlated with faster aging and support of the wear and tear theory of aging. For example, analysis of maximum life span potential (MLSP, [years]) as a function of specific metabolic rate (SMR, [calories/ gram/ day]) exhibits an exponential relationship, *i.e.*, as SMR decreases MSLP increases (Cutler, 1985).

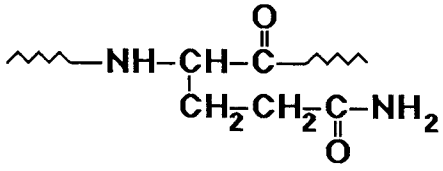
There is a growing body of evidence on the accumulation of "altered" or "abnormal" proteins in aging cells (Hayflick, 1965, Holliday *et al.*, 1974, Goldstein and Moerman, 1975, Tollefsbol *et al.*, 1982). "Abnormal" proteins are found to accumulate *in vivo* (*i.e.*, in cells isolated from donors of older age) and *in vitro* (*i.e.*, cells from young donors accumulate abnormal proteins as they grow towards senscence in tissue culture) (Goldstein and Moerman, 1975, Goldstein, 1979, Tollefsbol *et al.*, 1982, Tollefsbol and Gracy, 1983). The molecular basis of age related changes have been attributed either to loss of fidelity in synthesis or to post-translational modifications. Post-translational modifications have been well documented and include noncovalent conformational changes (Dovrat and Gershon, 1983), glycosylation (Gundberg and Gallop 1985), racemization (Helfman *et al.*, 1977), oxidation (Oliver *et al.*, 1985) and deamidation (Funakoshi and Deutsch, 1969).

Deamidation is the loss of ammonia from the side chain of an asparagine or glutamine by hydrolysis (Fig. 1). *In vitro* deamidation can occur during purification, storage or may be induced deliberately. *In vivo*, it may be due to specific enzymes but can also be spontaneous. *In vitro* deamidation has been observed with essentially all functional classes of proteins such as lysozyme (Tallan and Stein, 1953), growth hormone (Lewis *et*

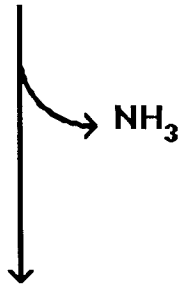
Figure 1. Deamidation of asparagines and glutamines.

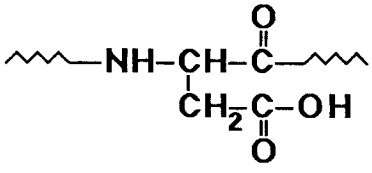


asparaginyl peptide

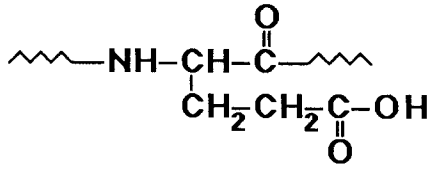


glutaminyl peptide





aspartyl peptide



glutamyl peptide

al., 1981), α-crystallin (Ikeda and Sawano, 1971), cytochrome C (Flatmark and Sletten, 1968), γ-immunoglobulin (Minta and Painter, 1972), calmodulin (Johnson *et al.*, 1984) and neocarzinostatin (Maeda and Kuromizu, 1977). These findings establish deamidation as a general phenomenon. One of the first examples of *in vivo* deamidation was the aging associated accumulation of the multiple forms of aldolase. Horecker and coworkers found that the origin of the age associated modifications of aldolase to be the specific deamidation of Asn-358. (Koida *et al.*, 1969, Lai *et al.*, 1970). The *in vivo* half life of this deamidation was calculated to be about eight days (Midelfort and Mehler, 1972). Later, the half life of a synthetic peptide with similar sequence was found to be 6.4 days (McKerrow and Rubinson, 1974).

Deamidation of asparagines and glutamines have been studied in model peptides. Robinson and coworkers (for review, see Robinson and Rudd, 1974) established that a) asparagines deamidate faster than glutamines, b) deamidation occurs more rapidly with increasing ionic strength, pH and temperature, and c) rates of deamidation are influenced by the primary sequence. Systematic studies with pentapeptides indicated the importance of the amino acid residues on the amino terminal side of Asn or Gln. Steric hinderance was observed for amino acids with bulky side chains. Deamidation appeared to be facilitated if the neighboring residue was charged. Comparison of amino acid sequences of proteins prone to deamidation indicate that in eight of the thirteen cases there is a charged amino acid one or two residues away on the amino terminal side the labile asparagine (Table I). Unfortunately, the effects of C-terminal neighbors were not studied. However, an important clue on the effect of residues toward the C-terminal side comes from an unrelated study (Bornstein, 1970). Asparaginylglycine bonds were found to be extremely labile in alkaline hydroxylamine. The proposed mechanism of action involves the formation and subsequent opening of a five membered cyclic imide ring (Fig. 2). The result is the deamidation of asparagine into aspartic acid. Since the two carbonyl groups are roughly equivalent, ring opening can occur at either and yields α-Asp and β-Asp peptides. In terms of protein structure these are different products. The β-form may be expected to strain the peptide bond, and in turn the protein structure.

Triosephosphate isomerase (TPI, EC 5.3.1.1) is a constitutive enzyme in all organisms. It is centrally located in the glycolytic/gluconeogenic pathways and catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Fig. 3). The enzyme catalyzed reaction is about 10^9 times faster than the spontaneous reaction

TABLE I

AMINO ACID SEQUENCES AROUND KNOWN DEAMIDATION SITES*

Protein	Sequence
Aldolase (rabbit muscle)	I S H N A Y
α-crystallin (bovine lens)	H N E R
Calmodulin (bovine brain)	D G N G T
Cytochrome C	A T N Q
	D A N D K N
	L D N P K
Growth hormone (human)	S H N D
	T G Q I F
Lysozyme (hen egg)	E S N F N
	L D N Y R
	L G N W V
Triosephosphate isomerase (human)	K V T N G A F
	W K M N G R K

* Deamidating residues are underlined.

Figure 2. Mechanism of spontaneous deamidation of Asn-Gly sequences as proposed by Bornstein (1970).

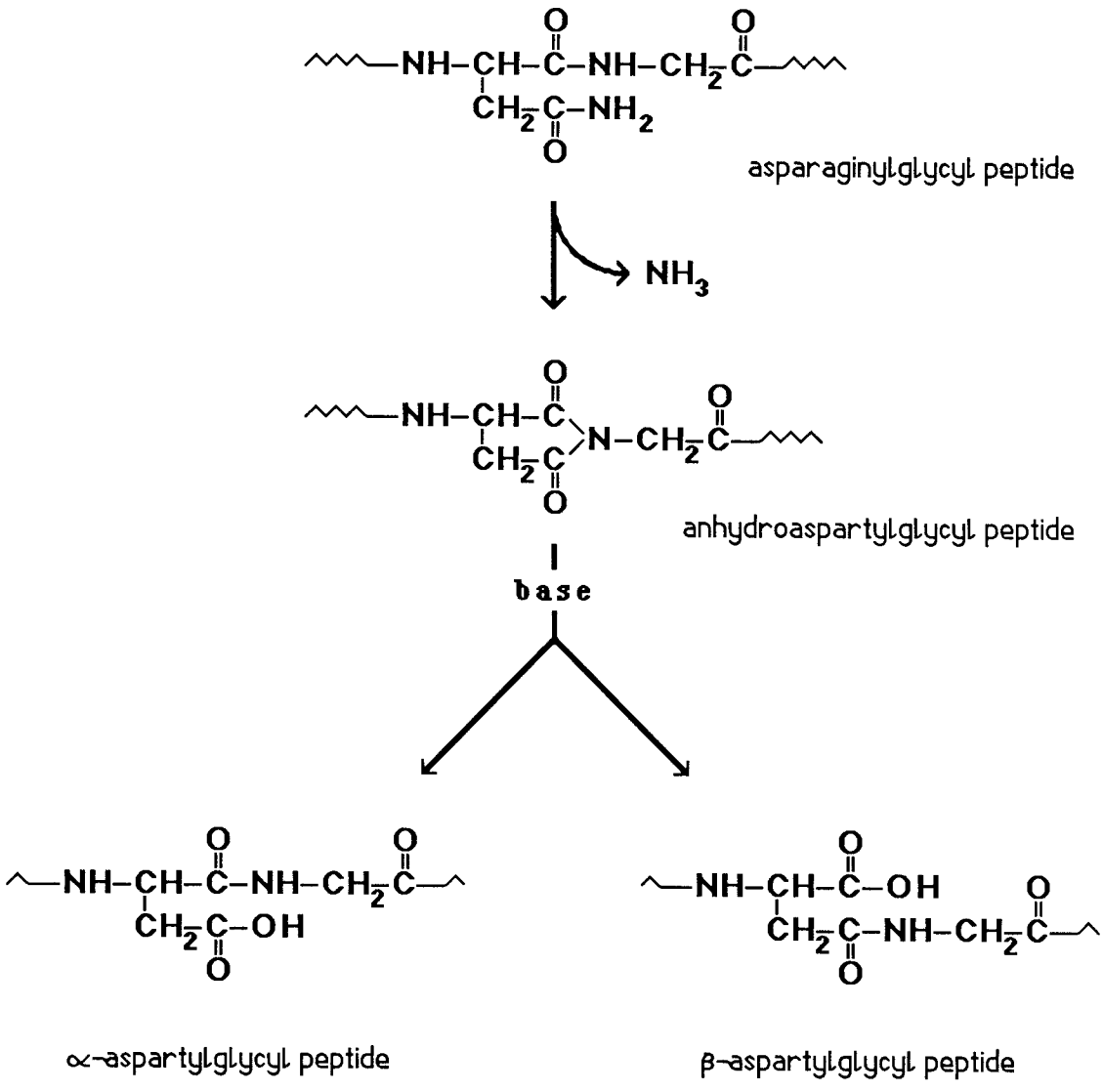
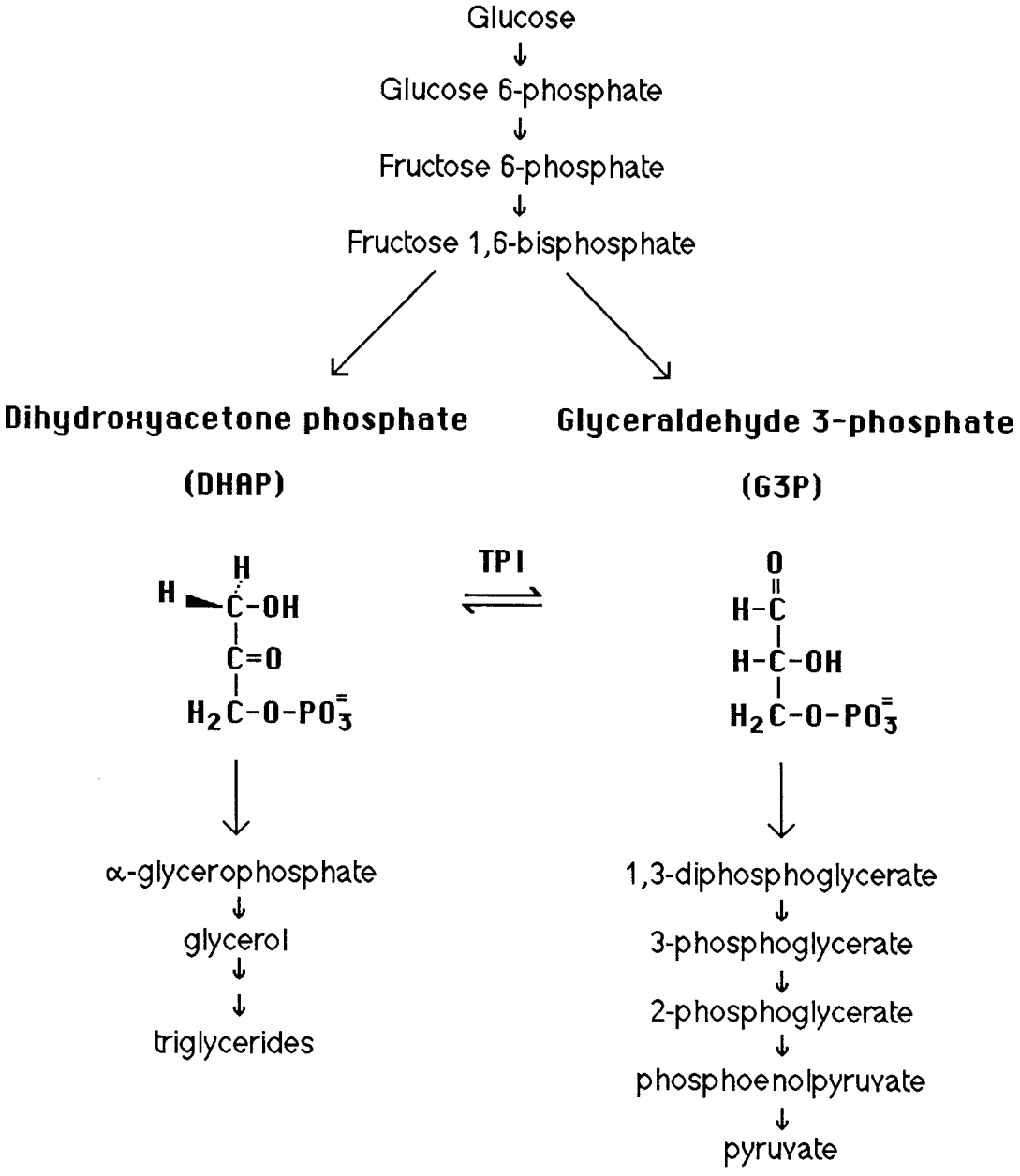

asparaginylglycyl peptide
NH3
anhydroaspartylglycyl peptide
base
α-aspartylglycyl peptide
β-aspartylglycyl peptide

Figure 3. Location of triosephosphate isomerase (EC 5.3.1.1) catalyzed reaction in glycolysis.


Glucose
Glucose 6-phosphate
Fructose 6-phosphate
Fructose 1,6-bisphosphate
Dihydroxyacetone phosphate
(DHAP)
Glyceraldehyde 3-phosphate
(G3P)
TPI
α-glycerophosphate
glycerol
triglycerides
1,3-diphosphoglycerate
3-phosphoglycerate
2-phosphoglycerate
phosphoenolpyruvate
pyruvate

(Albery and Knowles, 1976), and the rate is limited only by the diffusion of the substrate. This aldol-ketol isomerase has been purified to homogeneity from a large number of species and its amino acid sequence determined for several of these including human (Lu *et al.*, 1984), chicken (Banner *et al.*, 1975), rabbit (Corran and Waley, 1975), coelecanth (Kolb *et al.*, 1974), *Bacillus stearothermophilus* (Artavanis-Tsakonas and Harris, 1980). Sequence studies have shown an extraordinary high degree of conservation of TPI during evolution. The enzyme is calculated to have undergone only 2.8 accepted mutations per hundred residues in hundred million years (Asakawa and Mohrenweiser, 1982).

Triosephosphate isomerase exhibits several isozymes (Snapka *et al.*, 1974, Gracy, 1975). In mammalian tissue, age related changes in the isozyme pattern have been observed (Turner *et al.*, 1975, Gracy, 1982). Studies on the rabbit and human enzymes have shown the origin of these isozymic forms to be deamidation (Yuan *et al.*, 1981). The most basic isozyme [pI= 5.9 for human TPI, (Yuan *et al.*, 1979)] is the form synthesized in cells. The enzyme undergoes spontaneous, specific deamidations (Fig. 4) at asparagine 15 (Asn-15) and asparagine 71 (Asn-71) of each subunit and yields the acidic isozymes (Yuan *et al.*, 1981). In our nomenclature the most basic isozyme is designated as TPI-B. The more acidic, deamidated forms are designated as TPI-A_x, where the subscript ($x \geq 1$) represents the number of deamidations per dimeric molecule of TPI.

The deamidated isozymes exhibit similar kinetic constants for either substrate (Gracy, 1975, Yuan *et al.*, 1979, Eber and Krietsch, 1980) and are indistinguishable on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) or on molecular exclusion chromatography (Yuan *et al.*, 1979). However, the isozymes can be separated upon nondenaturing alkaline gel electrophoresis (PAGE) (Yuan *et al.*, 1981), isoelectric focusing on polyacrylamide gels (PAGIEF) or sucrose gradients (Sawyer *et al.*, 1972), and chromatofocusing (Oray *et al.*, 1983). An important feature of the deamidated forms is their lability to heat (Tollefsbol *et al.*, 1982) and chemical denaturants (Sawyer and Gracy, 1975, Yuan *et al.*, 1981). The deamidating residues Asn-15 and Asn-71 are located at the subunit contact sites. Introduction of up to four negative charges into the contact region (Fig. 5) is believed to be responsible for this loss of stability (Yuan *et al.*, 1981).

The age-related isozyme shift indicates the accumulation of the less stable, deamidated, acidic isozymes. Acidic isozymes also accumulate in cells with premature aging diseases, *e.g.* Hutchinson-Gilford Progeria and Werner's syndromes. This accumulation parallels the increase in the amount of thermolabile TPI activity in cells

Figure 4. Three dimensional structure of triosephosphate isomerase showing the deamidation sites. Peptide backbones of the two subunits are shown except for the side chains of deamidating Asn-17 and Asn-71 residues. For the sake of clarity, the homologous subunits are arbitrarily designated as "A" (left) and "B" (right).

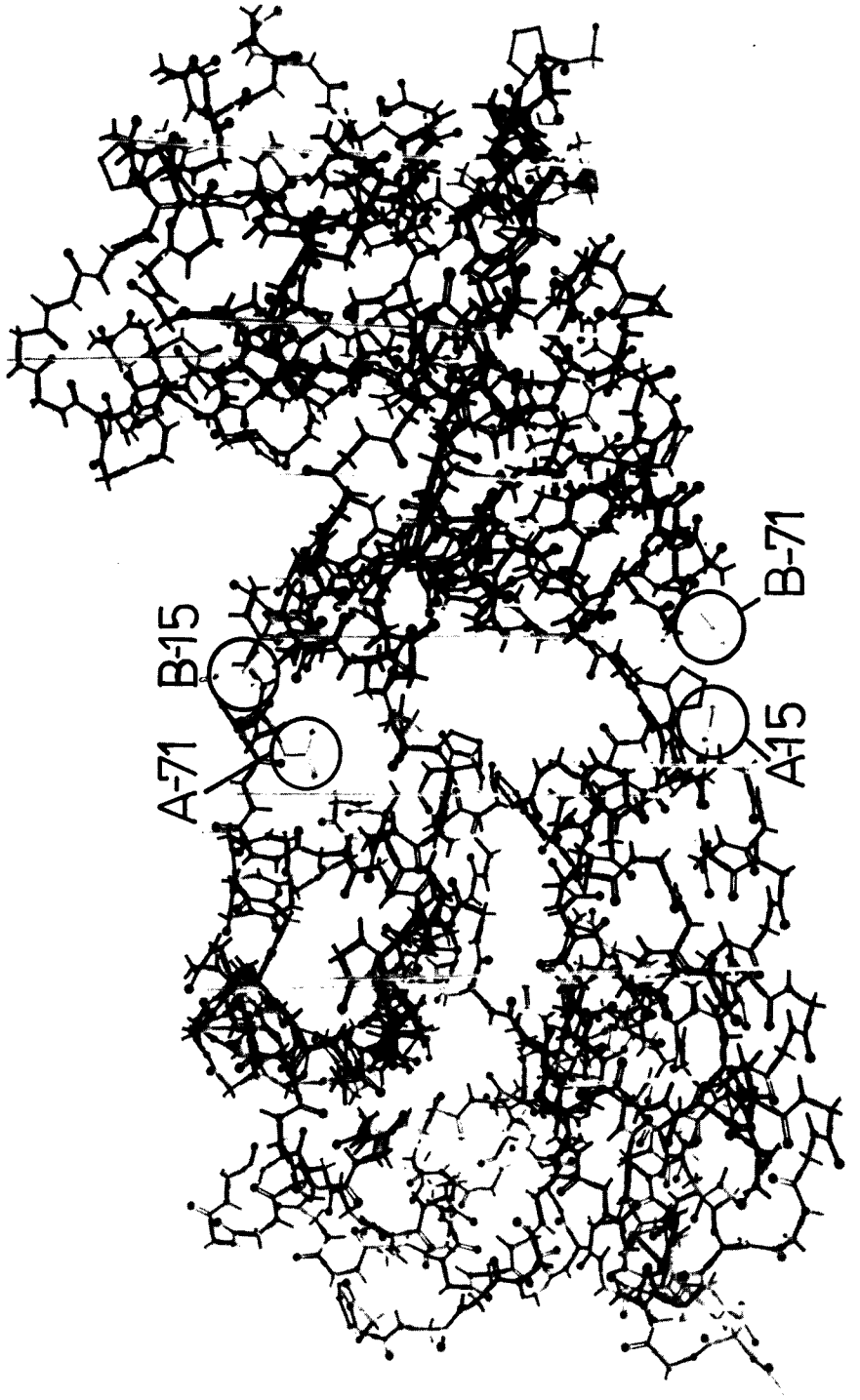
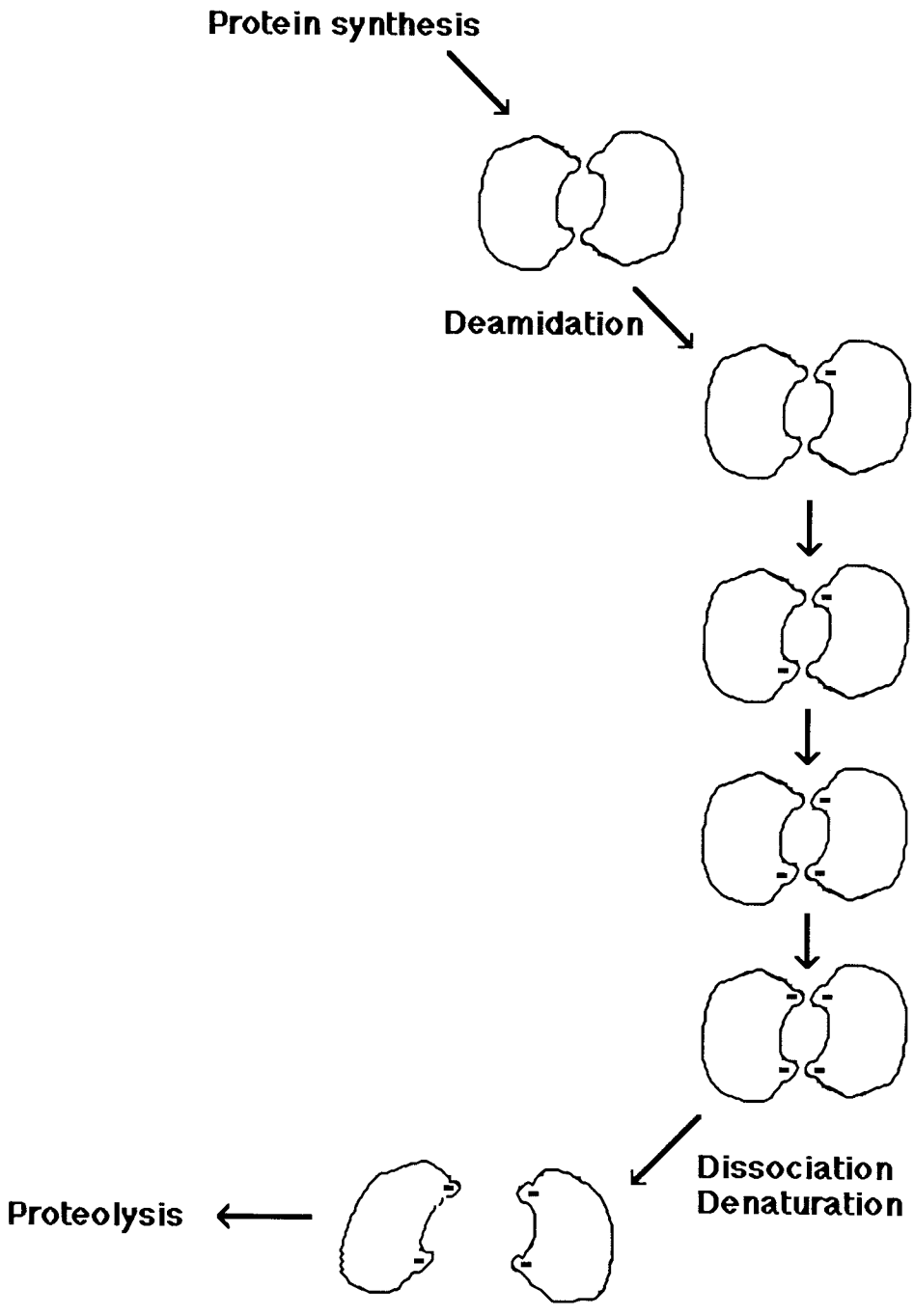

B-71
B-15
A-15
A-71

Figure 5. Proposed route of normal degradation of TPI. Deamidation was proposed to be part of the normal degradation mechanism of TPI (Yuan *et al.*, 1981).


Protein synthesis
Deamidation
Dissociation
Denaturation
Proteolysis

(Tollefsbol *et al.*, 1982). These data have been interpreted to mean that deamidation is a marker for the proteins to be catabolized and may constitute a first step in the normal degradation of TPI in the cell. Cells from aging individuals, premature aging diseases and cells "aged" in culture accumulate the deamidated isozymes presumably due to a failure of the old cells to "recognize" and dispose of the post-synthetically modified proteins.

In mammals, including human, there appears to be an additional class of TPI isozymes. These isozymes are detectible only in rapidly dividing cells (Kester and Gracy, 1975, Kester *et al.*, 1977, Decker and Mohrenweiser, 1981) and only in species recently evolved among the primates (Decker and Mohrenweiser, 1985). On the basis of the experiments carried out on TPI variants (Asakawa and Iida, 1985, Asakawa and Mohrenweiser, 1982) and limited structural analysis on TPI from a human lymphoblastoid cell line (Decker and Mohrenweiser, 1986), both classes of the isozymes are believed to be the product of the same gene locus (Decker and Mohrenweiser, 1981, 1985, 1986). Studies are currently underway to determine the mechanism of origin of these more acidic (>4 charge difference) isozymes (H. W. Mohrenweiser, personal communication).

Detection of Modified Proteins

As noted above, the number of known altered or "abnormal" proteins which accumulate in aging cells is limited, perhaps due to the difficulty in resolution and detection of these minor forms. The introduction of two dimensional electrophoretic separation (O'Farrell, 1975) and its application to different systems (Ames, 1976, O'Farrell *et al.*, 1977, Goldman *et al.*, 1985) made the resolution of a large number of proteins possible. This powerful procedure provided a tool to detect proteins of minute quantities. Protein staining with Coomassie Brillant Blue is about ten times more sensitive than amido black (Fishbein, 1972), and does not require continued attendance during the staining process. It stains most proteins, and the detection limit is around 1μg (Fishbein, 1972). This detection limit is generally adequate for most work; thus, Coomassie Blue has been the choice of many researchers. However, for the detection of "altered" or "abnormal" proteins and other biologically active peptides usually present in minute quantities, this is not sufficient. The electrophoretic system is limited in the amount of total protein and sample volume that

can be analyzed at one time. Thus, the use of more sensitive staining procedures are necessary. Silver staining of proteins as described by Switzer and coworkers (Switzer *et al.*, 1979) provides a more sensitive alternative. There are two chemically distinct silver staining techniques: the photochemical silver stain (Merril *et al.*, 1980) and the ammoniacal silver stain (Wray *et al.*, 1981). The detection limits of both silver stains are comparable and at least a hundred-fold more sensitive than the Coomassie Blue stain (Switzer *et al.*, 1979). The photochemical silver staining process is preferred because it requires fewer chemicals, the reagents are more stable, potentially less hazardous (Morrissey, 1981), and it is completed in a shorter time span. The principle of the photochemical stain is the selective uptake and retention of silver ions into protein containing regions of the polyacrylamide gel which are oxidized prior to incubation in silver nitrate. Imbedded silver ions are then reduced to metallic silver in a suitable alkaline medium. This "physical development" (Yudelson, 1984) is similar to the formation of the silver granules in photographic emulsions.

The intensity of the silver stain increases linearly with increasing protein concentration (Switzer *et al.*, 1979, Merril *et al.*, 1980, 1982). However, proteins exhibit different staining intensities (Poehling and Neuhoff, 1981, Merril *et al.*, 1982). Differential staining is not unique to silver staining, and is observed with other stains including Coomassie Blue (Fishbein, 1972). Attempts have been made to determine the cause of differential Coomassie staining. Recently, correlation between amount of dye bound (*i.e.*, staining intensity) and the number positive charges on the proteins was established (Tal *et al.*, 1984). In the case of silver stain, this effect is much exaggerated and can even result in a "negative stain" (Morrissey, 1981). Aside from differences in staining intensities, silver stained proteins exhibit differential spectral properties. These differences have actually proved to be advantageous. For example, Sammons and coworkers (Sammons *et al.*, 1981) have exploited the spectral characteritics and developed a staining procedure with perceptible differences in colors of stained proteins. The color differences were utilized to distinguish between proteins which were poorly resolved on two dimensional electrophoresis gels. Others have used the "negative stain" property to differentiate proteins by double staining them with silver and Coomassie Blue (Dzandu *et al.*, 1984).

Aims of this Project

Among the few proteins known to exhibit age related changes, triosephosphate isomerase is the best characterized: its kinetics, amino acid sequence and three dimensional structure are known. In addition, the sites of the age-related alterations and the chemical nature of these alterations are known. The conservation of the amino acid sequence during evolution indicates that further structural changes in this extremely efficient biological catalyst may be detrimental to its biological functions. These deamidated forms may be recognized as "abnormal" by the normal cellular proteolytic system and destined for clearance. In the "old" cells the "abnormal" (*i.e.,* deamidated) forms of TPI do not appear to be "recognized" and degraded appropriately.

While some studies have been carried out *in vitro* on the deamidation of synthetic peptides, *in vivo* deamidation has barely been studied. Although the deamidated forms of TPI are known to accumulate in aging cells, a cause and effect relationship has not been established. It is also not known whether the deamidated forms accumulate due to an age related defect in protein metabolism, an increase in the deamidation rates of TPI, or both.

This work was initiated to acquire a better understanding of the mechanism(s), regulation(s) and significance(s) of deamidation, as well as its role in the aging process. Using triosephosphate isomerase as a model system, the following goals were set:

I. *In vitro* studies:
   1. determination of the factors affecting the rates of the specific deamidations of triosephosphate isomerase
   2. effects of deamidation on the structure of TPI
   3. effects of structure on the deamidation of TPI

II. *In vivo* studies:
   1. determinations of half lives of deamidation
   2. effects of cell age on the rates of deamidation

III. Methods development for the determination of minute quantities of altered proteins in cell extracts

CHAPTER II

MATERIALS AND METHODS

Materials

Enzymes

Yeast hexokinase (EC 2.7.1.1.) specific activity 375 units/mg, yeast glucosephosphate isomerase (EC 5.3.1.9) specific activity 580 units/mg, rabbit muscle fructose 6-phosphate kinase (EC. 2.7.1.11) specific activity 115 units /mg, rabbit muscle aldolase (EC 4.1.2.13) specific activity 12 units /mg, yeast glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) and α-glycerophosphate dehydrogenase (EC 1.1.1.8) specific activity 165 units/mg were obtained from Sigma Chemical Company (St. Louis, Mo.). Pure chymopapain B was a gift from Pharmotex Laboratories (Fort Worth, Tx.). Triosephosphate isomerase (EC 5.3.1.1) was purified as described below.

Substrates

Dihydroxyacetone phosphate (dimethylketal cyclohexylammonium salt), *D,L*–glyceraldehyde 3-phosphate (diethylacetal monobarium salt), *D* (-)3-phosphoglyceric acid disodium salt, *D,L*-glyceraldehyde and adenosine 5'-triphosphate disodium salt were from Sigma Chemical Company. For comparison purposes, *D,L*-glyceraldehyde 3–phosphate (diethylacetal monobarium salt) and *D,L*-glyceraldehyde 3-phosphate

(diethylacetal dicyclohexylammonium salt) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, In.). 2-Phosphoglycollate tricyclohexylammonium salt was also from Boehringer Mannheim Biochemicals. *D*-[U-^{14}C]glucose (279 Ci/mole was from ICN-Radiochemicals (Irvine, Ca.).

Chromatographic and Electrophoretic Supplies

Phosphocellulose (exchange capacity 1.01 meq/g), DEAE-Sephacel (exchange capacity 1.4 meq/g) and Sephadex G-100 (particle size 40-120 μm) were from Sigma Chemical Company. Dowex-1 (AG 1X8 formate, 140-325 wet mesh), and all the polyacrylamide gel electrophoresis reagents were from Bio-Rad Laboratories (Richmond, Ca.). Ampholines and PAGplates for isoelectric focusing were from LKB instruments (Bromma, Sweden). Molecular weight standards for SDS-PAGE were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.), Sigma Chemical Company and BDH Chemicals (Poole, England). Isoelectric focusing marker proteins were from Pharmacia Fine Chemicals. Thin-layer chromathography cellulose sheets (No. 13255) were from Eastman Kodak Company (Rochester, N.Y.).

Cell Culture Supplies

Human skin fibroblasts from donors of different ages were obtained from the National Institutes of Aging - Aging Cell Repository. Eagle's minimum essential medium (MEM) and Dulbecco's phosphate buffered saline (PBS) were purchased from GIBCO (Grand Island, N.Y.). Fetal bovine serum (FBS) was from Irvine Scientific (Santa Ana, Ca.), Ser-Xtend from Hana Media Inc., Boston, Ma., and [^{3}H]leucine fron ICN Radiochemicals. Trypsin and glutamine were from Sigma Chemical Company and gentamycin was from Invenex Laboratories (Chagrin Falls, Oh.).

Miscellaneous Reagents

Chloroacetol phosphate was synthesized as described by Hartman (1970). Ammonium sulfate (Special Enzyme Grade) was purchased from Schwarz/Mann Biotech (Cleveland, Oh.). Silver standard solution was from Fisher Scientific Company (Fair Lawn, N.J.). All other chemicals were of the highest purity grades available.

Methods

Enzymatic Assays

Spectrophotometric assay of TPI activity.--Triosephosphate isomerase was routinely assayed at 30°C using the α-glycerophosphate dehydrogenase coupled spectrophotometric procedure described by Rozacky *et al.* (1971):

$$\text{G3P} \xrightarrow{\text{TPI}} \text{DHAP} \xrightarrow[\text{NADH}]{\alpha\text{-GDH}} \underset{\text{NAD}^+ + \text{H}^+}{\alpha\text{-glycerophosphate}} \qquad (1)$$

The assay medium consisted of 0.15 mM NADH, 1.5 mM *D,L*-glyceraldehyde 3–phosphate, 2 units glycerophosphate dehydrogenase (α-GDH), 50 mM triethanolamine/ HCl pH 7.6 and the triosephosphate isomerase sample to be assayed. One unit of triosephosphate isomerase activity is defined as the amount of enzyme required to convert one micromole of glyceraldehyde 3-phosphate into dihydroxyacetone phosphate per minute at 30°C as monitored by the oxidation of NADH at 340 nm (ε^{mM}= 6.22).

Assay of TPI activity on polyacrylamide gels.-- Triosephosphate isomerase activity on nondenaturing alkaline polyacrylamide electrophoresis gels was located by the formazan precipitation stain as described by Scopes (1968) and modified by Snapka *et al.* (1974). Nicotinamide adenine dinucleotide (NAD^+, 1 mg), glyceraldehyde 3-phosphate dehydrogenase (G3PDH, 25 units), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg) and phenazine methosulfate (PMS, 1 mg) were dissolved in 1.5 ml activity buffer (5 mM $MgSO_4$, 5 mM Na_3AsO_4, 50 mM TRIS/HCl; pH 7.8) and dihydroxyacetone phosphate (DHAP, 500 µl of 15 mM solution). This mixture was soaked onto Sephrapore III cellulose acetate sheets, the latter was then overlayed on the electrophoresed polyacrylamide gel. The sandwich was incubated at room temperature for 3-5 min in the dark. At the end of the incubation, the strip was removed and fixed with 7% acetic acid in water.

$$\begin{array}{l} \text{DHAP} \xrightarrow{\text{TPI}} \text{G3P} \\ \qquad\qquad \downarrow \\ \text{G3P} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{G3PDH}} \text{3-PGA} + \text{NADH} + \text{H}^+ \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \text{formazan blue (ppt.)} \leftarrow \text{MTT} + \text{PMS} + \text{NADH} \end{array} \qquad (2)$$

Spectrophotometric quantitation of dihydroxyacetone phosphate.-- To assess the extent of formation of [U-^{14}C]DHAP from [U-^{14}C]glucose and purity of [U-^{14}C]DHAP after ion exchange chtromatography, samples of [U-^{14}C]DHAP were quantitated spectrophotometrically by the same procedure as described above for TPI activity. However, *D,L*-glyceraldehyde 3-phosphate was replaced by isotopically labelled dihydroxyacetone phosphate and triethanolamine buffer (50 mM, pH 7.6) was substituted in place of triosephosphate isomerase. The reaction was allowed to proceed to completion and the amount of DHAP determined from the change in the absorbance at 340 nm.

Quantitation of glyceraldehyde 3-phosphate.-- Glyceraldehyde 3-phosphate (G3P) was assayed as outlined below:

$$\text{G3P} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{G3PDH}} \text{3-PGA} + \text{NADH} + \text{H}^+ \qquad (3)$$

and the reaction monitored at 340 nm following the increase in absorbance ($\varepsilon_{mM} = 6.22$). The final concentrations of the assay mixture (1.0 ml) containing the sample glyceraldehyde 3-phosphate was: 0.1 M Tris/HCl pH 8.5, 17 mM sodium arsenate, 3.3 mM cysteine, 20 mM sodium fluoride, 3.3 mM NAD^+, 1.5 units glyceraldehyde 3-phosphate dehydrogenase.

Protein Determination

During chromatographic fractionation, protein content was monitored by measuring the absorbance at 280 nm. The absorbance index ($\varepsilon^{1\%}$) of 12.9 reported by Rozacky *et al.* (1971) was used. Otherwise protein content was measured by absorbance at 280 nm as well as by the method of Bradford (1976).

Electrophoresis and Isoelectric Focusing

Non-denaturing alkaline gel electrophoresis.-- Nondenaturing alkaline electrophoresis gels were as described by Maizel (1971). Slabs gels (140 x 160 x 1.5 mm) consisted of 3% acrylamide stacking and 7.5% acrylamide resolving (120 mm high) portions. Electrophoresis was conducted at 4°C. Field strength was 20 mA until the tracking dye entered into the resolving gel and 30 mA thereafter. Activity stains were performed as described under "Enzyme Assays".

SDS-PAGE (phosphate system).-- For purity determinations, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was run according to Weber and Osborn (1969). Gels were 10% in acrylamide and were run at 5 mA per tube (5 mm inner diameter, 12 cm long).

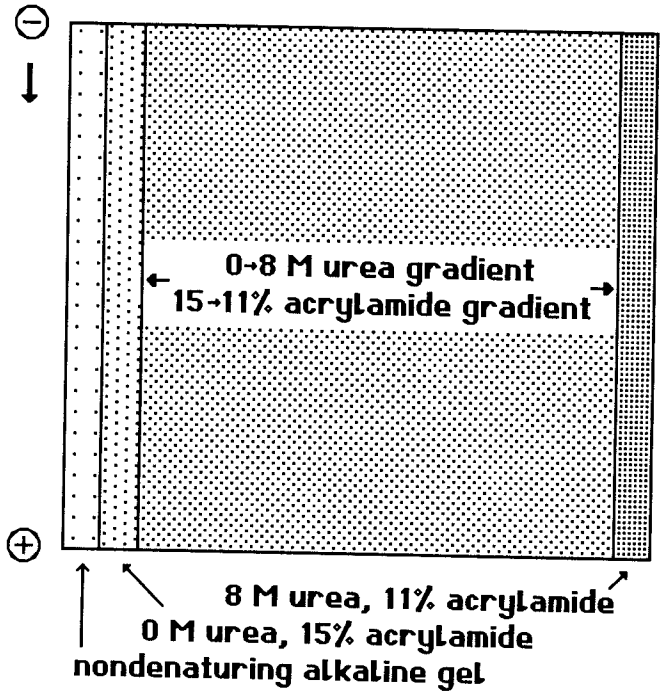
SDS-PAGE (Laemmli system).--During silver stain studies the SDS-PAGE procedure of Laemmli (1970) was used. Polyacrylamide slabs (140 x 160 x 1.5 mm; 10% acrylamide resolving gel, 12 cm high; 5% acrylamide stacking gel; 0.1 % SDS) were run at 30 mA or 60 mA per slab. SDS-PAGE were run at 10°C.

Isoelectric focusing.-- Preparative isoelectric focusing in sucrose gradients (pH 5-8) was carried out as described by Yuan *et al.* (1979) at 450 V for 72 h at 4°C. Analytical isoelctric focusing on polyacrylamide slabs (pH 3.5-9.5, 1.5 mm thick PAGplates) was run at 2°C at 1 W/cm width of gel. Gels were prefocused for 30 min, samples applied and focused for 90 min, sample wicks removed and focusing continued for another 30 min.

Transverse urea gradient gel electrophoresis.-- Transverse urea gradient gel electrophoresis was conducted according to Creighton (1979). Gels contained 0-8 M urea gradients orthogonal to the direction of elctrophoresis (Fig. 6). Furthermore, an inverse gradient of 10-15% acrylamide was superimposed on the urea gradient. Depending on the purpose of each run, gels were run at temperatures from 2°C to 20°C and at 10 mA or 100 mA per gel slab (140 x 160 x 1.5 mm), respectively. Samples were applied both in native and denatured states. Denaturation was carried out by incubating triosphosphate isomerase at room temperature in 50 mM triethanolamine buffer (pH 8.0) containing 8 M urea for two hours.

Two dimensional electrophoretic separation.-- During the *in vivo* deamidation studies, two dimensional gel electrophoresis was utilized to isolate triosephosphate isomerase from crude cell homogenates. The setup used differed from O'Farrell's (1975) procedure in its first dimension. To preserve the activity of the enzyme, nondenaturing alkaline gel electrophoresis was substituted for isoelectric focusing in 8 M urea. Thus, TPI activity from crude cell homogenates could be located on nondenaturing alkaline gels as described above. Activity bands were excised and stored frozen overnight at -20°C. Samples were thawed and equilibrated in 100 µl "high SDS-buffer" of O'Farrell (1975) in glass test tubes for 30 min at room temperature. The "high SDS-buffer" consisted of 25 mM Tris base, 192 mM glycine and 2% SDS. Equilibrated samples were placed into wells of the SDS slab gel, and to minimize protein loss, overlayed with the solution they had been incubated in.

Figure 6. Experimental setup of transverse urea gradient gels.


⊖
⊕
0→8 M urea gradient
15→11% acrylamide gradient
8 M urea, 11% acrylamide
0 M urea, 15% acrylamide
nondenaturing alkaline gel

In both dimensions, purified human TPI was run in parallel lanes as control. In the second dimension additional molecular weight markers were run in tandem. Protein staining of the second dimension gel with silver nitrate, its solubilization, and quantitation of the radioactivity are described below.

Isolation of Triosphosphate Isomerase

Triosephosphate isomerase was purified according to Lu *et al.* (1984) and consisted of the following steps (all steps were carried out at 0 - 4°C).

Preparation of the crude homogenate.-- Human placentae were obtained from a local hospital after full term pregnancies. Connective tissue was removed and placenta dissected into small pieces for homogenization. Homogenization was carried out in a Waring blendor containing 150 g tissue and 250 ml homogenization buffer (50 mM triethanolamine/HCl, pH 7.0, 1 mM Na-EDTA, and 0.1% 2-mercaptoethanol). The homogenate was centrifuged at 10,000 x*g* for 60 min at 4°C. The supernatant solution was filtered through glass wool to remove the lipids. This solution (1500 ml) was concentrated to 200 ml on a Minitan Ultrafiltration System (Millipore, Bedford, Ma.) equipped with 10^5 Dalton molecular weight cutoff polysulfone filters. TPI was recovered in the retentate.

Phosphocellulose filtration.-- Phosphocellulose was equilibrated to pH 7.0 with the homogenization buffer and placed into a sintered glass funnel. The retentate from the previous step was slowly overlaid on the phosphocellulose matrix and allowed to soak into the matrix. Filtration was aided by a light suction. This step removed the bulk of the contaminating hemoglobin and other proteins with apparent isolectric values greater than 7.0. TPI is found in the filtrate.

Ammonium sulfate fractionation.-- The eluate from the previous step was brought to 60% saturation in ammonium sulfate by slow addition of solid ammonium sulfate. This step was carried out in an ice bath, the mixture was constantly stirred and kept at pH 7.0.

After the addition of the ammonium sulfate was complete, the solution was stirred for an additional 30 min. Thereafter, the sample was centrifuged at 10,000 x *g* for 30 min and the pellet discarded. The supernatant solution was brought to 90% saturation with further addition of solid ammonium sulfate as described above. After centrifugation, the supernatant solution was decanted and the pellet dissolved in a minimum volume of dialysis buffer (10 mM triethanolamine/HCl pH 8.3, 1 mM Na-EDTA, 0.1% 2-mercaptoethanol). The solution was dialyzed for 24 h with three changes of buffer (10 liters each).

DEAE-Sephadex ion exchange chromatography.-- The dialysate was concentrated by vacuum dialysis against the same buffer, and the concentrate (≤5 ml) was loaded on a DEAE-Sephadex column (2.6 x 70 cm) pre-equilibrated in the same buffer. The column was first washed with 50 ml buffer, then TPI was eluted with an exponential gradient of 0–300 mM sodium chloride (both components 1.0 liter each). Fractions containing TPI activity were pooled and concentrated by vacuum dialysis.

Sephadex G-100 size exclusion chromatography.-- Concentrated samples were applied to Sephadex G-100 columns (2.6 x 90 cm) and the activity of fractions monitored. The column was pre-equilibrated and developed with the dialysis buffer. Activity containing fractions were analysed with nondenaturing alkaline PAGE stained for protein (Coomassie Blue stain) and TPI activity and on SDS-PAGE (Coomassie Blue stain). The enzyme was judged to be homogenous based on the comigration of the activity and protein stains on nondenaturing alkaline gels and the appearance of a single band corresponding to 27,000 Daltons on SDS-PAGE. Individual isozymes were obtained by preparative isoelectric focusing in sucrose gradients as described above.

Synthesis of [U-^{14}C]Dihydroxacetone Phosphate

In order to probe possible non-specific glcosylation of TPI by the substrate (see p:66) [U-^{14}C]dihydroxyacetone phosphate was synthesized as described by Yuan and Gracy (1977), except that carrier glucose was omitted to obtain the highest possible specific

radioactivity. A representative elution profile from the ion exchange column and a chromathographic run on cellulose sheet are shown on Fig. 7 and Fig. 8, respectively.

In Vitro Deamidation

All *in vitro* deamidation studies were performed on human triosephosphate isomerase purified to homogeneity as outlined above. Purified TPI-B was concentrated by vacuum dailysis against the dialysis buffer at 4°C. The concentrated sample was then dialyzed against the buffer of interest (4°C, three buffer changes over 24 h, 1:1000 volume to volume dialysis ratio). Following the determination of the TPI activity and protein concentration, aliquots were placed into Pierce Reactivials or Eppendorf centrifuge tubes.

The effect of temperature was studied at two temperatures, 4°C and at 37°C, at pH's 5.0, 7.0 and 10.0. The following buffers (50 mM) were utilized: boric acid/NaOH, pH 5.0; triethanolamine/HCl or sodium phosphate, pH 7.0; and sodium phosphate or triethanolamine/HCl or boric acid/NaOH, pH 10.0. Additional studies at pH 10.0 at 37°C were conducted with BICINE, ethanolamine, glycine, HEPES and TRIS buffers. Incubations were up to 7 h at pH 10.0 at 37°C, or up to sixty days under all the other conditions. Samples removed at predetermined times were analysed by alkaline gel electrophoresis. Gels were first stained for TPI activity, then with Coomassie Blue.

Deamidation in presence of substrates and inhibitors.-- The stock solutions of the substrates were at pH 4.5 and stored at -20°C. Immediately prior to the experiment, the pH of the substrate was adjusted to 10.0 with sodium hydroxide. When the substrate concentration was varied to change the G3P to TPI ratio, the substrate was diluted with the buffer of interest to keep sample volumes constant. Incubations were carried out in capped Eppendorf centrifuge tubes (600 µl) in thermostatted water baths. The effect of pH on the substrate effect was studied in the same manner except the pH of the buffers and the substrate were adjusted to the desired values.

Figure 7. Elution profile of [U-^{14}C]dihydroxyacetone phosphate from AG 1X8 ion exchange column. Enzymatic synthesis of [U-^{14}C]DHAP from [U-^{14}C]glucose and its subsequent purification was as described by Yuan and Gracy (1977). The sugar phosphates were fractionated according to Bartlett (1959) on an 1 x 20 cm column of AG 1X8 formate (140-325 mesh, pre-eqilibrated in water) with a 4 liter linear gradient of 0 to 1 N formic acid at 1.0 ml/min flow rate.

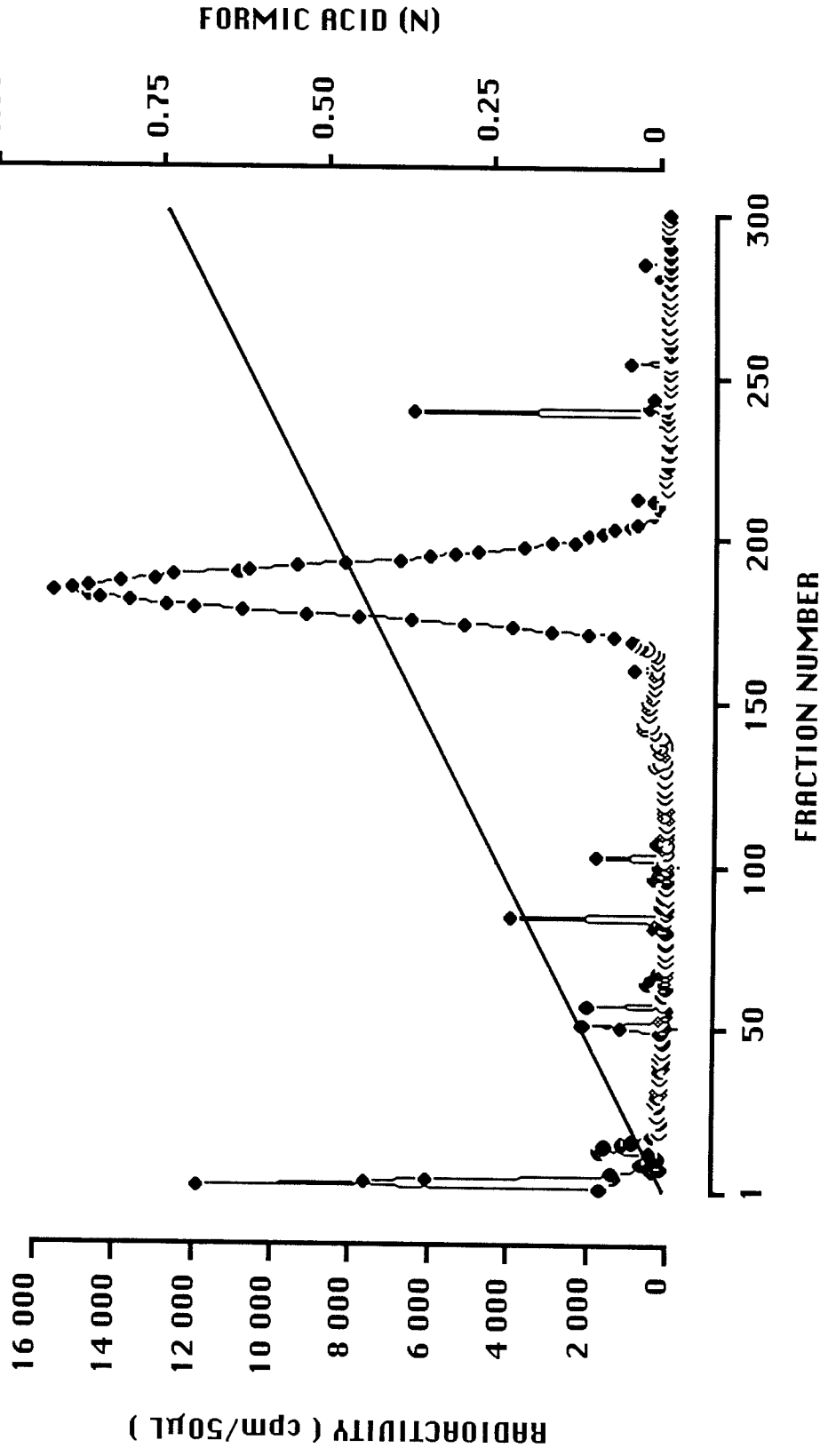
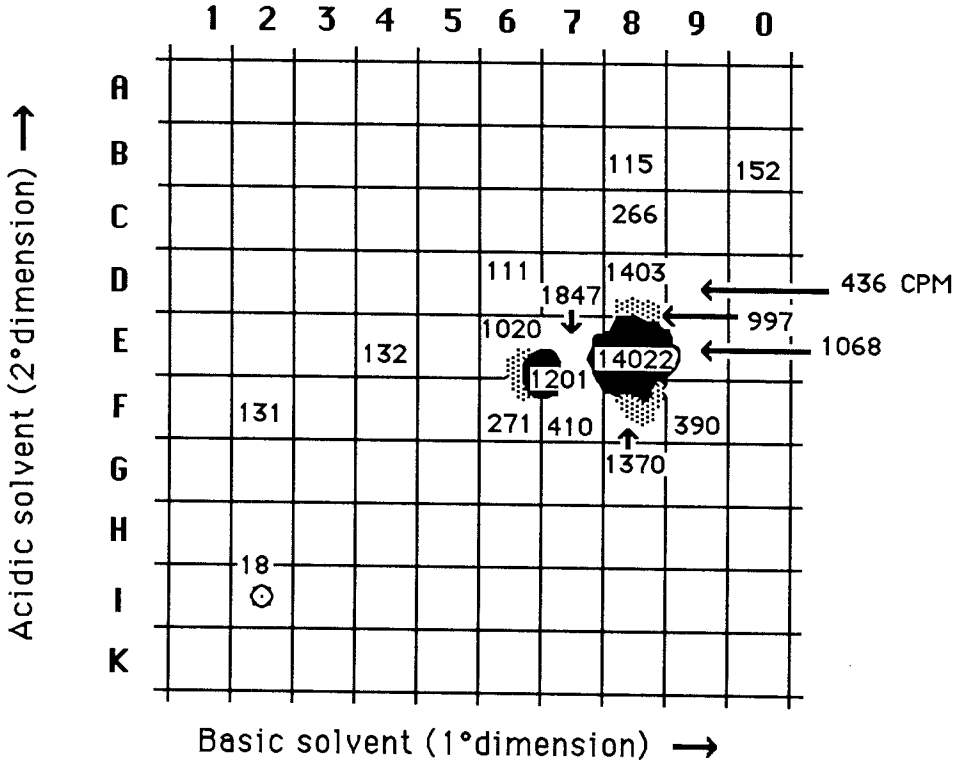

FORMIC ACID (N)
1.00
0.75
0.50
0.25
0
RADIOACTIVITY (cpm/50µL)
16 000
14 000
12 000
10 000
8 000
6 000
4 000
2 000
0
1
50
100
150
200
250
300
FRACTION NUMBER

Figure 8. Two dimensional chromatogram of [U-^{14}C]DHAP. The purity of [U-^{14}C]DHAP was assessed by thin layer chromatography on cellulose sheets (10 x 10 cm) according to Bandurski and Axelrod (1951). Sugar phosphates formed blue spots upon spraying with ammonium molybdate. Authentic DHAP was run on parallel plates. The radioactivity of the blue spots as well as the rest of the plate (divided into 1 cm squares) were determined. Figure shows radioactivity (cpm) and color gradation of the plate.


1 2 3 4 5 6 7 8 9 0
A
B
C
D
E
F
G
H
I
K
Acidic solvent (2°dimension)
Basic solvent (1°dimension)
115
152
266
111
1403
1847
436 CPM
997
1020
1068
132
14022
1201
131
271
410
390
1370
18
Color intensity of the spot
Origin

Quantitation of *in vitro* deamidations.-- Coomassie Blue stained gels were quantitated with computer aided scanning densitometry. The area under the densitometer tracing of the most basic TPI band was taken as the amount of TPI-B. In each of the time series, the amount of TPI-B was set as 100%, and the amount of TPI-B from successive time points expressed as a fraction thereof. Since the same amount of protein electrophoresed at any given time point, the total area under the densitometric scan was used to verify that no net protein loss due to chemical cleavage, precipitation, *etc.* had occured. The obtained data were fitted to first order exponential decay curves

$$N = N_0 \cdot e^{-kt} \qquad (4)$$

where t= time, N= % TPI-B at time t, N_0= % TPI-B at time zero, *i.e.*, 100% and k= the first order rate constant.

In Vivo Deamidation

Radiolabelling of Cells.-- Human skin fibroblasts were grown in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS) supplemented with Ser-Xtend (25 ml per liter of FBS). Cells were maintained at 37°C, 95% relative humidity, 5% carbon dioxide and were fed twice a week.

For the *in vivo* deamidation studies, fibroblasts were seeded at $3\text{-}5 \cdot 10^5$ cells/75 cm^2 tissue culture flasks and kept for 24 h at the conditions described above to allow for attachment. The cells were radiolabelled by introduction of medium containing 25 µCi (long term labelling, six to eight days) or 67 µCi (short term labelling, 24 h) [^{3}H]leucine (120 Ci/mmole). At the end of the labelling period, cells were washed six times with Dulbecco's phosphate buffered saline, trypsinized, seeded at $4\text{-}6 \cdot 10^5$ cells/75 cm^2 tissue culture flask, and chase medium consisting of Eagle's minimum essential medium, 10% FBS+Ser-Xtend and 2 mM unlabelled leucine introduced. Fibroblasts were cultured in the chase medium for the predetermined time periods, then trypsinized, rinsed twice with phosphate buffered saline, centrifuged, resuspended in cell homogenization buffer (10 mM triethanolamine, pH 7.6, 1 mM Na-EDTA, 0.05% 2-mercaptoethanol) and sonicated. The homogenate was then centrifuged in Eppendorf tubes (3 min at 4°C) and the supernatant

solution maintained at -60°C until two dimensional electrophoretic analysis was conducted as described above.

During preliminary experiments, cells were grown in leucine free medium to facilitate the uptake of [^{14}C]leucine. This created problems with cell growth and the possible gain in higher specific activity was offset by the smaller number of viable cells. For the rest of the experiments, cells were grown in MEM containing leucine. To minimize protein loss by surface adsorption and dilution due to rinsing, the cells were disrupted by sonication rather than crushing in a Potter-Elvehjem type homogenizer. The completeness of the homogenization by sonication was periodically checked by inspection of the homogenate under the microscope.

Quantitation of *in vivo* deamidation.-- Following electrophoresis samples were stained with silver nitrate as described below. Stained protein spots were cut and solubilized by incubation in 16 N nitric acid (0.5 ml) for 3 h at 85°C. Solutions were cooled over ice, neutralized with 10 N sodium hydroxide (0.5 ml), and transferred to scintillation vials for radioactivity determinations.

Silver staining

The photochemical silver nitrate staining procedure of Merril *et al.* (1981) was used after modifications as outlined in Table II. Silver stained gels were quantitated by scanning densitometry and by atomic absorption spectrophotometry.

Atomic absorption spectrophotometry.-- To determine the silver content in the protein bands, they were excised and solubilized in 16 N nitric acid (0.5 ml) by heating at 85°C for 3 h. After cooling to room temperature, samples were diluted with 4.5 ml water. Digests of large sample bands as well as control bands devoid of protein formed precipitates upon dilution. To avoid clogging of the aspiration orifice of the atomic absorption analyzer, it was essential to remove the precipitate. Since removal by filtration or centrifugation could have resulted in the loss of silver by entrapment in the precipitate, it was necessary to dissolve the precipitate. The samples were redissolved by reheating for 10 min at 85°C.

They did not reprecipitate when brought to room temperature. Silver concentrations were determined on a Perkin Elmer 4000 Flame Spectrophotometer at 328.1 nm using a silver lamp. All samples were corrected for the background noise arising from the nitric acid present in the solutions.

Densitometry.-- Silver stained gels were quantitated by computer interfaced scanning densitometry in the visual spectrum. High resolution optics with 0.1 mm slit width was in place. No color filters were used.

During electrophoresis, band widths of different proteins increase to different extents as protein concentrations are increased (Quitschke and Schechter, 1982). To minimize this effect, protein concentrations were corrected for the area of respective protein and expressed as ng/mm^2 (Berson, 1983). In case of PAGIEF staining intensities were calculated as the slopes of relative absorbance versus protein concentration (ng) since the bands were too narrow to allow accurate width measurements and area corrections.

TABLE II

SILVER STAINING PROCEDURES FOR PROTEINS ON POLYACRLAMIDE GELS*

Reaction	Reagents	Slab gels		Tube gels
		1.5 mm	0.75 mm**	1.5 mm ID***
Fixer 1	40% Methanol 10% Acetic acid	400 ml, 60 min	30 min	60 min
Fixer 2	10% Methanol	400 ml, 60 min	30 min	60 min
Oxidizer	3.7 mM $K_2Cr_2O_7$ 3.2 mM HNO_3	200 ml, 15 min	5 min	10 min
Wash	Water (4 times)	400 ml, 15 min	5 min	5 min
Sensitizer	12 mM $AgNO_3$ (at 45°C)	200 ml, 30 min	20 min	30 min
Wash	Water (twice)	400 ml, 1 min	1min	1min
Developer	*p*-formaldehyde (0.185 g/l) in 0.28 M Na_2CO_3	150 ml, 1min 200 ml, 5 min 150 ml, 3 min	1 min --	1 min --
Stopper	5% Acetic acid	as needed		

* All steps were carried out on a shaker at room temperature except the silver nitrate incubation at 45°C.

** 200 ml of each reagent was used in each step.

*** 10 ml of each reagent was used in each step.

CHAPTER III

RESULTS

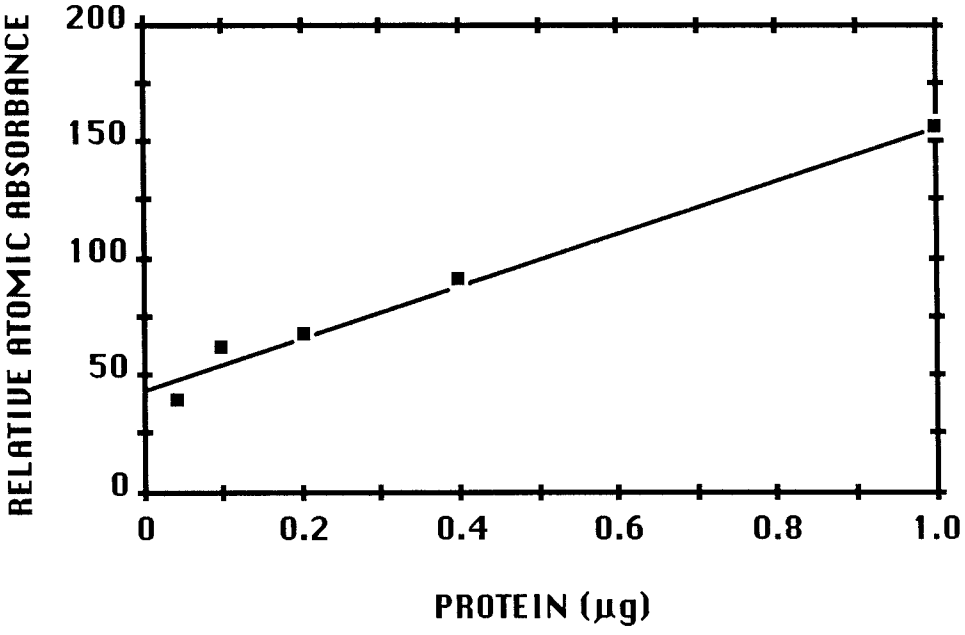
Quantitation of Silver Stained Proteins by Atomic Absorption Spectrophotometry

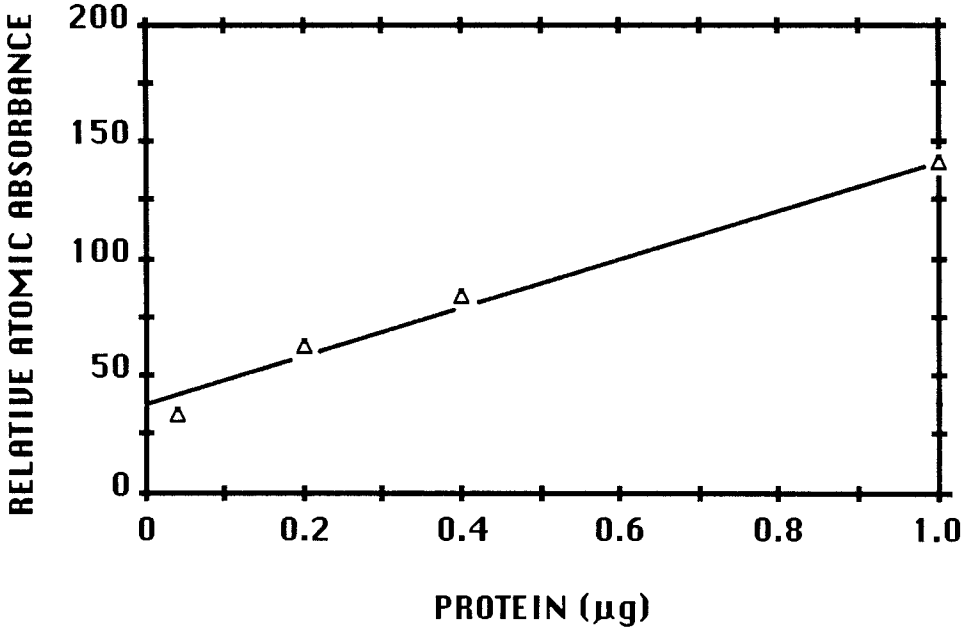
As discussed earlier, two dimensional electrophoresis on polyacrylamide gels resolves a large number of proteins, including those present in minute amounts. Quantitation of these gels has been problematic, partially because the sensitivity of the Coomassie Blue stain is not adequate to allow the detection and quantitation of minor proteins. The use of more sensitive silver stain has its own drawbacks: proteins stain to different extents, sometimes not at all . Results of the efforts to quantitate silver stained proteins on polyacrylamide gels by scanning densitometry and atomic absorption spectrophotometry, and to determine the factors affecting the staining of the proteins are presented.

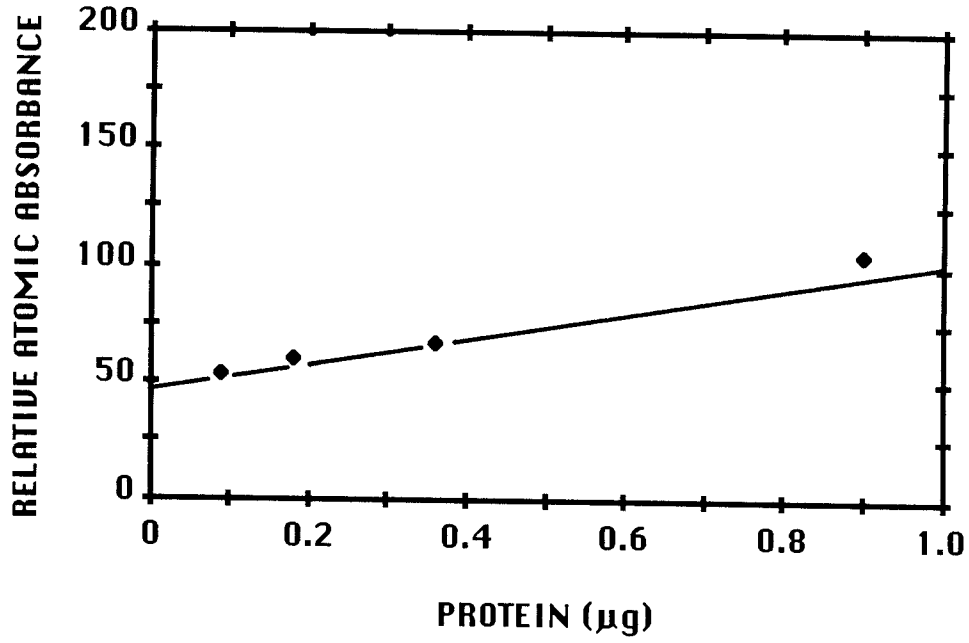
The relative atomic absorbance of silver stained, solubilized protein bands increased linearly with increasing protein concentration (Figure 9). Correlation coefficients for β–galactosidase (0.1-5.0 μg) and bovine carbonic anhydrase B (0.04-1.0 μg) were 0.991 and 0.992, respectively. The lower limit of detection was equivalent to 0.05 mg/l silver nitrate concentration. In this study the lowest concentration of protein utilized was 1.22 picomoles of cytochrome C per lane with a silver nitrate equivalent of 0.50 mg/l. All the samples were within the linear response range of the instrument as established by standard calibration curves.

Silver staining was examined with twenty five different proteins (Table III). The stains were compared in terms of "silver intensities" [silver intensity was defined as the slope of relative atomic absorbance at 328.1 nm as a function of protein concentration (ng)]. Silver intensities of several proteins are given in Table III.

Figure 9. Quantitation of silver stained proteins by atomic absorption spectrophotometry. Relative atomic absorbance of ovalbumin (■), bovine carbonic anhydrase (Δ), catalase (♦), and cytochrome C (●) are shown as functions of protein concentration.


RELATIVE ATOMIC ABSORBANCE
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1.0
PROTEIN (µg)


RELATIVE ATOMIC ABSORBANCE
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1.0
PROTEIN (µg)


RELATIVE ATOMIC ABSORBANCE
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1.0
PROTEIN (µg)

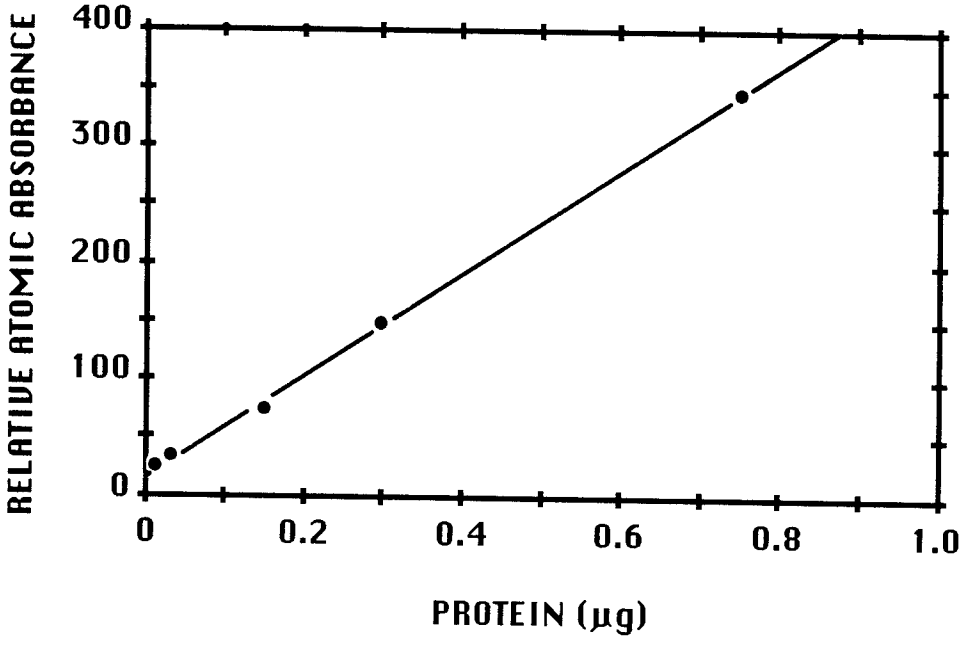

RELATIVE ATOMIC ABSORBANCE
400
300
200
100
0
0
0.2
0.4
0.6
0.8
1.0
PROTEIN (µg)

TABLE III

PROTEINS SILVER STAINED ON POLYACRYLAMIDE GELS

Protein	Silver intensity	Amino Acid Data*
I. Proteins quantified for silver intensity**		
Pepsin	10.37***	Croft, 1973
Myosin	12.48	Dayhoff *et al.*,1982
Lysozyme	18.14	Croft, 1973
β-galactosidase	44.35	Fowler and Zabin, 1977
Catalase	64.81	Schroeder *et al.*, 1982
Bovine carbonic anhydrase B	108.12	Sciacky *et al.*, 1976
Bovine serum albumin	111.72	Brown, 1975
Ovalbumin	114.18	Taborsky, 1974
Ovotransferrin	261.90	Phillips and Azari, 1972
Myoglobin	343.17	Dautrevaux *et al.*, 1969
Cytochrome C	440.32	Dayhoff *et al.*,1982
II. Other proteins stained with silver		
Amyloglucosidase		
Chymopapain B		
Ferritin		
Glyceraldehyde 3-phosphate dehydrogenase		
Horse myoglobin (two bands)		
Human carbonic anhydrase		
Lactate dehydrogenase		
Lentil lectin (three bands)		
Phosphorylase B		
Soybean trypsin inhibitor		
Triosephosphate isomerase (human)		
Trypsinogen		
α-lactalbumin		
β-lactoglobulin		

* Amino acid compositions of proteins were taken from references listed below.

** Silver intensity is defined as the slope of the line when relative atomic absorbance is plotted as a function of protein concentration.

*** Pepsin did not yield a visual stain. However, silver intensity was obtained from data presented in Fig. 10.

Of the twenty five proteins only pepsin did not yield a visible silver stain. This protein could be stained with Coomassie Blue prior or after the silver stain. Although pepsin exhibited "negative staining" characteristics, atomic absorption measurements indicated the presence of silver. Indeed, the silver content increased linearly with the amount of pepsin applied to the gels (Fig. 10).

Nonprotein Factors in Staining Intensity

Silver stain is a surface stain and does not stain the crossection of the gel as Coomassie blue does. In an attempt to increase the sensitivity of the stain, possible enhancement of crossectional staining was explored. First, the duration of the silver nitrate incubation was prolonged. Excised protein bands were microscopically inspected to determine the extent of crossectional staining. Prolonged incubation (200 min *versus* 20 min for 1.5 mm thick gels) did not yield complete staining. Second, the development period was prolonged to 22 h with intermittent replenishment of the developer. Silver nitrate incubation was the usual 20 min. This procedure resulted in almost complete crossectional staining (Fig. 11). However, the gels were too dark for quantitation by densitometry.

Prolonged incubation in the silver nitrate solution, on the other hand, increased the sensitivity of detection. The silver content of the bands was increased as shown in Fig. 12.

Staining Intensity and Protein Properties

Properties of proteins were examined for their possible effects on staining intensity. Stained gels were quantitated by scanning densitometry and relative absorbances plotted against protein concentration. Plots were essentially linear (Fig. 13) but exhibited different slopes. The densitometric staining intensities are presented in Table IV. Differential staining was explored in an attempt to understand the mechanism(s) of proteins staining

Figure 10. Silver concentration as a function of protein concentration in "negatively staining" pepsin band. Pepsin bands were located by Coomassie staining of parallel lanes and prolonged development of silver staining.

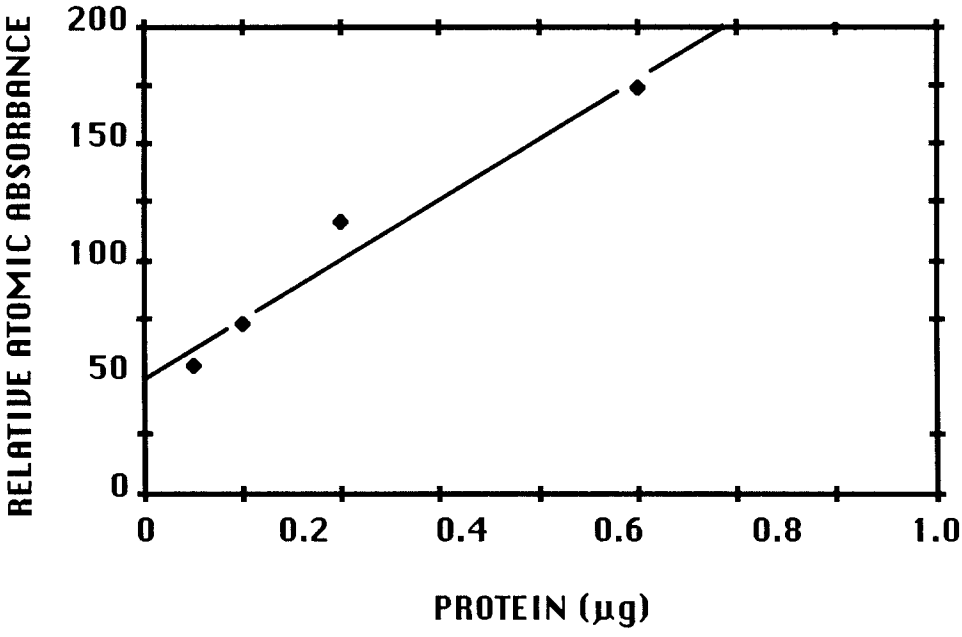

RELATIVE ATOMIC ABSORBANCE
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1.0
PROTEIN (µg)

Figure 11. Depth of crossectional staining as a function of development time. Silver stained polyacrylamide gels (1.5 mm thick) were kept in the developer solution for either (a) 10 min, or (b) 22 h. Protein bands were excised and the crossection of the gel photographed.

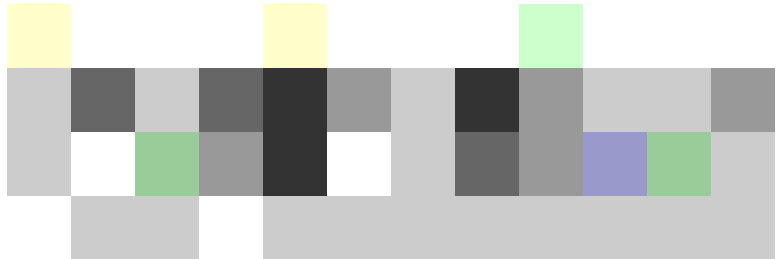


Figure 12. Extent of silver deposition as a function of silver nitrate incubation. Gels were treated identically except for the duration of silver nitrate was 20 min (♦), or 200 min (■).

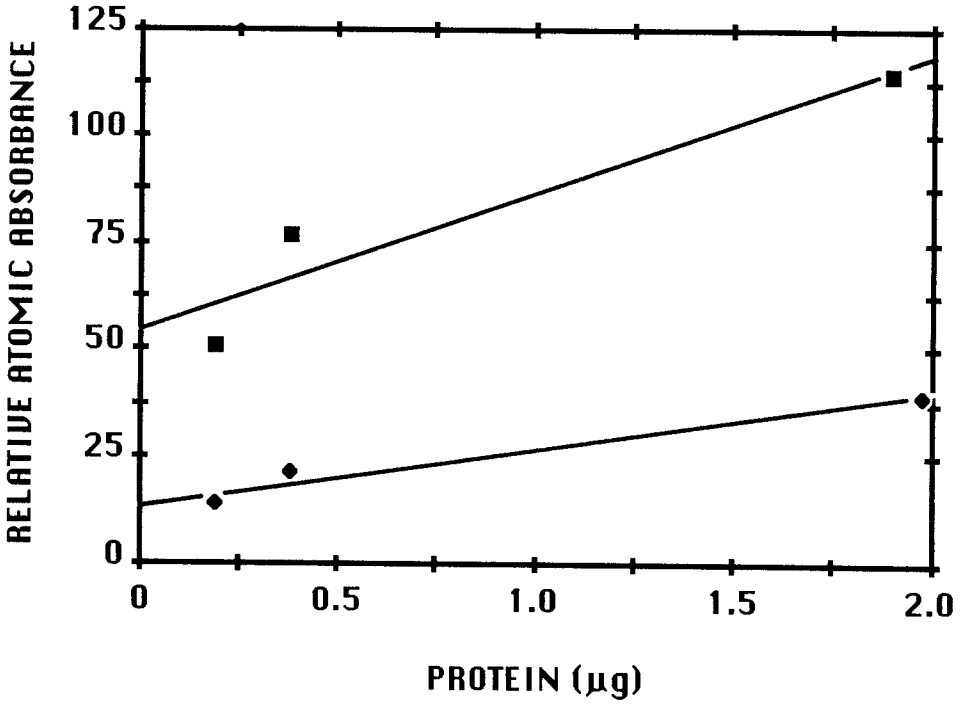
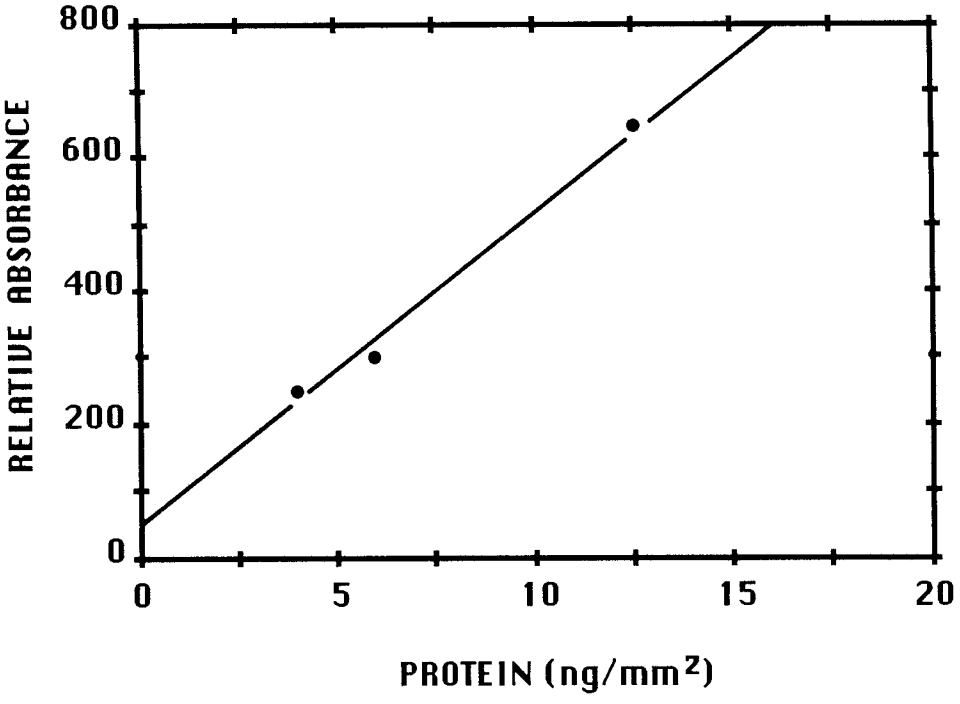
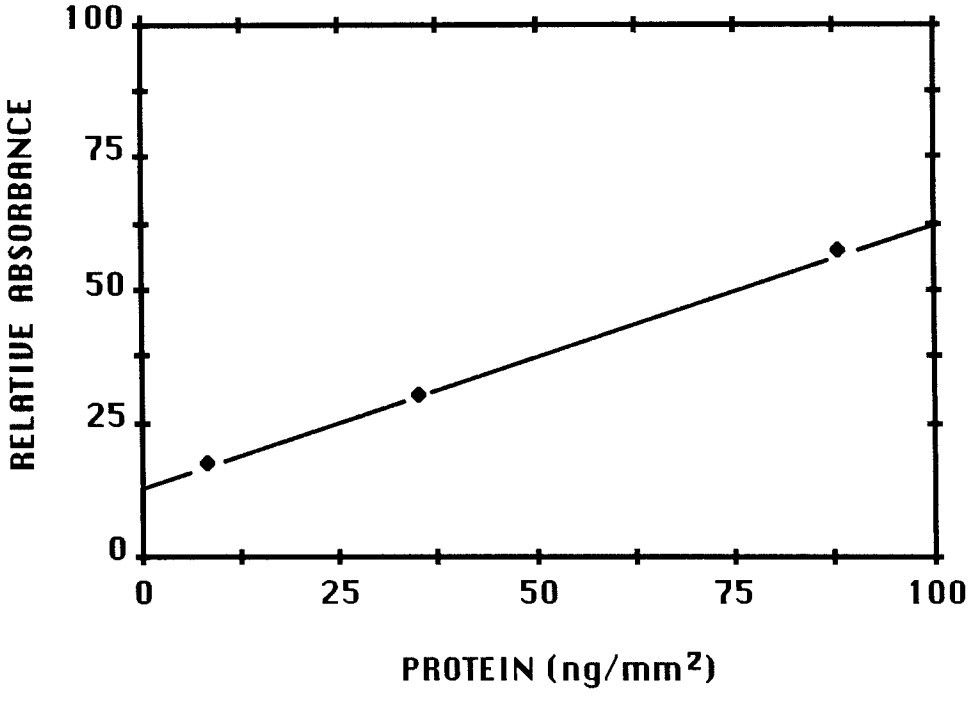
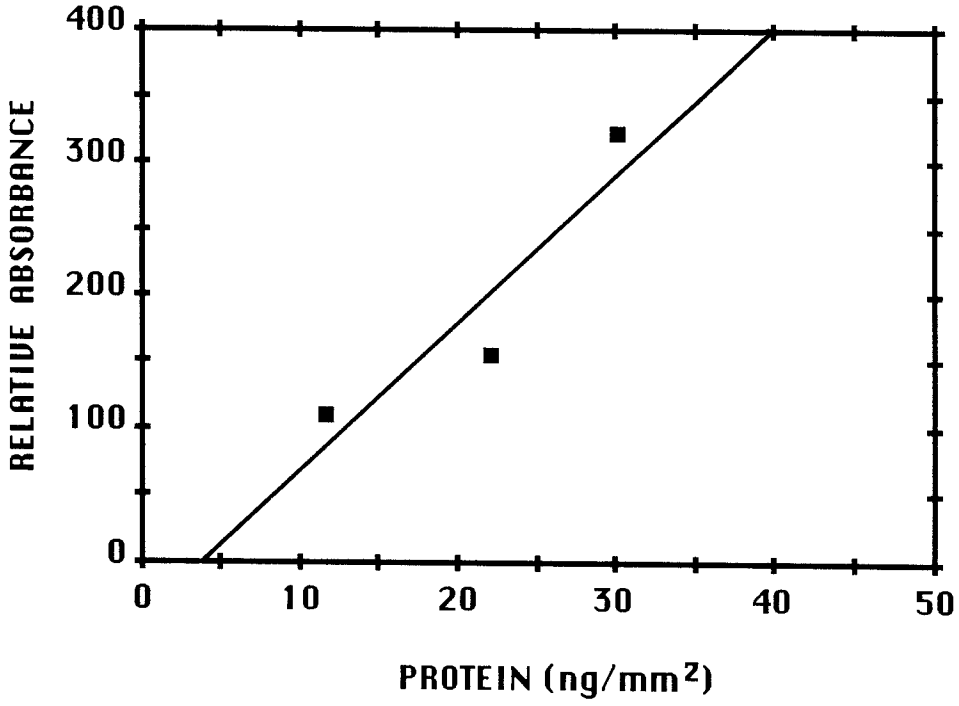

RELATIVE ATOMIC ABSORBANCE
125
100
75
50
25
0
0
0.5
1.0
1.5
2.0
PROTEIN (µg)

Figure 13. Quantitation of silver stained proteins by scanning densitometry. Proteins on silver stained gels were quantitated by scanning densitometry in visible spectrum using white light and 0.1 mm slit width high resolution optics. Cytochrome C (●), ovotransferrin (♦), ovalbumin (■) and bovine carbonic anhydrase B (Δ).


800
600
400
200
0
RELATIVE ABSORBANCE
0
5
10
15
20
PROTEIN (ng/mm²)


100
75
50
25
0
RELATIVE ABSORBANCE
0
25
50
75
100
PROTEIN (ng/mm²)


400
300
200
100
0
RELATIVE ABSORBANCE
0
10
20
30
40
50
PROTEIN (ng/mm²)

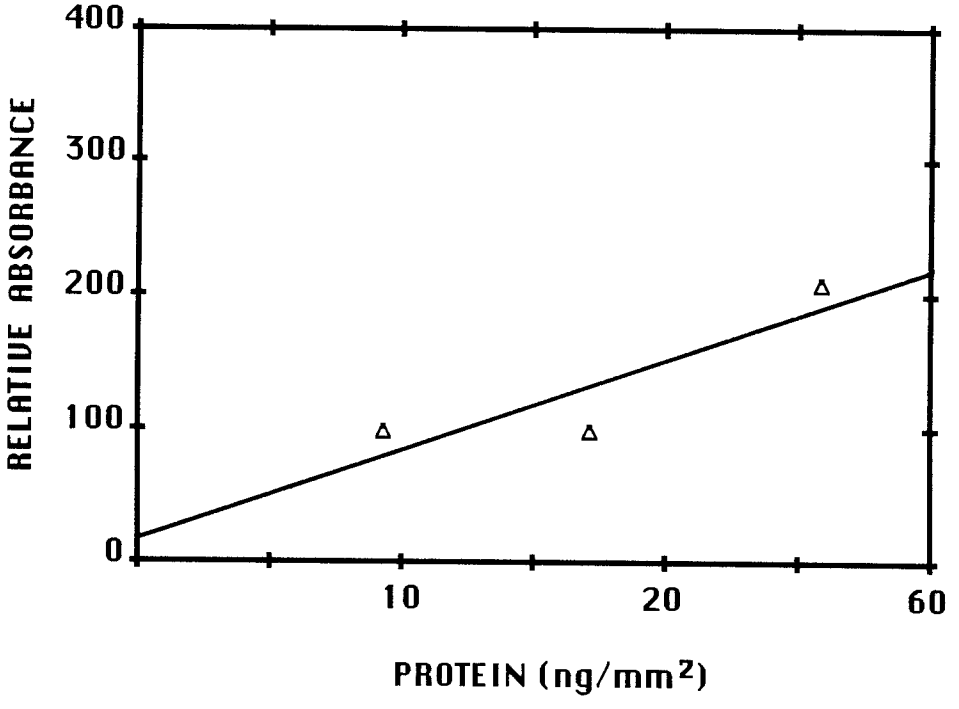

400
300
200
100
0
RELATIVE ABSORBANCE
10
20
60
PROTEIN (ng/mm²)

TABLE IV

DENSITOMETRIC STAINING INTENSITY OF PROTEINS FROM SILVER STAINED POLYACRYLAMIDE GELS*

Protein	Staining intensity	Subunit molecular weight
Cytochrome C	48.37	12 300
Ovalbumin	11.14	43 000
Bovine serum albumin	7.37	67 000
Carbonic anhydrase B	6.50	30 000
Catalase	1.43	60 000
Ovotransferrin	0.50	77 000

* Staining intensity is the slope of relative absorbance *versus* protein concentration (ng/mm^2) plots.

with silver. The intensity of the stain would be expected to depend on the number of sites in which silver can be deposited, which in turn would, among other factors, depend on the size, the amino acid composition and possibly the charge of the individual protein. Possible correlations were sought between the densitometric staining intensities and the three physical properties of proteins, *i.e.,* isoelectric point, subunit molecular weight and amino acid composition. Isoelectric points of four proteins, β-lactalbumin, human carbonic anhydrase B, bovine carbonic anhydrase B and amyloglucosidase showed no correlation with their densitometric staining intensities.

Densitometric studies. -- Densitometric staining intensities of proteins listed in Table IV were used to correlate them with their subunit molecular weights. A modest but statistically significant correlation was obtained ($r = -0.796$, $p < 0.05$). Linear regression analysis of the amino acid compositions of cytochrome C (Dayhoff *et al.*, 1982), ovalbumin (Taborsky, 1974), bovine serum albumin (Brown, 1975), bovine carbonic anhydrase B (Sciacky *et al.*, 1976), catalase (Schroeder *et al.*, 1982) and ovotransferrin (Phillips and Azari, 1972) indicated positive correlation to lysine and threonine contents, and negative correlation to aspartic acid, serine, valine and arginine contents. None of these were, however, statistically significant at the $p<0.05$ level. The same analyses were performed on the data published by Merril *et al.* (1982) for bovine serum albumin, phosphorylase B, ovalbumin, ferritin, carbonic anhydrase, ovotransferrin and soybean trypsin inhibitor. In this case no correlation was observed with molecular weights, but positive correlation to isoleucine ($r = -0.754$, $p<0.01$) and negative correlation to histidine contents ($r = 0.764$, $p<0.01$) were found.

Atomic absorption studies. -- As an alternative method for determining which amino acids are important in retaining silver, possible correlation between the silver intensities determined by atomic absorption spectrophotometry and the amino acid contents of the proteins were investigated. This method is independent of the color of the protein-silver complexes and is a true measure of the silver content. The analysis of the silver intensity data presented in Table III (excluding pepsin) revealed significant correlation ($p < 0.05$) to aspartic acid ($r = -0.775$) and to lysine ($r = 0.786$) contents. Inclusion of the data for pepsin resulted in slightly better correlation coefficients for aspartic acid ($r = -0.786$) as well as for lysine ($r = 0.801$).

In Vitro Deamidation Studies

Deamidation of triosephosphate isomerase was monitored on nondenaturing alkaline gels stained with Coomassie Brillant Blue. Time dependent generation of the progressively more acidic isozymes from TPI-B is shown on Fig. 14. The reaction profiles of deamidation under four different conditions are presented in Fig. 15. Half lives and rates of deamidations were calculated from exponential decay curves.

Effects of pH and Temperature

Changes in hydrogen ion concentration ($5.0 \leq pH \leq 10.0$) at 4°C had little effect on the observed rates of deamidation (Table V). At 37°C, hydrogen ion concentration exerted little effect in the range 10^{-5} to 10^{-7} M. Increasing the temperature from 4°C to 37°C at pH 5.0 and 7.0 enhanced the rates approximately three-fold. At higher pH (pH 10.0) and temperature (37°C) a different picture emerged. There was almost a 300-fold increase in the deamidation rates when the temperature was raised from 4°C to 37°C under alkaline conditions (pH 10.0). Similarly, when the temperature was kept constant (37°C) but the pH increased from 7.0 to 10.0, an approximately 150-fold increase in deamidation rates was observed.

The observed temperature related rate increases under the mildly acidic and neutral pH values were less than what one would have expected (e.g. $Q_{10}= 2$). On the other hand the temperature dependence under alkaline conditions is much higher. The rates of deamidation were measured utilizing a variety of buffers and similar results were obtained (Table VI). To eliminate the possibility that the observed increase was due to peptide bond cleavage, samples were analyzed on SDS-PAGE (Laemmli system). Samples were incubated in borate buffer (pH 10.0, 37°C) for up to 24 h and subjected to electrophoresis. After 18 h of incubation a slightly faster migrating peptide was observed. However, this peptide amounted to only 2% of the total protein at 24 h. Clearly, this cleavage is too slow and of very limited extent to account for the rapid loss of TPI-B.

Figure 14. Time dependent generation of acidic isozymes of triosephosphate isomerase. TPI-B was incubated at pH 10.0, 37°C in 50 mM triethanolamine buffer containing 0.5 mM *D*-glyceraldahyde 3-phosphate. Samples were analyzed by nondenaturing, alkaline polyacrylamide gel electrophoresis. Densitometric tracings for 0 h (left), 2 h (center) and 6 h (right) incubations are shown. Isozyme nomenclature is explained in the text. Electrode positions are shown on the left panel.

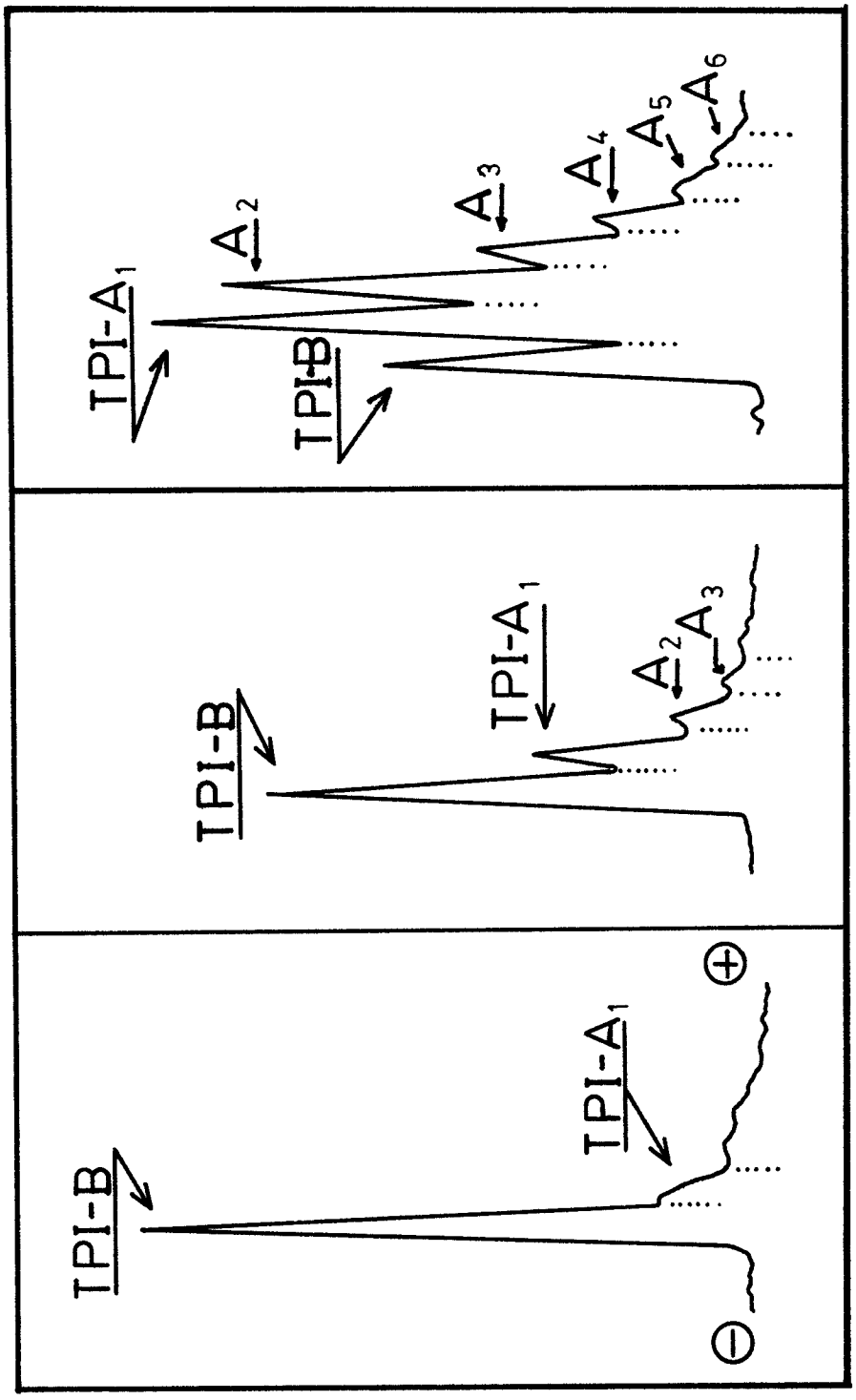
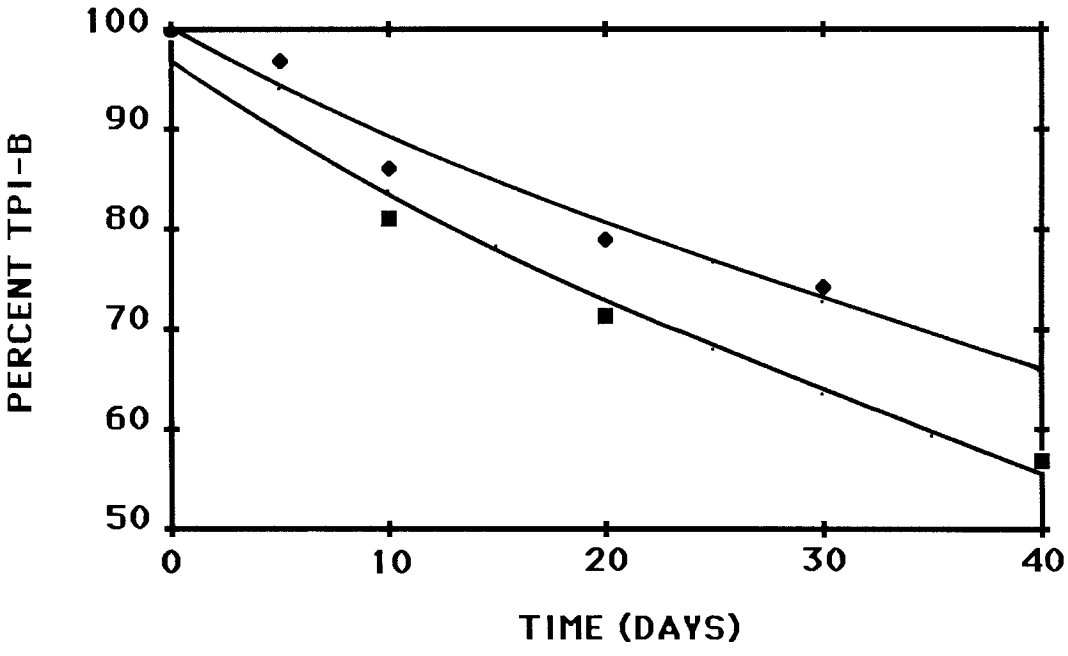

TPI-B
⊖
TPI-A1
⊕
TPI-B
TPI-A1
A2
A3
TPI-A1
A2
TPI-B
A3
A4
A5
A6

Figure 15. Reaction profiles of *in vitro* deamidation of human TPI-B. Purified, undeamidated human triosephosphate isomerase (TPI-B) was incubated at 4°C (top) at pH 5.0 in borate (♦) or at pH 7.0 in triethanolamine (■), and at 37°C, pH 10.0 in borate (bottom) in the absence (x) or persence (+) of glyceraldehyde 3-phosphate (0.5 mM). Buffers were of 50 mM concentration. Samples from each time point were analyzed electrophoretically on nondenaturing alkaline gels and stained with Coomassie Blue. Quantitation was carried out by scanning densitometry (595 nm, 0.1 mm slit width). Half lives and rates of deamidation were calculated from first order exponential decay curves (Eq. 4).


100
90
80
70
60
50
PERCENT TPI-B
0
10
20
30
40
TIME (DAYS)

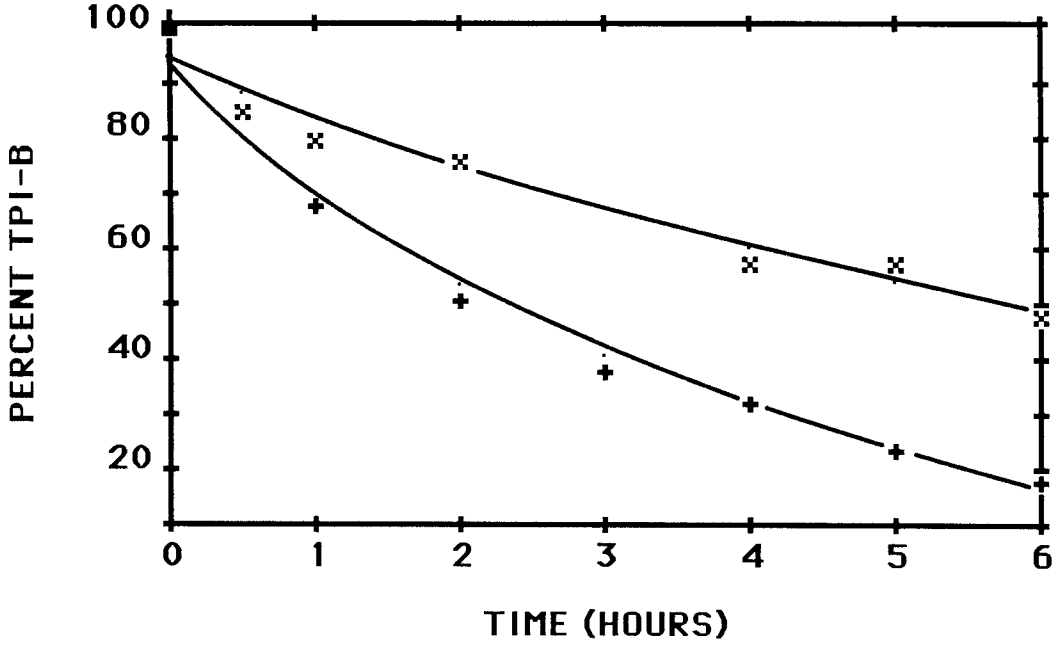

100
80
60
40
20
PERCENT TPI-B
0
1
2
3
4
5
6
TIME (HOURS)

TABLE V

IN VITRO DEAMIDATION RATE CONSTANTS AND HALF LIVES OF HUMAN TPI-B*

		$k_{deamidation} \times 10^4$ (day^{-1})		Half life (days)	
pH	Buffer	4°C	37°C	4°C	37°C
5.0	BO_3	4.3	13.8	66.8	20.9
7.0	TEA	5.6	13.5	51.2	21.7
	PO_4	5.1	7.6	57.8	37.8
10.0	BO_3	4.0	1112	71.9	0.26

* Samples were incubated in 50mM buffers for up to 6 h at pH 10.0, 37°C and for up to 60 days under other conditions. Quantitation of TPI-B and calculation of the rate constants of deamidation are described in the Methods section.

TABLE VI

EFFECT OF SUBSTRATE ON *IN VITRO* DEAMIDATION OF HUMAN TPI-B[a]

Buffer[b]	$k_{deamidation} \times 100$ (h^{-1}) (-) G 3 P (+)[c]		Half life (h) (-) G 3 P (+)[c]		Fold Increase
Phosphate	5.2[d]	15.2[d]	13.5	4.6	2.93
HEPES	7.9	21.1	8.8	3.3	2.67
TEA	7.5[e]	30.1[d]	15.3	2.3	4.00
TRIS	5.4	10.4	12.8	6.6	1.92
BICENE	11.9	77.5	8.4	0.9	6.94
Borate	11.1[d]	36.0[d]	6.2	2.8	3.24
$EtNH_2$	15.0	20.2	5.3	3.4	1.34
Glycine	18.1	52.8	3.8	1.3	2.92

[a] Quantitation of TPI-B and calculation of the rate constants were as decribed in the Methods section.

[b] All buffers were 50mM, pH 10.0 and samples incubated at 37°C.

[c] Each sample contained 0.5 mM *D*-glyceraldehyde 3-phosphate.

[d] Average of 3 experiments.

[e] Average of 2 experiments

Ion Effects

The effects of buffer ions have been noted in the literature, but no systematic studies have been reported. The results of the pH dependence experiments suggested a base catalysis mechanism. To assess the effects of the buffer ions, eight different nucleophiles were used: phosphate (pK_a= 7.21), HEPES (pK_a= 7.55), TEA (pK_a= 7.73), TRIS (pK_a= 8.10), BICINE (pK_a= 8.35), borate (pK_a= 9.24), ethanolamine (pK_a= 9.44) and glycine (pK_a= 9.60).

Brønsted plots of rates of deamidation (Fig. 16) at 37°C, pH 10.0 show good correlation for the best fitting line (correlation coefficient, r= 0.8762), thus suggesting a general base catalysis mechanism for the deamidation of TPI. As they are different nucleophiles than the rest, phosphate and borate data were not included in the calculations of the best fitting line and the correlation coefficient. TRIS data clearly deviate from the others. If excluded from the calculations, correlation is improved substantially (r=0.9788).

The effect of ionic strength on deamidation was studied at pH 10.0, 37°C in 50 mM triethanolamine buffer. Ionic strength was adjusted with addition of sodium chloride or ammonium chloride (0.125 to 1.0 M). The deamidation appeared arrested after the first deamidation. Similar results were obtained in borate and phosphate buffers. No correlations could be drawn from the ionic strength experiments.

Effects of Protein Concentration

The deamidation sites of TPI are located in the subunit contact sites (Yuan *et al.*, 1981). It is also known that the enzyme dissociates more readily at lower protein concentrations (Waley, 1973). The enzyme was incubated up to 6 h under conditions enhancing deamidation (50 mM borate buffer, pH 10.0, 37°C) and the rates of deamidation measured. A plot of the rates of deamidation as a function of protein concentration reveals an inverse relationship (Fig. 17).

Figure 16. Brønsted plots of in vitro deamidation rates of human TPI-B. Preparations of TPI-B were dialyzed (4°C) and incubated (37°C) in the indicated buffers (50 mM, pH 10.0). Rates of deamidation were calculted from data obtained at 0, 0.5, 1, 2, 3, 4, 5 and 6 h of incubation. Phosphate and borate were not included in the calculation of the best fitting line and the correlation coeffient (see text).

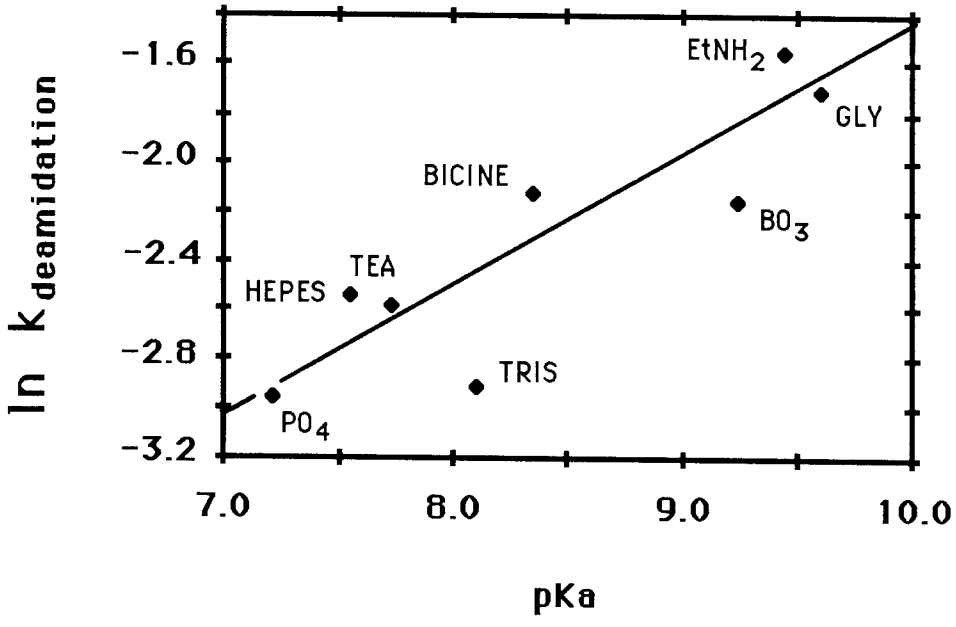
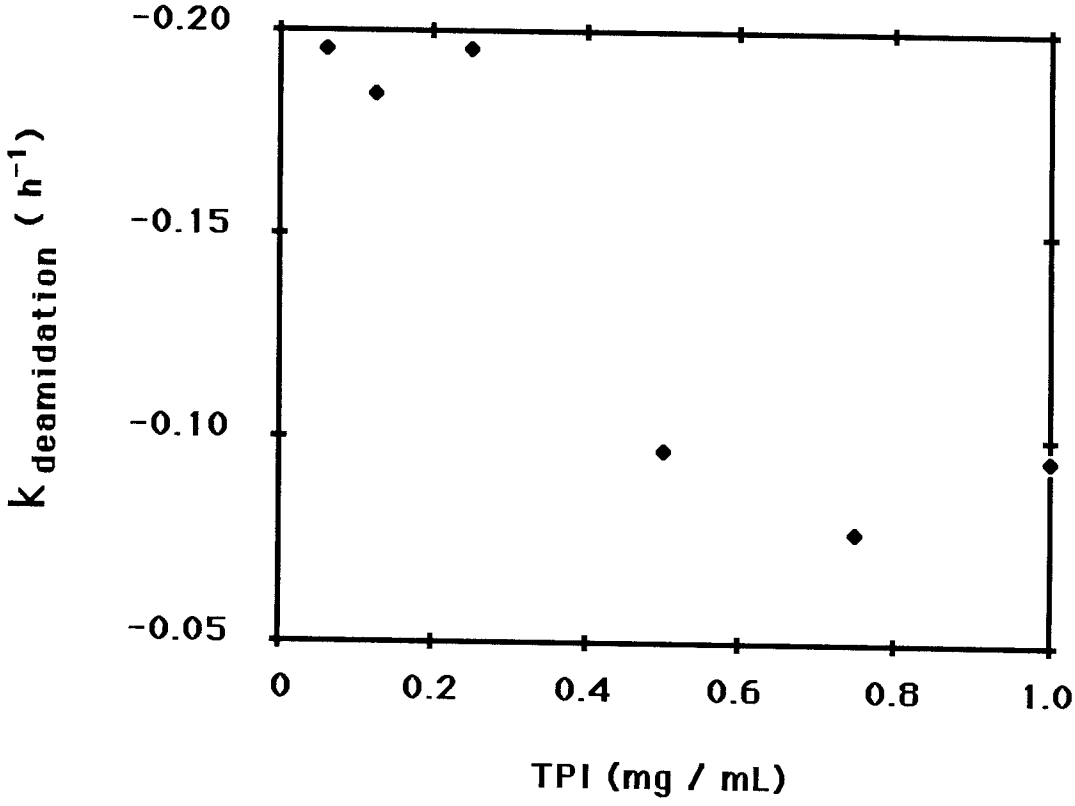

ln k deamidation
-1.6
-2.0
-2.4
-2.8
-3.2
7.0
8.0
9.0
10.0
pKa
EtNH2
GLY
BICINE
BO3
TEA
HEPES
TRIS
PO4

Figure 17. Rate constants of deamidation as a function of TPI concentration. Rates of deamidation were calculated from incubations (0 to 6 h) in borate buffer (50 mM, pH 10.0, 37°C) as described under the Methods section.


-0.20
-0.15
-0.10
-0.05
$k_{deamidation}$ (h^{-1})
0
0.2
0.4
0.6
0.8
1.0
TPI (mg / mL)

Effects of Substrates and Inhibitors

Incubation of the enzyme in the presence of the substrate (glyceraldehyde 3–phosphate) was initially conducted in borate buffer (50 mM, pH 10.0, 37°C). The observed deamidation rate was higher than in its absence. This surprising effect was further analyzed in other buffer systems under a variety of conditions (Table VI). The rates of deamidation were enhanced by substrates in all the buffers utilized.

To preclude possible artifacts due to contaminants of the substrate, preparation procedure or manufacturing, the substrate was obtained from two independent manufacturers in two different salt forms. Deamidation rates were similarly enhanced with these substrates (Fig. 18). Enhanced deamidation was also observed when the other substrate of the enzyme, dihydroxyacetone phosphate was employed (Fig. 18).

To determine if substrate binding alone was sufficient or if catalytic turnover was necessary to enhance the deamidation process, two inhibitors of the enzyme were utilized. Glyceraldehyde, a competitive inhibitor and also a slow substrate of the enzyme, effectively enhanced deamidation. Another competitive inhibitor (but not a substrate), 3–phosphoglyceric acid, did not enhance the deamidation rate (Fig. 19). Substrate effect was further investigated by varying the substrate to enzyme ratio. In separate experiments the substrate or the enzyme concentration were changed while the other was kept constant. Deamidation was enhanced with increasing substrate to enzyme molar ratio (Fig. 20). The effect of substrate was also investigated at different hydrogen ion concentrations. The substrate enhanced the rate of deamidation at all pH values between 7.0 and 10.0 The results are summarized in Fig. 21.

The substrates, DHAP and G3P are rather unstable in alkaline solutions. They undergo β-elimination and yield methylglyoxal (Richard 1984, 1985). Whether β–elimination also occurs during the the TPI catalyzed interconversion of DHAP and G3P is unclear (Iyengar and Rose, 1981, Rose, 1984). Therefore, it was necessary to determine if the observed rates were truely due to catalytic turnover of the substrates, or reflected an artifact such as covalent modification of the enzyme during the isomerization and/or elimination reactions. Nonspecific glycosylation of proteins is well documented in the literature (Acharya and Cho, 1986, Mori and Mannig, 1985, Rajaram *et al.*, 1986). Thus, TPI deamidated in the presence of [^{14}C]DHAP was analyzed by electrophoresis and the radioactivity contained in the TPI activity bands determined. The molar ratio of TPI:DHAP

Figure 18. Deamidation of TPI in the presence of dihydroxacetone phosphate and glyceraldehyde 3-phosphate. Samples were electrophoresed on nondenaturing alkaline polyacrylamide gels and stained with Coomassie Brillant Blue after incubation for 6 h at 37°C in triethanolamine buffer (50 mM, pH 10.0). Substrate was in molar excess over TPI: dihydroxyacetone phosphate (prepared from diethylketal dicyclohexylammoim salt, Sigma Chemical Co.) 590-fold (lane 1), 295-fold (lane 2) or 118-fold (lane 3) ; glyceraldehyde 3–phosphate (prepared from diethylacetal mono-barium salt, Boehringer Mannheim Biochemicals) 552-fold (lane 4), 276-fold (lane 5), or 110-fold (lane 6); glyceraldehyde 3-phosphate (prepared from diethylacetal dicyclohexylammonium salt, Boehringer Mannheim Biochemicals) 647-fold (lane 7), 324-fold (lane 8), or 130-fold (lane 9).

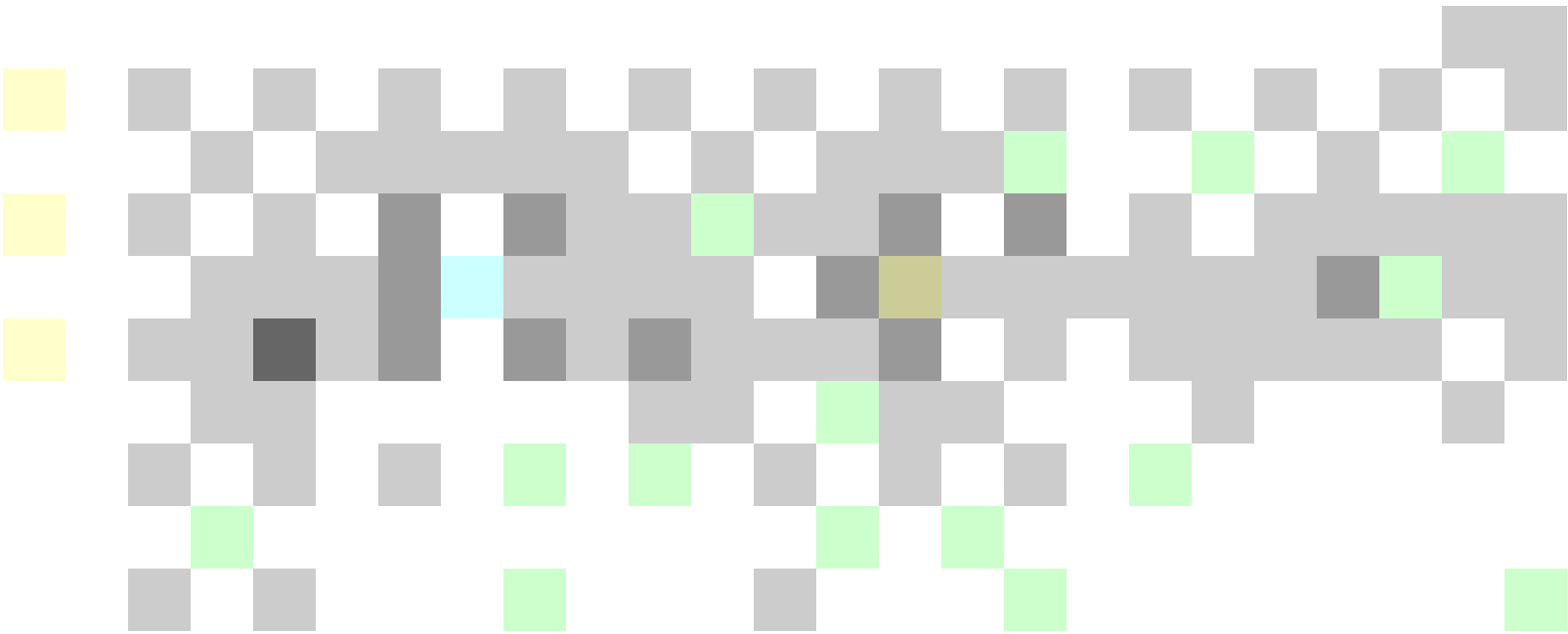


Figure 19. Deamidation of TPI in the presence of glyceraldehyde and 3-phosphoglyceric acid. Samples were electrophoresed on nondenaturing alkaline polyacrylamide gels and stained with Coomassie Brillant Blue after incubation in triethanolamine buffer (50 mM, pH 10.0) for 6 h at 37°C. Inhibitors were at 39-fold (lanes 1 and 4), 98-fold (lane 2 and 5) or 196 fold (lanes 3 and 6) molar excess over TPI.

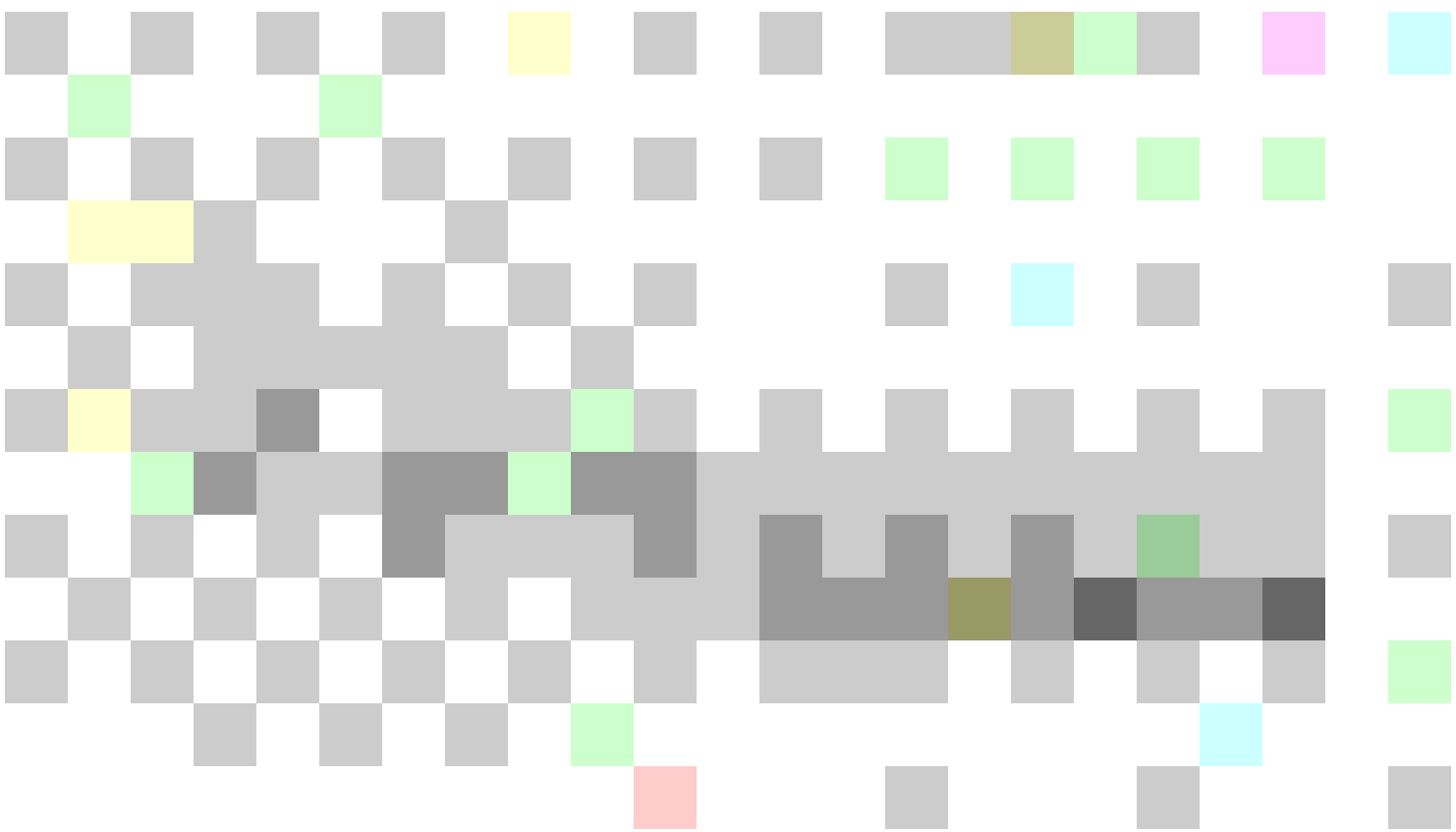
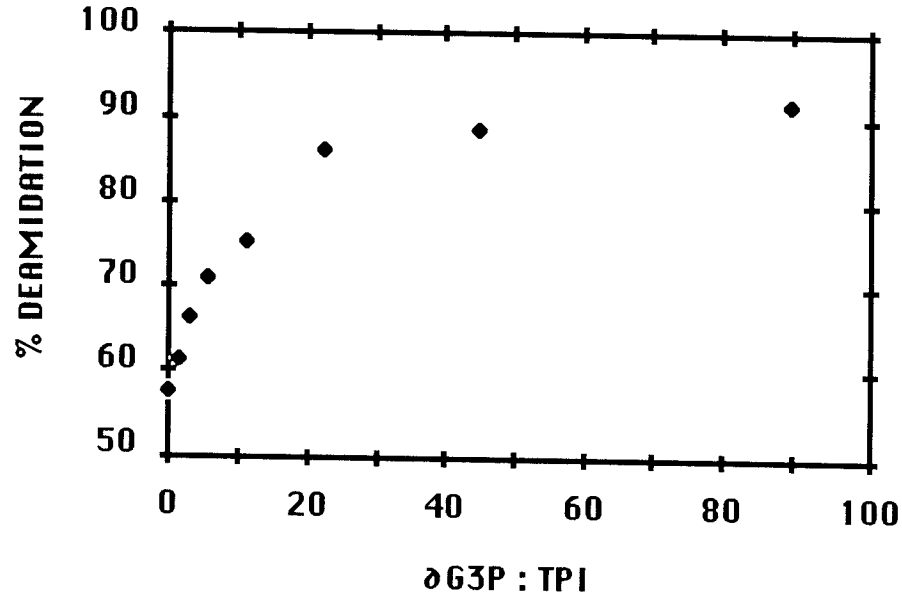


Figure 20. Effect of substrate to enzyme ratio on rates of deamidation. Extent of deamidation was calculated from loss of TPI-B in samples incubated in triethanolamine buffer (50 mM, pH 10.0, 37°C) for 7 h. Analysis of samples was as described in the Methods section. Top: Variation of G3P concentration (0 to 419 μM) at constant TPI concentration (4.68 μM). Bottom: Changing TPI concentration (0.6 to 37.4 μM) at fixed G3P concentration (300 μM).


100
90
80
70
60
50
% DEAMIDATION
0
20
40
60
80
100
∂G3P : TPI

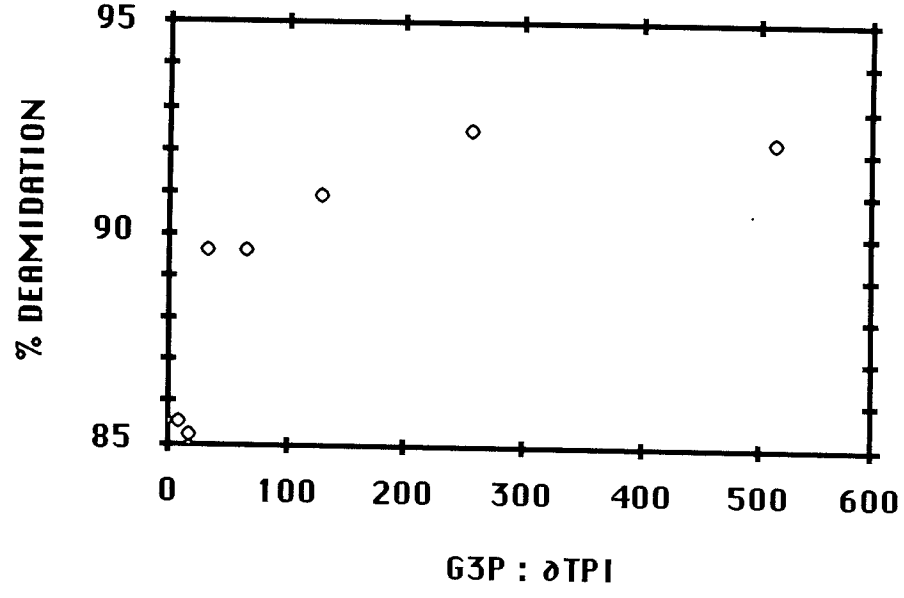
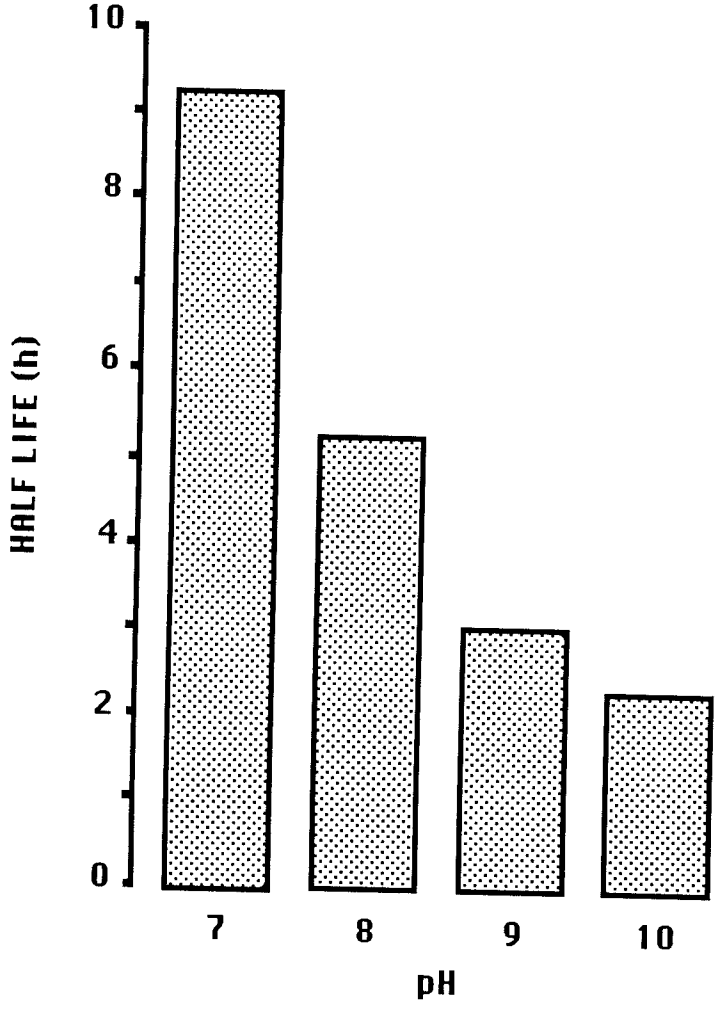

95
90
85
% DEAMIDATION
0
100
200
300
400
500
600
G3P : ∂TPI

Figure 21. Substrate effect at different pH values. TPI (36.8 μM) was mixed with glyceraldehyde 3-phosphate (25.0 mM) to give a final molar ratio of G3P:TPI= 50:1. Incubation at 37°C was up to 7 h at pH 9.0 and 10.0; and up to 24 h at pH 7.0 and 8.0. Experiments were conducted as described under the Methods section.


10
8
6
4
2
0
HALF LIFE (h)
7
8
9
10
pH

was found to be 500:1. Thus the effect of the substrateon deamidation is apparently not mediated by covalent binding of the substrate.

Effect of Deamidation on the Structure of TPI

Deamidation has been considered to be one of the marker events for proteins destined for proteolytic degradation (Gracy *et al.*, 1985). Deamidation, which introduces four negative charges into the subunit interaction sites of TPI and destabilizes the enzyme, was proposed to be one of the initial steps along the normal degradation route of TPI (Yuan *et al.*, 1981). The three dimensional structure of the chicken TPI (Alber *et al.*, 1981) is known from X-ray studies. Since chicken TPI does not deamidate, the effect of deamidation on the structure of TPI has not been studied. Thus, the possible effects of deamidation on the conformation of human triosephosphate isomerase was investigated using transverse urea gradient gels. A urea gradient (0-8 M), orthogonal to the direction of electrophoresis (Fig. 6) provides a two dimensional picture of conformational changes during the denaturation and renaturation processes. The experiment is considered to be "denaturing" if the sample is applied in its native form. During electrophoresis, molecules encountering high urea concentrations unfold and are retarded on the gel. The gels are termed "renaturing" if the sample is denatured in 8 M urea prior to the electrophoresis. In this case, molecules encountering low levels or no urea refold into a more compact and "native" form. Previously, transverse urea gradient gels have been used only for the analysis of monomeric proteins. Analysis of multimeric proteins have not previously been attempted as their patterns are expected to be more complex, especially if the subunits are not identical. In the case of TPI, the subunits are homologous and of relatively small size. Several preparations of TPI were treated under different conditions and then subjected to denaturing and renaturing electrophoresis. The enzyme was either freshly purified and exhibited a single isozyme (TPI-B) or it had been stored at 4°C for several months and contained several isozymes (TPI-B, A_1, A_2) or the fresh enzyme was aged under conditions promoting the deamidation (pH 9, 37°C).

Deamidated bands could be separated from each other at all urea concentrations. As an additional control, a nondenaturing alkaline gel (routinely used for the separation of TPI

isozymes) was appended on to the 0 M urea side of the gel. When samples were applied across the gel, deamidation bands could be distinguished in both systems. The results of the denaturing and renaturing gel studies from several treatments are presented below.

The gels were run at high electrical field (100 mA) to keep electrophoresis time short and to estimate the time frame of transitions. When denaturing electrophoresis was carried out at 100 mA current for 2 h, triosephosphate isomerase was found to exist in at least two non-native conformational states. One form migrated with the same retention as the native form (Fig. 22). The other form, highly retained on the gel, is belived to represent an unfolded form of the enzyme. The transition from the fast migrating form to the slow migrating form takes place between 3-4 M urea concentration. To determine if these forms would interconvert over a longer time span, gels were also run at 10 mA for 24 h. In this case no slow migrating form was observed (Fig. 23 b), but the shape of the curve took a more sigmoidal form, *i.e.,* differences in the migratory characteristics of the native and the fast migrating, denatured form became more apparant.

When renaturing gels were run at 100 mA for 2 h, the conversion of the slow migrating, uncoiled form to the faster migrating more compact form was more pronounced at higher urea concentrations (Fig. 23 c). Renaturing gels run at 10 mA for 24 h yielded patterns identical to their denaturing counterparts (Fig. 23 d).

These results suggest that the highly retained form is not an electrophoretic artifact but a denatured form of TPI. The interconversion of these forms is apparently not complete during the 2 h of electrophoretic run. However they were totally interconverted into a single form if electrophoresis was conducted over a longer period of time (24 h).

The slight change in the shape of the curve for the fast migrating band probably reflects the interplay between the sieving effect of the gel and the driving force of the electric field. A high electric field forces all the molecules to migrate so that small differences in size cannot be resolved. A weak field on the other hand, cannot overcome the sieving effect thus resolution is enhanced. These results indicate that the size of the fast migrating, denatured form is slightly larger than the native form.

The different isozymes were resolved from each other under all conditions, *i.e.,* they were visible as faster migrating bands below the fast migrating component of TPI-B (Fig. 23). This also means that the deamidated form(s) are not responsible for the slow migrating form. One might speculate that these two forms could reflect the difference between TPI-B and the cyclic imide form (Fig. 5), but the structural stresses on the peptide

Figure 22. Transverse urea gradient gel electrophoresis of TPI. Fresh TPI-B (in 20 mM TEA pH 8.0) was electrophoresed at 100 mA for 2 h on transverse urea gradient gels (Fig. 6) described earlier, and the gel stained with Coomassie Blue. Electrode positions are shown to the left of the gel. Electrophoresis was started at 2°C but temperature rose to 15°C at the end of the run. The urea gradient is shown separately underneath the gel.

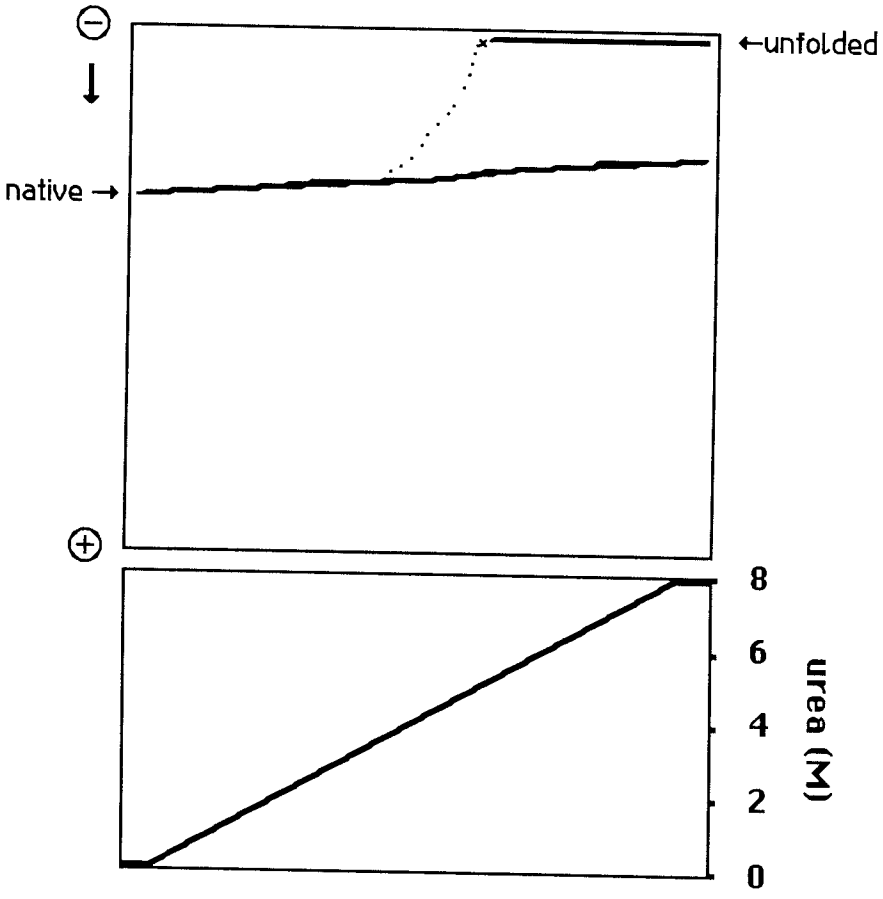
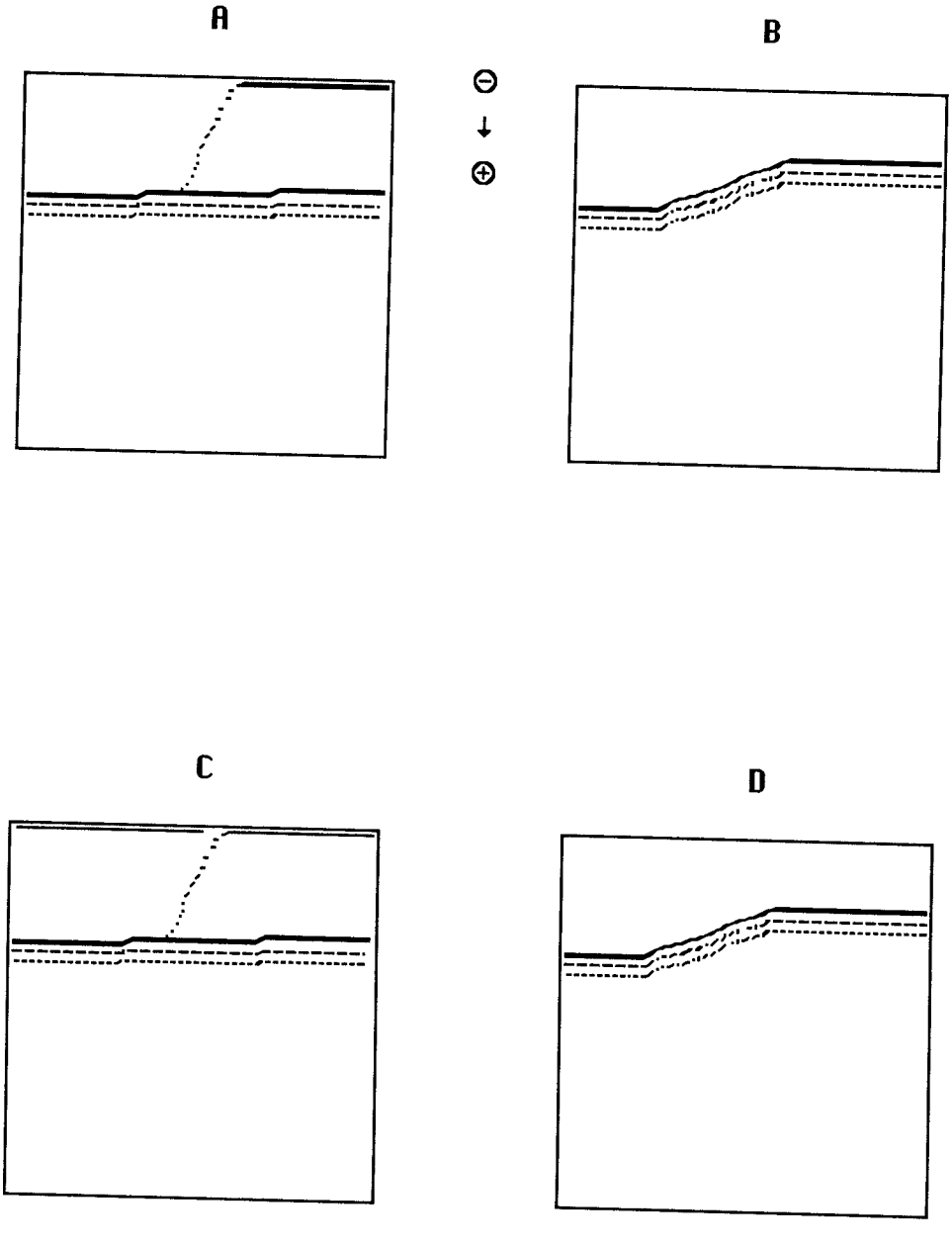

⊖
unfolded
native
⊕
8
6
4
2
0
urea (M)

Figure 23. Denaturation and renaturation profiles of TPI on urea gradient gels. TPI stored at 4°C was subjected to denaturing (panels A, B) and renaturing (panels C, D) transverse urea gradient electrophoresis. Electrophoresis was either for 2 h at 100 mA (panels A, C) or for 24 h at 10 mA (panels B, D) at 2°C. Direction of electrophoresis is shown in the center.


A
B
C
D

backbone would not appear to be large enough to warrant such a large change in the overall conformation of the enzyme.

The fast and slow migrating bands may represent TPI molecules which have dissociated prior to unfolding or TPI molecules which started unfolding before or simultaneously with dissociation and remained as tangled dimers, respectively. Since the molecule remains rather flexible at high urea concentrations, given the time, these dimers will try several conformations and may finally dissociate into unfolded monomers as implied by Fig. 23 b.

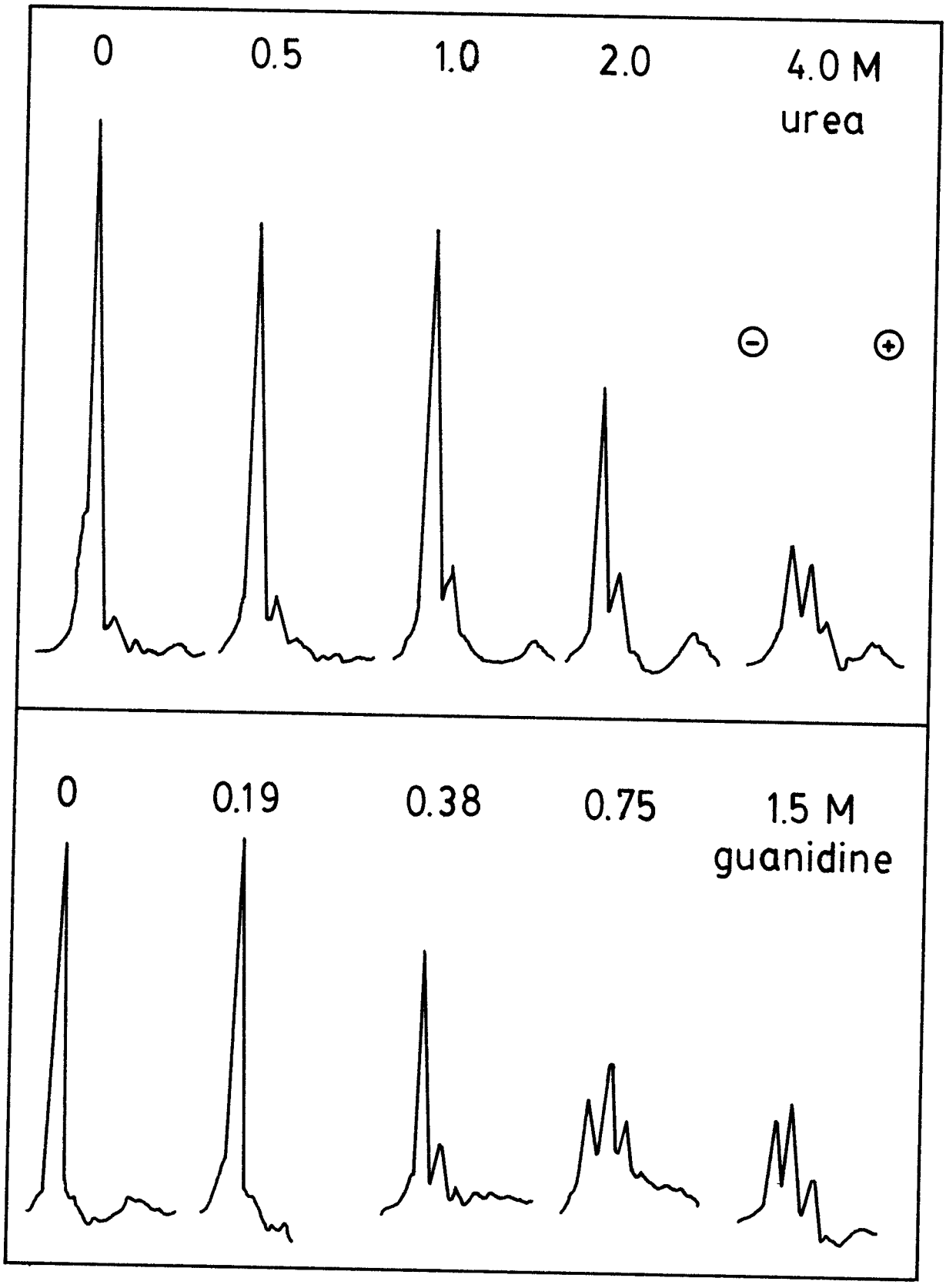
Effect of Structure on Deamidation of TPI

Three lines of evidence prompted the study of the effects of protein structure on the deamidation of TPI: a) the chicken enzyme which has Lys-71 instead of Asn-71 is not known to deamidate (Yuan *et al.*, 1981, Naidu *et al.*, 1984); b) Asn-71 appears to deamidate more readily than Asn-15 (Yuan *et al.*, 1981); and c) substrate, known to cause conformational changes upon binding (Alber *et al.*, 1981, Petsko *et al.*, 1984), also enhances deamidation (discussed above). These data may be interpreted that conformation plays some role in deamidation.

The contributions of tertiary and quartenary structure were examined by incubating triosephosphate isomerase in the presence of either urea or guanidium hydrochloride. After the incubation the denaturant was removed by dialysis, samples were electrophoresed on nondenaturing alkaline gels and gels stained for TPI activity and protein. All samples regained activity. Densitometric tracings of Coomassie Blue stained gels are presented in Fig. 24. A gradual increase of deamidated bands can be observed with increasing denaturant concentrations. This increase suggests improved access of the nucleophile (presumably water or the buffer) to the labile asparagines due to dissociation and unfolding of the enzyme enhances deamidation. These data are in accord with the effects of protein concentration on deamidation presented above (Fig. 17).

As it is evident from the densitometric tracings, some loss of protein was observed with samples incubated at higher denaturant concentrations. There was no visible precipitate in the vials, nor were there any additional bands on Coomassie Bluestained gels that did not correspond to the activity bands. The effect of structure and the requirement of

Figure 24. Effect of structure on deamidation: presence of denaturants. TPI-B was dialyzed overnight against triethanolamine buffer (50 mM, 1:1000 volume ratio, 4°C), then incubated for 5 h at 37°C in the same buffer containing either guanidium hydrochloride (0, 0.19, 0.38, 0.75 and 1.5 M; pH 9.9) or urea (0, 0.5, 1.0, 2.0 and 4.0 M; pH 9.7). Denaturants were removed by dialysis (three buffer changes over 24 h. in 50 mM TEA, pH 7.0, 1:1000 volume ratio, 4°C). Samples were analyzed by nondenaturing alkaline gel electrophoresis. Densitometric tracings of the Coomassie Blue stained gel are shown. Molar concentrations of denaturants are listed above each tracing. Electrode positions are shown on the right hand side of the upper panel.


0
0.5
1.0
2.0
4.0 M
urea
⊖
⊕
0
0.19
0.38
0.75
1.5 M
guanidine

Asn-71 for the deamidation needs further investigation (see "Outlook" under the Discussion section).

In Vivo Deamidation

The precise steps involved in the degradation of triosephosphate isomerase are incompletely understood. Known features of the deamidation of TPI include the accumulation of deamidated forms in aging cells (Gracy,1982, Tollefsbol *et al.*, 1982) and in cells known to exhibit little or no protein turnover such as the nucleus region of the eye lens (Gracy *et. al.*, 1985) and old erythrocytes (Turner *et al.*, 1975). To gain a better understanding of the physiological significance, the possible role of the deamidation(s) in the degradation and regulation of triosephosphate isomerase, the half lives of the different forms of TPI were studied *in vivo*. Human skin fibroblasts from donors of different ages (10-84 years) were utilized.

Two dimensional electrophoretic separation was chosen as the method for studying the *in vivo* deamidation of TPI. This method allows simultaneous analysis of several samples in a much shorter period than the conventional purification procedures, thus saving time and minimizing sample handling, eliminating the potential loss of one or more isozymes, and avoiding the possibility of deamidation during the isolation period.

Initially, the labelling period for the cells was chosen on the basis of *in vitro* deamidation studies in absence of substrate. Thus, deamidation was measured at 0, 2, 4, 7 and 10 days of chase after a 4 to 8 day labelling period. This schedule was selected to yield sufficient time points to monitor deamidation in culture and to allow enough radioactivity to be incorporated into TPI. Activity stains of the lanes containing the cell extracts usually exhibited two bands comigrating with TPI-B and A_2 of purified human TPI, respectively. The other deamidated forms, *viz.* TPI-A_1, A_3 *etc.* were usually not observed (Fig. 25). This was not a problem of resolution as the deamidated forms of purified human TPI run in parallel lanes could be separated. Samples from locations corresponding to the positions of TPI-A_1 and A_3 revealed far less radioactivity than the other forms, these isozymes were not studied any further. Data exhibited exponential decay for TPI-B and TPI-A_2 (Fig. 26).

Figure 25. *In vivo* deamidation of human skin fibroblasts. Homogenates of human skin fibroblasts were analyzed by nondenaturing alkaline gel electrophoresis and stained for activity. Lanes contain (from left to right): purified human TPI and homogenates of cells chased for 0, 4 and 8 days. Direction of electrophoresis is from top to bottom. Figure is redrawn from activity stain. The uniform darkness of the bands does not imply equal amounts of isozymes.

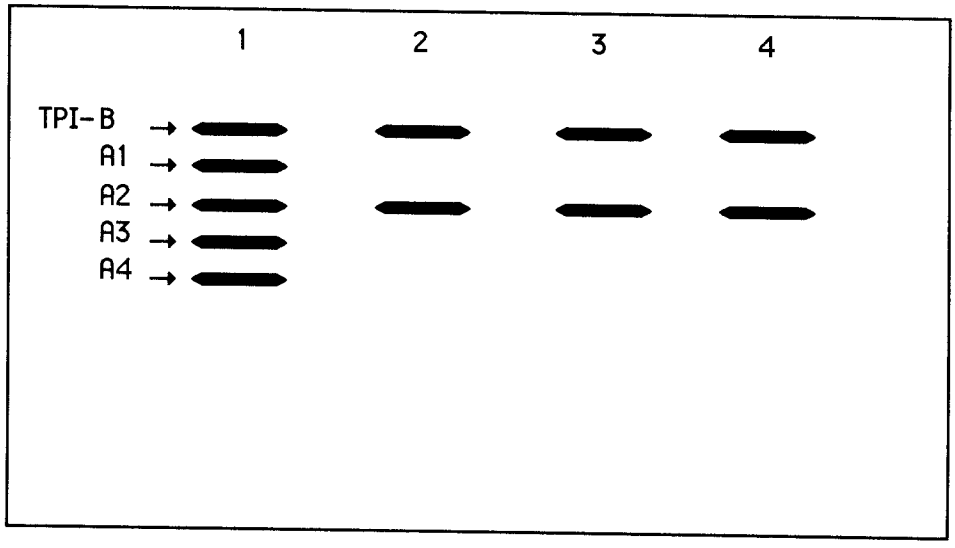
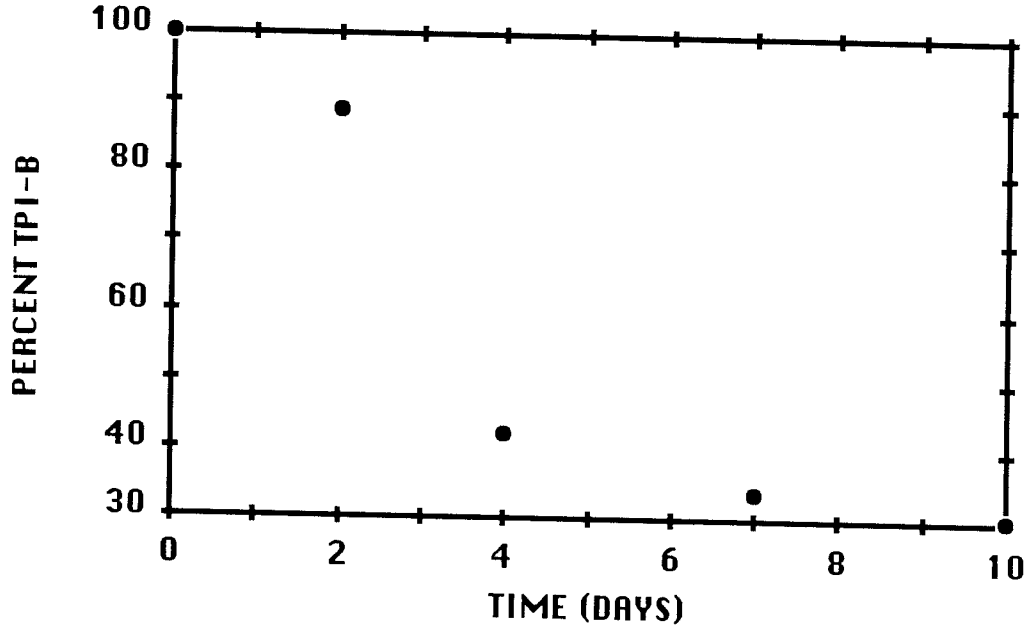
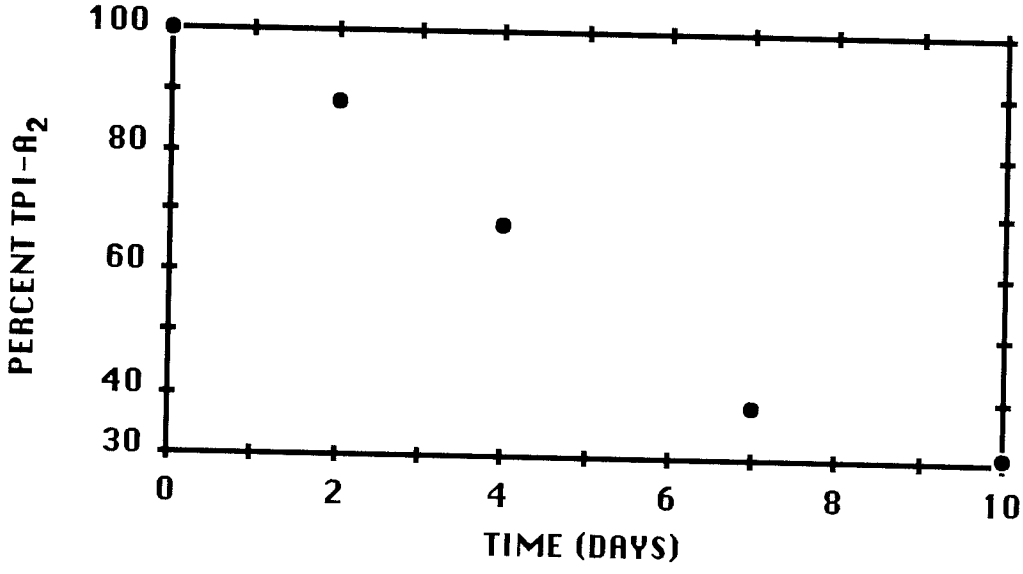

1
2
3
4
TPI-B
A1
A2
A3
A4

Figure 26. *In vivo* deamidation profiles of TPI. Human skin fibroblasts (cell line AG 6103, 29 year old donor, passage 17) were labelled with [^{3}H]leucine (25 μCi/ml medium) for 6 days. Deamidation profiles for TPI-B (top) and TPI-A_2 (bottom) are shown.


100
80
60
40
30
PERCENT TPI-B
0
2
4
6
8
10
TIME (DAYS)


100
80
60
40
30
PERCENT TPI-A2
0
2
4
6
8
10
TIME (DAYS)

The radioactivity content of TPI-A_2 was much higher than one would calculate from the observed deamidation half life of TPI-B (Table VII). This could mean that TPI-A_2 was accumulating during the long labelling period. In addition, it is possible that TPI-A_2 was also generated by some means other than successive deamidation of TPI-B. To test this possibility, the labelling period was shortened to 24 h and the sampling regime changed to 0, 6, 12, 24, 48, 96, 144 and 192 h of chase. The amount of radioactivity in the growth medium was also increased from 25 to 67 μCi/ml of culture medium.

Table VII lists the cell lines studied, data relevant to cell culture and the calculated half lives for both isozymes. The half life data for both, TPI-B and TPI-A_2 for donors with an age span of 10 to 84 years is graphically summarized in Fig. 27. For TPI-B, the data resemble a bell shaped curve with an increase in half life towards maturity, and a decline towards old ages. The data are rather similar for TPI-A_2. The relative ratio of the turnover of TPI-B and TPI-A_2 changes with the age of the donor. For example, in cells from younger individuals (age 10-29), deamidated TPI (TPI-A_2) has a shorter half life than TPI–B. At 32 years, both rates are essentially the same. However, in the cells from older donors, TPI-A_2 has a significantly longer half life than TPI-B. The possible implications of this curve are discussed in the next chapter.

TABLE VII

IN VIVO HALF LIVES OF TPI-B AND TPI-A_2

Exp[a]	Cell line	Age[b] (y)	P[c]	Label time	Half life[d] B	A2
2	AG6285	32	9	4	6.5	6.6
4	AG6103	29	12	6	5.6	3.8
5	AG6103	29	17	6	5.4	5.4
6	AG5274	84	15	8	2.3	3.4
8	GM0500	10	19	1	4.8	2.9
9	AG4382	81	11	1	1.9	2.4
10	AG4382	81	14	1	1.9	7.2
11	GM0500	10	22	1	2.9	3.6
14	AG6103	29	8	6	2.6	7.2
15	GM0500	10	15	6	4.1	(e)

(a) Experiments 2 through 6 represent initial sampling regime of 0, 2, 4, 7 and 10 days. The rest were sampled at 0, 6, 12, 24, 48, 96, 144 and 192 h of chase.

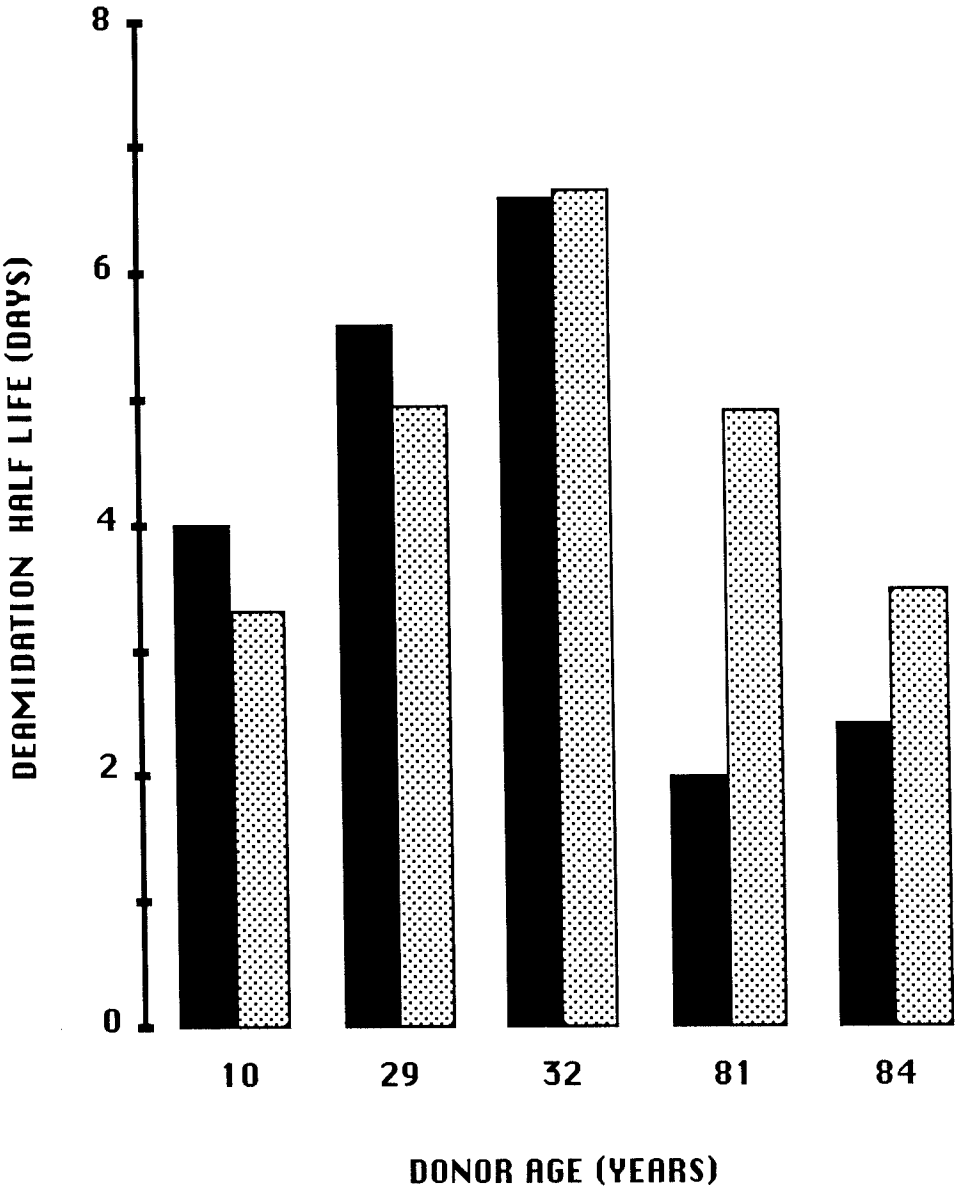
(b) Age of the donor as reported by NIA- Aging Cell Repository.

(c) Passage number; cells were split at 1:3 ratio.

(d) Half lives (days) were calculated from exponential decay curves. All points were included in experiments 2-6. Only points after the peak time were included for other experiments. "B" and "A_2" refer to issozymes TPI-B and TPI-A_2.

(e) Not determined.

Figure 27. Age dependence of half lives of *in vivo* deamidations of TPI. Average half lives were calculated from data presented in Table VII. TPI-B and TPI-A2 are represented by solid black and shaded bars, respectively.


8
6
4
2
0
DEAMIDATION HALF LIFE (DAYS)
10
29
32
81
84
DONOR AGE (YEARS)

CHAPTER IV

DISCUSSION

Detection and Quantification of Proteins on Polyacrylamide Gels

During the screening of different cell lines on polyacrylamide gels for altered proteins in aging, need for better quantitation arose. Atomic absorption spectrophotometry was explored not only as a possible means of increased sensitivity in quantitation, but also as a tool which would detect and quantitate silver present in the protein bands with the same accuracy irrespective of their color on the gel. In addition, atomic absorption spectrophotometry was used to investigate the factors governing the differential staining characteristics of proteins.

The spectroscopic method is certainly more expensive than densitometry as it requires highly specialized equipment. However, in all the analyses the silver concentration was in the linear response range of the instrument. Furthermore, sample dilution can be used as an effective variable to quantitate proteins found in miniscule amounts. This is a plus for the spectrophotometric method when proteins of vastly differing amounts, such as found in cell extracts resolved on two dimensional gels, need to be quantitated for comparison purposes. In contrast, with variable gain densitometers the attenuation is not always a linear function. The researcher may be required to empirically determine an elaborate exponential function if comparisons of this kind are to be made.

Silver stain in its present form, is a surface stain (Fig. 11, and Poehling and Neuhoff, 1981) as opposed to being a complete crossectional stain like Coomassie Blue. (It should be noted that since the extent of crossectional staining is diffusion controlled, Coomassie Blue can also be surface stain if staining periods are kept short.) Different degrees of crossectional staining with silver were achieved by varying the development time. The quantitation by atomic absorption spectrophotometry was not influenced by the degree of

crossectional staining (examined on 1.5 mm thick slab gels). Prolonged silver nitrate incubation, on the other hand, improved the detection limit due to both, specific (steeper slope) and nonspecific (higher intercept) deposition of silver (Fig. 11). These data contrast to that reported by Poehling and Neuhoff (1981), who observed complete staining of 0.7 mm thick gels upon prolonged incubations in glutaraldehyde and/or silver solutions. However, they utilized the glutaraldehyde enhanced ammoniacal silver staining procedure. The differences in chemical mechanisms of the two procedures could easily account for the differences. The possible mechanism of the photochemical stain will be discussed later.

To explain the differential staining intensities observed in this work and by others (Switzer *et al.*, 1979, Poehling and Neuhoff, 1981, Goldman *et al.*, 1980, Merril *et al.*, 1982), molecular properties of proteins, *i.e.*, isoelectric point, subunit molecular weight and amino acid composition were examined. Data from this study showed no linear correlation between densitometric staining intensity and isoelectric points of the proteins examined. Similarly, no statistically significant correlation ($p>0.05$) to amino acid composition was found. Only subunit molecular weight exhibited substantial and statistically significant correlation ($r = -0.795$, $p<0.05$). The same analysis was carried out on data published by Merril *et al.* (1982). In this case, no correlation was found with molecular weight but some significant correlation was observed with isoleucine and histidine contents. The apparent discrepancy between these two analyses could be attributed to differences in the densitometric staining intensities. These in turn may be the consequence of slightly different staining procedures employed and the difference in the expression of the data. Two additional sets of data, one from the same laboratory (Merril *et al.*, 1984) and the other from an independent laboratory (Berson, 1983; these data were obtained with ammoniacal silver stain) do not necessarily agree with the two sets of data analyzed here. This fact illustrates possible reproducibility problems associated with silver staining when analyzed by densitometry and suggests that the direct silver determination by atomic absorption spectrophotometry may also be superior in terms of its quantitative aspects. Great care needs to be exercised when quantitative data form different sources are compared.

Effects of Amino Acids on Staining Intensity

Special attention was paid to the role that specific amino acids may play in the reaction of proteins with silver. Experiments conducted during this work showed no linear correlation between the densitometric staining intensites and amino acid contents. Analysis of other data (Merril *et al.*, 1982) suggested correlation to isoleucine and histidine contents. On the other hand, Dion and Pomenti (1983) obtained correlation to lysine content when ammoniacal silver stain was used. Thus, we examined the participation of amino acids directly by quantitation with atomic absorption spectrophotometry. The advantage of this method is its independence of the color of the stain, which is attributed to differences in silver amino acid complexes (Nielsen and Brown, 1984). Analysis by atomic absorption revealed negative correlation to aspartic acid and positive correlation to lysine contents. The correlation to lysine agrees with the finding of Dion and Pomenti (1983). This may appear surprising as they had used ammoniacal silver stain with glutaraldehyde pretreatment. Glutaraldehyde is known to react with the ε-amino groups of lysine and believed to fixate the silver ions. Although the photochemical silver stain procedure used in the present study does not employ glutaraldehyde, it utilizes dichromate. Dichromate is strong enough to oxidize the residual methanol (from the preceeding fixing step) to formaldehyde. Trace amounts of formaldehyde present in commercial methanol have been reported to enhance the sensitivity of the ammoniacal silver stain (Wray *et al.*, 1981).

Investigation of the silver content of the protein bands revealed an interesting fact. The "negatively staining" pepsin bands indeed contained silver and the silver content increased with protein concentration (Fig. 10). Negative staining is not unique to pepsin and has been reported for other proteins (Morrissey, 1981, Merril *et al.*, 1982, Marshall, 1984, Nielsen and Brown, 1984). The silver content in the pepsin band indicates failure of color development rather than lack of silver deposition. This could mean that either (a) the silver complexes were colorless, and/or (b) the silver ions could not be reduced to metallic silver. Colorless silver amino acid complexes have been observed and color differences of these complexes attributed to differences in bond lengths, amino acid type and configuration (Nielsen and Brown, 1984). Colorless complexes were obtained with nonpolar and uncharged side chain amino acids, *i.e.*, tryptophan, phenylalanine, serine,

threonine and methionine. The high serine content of pepsin may contribute to the colorless complex.

The second possibility is supported by the findings of Merril *et al.* (1982) and Guevara *et al.* (1982). Merril *et al.* (1982) reported that an acidic protein would not stain unless the recycling process was performed, and attributed this to the presence of dichromate ions since during the recycling process dichromate was omitted. Guevara *et al.* (1982) suggested the surroundings of the protein band may effect the development of the silver deposition *via* the Tollen's reaction. High proton concentration could inhibit the Tollen's reaction, thus preventing the formation of silver granules. The formation of silver specks were proposed to follow the Gurney-Mott (Hamilton, 1966, James, 1966) and Mitchell (Hamilton, 1966, James, 1966, Mitchell, 1978) concentration theories for photographic emulsions. High proton concentration within the protein structure, which constitutes an unfavorable environment could, in the presence of dichromate ions, thus inhibit the development step. Strongly oxidative dichromate ions would compete with the weaker silver ions for electrons of formaldehyde. Since the photochemical silver stain employs the more neutral silver nitrate solution rather than Tollen's reagent, the electrochemical environment is probably more important during the redox reaction. As in pepsin, high amounts of serine residues could provide an unfavorable environment. Accordingly, cytochrome C with the least amount of serine stained the heaviest. These two observations are in accord with the finding that silver complexes are colorless. It should be noted however, the abundance of amino acids were considered in terms of mole percentages rather than absolute numbers. Proteins with higher total number of serines than found in pepsin still stain rather well. Nonetheless, the availability of amino acids is another contributing factor, *i.e.*, the structure of a given protein needs also to be considered. This has been demonstrated by comparison of staining intensities of proteins in their native and denatured states (Heukeshoven and Dernick, 1985). All proteins in the present study were denatured by boiling in presence of SDS to minimize the availability problem.

Comparison of silver intensities as measured by atomic absorption (Table III) with densitometric intensities (Table IV) shows a close parallel. Except for ovotransferrin, the same order of intensities are observed. Negatively staining pepsin has also the lowest silver intensity. This behavior could indicate that not enough silver was deposited to reach the critical density to allow the silver granules to grow large enough to become translucent. This is certainly a factor but in the case of pepsin, but is challenged by two lines of

evidence. First, the silver contents of proteins in Fig. 9 are comparable to that of pepsin (Fig. 10). Furthermore, if gels were developed long enough, control lanes without any protein stained into light shades of brown while pepsin bands remained colorless. Thus, even though a certain silver concentration may be required to overcome a critical size for silver speck formation, it is certainly not the only or sufficient factor for visible stain formation.

In Vitro Demidation of Human Triosephosphate Isomerase

In the first part of this work the *in vitro* deamidation of TPI was examined. Data were presented on how deamidation is affected by pH, temperature, ion type, ionic strength and presence of substrate; as well as by the protein (*i.e.*, TPI concentration and the structure of the enzyme). As a natural extention, the effect of deamidation on the enzyme structure was investigated.

At 4°C, changes in hydrogen ion concentration ($6.0 \leq pH \leq 10.0$) had no significant effect on the rates of deamidation. At 37°C, decreases in hydrogen ion concentration ($5.0 \leq pH \leq 7.0$) were equally ineffective. However, more alkaline conditions (pH 10.0) caused sharp increases in deamidation rates. When the pH was held constant and the temperature varied, a similar picture emerged. Under mildly acidic (pH 5.0) and near physiological (pH 7.0) conditions increases in temperature resulted in only moderate increases in the rates of deamidation. The observed increases (about 3-fold) are lower than one might predict since rates would have been expected to have doubled for each 10°C rise in temperature. The increase in the rates of deamidation at 37°C, pH 10.0 are much higher than expected (Table V). Use of different buffers and molecular size separation electrophoresis assured the reproducibility and authenticity of this phenomenon. It also corroborates the pH dependence, *i.e.*, base catalysis mechanism of deamidation.

Deamidation could proceed by two mechanisms. In one scheme, ammonia is released following direct nucleophillic attack on the carbonyl group of the extended side chain of asparagine. The second scheme proposed by Bornstein (1970), involves ring formation (Fig. 2). The nitrogen atom of the peptide bond attacks the side chain carbonyl group of asparagine and a cyclic imide is formed. Then a second nucleophilic attack takes place at either of the ring carbonyls causing ring opening. The result is an aspartic acid in either

case, but one is an α-Asp the other a β-Asp. The resulting molecules are clearly not identical. The cyclic imide bears the same charge as the native form (asparagine) and it would not be expected to be distinguished by electrophoresis on nondenaturing alkaline gels. The charge difference can only be observed after the ring opening step. Thus, it is quite possible the rates measured here are the rates of ring opening. Experimental conditions favoring extensive deamidation suggest that more than four deamidation products per dimeric molecule of TPI can be obtained after prolonged incubation (Fig. 14, panel 3). If the ring formation mechanism is the only route of deamidation then one would expect only four deamidations, since steric hinderance from the side chain of the neighboring residue is a consideration (Bornstein, 1970). The remaining asparagines of TPI are followed by tryptophan, valine, glutamine, cysteine, leucine and alanine. With the exception of alanine, all have rather bulky side chains (Table VIII). Even under conditions promoting deamidation, isozymes with more than four deamidations per dimeric molecule of TPI appear rather late in the course of the reaction, indicating (some) steric hinderance. Nevertheless, their presence suggests that ring formation may not be as sterically hindered as one might expect, or ring formation and opening is not the only mechanism for deamidation.

Recently, TPI was reported to be a substrate for protein carboxymethyl transferases (Aswad, 1986). Only peptides with iso-aspartyl bonds (peptide linkage *via* β-carboxyl gorup) are substrates of these transferases (Aswad, 1984, Murray *et al.*, 1984). Indeed, for the methylation of porcine adrenocorticotropin (ACTH), deamidation of Asn-25 was a prerequisite (Aswad, 1984). In the case of TPI, methylation gradually increased with increasing duration of alkaline pretreatment of TPI (Aswad, 1986). Progressive deamidations of TPI in alkaline solutions are shown by the present study as well as by previous work of Yuan *et al.* (1981). Taken together, they suggest that the cyclic imide formation is one mechanism of deamidation of TPI, especially for the most rapid deamidations of Asn-15 and Asn-71, both of which involve the Asn-Gly sequence.

Enhanced deamidation at lower protein concentrations (Fig. 17) and in the presence of denaturants (Fig. 24) support the interpretation that the labile asparagines become more freely exposed to the solvent as the dimer dissociates. These labile asparagines are at the contact sites and the dissociation of the dimer assists nucleophilic attack and deamidation *via* increased accessibility to solvent molecules.

The effect of deamidation on the structure of TPI was examined to determine if deamidation was accompanied by any gross changes in structure which might "cause" the

TABLE VIII

AMINO ACID SEQUENCES AROUND ASPARAGINES OF TRIOSEPHOSPHATE ISOMERASE(*)

V	G	G	N	011	W	K	M
W	K	M	N	015	G	R	K
R	K	K	N	020	L	G	E
G	A	Q	N	065	C	Y	K
K	V	T	N	071	G	A	F
H	A	L	N	118	E	G	L
A	Y	E	N	166	V	W	A
L	K	S	N	195	V	S	A
D	L	I	N	245	A	K	Q

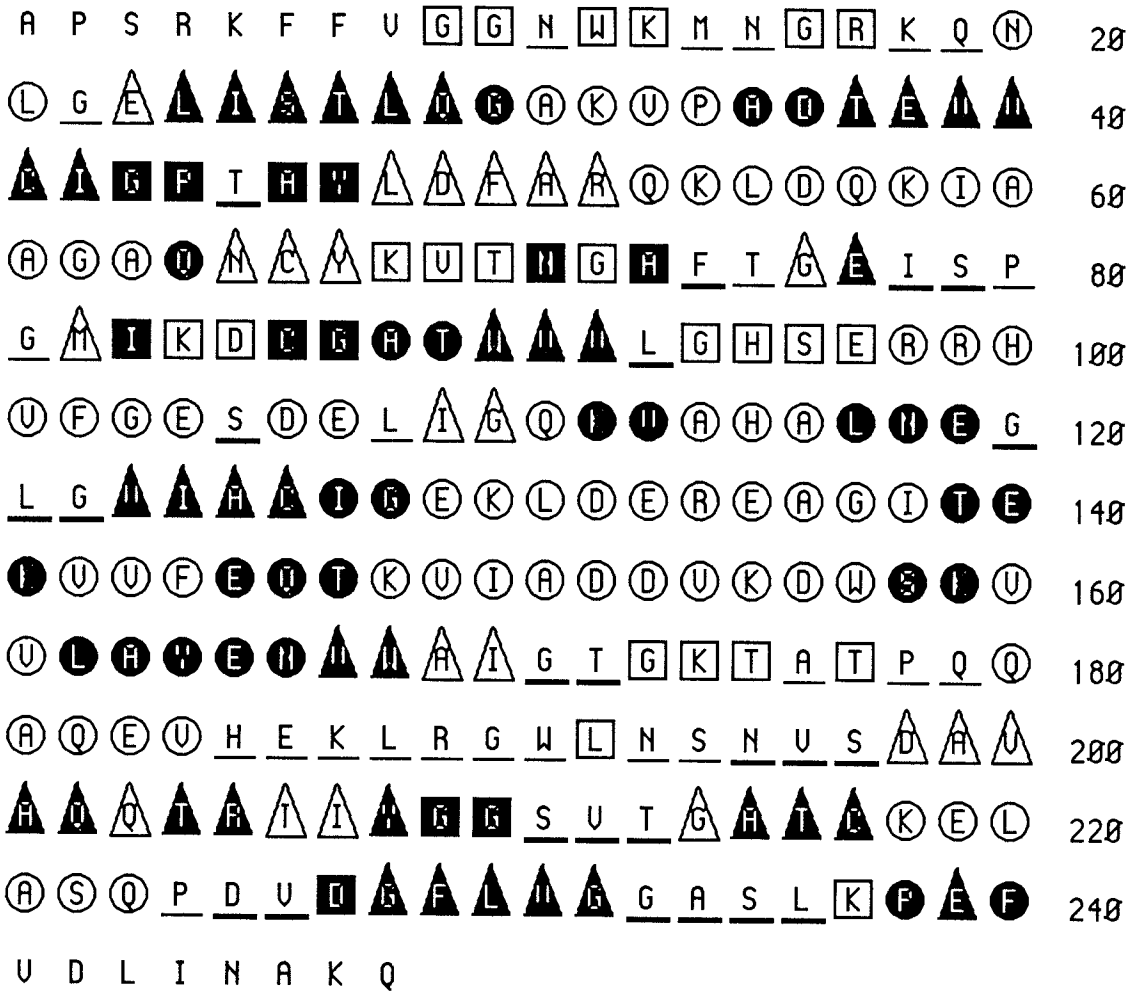
* Asparagines known to deamidate are underlined. The numbers in superscript are the positions of the amino acids in the published sequence (Lu *et al.*, 1984).

deamidated isozymes to become "recognized" as altered proteins. Denaturation and renaturation profiles of TPI on transverse urea gradient gels did not reveal any significant differences (Fig. 23). This suggests that deamidation did not invoke any major changes in the structure, *i.e.*, the potential energy profile of TPI. A computer simulation of the effects of deamidation on the secondary structure is shown in Fig. 28. Deamidation of Asn-15 changes the projected conformation (random coil→β-turn) along with that of Glu–23 (β-sheet→α-helix). Deamidation of Asn-71 on the other hand, does not effect its own predicted tendency, but is predicted to alter the positions of Asn-65 (β-sheet→β-turn) and Thr-75 (random coil→β-sheet). Apparently the folding and unfolding patterns of TPI isozymes were not influenced to an extent to become detectable by analysis on transverse urea gradient gels.

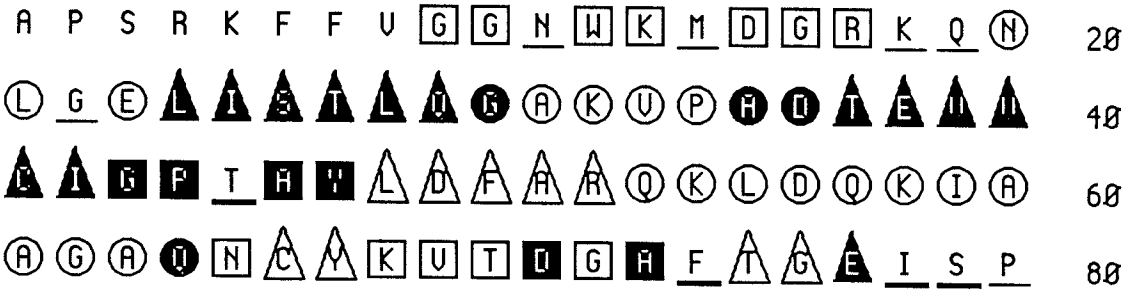
In general, substrates are considered to protect enzymes from proteolysis and denaturation, both *in vivo* and *in vitro* (Holzer, 1980). Surprisingly, the presence of the substrate destabilized TPI *via* deamidation. Deamidation increased as the number of catalytic turnovers increased. The facilitation by substrate was in addition to the increases observed due to the increases in pH. The exact mechanism of substrate involvement in deamidation is not known. However, a model can be proposed on the following basis: (a) the deamidating asparagines are at the subunit-subunit contact sites (Yuan *et al.*, 1981), (b) there is isologous contact between Met-14 of one subunit and the loop consisting of residues 70-80 of the other subunit (Alber *et al.*, 1981), (c) Lys-13 interacts with the carbonyl groups of the substrate, DHAP (Petsko *et al.*, 1984), and (d) substrate binding causes movements in the active site region, hence, changes the microenvironment around Asn-15 and Asn-71 (Alber *et al.*, 1981, Petsko *et al.*, 1984). Thus the proposed model is: the greater extent of deamidation observed with increasing substrate to enzyme ratio (Fig. 20) implies the geometry of the transition state makes the side chain amide groups of Asn-15 and Asn-71 particularly vulnerable to nucleophillic attack. That is to say that the side chains of these specific asparagines, either in the free amide or the cyclic imide form, are brought into spatial configurations favorable for deamidation. As the number of catalytic turnovers increases, *i.e.*, the enzyme passes through the transition state(s) more often, the greater the probability of a nucleophilic attack, and deamidation. In essence, the enzyme is being "worn out" as it performs its metabolic duty. This property makes TPI the first example of the "wear and tear" theory of aging at the molecular level.

Figure 28. Secondary structure prediction maps for human TPI. Secondary structure for human TPI-B and TPI-A_2 were predicted according to Garnier *et al.*(1978) using Mac Gene software on an Apple MacIntosh computer. Hydrophaty values are calculated according to Kyte and Doolittle (1982) using the same software. The structure of the first and last eight residues are not predicted due to algorithm constraints. The amino acid sequence of Lu *et al.* (1984) was used, except Lys-19 was corrected as Glu-19 based on independent sequencing studies during the course of this work. This change is in accord with the inferred sequence of Maquat *et al.* (1985) and the partial amino acid sequence of Decker and Mohrenweiser (1986). Legend: (o) α-helix, (Δ) β-sheet, (□) β-turn, underlined random coil, (black) inside, (white) outside. Amino acids are given in the standard single letter code.

PROTEIN STRUCTURE OF TPI-B



PARTIAL PROTEIN STRUCTURE OF TPI-A2



In Vivo Deamidation of Triosephosphate Isomerase

While the simultaneous study of the *in vitro* and *in vivo* aspects of the specific deamidations of TPI are useful, there appear to be some distinct differences in the *in vivo* and *in vitro* processes. For example, the half lives of deamidation *in vivo* are almost 10 times shorter than those under comparable *in vitro* conditions (in the absence of substrate). Secondly, the appearance of isozymes A_1 and A_3 are found only in trace amounts *in vivo* (Fig. 25). This contrasts the sequential behavior of deamidation *in vitro* and in aging erythrocytes (Gracy, 1982). One explanation is that in growing cells the deamidation rates of TPI-B and A_2 are substantially slower than the deamidations of TPI- A_1 and A_3, so the forms corresponding to TPI-A_1 and A_3 are not observed. This explanation is corroborated by the finding that the radioactivities of TPI-B and A_2 were more similar to each other than one would predict based on the isozyme ratios reported in the literature (Rozacky *et al.*, 1981, Snapka *et al.*, 1984) and the observed half life of deamidation for TPI-B (Table VII). Similarly, if the deamidation of TPI-A_2 is slower than TPI-B this would lead to the accumulation of TPI-A_2, hence higher levels of radioactive counts in this isozyme. This is based on the assumption that TPI-A_2 is in a dynamic equilibrium and is only formed as a transient intermediate before further degradation takes place. During the accumulation of TPI-A_2, radioactivity of TPI-B would decline steadily. Another explanation would be the cosynthesis of these two isozymes. Cosynthesis, however, would complicate the picture in that the observed radioactivity for TPI- A_2 would originate both from *de novo* synthesis of TPI-A_2 as well as from successive deamidations of TPI-B. Although cosynthesis cannot be ruled out entirely, there is no evidence for this phenomenon in the literature. As discussed in the Introduction section, a cell proliferation associated isozyme of TPI has been reported in lymphocytes. In the hands of Mohrenweiser and coworkers, this isozyme migrated more negatively than TPI-A_4. This appears not be the case here, as the observed bands comigrated with TPI-A_2 purified from human placenta, run on parallel lanes. As the electrophoretic procedures of these two studies are almost identical, resolution should not be a problem.

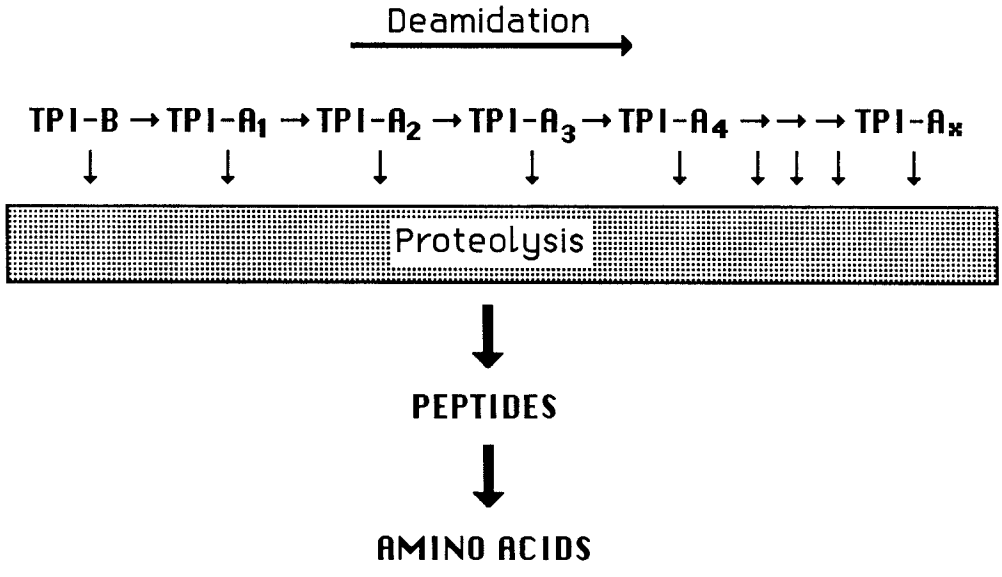
Comparison of half lives of the TPI isozymes in fibroblasts from donors aged 10 to 84 years (Fig. 27) indicates longer half lives for TPI-B and A_2 towards maturity and a decline in old ages. There are, however, differences in the ratios of the half lives of the isozymes at different ages. In fibroblasts from younger donors (*e.g.* 10 years) the rates of

deamidation for TPI-B are slower than for TPI-A_2. This difference disappears towards maturity (≈32 years) and reverses in cells from older donors (*e.g.* 81 years).

The shift in the isozyme ratios is probably affected by a multitude of factors. One is the decrease in the proteolytic activity in the older cells. As the cells age, the deamidated form(s) are probably not cleared as effectively as in young cells. Another explanation which may clarify both the change in ratio as well as the actual change in the half lives, can be formulated by combining the substrate effect on *in vitro* deamidation demonstrated in the present study with the findings of Tollefsbol *et al.* (1981). They reported that when cells were kept in culture, TPI activity per viable cell increased in young cells and decreased in old cells. Taken together the following can be postulated: as TPI activity decreases in old cells, each TPI molecule has to perform a larger number of catalytic reactions than their counterparts in the young cells. Higher number of catalytic turnovers enhance deamidation, thus resulting in more "worn out" molecules to be degraded. Concomitantly, slower proteolysis in older cells further enhances the accumulation of the worn out (*i.e.*, deamidated) isozyme(s). The end result is an apparent decrease in the half life of TPI-B concomitant with an apparent increase for TPI-A_2. This explanation is in accord with the results of the *in vitro* deamidation studies and consistent with the "wear and tear" theory of aging.

Starvation is known to stimulate proteolysis as observed in human fibroblasts (Gronostajski *et al.*, 1984, Berger and Dice, 1986, Slot *et al.*, 1986), in rat liver (Harikumar and Ninjoor, 1985) and rat skeletal muscle (Fagan and Tischler, 1986). This stimulation is counteracted by insulin (Trowbridge and Draznin, 1983, Maksoud *et al.*, 1984, Tischler *et al.*, 1986). Recently, Gracy *et al.* (1985) reported on the effects of starvation and insulin on the isozyme patterns of TPI in human fibroblasts. Starvation lowered the levels of TPI-A_2, while the addition of insulin raised TPI-A_2 concentration back to prestarvation levels in a dose dependent manner. In the same study an age dependent decline in cathepsin B was observed. This information suggests impaired proteolysis as one of the major reasons for the accumulation of postsynthetically modified proteins in aging cells. If impaired proteolysis and enhanced deamidation due to lower TPI levels in aging are considered together, the degradation scheme illustrated in Fig. 29 may be postulated. In other words, any of the TPI isozymes can be proteolytically degraded (probably each at a different rate). Also, each form is simultaneously deamidating (again at different rates). In the aging cell proteolysis may be slowed while the deamidations proceed without any changes or with increased rates (see previous paragraph). If there are

Figure 29. Proposed scheme of TPI degradation *in vivo*. Rates of deamidation and proteolysis of each isozyme is not implied to be the same.


Deamidation
TPI-B → TPI-A1 → TPI-A2 → TPI-A3 → TPI-A4 → → → TPI-Ax
Proteolysis
PEPTIDES
AMINO ACIDS

no changes in the deamidation rates, TPI-A_2 would accumulate only because it is not efficiently degraded. On the other hand, if TPI-B deamidates faster in aging cells due to lower TPI activity per cell, TPI-A_2 would accumulate because it is formed at higher rates and is degraded less efficiently. This would result not only in higher levels of deamidated forms in aging cells and under conditions of reduced proteolysis, but would also prolong the apparent half life of TPI-A_2. This is in concert with the theory presented in the previous paragraph.

If indeed, the deamidation of TPI-B is enhanced by higher number of catalytic turnovers in older cells, this suggests also the faster deamidation of TPI-A_1. TPI is a homodimer and there is no reason to believe that the same two asparagines (*i.e.*, Asn-71 of both subunits), under the same environmental conditions should deamidate with different rates. The amount of TPI-A_1 observed is probably a reflection of the statistical distribution of how many singly deamidated molecules are present at a given time. This also applies to TPI-A_2 and A_3. The miniscule amounts of the further deamidated forms present *in vivo* are probably the result of their instability or their recognition as "altered" proteins by the cellular system and thus, proteolytic cleavage.

Outlook

In this study the *in vivo* and *in vitro* deamidations of triosephosphate isomerase were examined. *In vitro,* the effects of pH, temperature, substrates, inhibitors, protein concentration and denaturants were analyzed. *In vivo,* the rates of deamidation in different cell lines and their dependence on the cell age were monitored. When the *in vivo* rates of deamidation were compared with *in vitro* rates of deamidation at 37°C, pH 7.0, a large difference was observed. However when the substrate was addaed to the incubation medium, this difference was largely eliminated. Thus, the major conclusions of this study were that *in vitro*, the enzyme deamidates more rapidly while performing its metabolic function; and the *in vivo* accumulation of the deamidated forms in the old cells is probably due to increased metabolic wear of the enzyme in addition to impaired proteolysis.

The results of this study together with the previous knowledge on TPI, makes the post-synthetic modifications of this enzyme far better understood than many others. There are however a number of other questions remaining in this regard.

At the molecular level, the possible interaction of the deamidation sites are of great interest. The chicken enzyme which contains Asn-15 but lacks Asn-71, does not exhibit deamidation bands. In the human enzyme, Asn-71 appears to deamidate more readily than Asn-15 (Yuan *et al.*, 1981). Thus, the question arises, is the deamidation of Asn-71 somehow a prerequisite for the further deamidation steps? To obtain a partial answer, TPI was deamidated in the presence of denaturants (Fig. 24). This revealed that availability of the labile asparagines to the solvent is a factor. One way of answering this question is by site specific mutagenesis, but a more practical way is the hybridization of the chicken enzyme with human and/or rabbit enzyme. The hybridization studies should reveal if Asn–15 of the chicken subunit will deamidate when it is juxtaposed by Asn-71, and also indicate if the converse is true, *i.e.*, if Asn-15 of the human subunit will deamidate when it lacks the opposing Asn-71.

Another point of interest is the sequential deamidation of TPI. It is not known if deamidation proceeds as Asn-71→15→71→15 or as Asn-71→71→15→15. Assuming there is no communication between the two sites, there is no reason to believe that two identical residues, one on each subunit, will deamidate at different rates. Ideally they should deamidate at the same rate, thus, TPI-A_1 and A_3 would not be observed. However, deamidation does not happen in discrete steps but represents the probability distribution of the nucleophilic attack. Experimental determination of the sequence of deamidation may be difficult. Direct determination of the presence of an aspartic acid or asparagine by protein sequencing is complicated by the fact that experimental conditions during protein sequencing themselves promote deamidation. Thus, one could not guarantee that deamidations of Asn-15 and Asn-71 would be effected equally. Less destructive methods such as nuclear magnetic resonance spectrophotometry, possibly after ^{13}C-tagging of the β-Asp with protein methyl transferases, could to be explored as a possible alternatives.

At the cellular level, it might be interesting to determine the nature of the deamidation of TPI in "immortal" cell lines. Deamidation half lives in transformed cells, especially if there is any relation to the chronological age of the cell line, may provide interesting insights into the deamidation process. The modulation of deamidation by metabolic rates can be investigated from several points. Initially, the rates of deamidation during

exponential growth and near confluency need to be studied. Secondly, the effect of starvation on the deamidation of TPI in growing and resting cells can be examined. Next, one might combine the effect of metabolic rates with the effect of aging by repeating the above exp[eriments in cells from aged donors. Finally, effects of insulin may be studied under the conditions listed above. The study can be further expanded by studying triglyceride formation under the same conditions. Double labelling experiments with [^{3}H]leucine and [U-^{14}C]glucose would provide simultanous monitoring of TPI and triglycerides.

The hypothesis of increased deamidation with incresing metabolism needs to be tested in animal models. A multi-faceted study can be set up by (a) first investigating the effect of physical activity (animals kept in groups without space restriction *versus* kept individually in confined spaces), then (b) imposing dietary restrictions under the conditions of (a), and finally (c) conducting experiments on animals from different ages. The knowledge gained from these studies can be greatly enhanced if samples from different tissues, such as those known to exhibit different modulation of proteolysis (*e.g.* extensor digitorum longus *versus* soleus muscles) are analysed. The study can be further expanded by analysing samples from animals treated with dihydroepiandosterone (DHEA) to mimic fasting (Schwartz *et al.*, 1985) and with streptozotocin to induce diabetes (Tischler *et al.*,1986).

In summary, the knowledge gained on *in vitro* and *in vivo* processes of this type of post-synthetic modifications in a single protein for which the structure is clearly understood can provide a model system to examine the basic mechanisms of the regulation of protein degradation and how these processes may be altered during aging.

Furthermore, one can determine the proteolytic products of TPI. What cellular fraction or what types of proteases are involved in the metabolism of TPI? Starvation studies suggested the involvement of the lysosomal pathway. One should bear in mind however, that starvation stimulated proteolysis proceeds *via* the lysosomal pathway and does not necessarily reflect the mechanism of the non-starved basal state. Incubation of purified TPI with subcellular fractions in the absence and presence of known protease inhibitors could shed light on this process. Microinjection of active site-labelled TPI and monitoring of the proteolytic fragments, possibly in concert with immunological methods could be very informative. Along the same lines, the site of initial proteolytic cleavage can be investigated. Labelling of the active site and monitoring of the resulting proteolytic fragments with simultaneous high sensitivity microsequencing could provide invaluable information on the catabolism of TPI and that of proteins in general.

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