Iodination of Glyceraldehyde 3-Phosphate Dehydrogenase

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(Received 31 March 1970)

1. A high degree of homology in the positions of tyrosine residues in glyceraldehyde 3-phosphate dehydrogenase from lobster and pig muscle, and from yeast, prompted an examination of the reactivity of tyrosine residues in the enzyme. 2. Iodination of the enzyme from lobster muscle with low concentrations of potassium tri- ^{125}I -iodide led to the identification of tyrosine residues of differing reactivity. Tyrosine-46 appeared to be the most reactive in the native enzyme. 3. When the monocarboxymethylated enzyme was briefly treated with small amounts of iodine, iodination could be confined almost entirely to tyrosine-46 in the lobster enzyme; tyrosine-39 or tyrosine-42, or both, were also beginning to react. 4. These three tyrosine residues were also those that reacted most readily in the carboxymethylated pig and yeast enzymes. 5. The difficulties in attaining specific reaction of the native enzyme are considered. 6. The differences between our results and those of other workers are discussed.

The accessibility to iodine of the tyrosine residues in glyceraldehyde 3-phosphate dehydrogenase was studied since the distribution of tyrosine between the interior and the exterior of the protein molecule should indicate something of the topography of the structure. Comparison of the amino acid sequences of the enzyme from lobster muscle (Davidson, Sajgó, Noller & Harris, 1967), pig muscle (Harris & Perham, 1968) and yeast (Jones & Harris, 1968; Jones, 1969) reveals a striking homology in the positions of the tyrosine and tryptophan residues, which contrasts with the degree of correspondence observed for other residues such as cysteine and histidine. All nine tyrosine residues per subunit in the lobster and pig enzymes occur in identical positions in the sequence; moreover, eight of these are homologous with eight of the 11 tyrosine residues in the yeast enzyme. Most of the iodination experiments were carried out on the lobster enzyme, which is of particular interest because it is being studied crystallographically (Watson & Banaszak, 1964; Wassarman & Watson, 1968), and were extended under certain conditions to the pig and yeast enzymes.

This paper describes detailed iodination studies with ^{125}I iodine, under conditions where specificity for tyrosine residues might be expected (Hughes & Straessle, 1950). For complete iodination to DIT†

2mol of iodine/mol of tyrosine is required. The radioactive peptides isolated from reactions in which differing degrees of specificity were obtained were characterized, thereby identifying the positions in the primary sequence of those tyrosine residues iodinated.

Brief labelling experiments were carried out on enzyme that had been carboxymethylated at the 'active thiol' (cysteine-149 in the sequence) (Harris, Meriwether & Park, 1963) before iodination, the aim being to minimize problems arising from possible oxidation of this group (Hughes & Straessle, 1950). Use of the monocarboxymethylated derivative to obtain information about the distribution of tyrosine residues in the native enzyme was justified by the crystallographic results (Wassarman & Watson, 1968), which showed that the 'native' conformation of the enzyme had not been altered to any detectable extent by alkylation of the essential thiol group.

MATERIALS

The pure crystalline enzyme was obtained from lobster (*Homarus vulgaris*) tail muscle by the method of Allison & Kaplan (1964a), from pig skeletal muscle by the procedure of Elődi & Szőrényi (1956), and from yeast by the method of Krebs (1955). The yeast enzyme was kindly provided by Dr G. M. T. Jones. $E_{280}^{1\%}$ 10 was taken as a measure of protein concentration in all three cases.

Reagents. ^{125}I Iodide was obtained, carrier-free, from The Radiochemical Centre, Amersham, Bucks., U.K., at a concentration of 80–100 mCi/ml. Iodine (resublimed before use), potassium iodide and sodium arsenite were all of A.R. grade. Iodoacetic acid was recrystallized from

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† Abbreviations: DIT, di-iodotyrosine; MIT, mono-iodotyrosine.

heptan-1-ol and was stored in the dark at 4°C. A 50mM solution that had been neutralized with NaOH was used for carboxymethylation of the (iodinated) protein. 2-Mercaptoethanol, obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., was redistilled and the middle fraction was used. Dithiothreitol was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Scintillation fluid. A solution (6g/l) in toluene of 2,5-bis-(5-*tert*.-butylbenzoxazol-2-yl)thiophen (BBOT) obtained from CIBA Ltd., Basle, Switzerland, was diluted in the ratio 3:1 (v/v) with 2-methoxyethanol.

Enzymes. Trypsin (twice recrystallized), di-isopropyl phosphorfluoridate-treated carboxypeptidase A, carboxypeptidase B, leucine aminopeptidase and soya-bean trypsin inhibitor were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Subtilisin was obtained from Novo Industri A/S, Japan, and pronase from Nomoto, Japan.

Sephadex. This was a product of Pharmacia, Uppsala, Sweden.

METHODS

Preparation of potassium tri- ^{125}I iodide. Resublimed iodine was dissolved, to a concentration of 0.025M, in potassium ^{125}I iodide (0.1M) prepared by dilution of a carrier-free solution with unlabelled potassium iodide, i.e. 4 mol of iodide/mol of iodine. In general, the reagent had a specific radioactivity of about 2.66mCi/ml; in experiments where 20 mol of iodine was used/mol of subunit, about 1 mCi (360 μl) was used for reaction with 1 μmol of subunit (36 mg of protein).

Iodination. The general procedure was adopted from Hughes & Straessle (1950). However, our experiments were carried out at pH 8.5 in 0.1M-tris-HCl buffer, containing 0.01M-EDTA, instead of 0.1M-glycine buffer, pH 9.2. Further details of procedure are given below.

Carboxymethylation. This was carried out in the same tris-EDTA buffer, pH 8.5. Where thiol reagent was present in the medium an equimolar amount of iodoacetate was used, in addition to the appropriate excess over the protein thiol groups to be reacted. Carboxymethylation in urea after iodination was terminated with an excess of either dithiothreitol or mercaptoethanol, and the solution was then dialysed.

Fractionation of proteolytic digest. A preliminary separation was achieved by gel filtration on Sephadex G-50 in 0.1M- NH_4HCO_3 , pH 7.9. The eluate was continuously monitored both for extinction at 254nm, with a Uvicord (LKB Producter AB, Stockholm, Sweden), and for radioactivity, with a scintillation counter (Nuclear-Chicago 6770 Chroma/Cell) equipped with an anthracene flow-cell. Peptides were purified further by high-voltage paper electrophoresis and were eluted with 0.1M- NH_3 solution.

Paper electrophoresis. High-voltage paper electrophoresis was carried out on Whatman 3MM or no. 1 paper in the apparatus described by Michl (1951), as modified by Ryle, Sanger, Smith & Kitai (1955). The buffer solutions and the conditions for electrophoresis of peptide mixtures were as follows: pH 6.5, 10% (v/v) pyridine-0.5% (v/v) acetic acid in water, 50 min at 3kV; pH 3.5, 5% (v/v) acetic acid-0.5% (v/v) pyridine in water, 50 min at 3kV; pH 8.9, 1% (w/v) $(\text{NH}_4)_2\text{CO}_3$ in water, 2 h at 1kV. Separation of MIT and DIT in total enzymic

digestion mixtures was achieved by electrophoresis at pH 2.1 [8% (v/v) acetic acid-2% (v/v) formic acid in water] for 20 min at 6kV. White spirit was used as the coolant at pH 3.5, 8.9 and 2.1; toluene containing 8% (v/v) of pyridine was used at pH 6.5 (Naughton, Sanger, Hartley & Shaw, 1960).

Radioautography. Overnight exposure to Kodak Kodirex Auto Process X-ray film was usually adequate.

Scintillation counting. The radioactivity of solutions was counted with a Nuclear-Chicago Unilux scintillation counter, in one of two ways, depending largely on the degree of radioactivity to be determined: (i) a small sample (approx. 5 μl) was injected directly into the scintillation fluid in a vial, which was then suspended in an empty vial for counting; (ii) an aqueous radioactive solution in the inner vial was surrounded by scintillation fluid in the outer vial. In both cases an analogous radioactive standard of known iodine content was used.

The relative radioactivity of peptides on ionograms was determined by counting excised strips immersed directly in the scintillation fluid; strips of the same size were counted in each case.

Determination of incorporation of ^{125}I iodine into the protein. Protein was estimated on the basis of arginine content, determined by amino acid analysis, and radioactivity was measured by scintillation counting. Both measurements were made on the protein solution before tryptic digestion.

Amino acid composition. Peptide samples that had been hydrolysed anaerobically for 20 h at 105°C in 6M-HCl containing 1% (w/v) of phenol (Sanger & Thompson, 1963) were analysed on a Beckman Spinco 120 amino acid analyser. Iodotyrosine residues appeared largely as tyrosine.

End-group determination. N-Terminal residues of peptides were identified by the 'dansyl' technique (Gray & Hartley, 1963; Gray, 1967).

Total enzymic digestion. A portion of the tryptic digest was digested further with subtilisin (1:50, w/w) for 6 h at 37°C, followed by pronase (1:50, w/w) for 6 h at 37°C, and then by a mixture of carboxypeptidases A and B and leucine aminopeptidase for 10-12 h at 20°C.

Determination of MIT and DIT. The total enzymic digest of the protein or a peptide was subjected to electrophoresis at pH 2.1 with authentic MIT and DIT as markers. Radioautography, followed by scintillation counting of the excised bands, permitted the allocation of radioactivity to MIT and DIT.

Iodination of glyceraldehyde 3-phosphate dehydrogenase: typical procedure. Iodination was carried out either on the native protein or on enzyme that had been carboxymethylated at the essential thiol group by alkylation at 0°C for 30 min. All reactions were carried out in an ice bath. Tri-iodide reagent was added to a magnetically stirred solution either in one batch or in portions, as indicated in the text for specific cases. After the appropriate time the reaction was terminated by addition of 200 μl of 0.5M-sodium arsenite solution. (Addition of 2-mercaptoethanol at this stage was avoided since it generated translucence in the reaction mixture.) The protein was separated from excess of reagents by gel filtration in the reaction buffer at 4°C on Sephadex G-25, and the radioactivity of the pooled eluate was counted. Solid urea was added to a concentration of 8M, and the

concentration of tris was maintained at 0.1 M by addition of 1.0 M-tris-HCl buffer, pH 8.0. The solution was incubated at 37°C for 1 h after addition of a 1.5-fold excess of dithiothreitol over total protein thiol groups. Iodoacetate was then added in amount sufficient to react with the added thiol and a further 4-fold excess over protein thiol groups. After 1 h at 37°C the reaction was terminated with an excess of dithiothreitol. The reaction mixture was dialysed at 20°C, first against 8 M-urea whose pH had been adjusted to pH 3 with conc. HCl, and then against mM-HCl. Solid NH_4HCO_3 was added to a concentration of 1% (w/v), giving a pH of about 8, followed by trypsin (2% by weight of iodinated protein). Digestion was allowed to proceed at 37°C for 4 h and was then terminated with soya-bean trypsin inhibitor in a twofold excess (w/w) over trypsin. The digest was freeze-dried; the distribution of radioactive peptides was examined by radioautography after electrophoresis of a small portion at pH 6.5. The digest was fractionated by gel filtration on Sephadex G-50. To determine the relative radioactive content of ^{125}I -labelled peptides, an equivalent portion of each radioactive peak was subjected to electrophoresis at pH 6.5, and the radioactivity of the excised bands was counted. Radioactive peptides were purified by preparative paper electrophoresis. Those that were acidic or basic at pH 6.5 were re-run at pH 3.5; radioactive origin material at pH 6.5 was run at pH 8.9. The purity of the eluted radioactive peptides was confirmed by determination of N-terminal groups. These, in conjunction with the amino acid composition, served to locate a given peptide in the known amino acid sequence of the protein chain. The MIT/DIT ratio of the whole digest and, in some cases, of the purified peptides was determined after total proteolytic digestion as described above.

In certain cases, described in the text, the product of tryptic digestion proved to be not completely soluble in 0.1 M- NH_4HCO_3 , pH 7.9. In such cases insoluble radioactive material was collected and washed by centrifugation; the supernatant was fractionated on Sephadex G-50 in the usual manner, and the insoluble material, suspended in 0.1 M- NH_4HCO_3 , was digested with subtilisin to give a soluble digest, which was fractionated similarly.

RESULTS

The degree of specificity attainable in the reaction was found to be markedly dependent on the conditions, as illustrated by the experiments described below.

Assuming that trypsin cleaves all, and only, lysyl and arginyl peptide bonds, lobster glyceraldehyde 3-phosphate dehydrogenase would be expected to give seven tryptic peptides containing tyrosine. [For the sequence of the lobster enzyme, see Davidson *et al.* (1967); the residues are, however, best numbered so as to maximize the homology with the sequence of the pig enzyme (Harris & Perham, 1968).] In practice, however, tryptic digestion of iodinated carboxymethylated protein gave as many as ten tyrosine-containing peptides (Table 1), including the seven that were predicted. The three additional peptides were extensions of

Table 1. *Tryptic peptides containing iodotyrosine from iodinated lobster glyceraldehyde 3-phosphate dehydrogenase*

Where two peptides contain the same tyrosine residue, the one listed first is the predicted tryptic peptide, and the other contains an intact lysyl bond (see the text). (The anomalous behaviour of the third and fourth peptides is probably due to their adsorption to paper.)

Peptide	Tyrosine residue	m_{obs}^*	$m_{\text{calc.}}^\dagger$
Tyr ₍₄₆₎ -Lys ₍₅₄₎	46	-0.15	~ -0.12
Tyr ₍₄₆₎ -Lys ₍₅₈₎	46	-0.14	~ -0.1
Ala ₍₁₈₎ -Tyr ₍₄₂₎	39 + 42	Origin	+0.35
Ala ₍₈₇₎ -Lys ₍₁₀₄₎	91	Origin	+0.29
Tyr ₍₁₃₇₎ -Lys ₍₁₃₉₎	137	-0.35	-0.41
Leu ₍₂₄₆₎ -Lys ₍₂₅₆₎	252	+0.37	+0.37
Glu ₍₂₄₉₎ -Lys ₍₂₅₆₎	252	+0.54	+0.56
Thr ₍₂₆₁₎ -Arg ₍₂₈₈₎	273	+0.48	> +0.44
Val ₍₃₀₅₎ -Arg ₍₃₂₀₎	311 + 317	Origin	0
Val ₍₃₀₇₎ -Arg ₍₃₂₀₎	311 + 317	+0.15	+0.17

* Expressed relative to $m_{\text{Asp}} + 1.0$.

† Calculated for the molecular weight of the MIT form by the method of Offord (1966).

three of the predicted ones (also found) due to slow cleavages of the bonds Lys₍₅₄₎-Gly₍₅₅₎, Lys₍₂₄₈₎-Glu₍₂₄₉₎ and Lys₍₃₀₆₎-Val₍₃₀₇₎. It was found (cf. Harris & Perham, 1968) that the peptide Ala₍₁₈₎-Tyr₍₄₂₎ was formed to the exclusion of Ala₍₁₈₎-Lys₍₄₅₎ by a chymotryptic-like cleavage after tyrosine-42 in the sequence of the lobster enzyme. The predicted mobilities at pH 6.5 are calculated for the monoiodinated forms of the peptides and are based on the observations of Offord (1966); no account is taken of the effect on mobility of the lowering of the pK of the phenolic group due to iodination of the aromatic ring.

Iodination of native lobster glyceraldehyde 3-phosphate dehydrogenase. The distribution of radioactive peptides at pH 6.5, after tryptic digestion of the iodinated protein carried out as described, is shown in Plate 1. In Plate 1(a) much of the radioactivity at the origin was due to insoluble material; in Plates 1(b) and 1(c) only the material soluble in ammonium hydrogen carbonate was run. Plate 1(a) refers to a reaction in which 20 mol of iodine/mol of subunit was added in portions over 5 min to protein at a concentration of 7 mg/ml; the two peptides, A and B, containing tyrosine-46 were the most radioactive, suggesting the preferential reaction of tyrosine-46 with iodine. The peptide containing tyrosine-137 contained least radioactivity; other peptides listed in Table 1 all contained varying amounts. These conclusions were confirmed in subsequent experiments.

Plates 1(b) and 1(c) refer to reactions carried out at a protein concentration of 0.7 mg/ml with a single

addition of 20 mol of iodine/mol of subunit; the time of reaction was (b) 30 s, (c) 5 min. Incorporation of iodine into the protein was 0.99 and 1.20 g-atoms/mol of subunit, and the MIT/DIT ratio was 5.4:1 and 4.2:1, for the reactions represented in Plate 1(b) and 1(c) respectively. Fractionation of the digests by gel filtration followed by paper electrophoresis, as described, permitted determination of the percentage of the soluble radioactivity in each peptide, and hence of the following order of reactivity for the tyrosine residues in the soluble fraction: 46 > 252, 273 > (39 + 42), 91, (311 + 317) > 137. Approximately 5% of the soluble radioactivity emerged at the void volume on Sephadex G-50 and was also shown (see below) to contain tyrosine residues 39, 42 and 46. In addition to the peptides given in Table 1, a minor peptide was found with mobility +0.78 (all mobilities are expressed relative to $m_{\text{Asp}} + 1.0$) and the composition of Leu₍₂₄₆₎-Lys₍₂₅₆₎, except that it lacked any derivative of cysteine. This suggests catalysis by iodine of the irreversible reaction of thiol groups in the native structure beyond the cysteic acid stage, with loss of sulphur. It also deserves comment that the peptide Val₍₃₀₅₎-Arg₍₃₂₀₎, containing several hydrophobic aromatic residues, was retarded on Sephadex G-50, and was eluted with the nine-residue peptide Tyr₍₄₆₎-Lys₍₅₄₎.

The fraction of the tryptic digest that was not soluble in ammonium hydrogen carbonate contained about 50% of the total radioactivity incorporated. Redigestion of this with large amounts of trypsin for extended periods, and with subtilisin, led us to conclude that it contained, almost exclusively, tyrosine residues 39/42 and 46. Treatment with trypsin gave the MIT and DIT forms of the peptide Tyr₍₄₆₎-Lys₍₅₄₎, distinguished by electrophoresis at pH 6.5, and a little of the peptide Ala₍₁₈₎-Tyr₍₄₂₎, which was mobile on electrophoresis at pH 8.9. Most of the radioactivity was, however, immobile at both pH values but could be eluted from the paper with 2M-ammonia and redigested with trypsin a second time to give additional amounts of the same radioactive peptides. Attempts to increase the efficiency of redigestion included progressive dilution of a solution of the radioactive material and trypsin in 8M-urea, since it is known that trypsin is reversibly inactivated in 8M-urea (Harris, 1956). However, no detectable increase in the extent of digestion was observed. The nature of peptides obtained by redigestion with trypsin, taken in conjunction with the single *N*-terminus (alanine) and the relative proportions of tyrosine, methionine and phenylalanine shown by the (albeit imperfect) amino acid analysis, suggested that the insoluble peptide began at alanine-18 in the sequence, and extended probably no further than lysine-86 since the peptide Ala₍₈₇₎-Lys₍₁₀₄₎

containing tyrosine-91 was not found, even under the most drastic conditions of redigestion. It seems likely that poor digestion of the *N*-terminal region by trypsin after treatment of the protein with iodine results from the iodination of tyrosine residues 39/42 and, especially, 46.

The nature of the 'insoluble' material was confirmed by digesting it with subtilisin when about 90% of the radioactivity became electrophoretically mobile. The two main radioactive peptides, each containing about 24% of the total radioactivity in this fraction, were Glu₍₃₈₎-Tyr₍₄₂₎ ($m_{\text{obs}} + 0.33$) and Lys₍₄₅₎-Tyr₍₄₆₎ ($m_{\text{obs}} - 0.45$). The radioactive neutral band (14% of the radioactivity) contained small amounts of peptides Lys₍₄₅₎-Asp₍₄₇₎ and Tyr₍₄₂₎-Phe₍₄₄₎, with traces of Lys₍₄₅₎-Thr₍₄₉₎; some free iodotyrosine was also found. Two acidic bands ($m_{\text{obs}} + 0.44$ and $+0.51$), which together accounted for about 12% of the radioactivity, had compositions corresponding to peptides Tyr₍₃₁₁₎-Tyr₍₃₁₇₎ and Asp₍₃₁₂₎-Ser₍₃₁₈₎; this was the only evidence that this region of the chain was represented in the insoluble material.

We thus conclude that the insoluble material present in the tryptic digest of iodinated carboxy-methylated lobster glyceraldehyde 3-phosphate dehydrogenase contained mainly the *N*-terminal region of the protein, apparently accompanied by relatively small (possibly contaminant) amounts of a region near the *C*-terminus. The overall order of reactivity of tyrosine residues (i.e. taking into account both the soluble and the insoluble fractions) was: 46 > (39 + 42) > 91, 252, 273, (311 + 317) > 137.

It appeared that the small proportion of the soluble radioactivity that emerged at the void volume on Sephadex G-50 (see above) was identical with this insoluble material, with respect both to the peptides obtained by redigestion and the MIT/DIT ratio. Since it was shown that this represented only a relatively small part of the polypeptide chain, it was thought that its anomalous behaviour on Sephadex might be due to aggregation. However, gel filtration both after attempted maleylation (Butler, Harris, Hartley & Leberman, 1969) of the excluded material and also after reduction with 2-mercaptoethanol showed the same elution position for the radioactivity. Cyanogen bromide and performic acid both failed to produce small radioactive fragments from the 'insoluble' and 'excluded' material, and led to loss of iodine (see 'Note added in proof').

Iodination of lobster glyceraldehyde 3-phosphate dehydrogenase in 8M-urea. Iodination in 8M-urea would be expected to result in reaction of all tyrosine residues. Although incorporation was low with addition of 20 mol of iodine/mol of subunit, presumably because iodine was consumed in the oxidation of the five thiol groups per molecule

EXPLANATION OF PLATE 1

Distribution of radioactive peptides in tryptic digests [in (a), total digest; in (b) and (c), fraction of digest that dissolved in $0.1\text{ M-NH}_4\text{HCO}_3$] of iodinated and carboxymethylated lobster glyceraldehyde 3-phosphate dehydrogenase after electrophoresis at pH 6.5. (a) 20 mol of iodine/mol of subunit, 5 min, 7 mg of protein/ml; (b) 30 s and (c) 5 min, both with 20 mol of iodine/mol of subunit, 0.7 mg of protein/ml. A is $\text{Tyr}_{(45)}\text{-Lys}_{(54)}$ and B is $\text{Tyr}_{(45)}\text{-Lys}_{(58)}$. For further details see the text.

EXPLANATION OF PLATE 2

Iodination (2 mol of iodine/mol of subunit) of monocarboxymethylated glyceraldehyde 3-phosphate dehydrogenase (10 mg/ml) from lobster (a), pig (b) and yeast (c and d): radioactive peptides in the tryptic digests of the completely carboxymethylated proteins after electrophoresis at pH 6.5. All reaction mixtures were run simultaneously on the same sheet of paper.

exposed in 8 M-urea, it was clear that no new radioactive peptides (cf Table 1) were present. However, the possibility of irreversible oxidation of thiol groups to cysteic acid on exposure to iodine, at least in urea, was shown by separation of approximately equal amounts of the *S*-carboxymethyl-cysteine and cysteic acid forms of the peptides containing tyrosine-252 (see Table 1) when a single band on electrophoresis at pH 6.5 was re-run at pH 3.5. The Sephadex G-50 elution profile of the soluble portion of the tryptic digest showed that the greatest increase in radioactivity occurred in the peptide Tyr₍₁₃₇₎-Lys₍₁₃₉₎, and to a lesser extent in Ala₍₈₇₎-Lys₍₁₀₄₎ and Val₍₃₀₅₎-Arg₍₃₂₀₎; this might suggest that the tyrosine residues in these peptides (residues 137, 91, 311 and 317) were the most buried in the native structure, and became completely accessible only after denaturation of the protein in urea.

When a sample iodinated in urea was desalted on Sephadex G-25 in the absence of urea, some of the protein was either irreversibly adsorbed on to the column (cf. Stevenson, 1968), or precipitated in it. This material could, however, be eluted with 2 M-ammonia solution, and it possessed a lower MIT/DIT ratio than the protein that was not retained on the column. It was nevertheless identical with this protein in the distribution of radioactive peptides both in the soluble part of the tryptic digest and in the subtilisin digest of the insoluble part. Possibly the lowering of the pK of the phenolic groups on iodination of tyrosine residues could be responsible for adsorption of part of the iodinated protein. It may be relevant that other workers (Lissitsky, Bismuth & Rolland, 1962) had shown that, whereas MIT and DIT could be separated on Sephadex G-25 in 0.05 M-tris buffer, pH 7.4, thyroxine and 3,3',5-tri-iodothyronine were adsorbed on the gel under these conditions, but could be eluted with ammoniacal 2-methylbutan-2-ol.

Iodination of pig glyceraldehyde 3-phosphate dehydrogenase. Results reported by Elődi, Libor & Móra (1968) suggested that iodination of the pig muscle enzyme occurred specifically at two tyrosine residues, and that no problems of insolubility, electrophoretically immobile material etc. had been encountered. We therefore studied the iodination of the pig enzyme under conditions similar to those described above for the lobster enzyme.

The reaction was carried out for 5 min after a single addition of 20 mol of iodine/mol of subunit, and with a protein concentration of about 6 mg/ml. Incorporation was rather low (0.1 g-atom/mol of subunit) and the MIT/DIT ratio was 3.4:1. One-third of the total radioactivity in the freeze-dried tryptic digest (1%, w/w, trypsin) was insoluble in ammonium hydrogen carbonate, and again some of the soluble radioactivity was not retarded by

Sephadex G-50. No single tyrosine residue in the pig enzyme had reacted preferentially with iodine under these conditions. Electrophoresis at pH 6.5 of the soluble portion revealed a strong radioactive neutral band containing Leu₍₂₄₆₎-Lys₍₂₅₆₎, with intact Lys-Pro and Lys-Tyr bonds, and rather less of Tyr₍₂₅₂₎ Lys₍₂₅₇₎ together with other (unidentifiable) components. Peptides found at the origin were Gly₍₂₆₉₎-Lys₍₃₀₆₎, and both the MIT and DIT forms of Leu₍₃₀₇₎-Arg₍₃₂₀₎ which separated at pH 8.9. The relatively strong acidic band ($m_{\text{obs}} + 0.33$) contained both the peptides Gly₍₂₆₉₎-Lys₍₃₀₆₎ and Tyr₍₂₅₂₎-Lys₍₂₅₆₎; a weaker basic band contained Met₍₄₃₎-Lys₍₅₂₎. The order of reactivity of tyrosine residues represented in the soluble portion of the tryptic digest in this experiment was: 252, 273 > (311 + 317) > 46. Radioactive peptides containing the other tyrosine residues in the sequence were not found. In contrast with the case of the lobster enzyme, prolonged incubation of the electrophoretically immobile ('insoluble') material with trypsin did not produce any mobile radioactive peptides (see the Discussion section).

Iodination of monocarboxymethylated enzyme. Prior alkylation of the essential thiol was investigated as a means of preventing the formation of disulphide bonds, and possible associated conformational changes, before substitution by iodine had occurred. Further, it permitted the use of small, limiting, amounts of iodine, in an attempt to increase the specificity of iodination.

In these experiments the lobster enzyme was first dialysed against reaction buffer containing 0.5 mM-dithiothreitol before treatment with iodoacetate (2 mol/mol of dithiothreitol and 3 mol/mol of protein active thiol group) at 0°C for 40 min; the solution was completely devoid of dehydrogenase activity. Iodine was added to this solution without further treatment. A preliminary experiment in which 20 mol of iodine/mol of subunit was slowly added to a protein solution (10 mg/ml) over a period of 30 min revealed some of the effects of carboxymethylating the active thiol group before exposure of the protein to iodine. A higher incorporation of iodine into the precarboxymethylated protein was observed (4.6 g-atom/mol of subunit, compared with 3.2 g-atom/mol of subunit for a control sample in which iodination had not been preceded by carboxymethylation). This was consistent with a higher effective concentration of iodine in the former case since it was not being consumed in the simultaneous oxidation of thiol groups, suggested also by the persistence of the iodine colour in the reaction mixture. The MIT/DIT ratio was 6.6:1 and 2.6:1 for the native (control) and carboxymethylated enzyme respectively i.e. the amount of DIT increased with increasing incorporation of iodine. The radioactive peptides produced in each

case were the same; tyrosine-46 was again found to be the most reactive one. Pre-alkylation did, however, decrease the amount of insoluble radioactivity in the tryptic digest to about 5%, although about 15% of the soluble radioactivity was still excluded by Sephadex G-50. In both reactions these fractions had a MIT/DIT ratio that was half that for the whole digest, i.e. they contained relatively more DIT. This higher degree of iodination might perhaps account for the rather refractory nature of the 'insoluble' and 'breakthrough' fractions. Prolonged redigestion with trypsin again gave the MIT and DIT forms of Tyr₍₄₆₎-Lys₍₅₄₎ (m_{obs} -0.21 and -0.14 respectively; relative values are more significant than absolute values since the peptides contain histidine) as the only mobile peptides after electrophoresis at pH 6.5.

Reactions on carboxymethylated enzyme with very limited amounts of iodine. (a) The lobster enzyme was carboxymethylated as described above, and then iodinated at a concentration of 10 mg/ml, with a single addition of 4 mol of iodine/mol of subunit (i.e. one-fifth the amount previously used, and sufficient to iodinate completely about two tyrosine residues/subunit); the reaction time was 45 s. After complete carboxymethylation and tryptic digestion (2%, w/w, trypsin) less than 8% of the total radioactivity in the digest was now insoluble, and the soluble radioactive fraction contained no material that was excluded by Sephadex G-50. About 0.1 g-atom of iodine/mol of subunit was incorporated, and the MIT/DIT ratio was 1.2:1. The distribution of radioactive peptides in the total tryptic digest after electrophoresis at pH 6.5 and analysis of the products after fractionation on Sephadex showed that virtually all of the radioactivity in the protein resided in the two basic peptides Tyr₍₄₆₎-Lys₍₅₄₎ and Try₍₄₆₎-Lys₍₅₈₎, and in three weaker bands containing respectively tyrosine residues 39/41, 91 and 311/317; these were mobile at pH 8.9 and accounted for nearly all the radioactivity that remained at the origin at pH 6.5. Prolonged radioautography revealed small amounts of all the previously observed radioactive peptides. The following order of reactivity could thus be established for the tyrosine residues under conditions of rather limited iodination (with, in parentheses, the approximate radioactive contents expressed as percentages of the total soluble radioactivity, which, in this case, was over 92% of the total incorporated into the protein): 46 (50%) $\gg$ (39+42) (12%), 91 (10%), 252 (9%), 273 (8%) $>$ (311+317) (5%), 137 ($<$ 5%). Thus, in conditions where some degree of true specificity was at last observed, one tyrosine residue (46) in the lobster enzyme appeared to have some special reactivity, and three (137 and 311/317) appeared not to react to any significant extent; the remaining five were all

relatively reactive but, clearly, less so than tyrosine-46.

(b) The lobster, pig and yeast enzymes were treated with even less iodine than in the previous experiment; the reactions were carried out in parallel with 0.5 μ mol of protein subunit (18 mg of protein). The yeast enzyme, which, unlike the muscle proteins, crystallizes without NAD⁺, was iodinated both in the absence and in the presence of added NAD⁺ (4 mol/mol of protein).

Carboxymethylation of the active thiol group as above was followed by iodination at a protein concentration of 10 mg/ml with 2 mol of iodine/mol of subunit, i.e. sufficient to iodinate completely only one tyrosine residue/monomer. The reaction was terminated after 20 s and the usual sequence of operations was carried out through to tryptic digestion of the carboxymethylated iodinated proteins. In several experiments the incorporation of radioactive iodine for the muscle enzymes was in the range 0.013–0.020 g-atom of iodine/mol of subunit; for the yeast enzyme in the absence of added NAD⁺ it was as much as 0.045, but was decreased to 0.025 g-atom/mol of subunit when 4 mol of NAD⁺/mol of protein was present in solution. In all cases only MIT, and no DIT, was present; this was probably due to the very limited amount of iodine used and to the short duration of the reaction.

The freeze-dried tryptic digest was now in all cases entirely soluble in 0.1 M-ammonium hydrogen carbonate solution and none of the radioactive material was excluded from Sephadex G-50. It should thus be possible to assign, unambiguously, all the radioactivity in the protein to particular tyrosine residues. Plate 2 shows the radioactive peptides for enzyme from lobster (a), pig (b), yeast with NAD⁺ (d) and yeast without NAD⁺ (c). For the lobster enzyme, virtually all the radioactivity occurred in the two basic peptides, A and B, containing tyrosine-46, i.e. Tyr₍₄₆₎-Lys₍₅₄₎ and Tyr₍₄₆₎-Lys₍₅₈₎ respectively, the former as usual predominating. The little radioactivity remaining at the origin on electrophoresis at pH 6.5 ran at pH 8.9 as a single band corresponding to the peptide Ala₍₁₈₎-Tyr₍₄₂₎.

The presence in the tryptic digests from the pig (Plate 2b) and yeast (Plates 2c and 2d) enzymes of a basic radioactive peptide of approximately the same mobility as that containing tyrosine-46 in the digest from the lobster enzyme was striking. The peptide was identified in the former case (band X) as Met₍₄₃₎-Lys₍₅₂₎ (m_{obs} -0.20) (residue 45 in the amino acid sequence of the pig enzyme is glutamine, and not glutamic acid as reported by Harris & Perham, 1968), arising from chymotryptic-like cleavage after tyrosine-42, and in the latter case (band Y) as Tyr₍₄₆₎-Arg₍₅₂₎ (m_{obs} -0.25). In the

case of the pig enzyme the radioactivity at the origin at pH 6.5 moved at pH 8.9 as a single band shown to be Leu₍₁₄₎-Tyr₍₄₂₎, containing tyrosine residues 39 and 42. The neutral and acidic peptides containing tyrosine residues 137 and 252, found by Libor & Elődi (1970b) to be the most reactive, were absent.

The stronger of the two radioactive acidic bands for the yeast enzyme (Plate 2d) was the peptide Ile₍₁₈₎-Lys₍₄₅₎ ($m_{\text{obs}} + 0.25$), containing tyrosine residues 39 and 42; the origin, which was weaker, appeared to be almost entirely the peptide Asp₍₂₉₃₎-Arg₍₃₂₀₎, containing tyrosine residues 311 and 317, which was mobile at pH 8.9. The greater incorporation of iodine, and the slightly greater intensity of certain bands in (c) compared with (d), suggested that the additional NAD⁺ present in solution in (d) was sufficient to cause a 'tightening' in the enzyme conformation; this would be compatible with evidence for changes in apoenzyme structure on binding NAD⁺ (Listowsky, Furfine, Bethel & Englund, 1965; Havsteen, 1965; Kirschner, Eigen, Bittman & Voigt, 1966).

Thus when the monocarboxymethylated lobster enzyme was iodinated very briefly with potassium tri-¹²⁵Iiodide, almost all of the radioactivity was found, as MIT, in a nine-residue basic peptide (with a little in a related 13-residue peptide) containing tyrosine-46; the next tyrosine residue(s) to react was 39 or 42, or both. Under the same conditions these three tyrosine residues were also the most reactive in the pig and yeast enzymes.

The experiments showed that the degree of specificity was very markedly dependent on the reaction conditions. The amount of iodine incorporated was directly proportional to the amount of reagent used, and to the reaction time if there was an excess of iodine present (i.e. if it had not all been used up in the oxidation of thiol groups). More iodine was incorporated, for a given amount of reagent, if the essential thiol was first carboxymethylated, confirming that oxidation of this group did diminish the amount of iodine available for substitution into tyrosine residues. Insolubility in the tryptic digest was increased on increasing the amount of iodine or the reaction time; it was somewhat decreased by prior carboxymethylation of the active thiol. Significant specificity was attainable only when the amount of iodine was severely limited and the reaction period was brief. For such experiments the active thiol group was blocked by carboxymethylation.

DISCUSSION

One tyrosine residue, tyrosine-46, in glyceraldehyde 3-phosphate dehydrogenase from lobster, is iodinated much more readily than any of the others.

Most of the others react to varying extents depending on the conditions, tyrosine-137 being the least reactive. Iodination experiments have shown how the specificity of reaction varies with the conditions (time, amount of reagent etc.). Although it was eventually possible to arrive at conditions that permitted unambiguous identification of the most reactive tyrosine residue, some attention to earlier difficulties might be useful since these could apply in other cases where thiol enzymes are exposed to iodine.

Dependence of specificity on reaction time and amount of reagent could be a consequence of slow conformational changes within the enzyme structure. This might be an effect of the initial iodination of tyrosine residue(s) (possibly due to the lowered *pK* of the phenolic group or to the bulk of the iodine atom), or it could be a result of modification of the active thiol, cysteine-149, in the presence of iodine. Improved specificity of iodination of the carboxymethylated enzyme, which still retains the 'native' conformation (Wassarman & Watson, 1968), might be a direct consequence of preventing reaction of the active thiol group, or solely the result of using very small amounts of iodine, which itself requires that the thiol groups be in some way masked to avoid the rapid consumption of iodine in oxidative reactions.

Iodine can react with thiol groups to give sulphenyl iodides; these give disulphides by reaction with other thiol groups:

Hughes & Straessle (1950) observed that oxidation of thiol groups beyond the disulphide stage could occur, and this was also apparent from the iodination performed in urea, described above. Evidence on the nature of the changes arising from oxidative reactions at the active thiol in glyceraldehyde 3-phosphate dehydrogenase has come from the work of Parker & Allison (1969). They conclude that when the enzyme is treated with tetrathionate or iodine monochloride at 0°C, or *o*-iodosobenzoate at 26°C, inactivation proceeds through a sulphenic acid derivative of the essential cysteine residue, and that only at this stage may enzymic activity be regained by addition of thiol; the second stage, which occurs at a higher temperature, is disulphide bridge formation accompanied by irreversible inactivation. It has also been shown that cysteine residues 149 and 153 can no longer be carboxymethylated in urea when the pig enzyme is first treated with *o*-iodosobenzoate (Perham, 1964), and the lobster enzyme is treated with tetrathionate (Parker & Allison, 1969), indicating that a disulphide bridge is formed between these two residues. An earlier study by Allison & Kaplan (1964b) had

also suggested that the irreversible inactivation of the enzyme from various sources by tetrathionate proceeded through a reversibly inactivated form; immunological techniques suggested that conformational changes occurred at both stages, being most marked on irreversible inactivation. It is thus very likely that initial reaction of iodine with thiol groups at alkaline pH will induce structural changes, even before disulphide bonds form; this could obviously affect the site of substitution of iodine into the protein. [At pH 6.5, however, it appears that if conformational changes do accompany disulphide bond formation when the pig enzyme is treated with small amounts of iodine, these are fully reversible (Móra, Hűvös, Libor & Elődi, 1969).] Further evidence of conformational changes presumably resulting from the formation of a disulphide bridge between the active thiol and the neighbouring cysteine residue came from a study of the reaction of the lobster muscle enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) (Wassarman & Major, 1969). If initial reaction with cysteine-149 led to the formation of a disulphide bridge with cysteine-153, all remaining thiol groups, previously buried, could be titrated with 5,5'-dithiobis-(2-nitrobenzoic acid); carboxymethylation of the active thiol group prevented this. It could be expected that carboxymethylation would serve a similar purpose in the reaction of the enzyme with iodine described in this paper.

Insolubility in the tryptic digest was decreased by carboxymethylation of the active thiol but, even then, prolonged reaction times or large amounts of tri-iodide still generated material excluded by Sephadex G-50, which appeared to be identical with the insoluble material. It is suggested (see above) that this might be an aggregated form of a portion of the polypeptide chain near the *N*-terminus. The increase in the amount of this material under conditions leading to increased incorporation of iodine is reminiscent of the effect of iodination on thyroglobulin. Work by Tarutani & Ui (1969) confirmed earlier observations (cited by them) that the dissociability of normal thyroglobulin into two subunits on treatment with sodium dodecyl sulphate was directly related to the degree of iodination of the molecule: the molecule became more resistant to dissociation with increased iodination. The nature of the change involved is unknown. Variation in the iodine content of thyroglobulin molecules within the range 0.2–0.8% (Tarutani & Ui, 1969) is sufficient to cause differences in the dissociability of the molecules. An iodine content of 0.2% for glyceraldehyde 3-phosphate dehydrogenase represents 0.58g-atom of iodine/mol of subunit, so some form of aggregation consequent on iodination cannot be excluded in the present work. Thyroxine is not being produced since total

enzymic digestion revealed MIT and DIT as the only iodinated species. However, it is not impossible, especially in a region of the chain containing three iodine-susceptible tyrosine residues close together in the primary structure, that some inter- or intra-chain interaction, possibly covalent, might occur between tyrosine residues (see 'Note added in proof').

There were certain differences between our results and those of Elődi & Libor (1969) and Libor & Elődi (1970*a,b*) for iodination of the enzyme from pig muscle. In each case, however, it was apparent that more tyrosine residues were relatively accessible to iodine than the earlier iodination studies by Elődi *et al.* (1968) had suggested, and would be in accord with results based on difference spectra (Libor, Elődi & Nagy, 1965). Libor & Elődi (1970*b*) carried out the reaction with NAD^+ -free enzyme in 0.2M-borate buffer, pH 9.2, at a protein concentration of 7–14mg/ml. At 30s after the addition of 33mol of iodine/mol of subunit they found that all the radioactivity in the protein resided in three small tryptic peptides, which were identified, on the basis of the known sequence (Harris & Perham, 1968), as Tyr₍₂₅₂₎-Lys₍₂₅₆₎, Tyr₍₂₅₂₎-Lys₍₂₅₇₎ and Tyr₍₁₃₇₎-Lys₍₁₄₂₎. This result indicated that tyrosine residues 252 and 137 in the apoenzyme had reacted preferentially with iodine. However, after 90s electrophoretically immobile material now contained as much as 60% of the total radioactivity (Elődi & Libor, 1969). Indirect evidence (Libor & Elődi, 1970*b*) suggested that this was the peptide Val₍₂₅₎-Lys₍₅₂₎, showing that tyrosine residues 39, 42 and 46 together contained 60% of the radioactivity incorporated after 90s, with only 40% now residing in tyrosine residues 137 and 252, which had appeared to be the sites of initial reaction. Since the peptide Val₍₂₅₎-Lys₍₅₂₎ was found only as the di-iodinated form under conditions that gave both the MIT and the DIT forms of the other peptides, determined spectroscopically, they deduced (cf. van Zyl & Edelhoch, 1967) that tyrosine residues 137 and 252 are in a polar environment favouring the formation of the moniodotyrosyl derivative (unpublished work by W. E. Mayberry, cited by van Zyl & Edelhoch, 1967), whereas tyrosine residues 39, 42 and 46 are in a hydrophobic environment.

We found, with both the carboxymethylated lobster enzyme and the carboxymethylated pig enzyme, which contained bound NAD^+ , that radioactive material that was immobile on electrophoresis at pH 6.5 appeared even after 30s, and specific reaction was difficult to attain. We found the pig enzyme to be similar to the lobster enzyme in that tyrosine residues 39/42 and 46 reacted preferentially in the presence of small amounts of iodine; only under more drastic conditions were peptides containing tyrosine residues 252, 273 etc. found, and then they did, in fact, appear to contain

more iodine than did tyrosine-46. However, it is possible that the strongly radioactive immobile material also observed under these conditions contains more of the *N*-terminal region, which, as a result of iodination, has become resistant to digestion with trypsin, so that the chymotryptic-like cleavage that produced the basic peptide Met₍₄₃₎-Lys₍₅₂₎ does not occur. (It may be significant that the most readily iodinated tyrosine residue, tyrosine-46, is at the *N*-terminus of the peptide slowly released from the 'insoluble' material from the lobster enzyme on prolonged, and repeated, redigestion with trypsin.) Moreover the absence of neutral or acidic radioactive peptides in the limited iodination experiment showed that tyrosine residues 137 and 252 had not reacted under these conditions.

Thus tyrosine residues 39/42 and 46, which we found to be the most rapidly reacting in the mono-carboxymethylated, native, pig enzyme were also found by Elődi & Libor (1969) and Libor & Elődi (1970b) to react in the apoenzyme, but less readily than did tyrosine residues 137 and 252. Of these, we found tyrosine-252 to be quite reactive under certain conditions, but tyrosine-137 was the least reactive of all. Moreover, in our experiments, only very brief iodination (of the carboxymethylated enzyme) gave MIT alone; in longer, but still limited (45s), exposure to iodine both MIT and a little DIT were detected. When tyrosine residues 39, 42 and 46 appeared in the 'insoluble' fraction of the tryptic digest, they contained relatively more DIT than when they occurred in peptides released initially by trypsin.

It is possible that the differences between our results and those of Elődi and Libor are due to a different disposition of tyrosine residues in the holoenzyme used by us and the apoenzyme that they studied. This is plausible, since the binding of NAD⁺ has been shown to cause conformational changes in the structure of the apoenzyme both in the muscle proteins (Listowsky *et al.*, 1965) and in the yeast enzyme (Havsteen, 1965; Kirschner *et al.*, 1966). Moreover, since it is known that the pig enzyme is less stable when the coenzyme is removed (Elődi & Szabolcsi, 1959), it is likely that the apoenzyme would be more susceptible than the holoenzyme to conformational changes arising from oxidative reactions at the active site. However, in

our experiments with carboxymethylated enzyme, reaction of iodine at the active thiol and any accompanying conformational changes are precluded and the results that we report for the pig and yeast enzymes are consistent with those for the lobster enzyme.

The superior reactivity of tyrosine-46 in the lobster enzyme and, to a smaller extent, in the pig and yeast enzymes shows that this residue is particularly susceptible to reaction with iodine. It also occurs in a highly conserved sequence in all three species (Fig. 1). However, this degree of homology alone may not be significant in distinguishing tyrosine-46 from the others, since sequences around, for example, tyrosines 311 and 273, although not 137, are also quite well conserved.

Despite the fact that some of the earlier reactions were rather unspecific, each iodination experiment carried out on the active lobster enzyme led to the conclusion that tyrosine-46 was the most reactive towards iodine. Limited iodination of the lobster enzyme, carboxymethylated at the active site, occurred virtually exclusively at tyrosine-46. The next residue(s) to be iodinated was 39 or 42, or both. The order of reactivity towards iodine of tyrosine residues in the lobster enzyme is: 46 > (39 + 42) > 91, 252, 273, (311 + 317) > 137. This suggests three accessible tyrosine residues (one especially so), five of intermediate reactivity and one buried.

With the enzyme from pig muscle and from yeast, iodination studies again suggested the accessibility of the three tyrosine residues near the *N*-terminus, as well as the inaccessibility of tyrosine-137. Other tyrosine residues, as in the lobster enzyme, appeared to possess intermediate reactivity.

Note added in proof. Material excluded by Sephadex G-50 during fractionation of tryptic digests of lobster glyceraldehyde 3-phosphate dehydrogenase that had been iodinated and then carboxymethylated (see under 'Iodination of native lobster glyceraldehyde 3-phosphate dehydrogenase' in the Results section) was shown by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate to be homogeneous and to have a molecular weight of approx. 12 000 (i.e. roughly double the expected molecular weight for the region of the chain implicated).

	46												
Lobster:	Tyr	Met	Val	Tyr	Met	Phe	Lys	Tyr	Asp	Ser	Thr	His	Gly-Val-Phe
Pig:	-	-	-	-	-	-	Gln	-	-	-	-	-	Lys -
Yeast:	-	Ala	Ala	-	-	-	Lys	-	-	-	-	-	Arg-Tyr

Fig. 1. Amino acid sequence around tyrosine-46 in glyceraldehyde 3-phosphate dehydrogenase from lobster, pig and yeast (the residues are the same in the three sequences unless otherwise indicated).

J.O.T. is pleased to acknowledge tenure of a Beit Memorial Fellowship for Medical Research throughout this work. We also acknowledge the collaboration of Dr R. D. Cole, who participated in the initial studies with the pig enzyme.

REFERENCES

- Allison, W. S. & Kaplan, N. O. (1964a). *J. biol. Chem.* **239**, 2140.
- Allison, W. S. & Kaplan, N. O. (1964b). *Biochemistry, Easton*, **3**, 1792.
- Butler, P. J. G., Harris, J. I., Hartley, B. S. & Leberman, R. (1969). *Biochem. J.* **112**, 679.
- Davidson, B. E., Sajgó, M., Noller, H. F. & Harris, J. I. (1967). *Nature, Lond.*, **216**, 1181.
- Elődi, P. & Libor, S. (1969). In *Pyridine Nucleotide Dependent Dehydrogenases*, p. 175. Ed. by Sund, H. Berlin, Heidelberg and New York: Springer-Verlag.
- Elődi, P., Libor, S. & Móra, S. (1968). *Proc. FEBS 5th Meet., Prague*, vol. 18, p. 17.
- Elődi, P. & Szabolcsi, G. (1959). *Nature, Lond.*, **184**, 56.
- Elődi, P. & Szörényi, E. (1956). *Acta physiol. Acad. sci. hung.* **9**, 339.
- Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 139. Ed. by Hirs, C. H. W. New York and London: Academic Press.
- Gray, W. R. & Hartley, B. S. (1963). *Biochem. J.* **89**, 59P.
- Harris, J. I. (1956). *Nature, Lond.*, **177**, 471.
- Harris, J. I., Meriwether, B. P. & Park, J. H. (1963). *Nature, Lond.*, **198**, 154.
- Harris, J. I. & Perham, R. N. (1968). *Nature, Lond.*, **219**, 1025.
- Havsteen, B. H. (1965). *Acta chem. scand.* **19**, 1643.
- Hughes, W. L. & Straessle, R. (1950). *J. Am. chem. Soc.* **72**, 452.
- Jones, G. M. T. (1969). Ph.D. Thesis: University of Cambridge.
- Jones, G. M. T. & Harris, J. I. (1968). *Abstr. FEBS 5th Meet., Prague*, p. 185.
- Kirschner, K., Eigen, M., Bittman, R. & Voigt, B. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 1661.
- Krebs, E. G. (1955). In *Methods in Enzymology*, vol. 1, p. 407. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Libor, S. & Elődi, P. (1970a). *Eur. J. Biochem.* **12**, 336.
- Libor, S. & Elődi, P. (1970b). *Eur. J. Biochem.* **12**, 345.
- Libor, S., Elődi, P. & Nagy, Z. (1965). *Biochim. biophys. Acta*, **110**, 484.
- Lissitzky, S., Bismuth, J. & Rolland, M. (1962). *Clinica chim. Acta*, **7**, 183.
- Listowsky, I., Furfine, C. S., Betheil, J. J. & England, S. (1965). *J. biol. Chem.* **240**, 4253.
- Michl, H. (1951). *Monatsh. Chem.* **82**, 489.
- Móra, S., Hűvös, P., Libor, S. & Elődi, P. (1969). *Acta biochim. biophys. Acad. sci. hung.* **4**, 151.
- Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C. (1960). *Biochem. J.* **77**, 149.
- Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
- Parker, D. J. & Allison, W. S. (1969). *J. biol. Chem.* **244**, 180.
- Perham, R. N. (1964). Ph.D. Thesis: University of Cambridge.
- Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
- Sanger, F. & Thompson, E. O. P. (1963). *Biochim. biophys. Acta*, **71**, 468.
- Stevenson, G. T. (1968). *J. Chromat.* **37**, 116.
- Tarutani, O. & Ui, N. (1969). *Biochim. biophys. Acta*, **181**, 136.
- Van Zyl, A. & Edelhoch, H. (1967). *J. biol. Chem.* **242**, 2423.
- Wassarman, P. M. & Major, J. P. (1969). *Biochemistry, Easton*, **8**, 1076.
- Wassarman, P. M. & Watson, H. C. (1968). *Proc. FEBS 5th Meet., Prague*, vol. 18, p. 51.
- Watson, H. C. & Banaszak, L. J. (1964). *Nature, Lond.*, **204**, 918.