

The Active Centre of Triose Phosphate Isomerase

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The molecular weight and amino acid composition of triose phosphate isomerase have been determined. The molecular weight (43 000) is lower and the molecular activity (500 000) higher than those of most other glycolytic enzymes. Reaction with iodoacetate (studied with radioactive reagent) takes place in two phases: in the first phase, at pH 6.3, cysteine and methionine groups react and enzymic activity is unimpaired; in the second phase, histidine reacts and enzymic activity is lost. Photo-oxidation leads to inactivation, with loss of cysteine, of histidine and of tryptophan, but little loss of tyrosine. The mechanism postulated for the action of the enzyme demands the intervention of a group functioning as a base, and the results obtained are consistent with histidine's being the basic group in the active centre.

Triose phosphate isomerase (D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1) has been crystallized from calf muscle (Meyer-Arendt, Beisenherz & Bücher, 1953; Beisenherz, 1955) and from rabbit muscle (Czok & Bücher, 1960), but there is little published information about the enzyme as a protein. The catalytic properties of triose phosphate isomerase have received rather more attention. Oesper & Meyerhof (1950) studied the rate of approach to equilibrium, and noted inhibition by phosphate ions. The enzyme also catalyses the incorporation of one atom of tritium into dihydroxyacetone phosphate in tritiated water (Rieder & Rose, 1959). The tritium is incorporated stereospecifically into the carbinol group. This tritium atom is lost to the medium in the presence of triose phosphate isomerase even when the conversion into glyceraldehyde 3-phosphate is made irreversible. From these and other experiments Rieder & Rose (1959) concluded that the reaction proceeded by a proton transfer, and that an enzyme-bound enediol was an intermediate.

In the related reaction catalysed by glucose 6-phosphate isomerase there is good evidence that the enzyme itself abstracts a proton from the substrate (Rose & O'Connell, 1961; Rose, 1962) and so we may expect that a basic group in these enzymes plays a crucial role in catalysis. The experiments described below were directed towards identifying this basic group.

METHODS

Assay of triose phosphate isomerase. The enzyme from rabbit muscle, obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) or C. F. Boehringer und Soehne G.m.b.H.

(Mannheim, Germany), was assayed by a slight modification of the method of Beisenherz (1955). The reaction mixture contained the following constituents (final concentrations) in a volume of 3 ml.: NADH (83 μ M), DL-glyceraldehyde 3-phosphate (0.4 mM) and α -glycerophosphate dehydrogenase (0.002 mg./ml.) in 0.02 M-triethanolamine hydrochloride buffer, pH 7.9, containing EDTA (5.4 mM). Triose phosphate isomerase (30–50 μ g. of protein in 20 μ l.) was added, and the decrease in E_{340} measured for 4 min. The DL-glyceraldehyde 3-phosphate was prepared (Racker, Klybas & Schramm, 1959) by adding 2 ml. of Dowex 50 (H⁺ form) to 50 mg. of the barium salt of the diethyl acetal (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., or Sigma Chemical Co.) in 2 ml. of water. The suspension was kept for 3 min. at 100° with a stream of N₂ passing through, and then filtered and the resin washed. The solution (volume about 3 ml.) was assayed by measuring P_i before and after treatment with alkali; the concentration of alkali-labile P_i was about 30 mM.

The unit of activity is the number of μ moles of NADH transformed/min. at 25°. One μ mole of NADH had $E_{340}^{1\%}$ 2.07 under these conditions (Dawson, Elliott, Elliott & Jones, 1959). The specific activity of the enzyme was usually in the range 1200–2000 units/mg., but values as high as 3000 units/mg. were sometimes obtained. The value of the specific activity depended on the batch of glyceraldehyde 3-phosphate, but varied little for different batches of the enzyme. We have not been able to ascertain the cause of the variability of the substrate. The unit as defined here is related to the conditions of assay, and is comparable with the unit used by previous workers and by suppliers of the enzyme; the value of the unit should be multiplied by 3.5 to obtain values at an optimum concentration of D-glyceraldehyde 3-phosphate. This factor was determined by carrying out assays with various concentrations of the substrate.

Detection and measurement of radioactivity. An end-window Geiger-Müller tube was used, and sufficient counts were recorded for the standard error to be less than 5%; under the usual conditions, 1 μ C of ¹⁴C gave about 2000

counts/sec. Radioautography was carried out with Kodak Royal Blue X-ray film for 10–50 days.

Estimation of thiol groups. This was carried out by the method of Ellman (1959). Samples were dissolved in 0.75 ml. of 4M-guanidine hydrochloride containing EDTA (1mM), and 0.25 ml. of a solution of di-(5-carboxy-4-nitrophenyl) disulphide (3.96 mg./ml.) in 0.1M-phosphate buffer, pH 7.6, was added. After 15 min. E_{412} was measured in a Bausch and Lomb Spectronic 20 spectrophotometer. The molar extinction coefficient of the coloured thiol formed had the usual value of 13600 in this medium, as has also been found by Miller & Metzger (1965).

Estimation of tryptophan. The method of Spies & Chambers (1949) was used on a smaller scale; 0.4 ml. of 23.7N-H₂SO₄ and 0.05 ml. of *p*-dimethylaminobenzaldehyde (30 mg./ml. in 2N-H₂SO₄) were mixed and cooled, and then 0.05 ml. of sample was added. The mixture was kept at 28° overnight in the dark, and then treated with 5 μ l. of 0.045% NaNO₂, and after 30 min. E_{590} was measured.

Paper chromatography. Two (descending) systems were used: butan-1-ol-acetic acid-water (40:9:20, by vol.) (Waley, 1956) on Whatman no. 4 paper, and butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953) on Whatman no. 1 paper.

Paper electrophoresis. This was carried out at 10 v/cm. on a ridge-pole apparatus on 39 cm.-long strips of Whatman no. 3 paper in pyridine-acetate buffer, pH 4 (Grassmann, Hannig & Plöckl, 1955), or collidine-acetate buffer, pH 7 (Newton & Abraham, 1954).

Estimations of histidine and of tyrosine. These were carried out after paper chromatography of hydrolysates by the method of Fraenkel-Conrat & Singer (1956). Leucine and isoleucine were estimated together after paper chromatography by Bode's (1955) method.

Carboxymethylhistidines. *N*^c-Acetylhistidine (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) was alkylated with iodoacetate, and the acetyl group removed by hydrolysis, as described by Crestfield, Stein & Moore (1963). The acid solution was extracted five times with chloroform before evaporation; water was then added and the evaporation repeated, three times. The residue, in 2 ml. of water, was fractionated by paper electrophoresis at pH 4: unchanged histidine migrated towards the cathode, carboxymethylhistidine did not migrate and dicarboxymethylhistidine migrated towards the anode with a mobility slightly greater than that of aspartic acid. The bands were eluted and the eluates concentrated. The two monocarboxymethylhistidines were apparently not resolved by paper chromatography in the solvents used but were separated by paper electrophoresis at pH 7; from the results of Crestfield *et al.* (1963) the isomer formed in larger amounts is 3-carboxymethylhistidine.

Carboxymethyl-lysines. Polylysine hydrobromide (Yeda Research and Development Co., Rehovoth, Israel) was alkylated at 40° at pH 10 for 4 hr., as described by Gundlach, Stein & Moore (1959). The solution was dialysed for 2 days, and the non-diffusible fraction was evaporated and hydrolysed in 6N-HCl at 110° for 16 hr. After removal of HCl, the residue was fractionated by paper electrophoresis at pH 4; monocarboxymethyl-lysine did not migrate and dicarboxymethyl-lysine migrated towards the anode.

Carboxymethyltyrosine. This was prepared by the method of Korman & Clarke (1956), except that *N*-formyltyrosine (Waley & Watson, 1954) replaced *N*-acetyltyrosine. After

hydrolysis, the *O*-carboxymethyltyrosine was isolated by paper electrophoresis at pH 4.

Carboxymethylhomocysteine. A solution containing *S*-carboxymethylhomocysteine was obtained by hydrolysis of methionine carboxymethyl sulphonium iodide (Gundlach, Moore & Stein, 1959), followed by paper electrophoresis at pH 4. The products of hydrolysis are homoserine, homoserine lactone, methionine and *S*-carboxymethylhomocysteine, but only the last of these will migrate towards the anode at pH 4. Carboxymethyl methyl sulphide may also be present, but is not ninhydrin-positive.

Starch-gel electrophoresis. This was carried out in a discontinuous buffer system (Poulik, 1957). The gel from hydrolysed potato starch (Connaught Medical Research Laboratories, Toronto, Canada) was cast in a block (10 cm. \times 18.5 cm. \times 0.6 cm.) in 76 mM-tris-5 mM-citric acid buffer, pH 8.8, and the buffer in the electrode vessels was 0.3M-boric acid-0.05N-NaOH, pH 8.0. The samples of enzyme were dialysed against the gel buffer and then mixed to a thick paste with (unhydrolysed) starch grains (Smithies, 1955) and introduced into a 6 cm. \times 0.3 cm. slot cut 6 cm. from the end of the gel. After electrophoresis for 7 hr. at 17 v/cm., one side of the gel was cut off and stained with Naphthalene Black; segments from the rest of the block were eluted with 0.05M-borate buffer, pH 7.9.

Photo-oxidation. This was carried out in flat-bottomed tubes, 5 cm. \times 2 cm. diam., or 6.5 cm. \times 3.3 cm. diam., illuminated by a 150 w lamp clamped immediately above the tube. The tube was maintained at a constant temperature in a water bath at about 20° by a copper cooling coil, and the contents were stirred.

RESULTS

Properties of triose phosphate isomerase

Starch-gel electrophoresis. Electrophoresis in starch gels at pH 8.6 and pH 5.6 was investigated. There was one band migrating as a cation at pH 5.6 and moving very little towards the anode at pH 8.6. These results are consistent with those of Czok & Bücher (1960), who used starch-block electrophoresis at pH 8.2, and suggest that triose phosphate isomerase is a fairly basic protein. The best results were obtained with the discontinuous system of Poulik (1957) (see the Methods section.).

The side of the gel stained for protein showed one strong band, and two faint bands just ahead (Fig. 1). The results of elution and enzymic assay showed that the activity paralleled the amount of protein; the small bands were also active, and may represent modified enzymic species altered during isolation, or genetic variants. The results indicated that the preparation was nearly homogeneous, and no further purification has been attempted.

Molecular weight. The molecular weight of triose phosphate isomerase was estimated by exclusion chromatography on Sephadex G-100 (Andrews, 1965). Cytochrome *c* (4.7 mg.) (British Drug Houses Ltd., Poole, Dorset), bovine serum albumin (7 mg.) (Armour Pharmaceutical Co., Eastbourne,

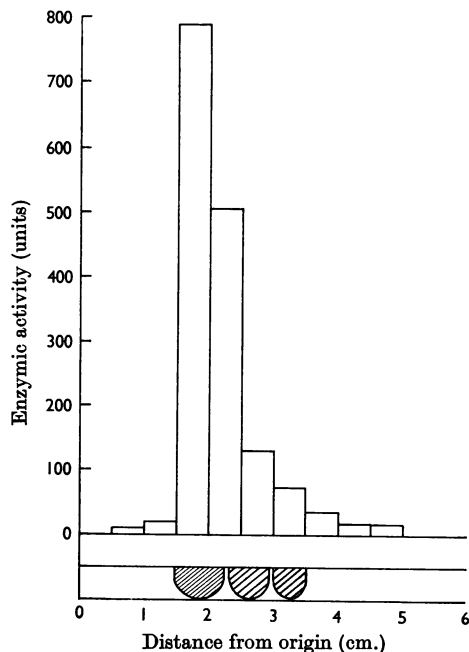


Fig. 1. Starch-gel electrophoresis of triose phosphate isomerase. The enzyme (5 mg.) was introduced into a slot 6 cm. from the end of the gel and electrophoresis carried out for 7 hr. at 17 v/cm.; details are given in the text. The lower part of the Figure shows the results of staining one side of the block for protein with Naphthalene Black. The upper part shows the enzymic activity in eluates of successive segments of the block.

Sussex), bovine γ -globulin (7 mg.) (Armour Pharmaceutical Co.) and ovalbumin (6 mg.) (grade V; Sigma Chemical Co.) were dissolved in 2 ml. of buffer [0.02M - triethanolamine hydrochloride, pH 7.8, containing EDTA (5.4 mM)]. A portion (20 μ l., containing 0.2 mg. of protein) of the stock suspension of triose phosphate isomerase was added, and the mixture applied to a column (52 cm. long \times 3 cm. diam.) of Sephadex G-100 and eluted with the same buffer. The albumins and globulin were detected by E_{280} , cytochrome *c* by E_{412} and the enzyme by assays (Fig. 2). A plot of elution volume against the logarithm of the molecular weight (Andrews, 1965) gave the molecular weight of the enzyme as 43 000.

Amino acid analysis. A portion (0.6 ml.) of the suspension of (Sigma Chemical Co.) enzyme (nominally 10 mg./ml.) in ammonium sulphate was dialysed against water for 3 days and evaporated to dryness, and the residue was hydrolysed with redistilled 6N-hydrochloric acid at 110° for 23 hr. under the conditions recommended by Moore & Stein (1963). The results are given in Table 1.

Peptide maps. A salt-free solution of triose phosphate isomerase (1%) was heated at 100° for 4 min., and the mixture then made 1% in ammonium hydrogen carbonate and 0.01% in trypsin (twice crystallized; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and incubated at 40° for 1.5 hr. Further trypsin was added to make the solution 0.015% and incubation continued for 1 hr. more. The clear solution was evaporated to

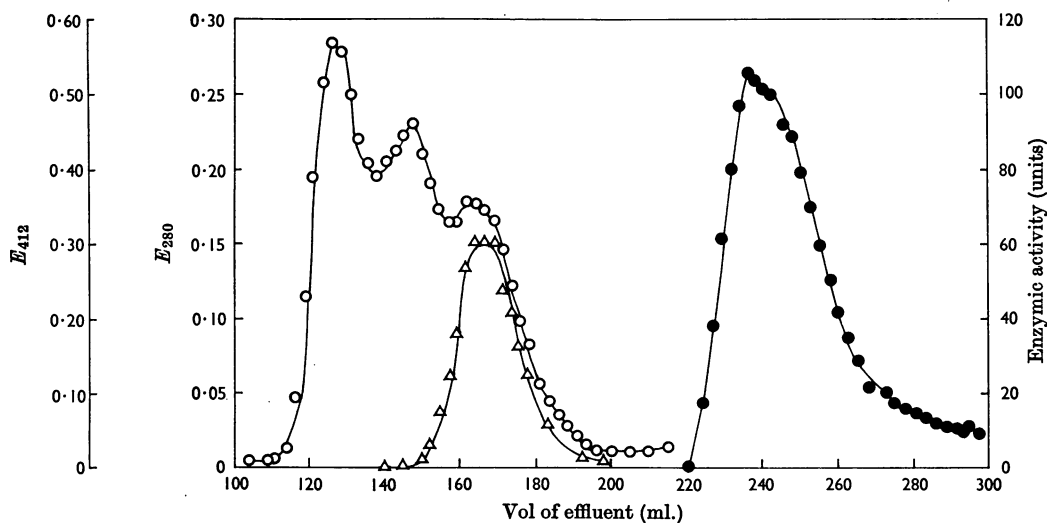


Fig. 2. Exclusion chromatography of triose phosphate isomerase and marker proteins on Sephadex G-100. Fractions (about 3 ml.) were collected, and E_{280} (O), E_{412} (●) and isomerase activity (Δ) were measured. Details are given in the text.

Table 1. *Amino acid analysis of triose phosphate isomerase*

Experimental details are given in the text. The integral number of residues in the last column was calculated assuming that there are 39 residues of lysine; this value was chosen to make the molecular weight calculated for the amino acid composition agree with the experimental value of 43000.

Amino acid	Analysis (μ moles found)	No. of residues
Lys	1.907	39
His	0.423	9
Arg	0.717	16
Asp	1.695	35
Thr	1.226	25
Ser	0.970	22*
Glu	2.041	42
Pro	0.580	12
Gly	2.126	43
Ala	1.964	40
Val	1.668	34
Met	0.172	4
Ile	0.951	19
Leu	1.312	27
Tyr	0.371	8
Phe	0.654	13
Trp		4†
CyS/2	0.370	8 (7‡)

* Corrected assuming 10% loss on hydrolysis.

† Determined colorimetrically on unhydrolysed protein (see the Methods section).

‡ No. of thiol groups (see the Methods section).

dryness and the residue dissolved in water (there was little insoluble 'core'); portions corresponding to 0.5–1 mg. of peptides were examined by electrophoresis on Whatman no. 3 paper at pH 4 (Grassmann *et al.* 1955) for 5 hr. at 8 v/cm. followed by chromatography for 16 hr. in butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953). The ninhydrin reagent of Bode (1955) revealed about 28 spots; the Ehrlich reagent (Smith, 1960a) revealed four or five spots containing tryptophan and a small spot at the origin, and the Sakaguchi reagent (Smith, 1960b) revealed six or seven spots containing arginine. These reagents were applied successively; the paper must be aerated thoroughly after the Ehrlich reagent. The Pauly reagent was prepared by adding 1.5 ml. of 3.7% (w/v) sodium nitrite dropwise to 15 ml. of 0.05 M-sulphanilic acid in 0.01 N-hydrochloric acid and then diluting to 50 ml. with pH 11 buffer (0.05 M in sodium carbonate and 1 mM in sodium tetraborate); after the paper had been sprayed with this reagent, it was suspended in a tank containing ammonia vapour. Five spots showed the colour of histidine peptides. These results suggest the possibility of there being two similar sub-units in the protein.

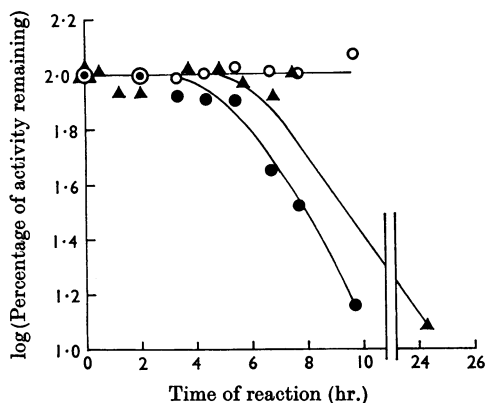


Fig. 3. Reaction of triose phosphate isomerase with sodium iodoacetate in the presence (○) or absence (●) of phosphate. A portion (0.8 ml.) of the stock (10 mg./ml.) suspension of the enzyme in $(\text{NH}_4)_2\text{SO}_4$ soln. was treated with 0.08 M-iodoacetate in 33 mM-triethanolamine hydrochloride buffer, pH 7, at 40°, in the presence of 0.1 M-sodium phosphate; a similar reaction mixture lacked phosphate. Portions (1 μ l.) of each mixture were taken for assays. After 10 hr., the mixture containing phosphate was dialysed against 0.14 M-triethanolamine hydrochloride buffer, pH 7, and then treated with sodium iodo[2- ^{14}C]acetate (72 mM, specific activity 0.52 $\mu\text{C}/\mu\text{mole}$), and the enzymic activity followed (▲) as before.

Alkylation of triose phosphate isomerase

The enzyme did not appear to react rapidly with iodoacetate but, since it was not readily denatured by heating at 40° (pH range 5.5–7) for several hours, prolonged incubations could safely be carried out. Ammonium sulphate was normally present in the reaction mixture; if it was removed by dialysis of the stock suspension of enzyme the rate of inactivation by iodoacetate decreased markedly.

The most marked feature in the alkylation of triose phosphate isomerase with either iodoacetate or iodoacetamide at pH 5.5–7 was an apparent induction period, i.e. inactivation did not start at once, but only after an interval that was sometimes of several hours. This suggested that the fastest reaction was with inessential amino acid residues, to give an alkylated but still active enzyme. Moreover, phosphate ions, which inhibit triose phosphate isomerase (Oesper & Meyerhof, 1950), prevented the inactivation at pH 7. This suggested that the active site might be labelled by the 'two-stage' method now described. The enzyme was treated with (cold) iodoacetate for 10 hr. in the presence of 0.1 M-phosphate; no activity was lost (Fig. 3). The phosphate was then removed by dialysis, and the enzyme now treated with iodo[2- ^{14}C]acetate until 90% of the enzymic activity had been lost. The mixture was then dialysed against water until the

diffusate was no longer radioactive and evaporated to dryness. The residue was hydrolysed with 6*N*-hydrochloric acid for 21 hr. at 110°. The main radioactive product was identified by paper electrophoresis (Fig. 4, right-hand side) followed by paper chromatography as carboxymethylhistidine.

Although this experiment certainly pointed towards the presence of histidine in the active centre, it was not altogether satisfactory. Prolonged incubation (25 hr.) was necessary for inactivation of the dialysed partially alkylated enzyme. There was also the possibility that part of the effectiveness of phosphate in preventing inactivation might be due to its action as a buffer, although this does not seem likely since control experiments showed that the fall in pH was insufficient to cause a marked change in the rate of reaction with iodoacetate. It did not seem advisable to use a high concentration of triethanolamine buffer to maintain the pH at 7, because alkylating agents react with amines. Further experiments were therefore carried out in 0.1*M*-3,3-dimethylglutarate buffer at pH 6.3, the pH where the buffer capacity is a maximum. At this pH, there was little protection against inactivation by phosphate ions, and so the relation between

loss in activity and alkylation of various groups was studied directly.

Four 0.05 ml. portions of the stock (10 mg./ml.) suspension of the enzyme were incubated at 40° with 73 mM-sodium iodo[2-¹⁴C]acetate (specific activity 0.54 $\mu\text{C}/\mu\text{mole}$) in 0.1 *M* - 3,3-dimethylglutarate buffer, pH 6.3; the final volume in each tube was 0.25 ml. Portions (1 $\mu\text{l.}$) were taken and diluted with the triethanolamine-EDTA buffer for assay, and at the times shown in Fig. 5 the protein in a tube was precipitated with 0.25 ml. of 20% (w/v) trichloroacetic acid, washed five times with 10% (w/v) trichloroacetic acid and dried. The radioactivity of the protein was measured by dissolving the residue in 0.1 ml. of 90% (w/v) formic acid and taking 0.05 ml. for counting. The remainder was evaporated and then hydrolysed with 6*N*-hydrochloric acid in a sealed evacuated tube at 110° for 20 hr.; the products were examined by paper electrophoresis (Fig. 4).

Although little or no activity is lost during the first 6 hr. of incubation, nearly one-third of the total radioactivity is introduced during this time (Fig. 5). Thus the original surmise that the rapidly alkylated groups are inessential is confirmed. These groups are mostly thiol groups; they are mainly in fraction *c* (Fig. 4), which consisted of carboxymethylcysteine. Although they are more reactive than the other groups, the absolute reactivity of the thiol groups is low (see the Discussion section). Fraction *b* was identified as carboxymethylhomocysteine, formed on hydrolysis of alkylated methionine residues, and the methionine too reacts relatively rapidly (Fig. 6). Fraction *d*, which migrates rapidly on electrophoresis, is not identical with any of the

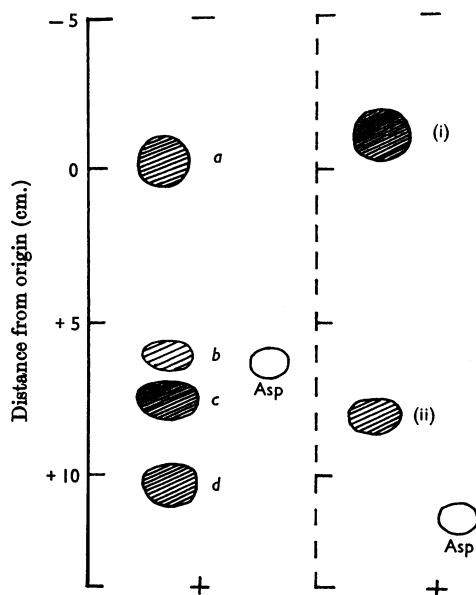


Fig. 4. Electrophoresis at pH 4 at 10 v/cm. of hydrolysis products of triose phosphate isomerase treated with sodium iodo[2-¹⁴C]acetate at pH 6.3 and 40°. The right-hand side of the Figure shows the products obtained by hydrolysis in the reaction of Fig. 3. The depth of hatching indicates the extent of blackening of the X-ray film after radioautography.

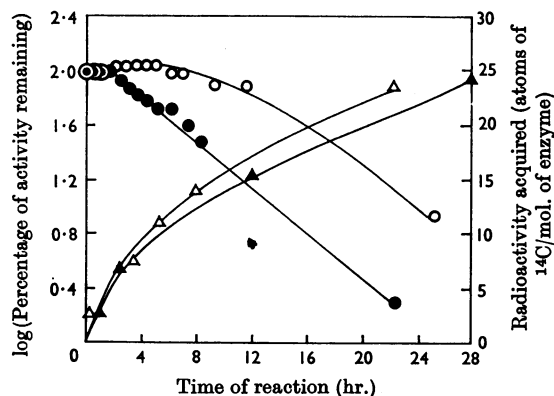


Fig. 5. Reaction of triose phosphate isomerase with 73 mM-sodium iodo[2-¹⁴C]iodoacetate at 40° at pH 6.3 (○, △) and at pH 5.5 (●, ▲). The circles (○, ●) show the enzymic activity remaining and the triangles (△, ▲) show the radioactivity acquired.

expected products, and could be an additional cleavage product from the alkylated methionine or a derivative of the *N*-terminal amino acid. Fraction *a* is mainly 1-carboxymethylhistidine (some 3-carboxymethylhistidine and carboxymethyl-lysine are also present); carboxymethyltyrosine was not formed. The extent of inactivation paralleled the amount of radioactivity in spot *a* (Fig. 6) and this suggests, although it does not prove, that the loss in activity is due to the carboxymethylation of a histidine residue.

There is a striking contrast between the relative amounts of carboxymethylhistidine from the experiment described above in the presence of phosphate and in this experiment (Fig. 4). Here only about one-quarter of the radioactivity was in the form of derivatives of histidine, but in the earlier experiment most was in this form.

There was a slight apparent gain in activity during the first 2 hr. of incubation, and this complicates interpretation of the results in this period. Further experiments were carried out at pH 5.5 in 0.08M-sodium acetate buffer; here there was apparently no early gain in activity, but the enzyme tended to be precipitated. It seems probable that the activity remained unchanged initially (Fig. 5) because the partial precipitation was balanced by an increase in activity of the enzyme that remained in solution.

Photo-oxidation of triose phosphate isomerase

Photo-oxidation was carried out in 0.09M-sodium borate buffer, pH 7.9, in the presence of 0.01% Methylene Blue; the dye, in the dark, did not inactivate the enzyme. The loss of enzymic activity was compared with the destruction of the amino acids histidine, tyrosine, cysteine and tryptophan. Histidine and tyrosine were estimated after hydrolysis as follows. Portions of the reaction mixture containing about 1mg. of protein were treated with an equal volume of 20% (w/v) trichloroacetic acid, and the precipitates, after centrifugation, were washed twice with 0.8ml. of ethanol to remove the dye and then dried. After hydrolysis in 6N-hydrochloric acid at 110°, the acid was removed by evaporation, followed by the addition of water and evaporation three times. The

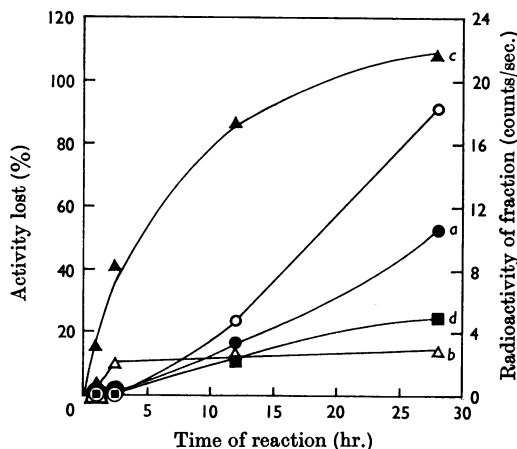


Fig. 6. Rate of formation of carboxymethylamino acids in the reaction of triose phosphate isomerase with sodium iodo[2-¹⁴C]acetate at pH 6.3. The open circles (O) show the enzymic activity lost, and the other symbols (●, Δ, ▲, ■) show the radioactivity of the fractions obtained by paper electrophoresis at pH 4 (see Fig. 4). The nature of the products is: *a* (●), 1- and some 3-carboxymethylhistidine (and possibly carboxymethyl-lysine); *b* (Δ), *S*-carboxymethylhomocysteine; *c* (▲), *S*-carboxymethylcysteine; *d* (■), unknown.

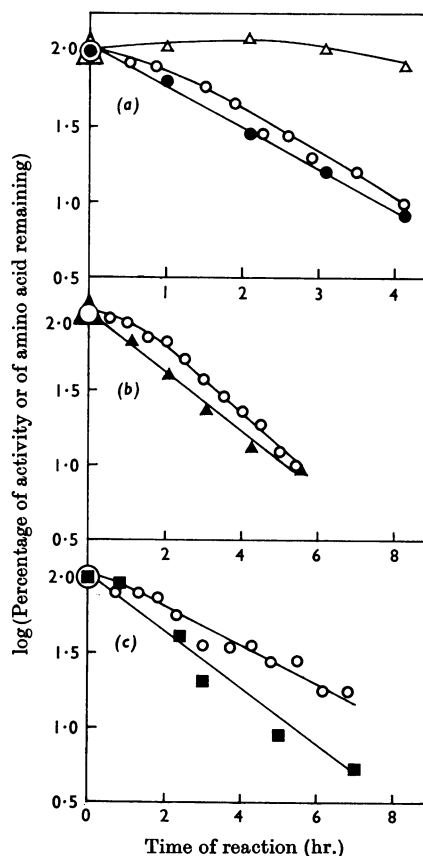


Fig. 7. Photo-oxidation of triose phosphate isomerase in 0.09M-sodium borate, pH 7.9, in the presence of 0.01% Methylene Blue: O, enzymic activity; ●, content of histidine; Δ, content of tyrosine; ▲, content of tryptophan; ■, content of cysteine.

amino acids were estimated after paper chromatography, the recoveries of histidine and tyrosine being based on the amounts of leucine plus isoleucine. Cysteine and tryptophan were estimated colorimetrically on the unhydrolysed protein. The protein was separated from Methylene Blue by exclusion chromatography on 5 cm. \times 0.5 cm. diam. columns of Sephadex G-25 equilibrated with 0.01 M-phosphate buffer, pH 7.6. Fractions (0.5 ml.) were collected and tested for protein by spotting 5 μ l. on to cellulose acetate strips and staining with 1% Naphthalene Black followed by washing with 10% (v/v) acetic acid. The fractions that contained protein were combined and evaporated. Estimations of thiol groups and of tryptophan were then carried out (see the Methods section).

The deviation from linearity in the (logarithmic) plots of activity remaining against time (Fig. 7) is probably partly due to variations in the conditions of assay; the temperature in the cell compartment of the spectrophotometer rose, and thus the loss of activity was partly offset. This complication prevents any precise correlation between the rate of inactivation and the rate of destruction of residues of a particular kind. Perhaps the most clear-cut feature is that the loss in tyrosine is too slow to account for the loss in enzymic activity (Fig. 7a). There is not a great deal of difference in the rates of destruction of histidine (Fig. 7a), tryptophan (Fig. 7b) and cysteine (Fig. 7c), and all the residues of each kind are destroyed at the same rate, as far as one can tell by the linearity of the logarithmic plots (although values for cysteine show appreciable scatter). Destruction of cysteine is more rapid than inactivation, and it seems likely that thiol groups are inessential. If the enzymic activity depended on the integrity of two groups, say a tryptophan residue and a histidine residue, then the fraction of activity remaining (F_A) at any time would be given by:

$$\log F_A = \log f_{\text{Trp}} + \log f_{\text{His}}$$

where f_{Trp} and f_{His} represent the fractions of tryptophan and of histidine residues respectively still intact at this time (cf. Ray & Koshland, 1961). Since the rate of inactivation is similar to the rate of loss of either tryptophan or of histidine, and rather less than the rate of loss of cysteine, it follows that if any one of these (types) is essential then the others are inessential. It thus seems likely that it is loss of histidine that is causing inactivation since the results of alkylation pointed towards histidine as an essential group, and the rate of loss of histidine can be correlated well with the rate of inactivation.

Photo-oxidation in the presence of the anionic dye Rose Bengal (British Drug Houses Ltd.) led to inactivation at a rate not appreciably greater than

that in the presence of Methylene Blue. Westhead (1965) suggested that an anionic dye might be a more effective photosensitizing agent than a cationic one for an enzyme whose substrate is anionic, and found this to be so for yeast enolase. It may also be a help with enolase, however, that the substrate is a carboxylate ion, as is the dye.

DISCUSSION

Characterization of triose phosphate isomerase. Triose phosphate isomerase (from rabbit muscle) is a basic protein of molecular weight about 43 000. It is one of the most active enzymes, and the molecular activity is about 500 000 (the molecular activity is defined by the Enzyme Commission as the number of molecules of substrate transformed/min. by one molecule of enzyme at optimum substrate concentration). A similar value was obtained by Meyer-Arendt *et al.* (1953) for the enzyme from calf muscle (assuming that the enzyme from the two sources has the same molecular weight); the lower value quoted by Czok & Bücher (1960) for the enzyme from rabbit muscle may not refer to an optimum concentration of substrate. The number of peptides obtained after digestion with trypsin suggests that there may possibly be two similar sub-units, but this conclusion must be regarded as tentative and needs confirming by other methods. The protein contains about seven thiol groups/mol., and there are probably no disulphide bonds; these are becoming recognized as common features of intracellular enzymes, which moreover often consist of sub-units that are not covalently bonded to each other. The thiol groups are presumably 'buried' in the interior of the molecule, as they are unreactive towards di-(5-carboxy-4-nitrophenyl) disulphide except in the presence of guanidine hydrochloride. Similarly, the order of magnitude of the bimolecular rate constant for the formation of carboxymethylcysteine was $10^{-41} \text{ mole}^{-1} \text{ sec}^{-1}$ at 40°, which is low; for example, the rate constant for the reaction of the thiol group of yeast alcohol dehydrogenase with iodoacetamide is $0.51 \text{ mole}^{-1} \text{ sec}^{-1}$ (Whitehead & Rabin, 1964).

Evidence for histidine in the active site. Studies on the mechanism of enzymic keto-enol tautomerisms have led to the idea that a *cis*-enediol is an intermediate (Rose, 1962; Topper, 1961) for all of the four enzymes studied, namely phosphoglucose isomerase, phosphomannose isomerase, phosphopentose isomerase and triose phosphate isomerase. A basic group in the enzyme participates by detaching a proton from the substrate, and the identity of this basic group will now be considered. For glucose 6-phosphate isomerase, Rose & O'Connell (1961) have shown that the basic group must be one

that in the form of its conjugate acid has but one hydrogen atom, i.e. the basic group cannot be an amino group. The dependence of V_{\max} on pH may be interpreted in terms of groups with apparent pK values about 6.5 and 9.5 (Rose 1962; Hines & Wolfe, 1963), and although this only enables one to make tentative identifications the obvious conclusion is that a histidine residue of pK 6.5 is required in the unprotonated form. Rose (1962) also suggests that the mechanisms of the triose phosphate isomerase and glucose 6-phosphate isomerase reactions are similar. Hence the same basic group may well be involved in both reactions.

The inactivation of triose phosphate isomerase by photo-oxidation provides some information about the nature of the basic group. Only five amino acids are susceptible to photo-oxidation (Weil, 1965), namely cysteine, histidine, tyrosine, tryptophan and methionine, and of these only the first three can function as bases. The results obtained (Fig. 7) rule out tyrosine, and possibly cysteine; the results (discussed below) of alkylation tend to exclude thiol groups. Histidine, then, emerges as the most likely candidate for the basic group, unless photo-oxidation were destroying an essential residue that plays some other function.

Although iodoacetate has often been used to inactivate enzymes, detailed knowledge of the amino acid residues attacked is only available in a few instances. Reactive thiol groups (as present, for example, in several dehydrogenases and kinases) are the most susceptible. Alkylation of proteins lacking thiol groups, at pH 5.5–7, may lead to reaction of histidine residues; in ribonuclease this reaction is much more rapid than is the reaction of the free amino acid (Henrikson, Stein, Crestfield & Moore, 1965), but in myoglobin, under rather different conditions, although some of the histidine residues are reactive others are inaccessible in the native protein (Banaszak, Andrews, Burgner, Eylar & Gurd, 1963). Iodoacetate reacts with both thiol groups and with histidine residues in β -galactosidase, but here imidazole in the denatured protein does not react (Wallenfels, Müller-Hill, Dabich, Streffer & Weil, 1964).

Triose phosphate isomerase reacted with iodoacetate at 40°; the susceptible amino acid residues were cysteine, methionine and histidine, in roughly that order. The nature of the products formed was elucidated by paper electrophoresis and chromatography of the carboxymethylamino acids; the more usual procedure is to study the amino acid composition of the modified protein. Both methods have their advantages and drawbacks, but the method used here needs less protein.

There was a marked lag before activity was lost during the reaction, and most of the thiol groups reacted during this lag (Fig. 6). Most (perhaps all) of

the thiol groups can therefore be alkylated, and it is the alkylated, still active, enzyme that reacts further. The reaction of methionine also did not parallel inactivation, but the formation of carboxymethylhistidine was proportional to the loss in activity.

Moreover, histidine was the main amino acid reacting after the reactive 'inessential' groups had been alkylated in the presence of phosphate (Fig. 4). Glucose 6-phosphate isomerase reacts with iodoacetate in a somewhat similar way (Noltmann, 1964). Cysteine (but not histidine) reacted without loss in enzymic activity at pH 8.5; at pH 5.5–6, methionine and possibly histidine also reacted, and activity was lost. It was not possible to decide whether inactivation was caused by reaction of methionine, which presumably plays a structural role, or of histidine.

The conclusion then is that several lines of evidence point towards histidine as the basic amino acid in triose phosphate isomerase that abstracts a proton from the substrates and this may turn out to be a general feature of H–C cleavage. This is also essentially the part that histidine may play in chymotrypsin and in ribonuclease, although here the proton is being abstracted from oxygen rather than from carbon.

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