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Functional Groups in the Activity and Regulation of *Escherichia coli* Citrate Synthase

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1. Citrate synthase has been purified from *Escherichia coli* and shown to exist at an equilibrium between three forms: monomer (mol.wt. 57000), tetramer (mol.wt. 230000) and, possibly, octamer. Modification of the enzyme by photo-oxidation and by treatment with specific chemical reagents has been carried out to gain information on the amino acid residues involved in enzymic activity and in the inhibition of activity by NADH and α-oxoglutarate. 2. Several photo-oxidizable amino acids appear to be involved in activity. The nature of the pH-dependence of their rates of photo-oxidation with Methylene Blue suggests that these are histidines, a conclusion supported by the greater rate of photo-inactivation with Rose Bengal and the destruction of activity by diethyl pyrocarbonate. 3. The participation of histidine at the α-oxoglutarate effector site is indicated by photo-oxidation and the participation of cysteine at the NADH effector site suggested by photo-oxidation is confirmed by the desensitization to NADH produced by treatment with 5,5′-dithiobis-(2-nitrobenzoate). Inactivation of the enzyme after modification with this reagent suggests the additional involvement of cysteine in catalytic activity. 4. Amino acid analyses of native and photo-oxidized enzyme are consistent with these conclusions. 5. Modification with 2-hydroxy-5-nitrobenzyl bromide indicates the participation of tryptophan in the activity of the enzyme.

Citrate synthase (EC 4.1.3.7) catalyses the introduction of acetyl-CoA to the tricarboxylic acid cycle, and may thus be regarded as the initial enzyme of that cycle and hence as an important potential site for its control. The dual role of the tricarboxylic acid cycle in providing both energy and biosynthetic precursors is reflected in the regulatory behaviour of citrate synthase from the Enterobacteriaceae. In these organisms citrate synthase is inhibited by NADH (Weitzman, 1966*a*,*b*; Weitzman & Jones, 1968) and by α-oxoglutarate (Wright *et al*., 1967; Weitzman & Dunmore, 1969), both inhibitors probably acting in an allosteric manner.

We have previously shown that citrate synthase may be purified from *Escherichia coli* extracts as a stable enzyme retaining sensitivity to feedback inhibition (Weitzman, 1969). A full understanding of the molecular processes involved in catalysis and its regulation requires knowledge of the chemical structure of the enzyme, the functional groups involved in the binding of ligands (substrates and effectors) and the interactions leading to modulation of activity. As a start in this direction we have investigated the effects of modification of the enzyme by photo-oxidation and by specific chemical reagents on catalytic activity and sensitivity to inhibition. The results presented here clearly implicate particular amino acid residues in the functional behaviour of the enzyme. Complementary studies by other investigators on the kinetic, physicochemical and binding properties of the enzyme have been reported (Faloona & Srere, 1969; Wright & Sanwal, 1971).

Experimental

Materials

Chemicals used were analytical grade or the finest grade commercially available. DEAE-cellulose was Whatman grade DE-11 obtained from H. Reeve Angel and Co., London, U.K. Sephadex G-200 and A-50 were obtained from Pharmacia (Great Britain) Ltd., London, U.K.; Rose Bengal, 5,5′-dithiobis-(2-nitrobenzoic acid) and 2-hydroxy-5-nitrobenzyl bromide were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; Methylene Blue and diethyl pyrocarbonate were from BDH Chemicals Ltd., Poole, Dorset, U.K.

The organism used in this work was *Escherichia coli* K12 strain CA 244. Glycerol-grown cells were obtained as a frozen paste from the Microbiological Research Establishment, Porton Down, Wilts., U.K.

Methods

Assay of citrate synthase. Citrate synthase was assayed spectrophotometrically at 412nm and 25°C by the method of Srere *et al*. (1963). Unless otherwise

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stated, assay mixtures contained 20mM-Tris–HCl, pH 8.0, 1mM-EDTA, 0.1mM-oxaloacetate, 0.15mM-acetyl-CoA and 0.1mM-5,5′-dithiobis-(2-nitrobenzoate).

The concentration of protein in impure preparations was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as standard, and in purified preparations from the values of absorbance at 260 and 280nm (Layne, 1957). Specific activity is expressed as μmol of CoASH produced/min per mg of protein.

Purification of citrate synthase. The purification procedure was based on that described by Weitzman (1969). A 200g batch of frozen cells was thawed, washed and suspended to a total volume of 400ml in 20mM-Tris–HCl, 1mM EDTA, pH 8.0 (hereafter referred to as 'Tris buffer'), containing 0.1M-KCl. This suspension was passed once through a French press at 12000lb/in^2 (8.3×10^4kPa) and cell debris was removed by centrifugation at 25000g for 3h. This and all subsequent steps were carried out at 4°C.

An aqueous 27% (w/v) solution of protamine sulphate was added to the supernatant solution (1mg of protamine sulphate/10mg of protein) and the mixture stirred for 15min. The precipitate was removed by centrifugation at 25000g for 20min and to the supernatant solution was added finely ground $(NH_4)_2SO_4$ (35.2g added to each 100ml). The mixture was stirred for 20min and the precipitate then removed by centrifugation as before. A further 10.3g of $(NH_4)_2SO_4$ was then added per 100ml of the supernatant solution and the mixture stirred for 30min. The precipitate was collected by centrifugation, dissolved in 20ml of Tris buffer containing 0.1M-KCl and dialysed overnight against similar buffer. The dialysed enzyme solution was diluted with an equal volume of Tris buffer to decrease the KCl concentration to 50mM, and was then applied to a DEAE-cellulose column (35cm × 2.5cm) previously equilibrated with Tris buffer containing 50mM-KCl. The column was washed with 100ml of the equilibrating solution and then with a solution of Tris buffer containing 0.1M-KCl until no more protein was eluted. A linear gradient of KCl (400ml) from 0.1–0.3M in Tris buffer was then applied and the effluent collected in 6ml fractions. Citrate synthase was eluted at about 0.15M-KCl and those fractions containing the enzyme of a specific activity greater than 30units/mg were pooled and $(NH_4)_2SO_4$ was added (0.56g to each ml). The precipitate so formed was collected by centrifugation and dissolved in 1.5ml of Tris buffer containing 0.1M-KCl. This solution, made 10% in sucrose, was applied to a Sephadex G-200 column (30cm × 2.5cm) equilibrated in Tris buffer with 0.1M-KCl and the protein eluted with the same buffer, 2ml fractions being collected. Those fractions containing citrate synthase of a specific activity greater than 50 units/mg were pooled and applied, again with sucrose, to a DEAE-Sephadex A-50 column (35cm × 2.5cm) equilibrated with Tris buffer containing 0.1M-KCl. The column was washed with this buffer until no more protein was removed and citrate synthase was then eluted with a linear gradient of KCl (300ml) from 0.1 to 0.4M, 3ml fractions being collected. The best fractions contained enzyme of a specific activity greater than 80 units/mg. These were pooled, the enzyme was precipitated at 80% saturation with $(NH_4)_2SO_4$ and collected by centrifugation and redissolved in Tris buffer to a protein concentration of 1mg/ml. The $(NH_4)_2SO_4$ was removed by dialysis against Tris buffer and the enzyme solution so obtained was used in the studies described. This procedure achieved a 100-fold purification of the enzyme and was found to be reproducible (Table 1).

Polyacrylamide-gel electrophoresis. Disc gel electrophoresis at pH 8.9 was performed as described

Table 1. *Purification of E. coli citrate synthase*

Step	Volume (ml)	Total enzyme activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
1. French-press extract	475	7450	7830	0.95	—
2. Supernatant from protamine sulphate	495	7560	5520	1.38	102
3. 55–70%-satd.-$(NH_4)_2SO_4$ precipitate	32	5180	830	6.2	69.6
4. After dialysis	50	4410	830	5.3	59.3
5. Pooled selected fractions from DEAE-cellulose column	30	1990	52.4	38.0	26.8
6. Pooled selected fractions from Sephadex G-200 column	18.1	880	15.6	56.3	11.2
7. Pooled selected fractions from DEAE-Sephadex column	22.4	630	7.1	88.4	8.5

by Davis (1964), with 7% polyacrylamide gels. Purified protein (50 μg) was applied to the gels and, after electrophoresis, protein was detected by staining with 1% Coomassie Brilliant Blue in 12.5% trichloroacetic acid by the method of Chrambach *et al.* (1967). Electrophoresis in the presence of sodium dodecyl sulphate was performed in 10% polyacrylamide gels according to the procedure of Weber & Osborn (1969) and the gels were stained with Coomassie Brilliant Blue.

Analytical ultracentrifugation. Sedimentation-velocity measurements were made in an MSE analytical ultracentrifuge. Protein peaks were detected at 280nm with a u.v. scanner and the observed sedimentation coefficients were corrected to the density and viscosity of water ($s_{20,w}$ values). All protein solutions used in the ultracentrifugation experiments had an absorbance of 1.0 at 280nm.

Analytical band centrifugation of the active enzyme–substrate complex was carried out according to the method described by Cohen & Mire (1971). Into a Vinograd centrifuge cell was introduced the substrate solution consisting of 5mM-acetyl-CoA (approx. $65\times K_m$), 2mM-oxaloacetate (approx. $50\times K_m$) and 1mM-5,5′-dithiobis-(2-nitrobenzoate) in Tris buffer containing 0.1M-KCl. The high salt concentration was included to prevent inactivation of the citrate synthase by 5,5′-dithiobis-(2-nitrobenzoate) (Weitzman, 1966*b*) throughout the 20min centrifugation. Enzyme solution (15 μl), at a protein concentration of 10 μg/ml, was placed in the well of the Vinograd cell and transfer to the surface of the substrate solution occurred at approx. 10000rev./min. Enzyme activity was measured by following the increase in absorbance at 412nm.

Photo-oxidation. Photo-oxidation was performed at 20°C with a 150W Crompton spot-light at a distance of 11cm from the top of a glass conical centrifuge tube containing the enzyme solution and photosensitive dye. At intervals, samples were withdrawn and immediately diluted ten times with Tris buffer in small glass tubes covered with aluminium foil to prevent further reaction. In all photo-oxidation experiments the concentration of protein was 0.15mg/ml and that of the photo-sensitive dye was 3 μM; i.e. the protein subunits and dye were about equimolar.

Amino acid analyses. Amino acid analyses were carried out on samples of native enzyme and of photo-oxidized enzyme by the method of Atkin & Ferdinand (1970, 1971). Hydrolyses were performed for 24, 48 and 72h in 6M-HCl under vacuum at 110°C.

The tryptophan and tyrosine contents of the enzyme were estimated spectrophotometrically by the method of Bencze & Schmid (1957) and the determination of cysteine residues was carried out at 412nm with 5,5′-dithiobis-(2-nitrobenzoate) in the presence of 5M-urea.

Treatment of the enzyme with specific chemical reagents. (1) Diethyl pyrocarbonate. The concentration of diethyl pyrocarbonate in commercial preparations was determined from the increase in E_{240} with 0.3mM-imidazole, pH8.0, on the basis of a molar extinction coefficient for the product, *N*-ethoxyformylimidazole, of 3200 litre·mol^{-1}·cm^{-1} (Ovádi *et al.*, 1967).

Purified citrate synthase, 0.16mg/ml, in 5mM-sodium acetate buffer, pH6.0, was treated with diethyl pyrocarbonate over a concentration range 0–17.5 μM, representing a molar ratio range (reagent to enzyme, mol. wt. 230000) of 0–22. The diethyl pyrocarbonate, in aq. 10% ethanol, was added to the enzyme and the mixtures were incubated at 0°C for 12h, during which time any unchanged reagent should decompose completely to ethanol and CO_2 (Mühlrad *et al.*, 1969). The treated enzyme was then assayed for activity, and the inhibitions produced by NADH (0.5mM) and by α-oxoglutarate (2.0mM) were measured.

(2) 2-Hydroxy-5-nitrobenzyl bromide. The purified enzyme was diluted to 14 μg/ml in 5mM-sodium acetate buffer, pH4.6, and the temperature brought to 25°C. To 1ml of this solution was added 6nmol of 2-hydroxy-5-nitrobenzyl bromide in 1 μl of dry ethanediol to produce a molar ratio (reagent to enzyme) of 100. The mixture was vigorously mixed for 30s, after which the enzyme activity and inhibitions by NADH (0.1mM) and by α-oxoglutarate (2.0mM) were measured. Owing to the high absorbance of the reagent at 412nm it was necessary to dilute the treated enzyme 50-fold before assay.

(3) 5,5′-Dithiobis-(2-nitrobenzoate). The purified citrate synthase was incubated in 1.0ml of Tris buffer with 0.1mM-5,5′-dithiobis-(2-nitrobenzoate) at 20°C in the dark. At intervals samples were removed and measurements were made of the activity and the inhibitions by NADH (0.2mM) and by α-oxoglutarate (2.0mM).

Results and Discussion

Homogeneity of the enzyme

The enzyme showed three electrophoretically distinguishable protein bands at pH8.9 (Fig. 1). The individual bands were obtained from an unstained gel by cutting 1mm slices and each of the three was transferred to the top of a fresh gel, covered with a thin layer of pH8.9 buffer, left for 15min and then subjected to electrophoresis as before. In each case three protein bands were again observed with mobilities identical with those on the initial gel. These results indicate an equilibrium between different forms of the enzyme rather than the presence of an

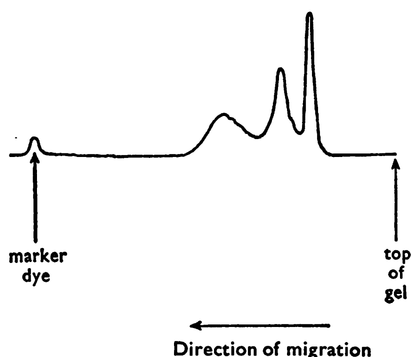

Fig. 1. *Densitometer trace of purified citrate synthase after polyacrylamide-gel electrophoresis*

Electrophoresis at pH8.9 and staining were carried out as described in the Experimental section and the gels scanned with a Joyce–Loebl recording microdensitometer (mark IIIC).

impurity and this method of fractionation has been suggested by Cann & Goad (1968) to provide an unambiguous distinction between the two possibilities. These authors have also discussed how electrophoretically distinct bands can arise from interconvertible forms of a protein. Extraction of the three protein bands into Tris buffer followed by enzyme assay showed citrate synthase activity to be present in each extract, consistent with there being an equilibrium between the three species.

Electrophoresis in sodium dodecyl sulphate at pH8.9 gave a single protein band, confirming the homogeneity of the preparation and, in addition, suggesting that the enzyme is composed of subunits of similar molecular weight. The molecular weight of citrate synthase treated with sodium dodecyl sulphate was determined by electrophoresis in 10% polyacrylamide gels and comparison with standard proteins treated in the same way.

The standards used were bovine serum albumin (mol.wt. 68000), ox liver catalase (mol.wt. 62500), rabbit muscle pyruvate kinase (mol.wt. 57000), pig heart fumarase (mol.wt. 49000), rabbit muscle aldolase (mol.wt. 40000) and rabbit muscle glyceraldehyde phosphate dehydrogenase (mol.wt. 36000). The plot of log mol.wt. against mobility was linear and a value of 55000 ± 4000 was obtained for citrate synthase.

Ultracentrifugation studies at pH8.0 demonstrated a size difference between the three species, and although the fastest sedimenting peak was too diffuse for an accurate sedimentation coefficient to be obtained, $s_{20,w}$ values for the two slower species were calculated to be 4.3S and 10.1S. At a total concentration of 1mg/ml the percentages of protein in each peak were 4% for the fastest, 75% for the 10.1S species and 21% for the 4.3S species. This distribution is in essential agreement with that reported by Wright & Sanwal (1971). Taking $\bar{v}$ as 0.73ml/g (calculated from the amino acid composition data below) and $D_{20,w}$ as 6.9×10^{-7}ml/s (Wright & Sanwal, 1971), the molecular weight of the slowest sedimenting species was calculated to be 57000, and by comparison with the sedimentation behaviour of catalase (mol.wt. 244000; Barlow & Margoliash, 1969) the molecular weight of the 10.1S species was calculated to be approx. 230000.

Ultracentrifugation studies by Wright & Sanwal (1971) indicated that at pH11.0 the enzyme dissociates to form monomers. We have confirmed this and have observed a single symmetrical peak at this pH of $s_{20,w}$ value 4.1S. Wright & Sanwal (1971) also reported that at pH7.0 in the presence of 2mM-dithiothreitol only the octameric species was present. However, our experiments under these conditions indicated that the tetrameric form predominated. The discrepancy between our results and those of Wright & Sanwal (1971) is perhaps due to the different protein concentrations employed (1 and 8.4mg/ml respectively).

Nevertheless our results are in essential agreement with those of Wright & Sanwal (1971), indicating that the enzyme exists in an association–dissociation equilibrium. These authors suggested that the tetramer exhibits catalytic activity. We examined this by the method of analytical band centrifugation of the active enzyme–substrate complex (Cohen & Mire, 1971) and obtained a sedimentation coefficient for the active species of 9.9S. This is in close agreement with the value of 10.1S determined above and indicates that the tetramer possesses catalytic activity. This technique necessitates the use of very low concentrations of enzyme. Under such conditions the concentration of octamer is likely to be extremely small and any activity associated with it undetectable. Moreover, the high activity of the tetramer prevents detection of any activity in the monomer. Wright & Sanwal (1971) concluded from their studies that both monomer and octamer are inactive.

Photo-oxidation

Effects on catalytic and regulatory properties. Photo-oxidation of citrate synthase at pH8.0 with Methylene Blue as the photo-sensitive dye resulted in a decay of enzymic activity, which, when plotted in a semi-log fashion, was non-linear (Fig. 2). In addition, photo-oxidation produced a rapid loss of NADH inhibition, indicating a photo-oxidizable residue at this effector site, and a slower decay of inhibition by α-oxoglutarate (Fig. 3).

The curved semi-log plot (Fig. 2) indicates that more than one first-order process is involved in the

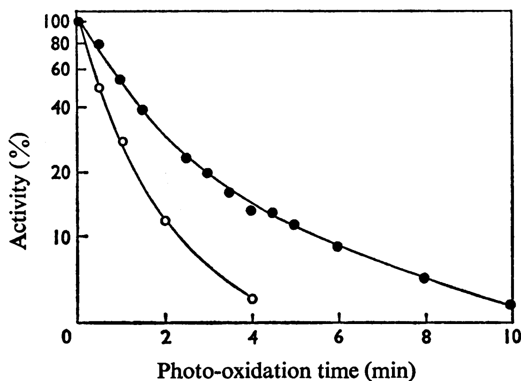

Fig. 2. *Effect of photo-oxidation on enzyme activity*

Photo-oxidation was carried out as described in the text in 20mM-Tris–HCl, 1mM-EDTA, pH8.0, in the presence of 3μM-Methylene Blue (●) or 3μM-Rose Bengal (○). For Methylene Blue only, the line through the experimental points is a theoretical one constructed on the basis of three first-order reactions with rate constants 1.4, 0.68 and 0.17min^{-1} calculated from the experimental data.

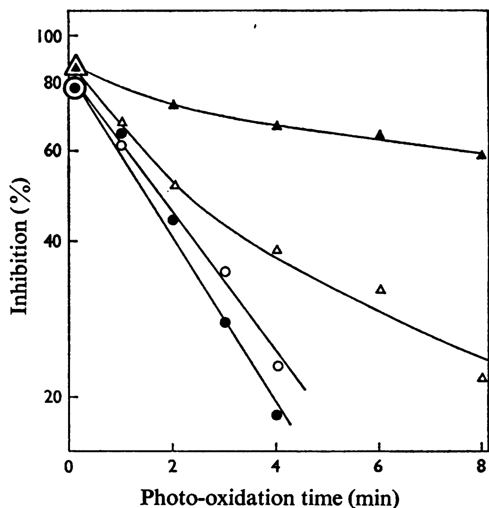

Fig. 3. *Effect of photo-oxidation on the inhibition of enzyme activity by NADH and α-oxoglutarate*

Photo-oxidation was carried out as described in the text. Samples were assayed in the absence and presence of NADH or α-oxoglutarate and the percentage inhibition was thereby determined and plotted against the time of photo-oxidation. In the presence of 3μM-Methylene Blue: ●, inhibition by 0.4mM-NADH; ▲, inhibition by 2mM-α-oxoglutarate. In the presence of 3μM-Rose Bengal: ○, inhibition by 0.4mM-NADH; △, inhibition by 2mM-α-oxoglutarate.

photo-inactivation. We first analysed the data on the assumption that the curve is biphasic. The two first-order rate constants thereby calculated (1.0 and 0.17min^{-1}) allowed reconstruction of a decay curve that closely fitted the experimental data. However, analysis of curves obtained by photo-oxidation at pH values between 6 and 8 showed marked inconsistency between the activity losses associated with the two phases, even though all measurements of activity were made at pH8.

We therefore re-analysed the data on the assumption of a triphasic decay of activity and determined the three first-order rate constants to be 1.4, 0.68 and 0.17min^{-1}. Again, the reconstructed curve fitted the experimental data well (Fig. 2) and, moreover, the activity losses associated with the three phases were consistent over the pH range investigated. Hence a triphasic decay is favoured as the simplest interpretation of the photo-inactivation kinetics, though clearly the process may be even more complex. The three phases are designated (a), (b) and (c), in order of decreasing photo-oxidation rate constants. It should be noted that each of (a), (b) and (c) may represent a single amino acid residue or a set of residues undergoing photo-oxidation at identical rates.

Of the amino acids found in proteins, only five are susceptible to photo-oxidation: histidine, tryptophan, tyrosine, methionine and cysteine (Weil & Maher, 1950; Weil *et al.*, 1951). In studies on free amino acids, Weil (1965) showed that the rate of photo-oxidation of tyrosine, tryptophan, histidine and methionine depended characteristically on the pH of the solution. For histidine, the rate was entirely dependent on the ionization state of the imidazole ring, only the uncharged form being photo-oxidized. For tyrosine, tryptophan and methionine, other factors in addition to the state of ionization appeared to determine the rate of photo-oxidation. Similar pH profiles have been observed for amino acids incorporated into proteins, as reported, for example, by Westhead (1965) and Martinez-Carrion *et al.* (1967). Weil (1965) did not report studies on the pH dependence of cysteine photo-oxidation. We have investigated the pH-dependence of photo-oxidation of the cysteinyl thiol group in the tripeptide glutathione as a better model for protein thiol groups than cysteine itself. The results presented in Fig. 4 are consistent with the photo-oxidation of a group with $pK_a \simeq 8.5$. This is in fair agreement with reported values for the thiol group of glutathione (Calvin, 1954; Isherwood, 1959).

The pH-dependence of the rates of photo-oxidation of the amino acids of citrate synthase was investigated. For the triphasic decay of activity, calculation of the percentage of activity lost by photo-oxidation of each group indicated that their relative rates of photo-oxidation were maintained at each pH.

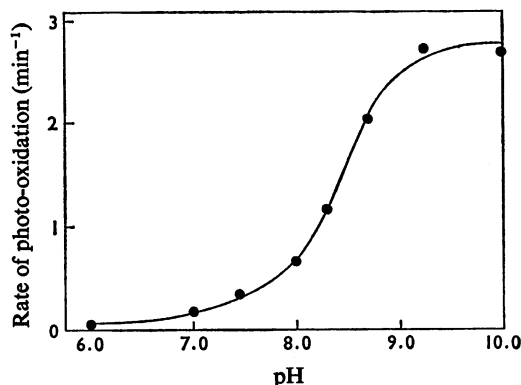

Fig. 4. *pH dependence of the rate of photo-oxidation of glutathione*

Reduced glutathione (0.6mM) was photo-oxidized as described in the text in buffers of composition 20mM-Tris–HCl, 1mM-EDTA with 3μM-Methylene Blue. At intervals, 0.1ml samples were removed, diluted 10-fold with buffer at pH8.0 and the concentration of thiol present was determined at 412nm with 5,5′-dithiobis-(2-nitrobenzoate). The rate of photo-oxidation is in arbitrary units.

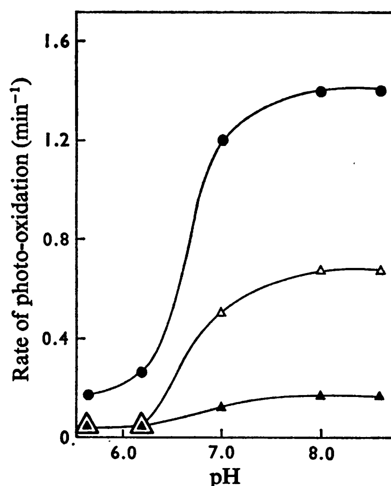

Fig. 5. *pH dependence of the rates of photo-oxidation of the three photo-oxidizable amino acids implicated in the activity of citrate synthase*

Photo-oxidation was carried out at various pH values in buffers of composition 20mM-Tris–HCl, 1mM-EDTA with 3μM-Methylene Blue. Rate constants for the photo-oxidation of the three amino acids were calculated as described in the text. ●, Residue (a); △, residue (b); ▲, residue (c).

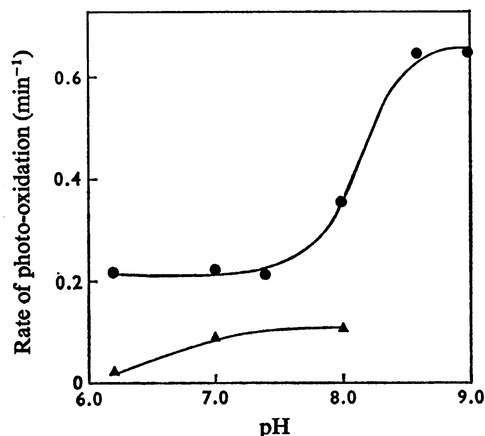

Fig. 6. *pH dependence of the rates of photo-oxidation of the photo-oxidizable amino acids implicated in the regulation of citrate synthase*

Photo-oxidation was carried out at various pH values in buffers of composition 20mM-Tris–HCl, 1mM-EDTA with 3μM-Methylene Blue. The effects on the NADH and α-oxoglutarate inhibitions were determined as described in the legend to Fig. 3; the rate constants for photo-densitization to NADH (●) and α-oxoglutarate (▲) were thereby calculated and plotted against pH.

We have therefore been able to plot the rates of photo-oxidation against pH for the amino acids (a), (b) and (c) (Fig. 5). Comparison of these pH profiles with those obtained by Weil (1965) for histidine, tyrosine, tryptophan and methionine, and that reported here for cysteine residues suggests that the amino acids (a), (b) and (c) associated with activity may be histidines. It is important to note that although, as discussed above, the triphasic photo-inactivation is favoured over a biphasic process, nevertheless similar analysis of the results on the basis of the latter assumption also implicates histidines. Additionally, the photo-oxidizable residue at the α-oxoglutarate effector site may also be histidine, whereas that at the NADH site may be tyrosine or cysteine (Fig. 6.).

Further evidence for the presence of photo-oxidizable histidine residues involved in activity and regulation has been obtained by the use of Rose Bengal as the photo-sensitive dye in place of Methylene Blue. This anionic dye was investigated by Westhead (1965) in the hope that it would catalyse photo-oxidation of enzymes with greater specificity than does the cationic Methylene Blue. Preferential photo-oxidation of histidine in the presence of Rose Bengal was indeed observed, and has also been found by Groudinsky (1971) and by

Coulson & Yonetani (1972). Consistent with the inferences from the pH-dependence studies reported above we found that the rates of loss of citrate synthase activity (Fig. 2) and of α-oxoglutarate inhibition (Fig. 3) were greater with Rose Bengal than with Methylene Blue, but the rate of photo-oxidation of the amino acid at the NADH site remained essentially unchanged (Fig. 3).

Protective effects of substrates and inhibitors. Both substrates of the enzyme afforded some protection against photo-inactivation, as did the inhibitor α-oxoglutarate (Fig. 7). Further, the protective effects of acetyl-CoA and α-oxoglutarate were additive (Fig. 7). Although photo-oxidation in the presence of NADH appeared to indicate complete protection of the enzyme, subsequent experiments showed that the NADH is itself photo-oxidized and that protection of the enzyme is lost when all the NADH has been photo-oxidized. Although it is possible that binding of NADH to the enzyme produces protection against inactivation it may well be that protection arises simply from a quenching effect by the more rapid photo-oxidation of the NADH. Our results should be borne in mind in the examination of photo-oxidative effects on other enzymes for which NADH is a substrate or effector. Photo-oxidation of NADH produces NAD^+ (Wagner-Romero *et al.*, 1966); oxaloacetate, acetyl-CoA and α-oxoglutarate were not affected by photo-oxidation.

Inhibition of the enzyme by NADH is competitive with acetyl-CoA and independent of oxaloacetate (Weitzman, 1966*a*). This is reflected in the observation that acetyl-CoA considerably decreased the photo-oxidative destruction of the NADH site whereas oxaloacetate provided little protection (Fig. 8). Both acetyl-CoA and oxaloacetate protected the enzyme against loss of α-oxoglutarate inhibition, though only to a small extent.

Effects on molecular structure. The amino acid composition of the enzyme is given in Table 2. Whereas the contents of tryptophan and cysteine, both determined spectrophotometrically, are in close agreement with those quoted by Wright & Sanwal (1971), the values they gave for the other amino acids are lower than the corresponding values in Table 2. Moreover, the molecular weight calculated from the amino acid composition given by Wright & Sanwal (1971) is 173000, which is considerably lower than the value they quoted of 245000. There thus appears to be some discrepancy in their results.

Amino acid analyses of the five potentially susceptible residues in native and photo-oxidized enzyme are presented in Table 3. These show substantial losses of cysteine, histidine and methionine with a smaller loss of tryptophan. There appears to be no photo-oxidation of tyrosine residues. The extent of methionine photo-oxidation may be greater than the data suggest since the oxidation product, methionine sulphoxide (Weil *et al.*, 1951), may undergo partial reversion to methionine during acid hydrolysis (Ray & Koshland, 1962). These observations are consistent with the conclusion drawn from

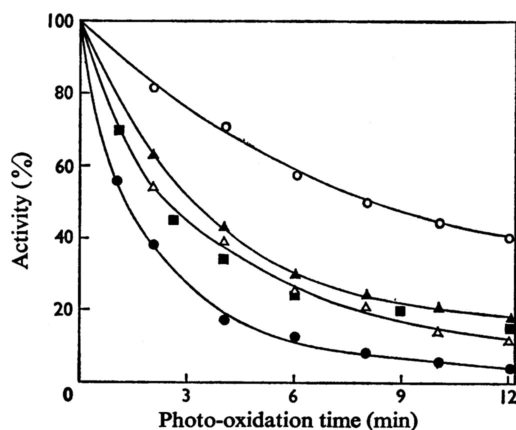

Fig. 7. *Protection against photo-inactivation by substrates and effector*

Conditions for photo-oxidation were as described for Fig. 2 with 3 μM-Methylene Blue. ●, Enzyme alone; △, with 0.6 mM-acetyl-CoA; ■, with 1 mM-oxaloacetate; ▲, with 10 mM-α-oxoglutarate; ○, with 0.6 mM-acetyl-CoA + 10 mM-α-oxoglutarate.

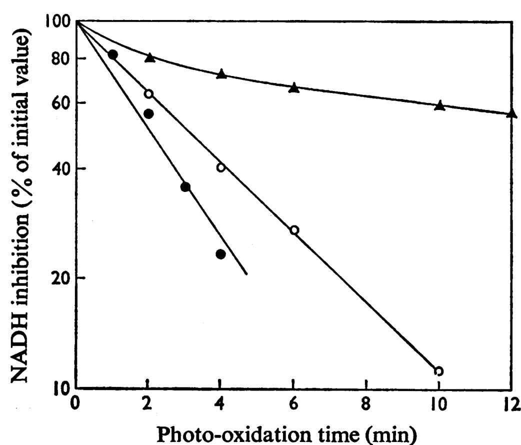

Fig. 8. *Protection by substrates against photo-desensitization of the enzyme to NADH*

Conditions for photo-oxidation were as described for Fig. 2 with 3 μM-Methylene Blue. Inhibition was tested at 0.4 mM-NADH. ●, Enzyme alone; ○, with 1 mM-oxaloacetate; ▲, with 0.6 mM-acetyl-CoA.

Table 2. *Amino acid analysis of E. coli citrate synthase*

Amino acid	Residues/molecule (mol.wt. 230000) ±S.E.M. ($n = 3$)
Tryptophan	25±0.6*
Cysteine	32±1.7
Tyrosine	65±1.4*
	64±0.7
Histidine	72±0.6
Methionine	72±1.0
Proline	84±2.5
Valine	98±0.4
Phenylalanine	106±1.5
Lysine	116±2.1
Arginine	116±2.2
Serine	122±2.8
Isoleucine	128±1.9
Threonine	132±0.1
Glycine	146±1.1
Leucine	168±0.6
Glutamate	176±0.1
Alanine	184±0.4
Aspartate	220±1.6

* These values were determined spectrophotometrically as described in the Experimental section. All other determinations were performed with an automatic amino acid analyser and the values arrived at by a method of integral fitting (W. Ferdinand, personal communication).

Table 3. *Comparison of the amino acid analyses of native and photo-oxidized citrate synthase for the potentially photo-oxidizable residues*

Amino acid analyses were carried out on native enzyme and on enzyme photo-oxidized for 12min in 20mM-Tris–HCl, 1mM-EDTA, pH8.0, 3μM-Methylene Blue. The values for tryptophan, cysteine and tyrosine* were determined spectrophotometrically (see the Experimental section); the other values were obtained by automatic amino acid analysis (see Table 2).

Amino acid	Residues/molecule (mol.wt. 230000) ±S.E.M. ($n = 3$)	
	Native enzyme	Photo-oxidized enzyme
Tryptophan	25±0.6	22±0.4
Cysteine	32±1.7	23±1.5
Histidine	72±0.6	52±1.0
Methionine	72±1.0	60±1.9
Tyrosine	64±0.7	70±1.0
Tyrosine*	65±1.4	64±1.0

the photo-oxidation experiments and suggest that the photo-oxidizable amino acid at the NADH site is a cysteine rather than a tyrosine residue. The absence of tyrosine photo-oxidation was not entirely surprising as destruction of this amino acid often occurs only when most of the available histidine and tryptophan residues have been oxidized (Weil & Buchert, 1951; Fowlks, 1959; Vodrážka, 1959).

Photo-oxidation of the enzyme was accompanied by a progressive loss in absorbance at 277.5nm, with a concomitant increase in absorbance in the region of 240–260nm. As tyrosine remains unaffected, the loss at 277.5nm must be due to the photo-oxidation of tryptophan residues in the enzyme. Presumably the increase in the 240–260nm region of the spectrum is caused by the generation of u.v.-absorbing products from the amino acids. A similar increase has been observed in enzymes in which only histidine has been photo-oxidized (e.g. Weil & Seibles, 1955; Weil *et al.*, 1965; Martinez-Carrion *et al.*, 1970) and also when tryptophan is photo-oxidized either as the free amino acid (Weil *et al.*, 1951) or as a component of small peptides (Benassi *et al.*, 1967).

Electrophoresis of a sample of enzyme that had been photo-oxidized for 12min with Methylene Blue revealed no changes in the electrophoretic mobilities of the monomer, tetramer or octamer. Vodrážka *et al.* (1961) have provided evidence that no polar groups are formed in the photo-oxidation of imidazole and phenolic nuclei, but that the indole nucleus of tryptophan probably gives rise to carboxyl groups. Cysteine is photo-oxidized to cysteic acid (Weil *et al.*, 1951). In the 12min photo-oxidation one tryptophan and two cysteine residues per 57000 mol.wt. were destroyed and these changes appear to be too small to affect the electrophoretic behaviour.

Chemical modification of the enzyme

The tentative conclusions concerning the identity of amino acid residues in the enzyme participating in catalytic activity and regulatory behaviour, which we have drawn from the photo-oxidation studies, amino acid analyses and other results described above have been further strengthened by studies on the modification of the enzyme with specific chemical reagents.

Diethyl pyrocarbonate. Diethyl pyrocarbonate carbethoxylates amino acid residues of proteins, and at pH6.0 histidine residues are specifically attacked (Mühlrad *et al.*, 1967, 1969). Treatment of citrate synthase with this reagent caused a loss of activity, the extent of inactivation being linear with increasing concentration of reagent up to a molar ratio of 22 (Fig. 9). Evidence concerning the specificity of attack of the enzyme by diethyl pyrocarbonate is derived from the following observations on enzyme that had been 80% inactivated by the reagent. A difference spectrum versus native enzyme showed a

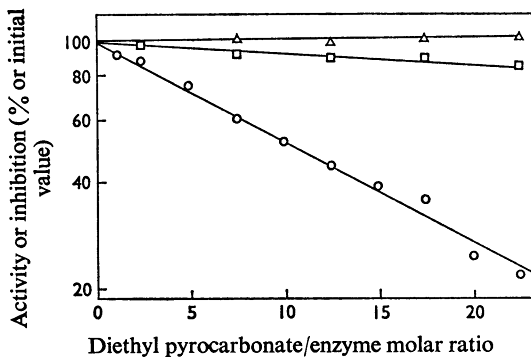

Fig. 9. *Effects of diethyl pyrocarbonate on the activity and inhibition of citrate synthase*

Treatment with diethyl pyrocarbonate was carried out as described in the text. ○, Enzyme activity; □, inhibition by 0.5mM-NADH; △, inhibition by 2mM-α-oxoglutarate.

peak at 243nm characteristic of *N*-carbethoxy-imidazole (Mühlrad *et al.*, 1967; Elödi, 1968). Taking the value $\epsilon_{240} = 3.2\times10^3$ litre·mol^{-1}·cm^{-1} the spectra obtained from several experiments indicated the modification of 2–3 histidine residues per enzyme subunit (mol.wt. 57000). The absence of any change in absorbance at 280nm suggests no loss of tyrosine or tryptophan, and determination of thiol groups with 5,5′-dithiobis-(2-nitrobenzoate) indicated no modification of cysteine residues. Enzyme activity was restored completely by treatment of the modified enzyme with 0.5M-hydroxylamine at pH7 for 15min, a procedure which removes carbethoxy groups from histidine but not from lysine or arginine (Melchior & Fahrney, 1970).

These observations are consistent with the results of photo-oxidation experiments implicating histidine residues in the activity of the enzyme. Studies on the citrate synthase reaction (Buckel & Eggerer, 1969) suggest that citryl-CoA may be an intermediate and that its hydrolysis to citrate may proceed via the formation of an acyl-imidazole.

Treatment with diethyl pyrocarbonate produced no appreciable loss of NADH inhibition (Fig. 9). Surprisingly, α-oxoglutarate inhibition was also unaffected despite the indication from photo-oxidation experiments that histidine is involved in this inhibition. Nevertheless, the very slow rate of photo-oxidation of this residue ($k = 0.104$ min^{-1} at pH8.0, see Fig. 3) suggests that it may be partly buried within the tertiary or quaternary structure of the enzyme and thus may be unavailable to attack by the diethyl pyrocarbonate. Attempts to investigate this possibility by unfolding the protein with urea before treatment with diethyl pyrocarbonate did not

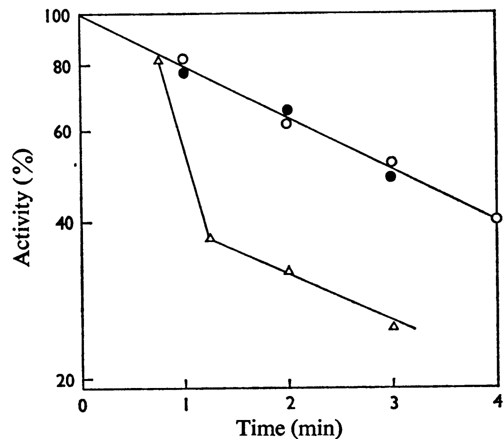

Fig. 10. *Inactivation of citrate synthase by 2-hydroxy-5-nitrobenzyl bromide*

Treatment with 2-hydroxy-5-nitrobenzyl bromide was carried out as described in the text. ●, Enzyme alone at pH4.6; ○, with 1μl of ethanediol; △, with a 100-fold molar excess of 2-hydroxy-5-nitrobenzyl bromide in 1μl of ethanediol added at $t = 45$s.

prove feasible, owing to irreversible urea inactivation of the enzyme.

2-Hydroxy-5-nitrobenzyl bromide. Koshland *et al.* (1964) introduced the reagent 2-hydroxy-5-nitrobenzyl-bromide and demonstrated its specificity for tryptophan residues, although it did react to a small extent with thiol groups. The latter reaction was minimized at low pH and in the presence of susceptible tryptophan residues, and under these conditions the specificity for tryptophan has deen demonstrated with several proteins (e.g. Barman & Koshland, 1967; Poulos & Price, 1971; Ford-Hutchinson & Perkins, 1972).

Treatment of citrate synthase at pH4.6 with a 100-fold molar excess of 2-hydroxy-5-nitrobenzyl bromide caused a rapid loss in activity over a period of less than 30s (Fig. 10) but had no effect on the NADH or α-oxoglutarate inhibitions. In view of the very rapid hydrolysis of the reagent (Horton & Koshland, 1965) a further 100-fold molar excess was added and resulted in an overall loss of more than 90% of the initial activity. After extensive dialysis against Tris buffer the number of hydroxynitrobenzyl groups incorporated was determined from the absorbance at 410nm (Koshland *et al.*, 1964). It was found that approx. 1.3 tryptophan residues per enzyme subunit (mol.wt. 57000) had been modified; no loss of thiol groups was detected. The value in excess of 1.0 may be due to di-substitution of tryptophan (Barman & Koshland, 1967; Barman, 1972). The observations suggest the involvement in enzyme

activity of a specific tryptophan residue in each subunit.

5,5′-Dithiobis-(2-nitrobenzoate). Treatment of the enzyme with 0.1 mM-5,5′-dithiobis-(2-nitrobenzoate), a reagent specific for thiol groups, resulted in a time-dependent loss of enzyme activity and sensitivity to NADH inhibition (Fig. 11) but no loss of α-oxoglutarate inhibition. Whereas the time-course curve for the inactivation was exponential, that for the loss of NADH sensitivity showed a definite lag and may be sigmoidal. Measurement of the time-course of thiol modification showed that two cysteine molecules per monomer (mol.wt. 57000) reacted with 5,5′-dithiobis-(2-nitrobenzoate) and that modification of the first proceeded more rapidly than that of the second and was complete within 4min. These results are similar to those of Wright & Sanwal (1971). Assays performed by allowing the catalytic reaction to proceed in the absence of 5,5′-dithiobis-(2-nitrobenzoate), stopping the reaction with sodium dodecyl sulphate and then adding the 5,5′-dithiobis-(2-nitrobenzoate) to estimate the CoA released indicated that this component of normal assay mixtures produces an extremely fast inactivation of the enzyme. Thus, under the experimental conditions relating to Fig. 11, the activity at zero time already represents an inactivation of approx. 26%. [Note that this rapid inactivation is not a feature of normal assays. When both substrates are added to the enzyme before the 5,5′-dithiobis-(2-nitrobenzoate) complete protection is achieved.] It therefore appears that the rapid modification of cysteine results in a loss of nearly half the activity, but no decrease of the NADH sensitivity, whereas the modification of additional cysteine residues leads to further inactivation and complete desensitization to NADH. These results support the photo-oxidation experiments in implicating cysteine in NADH inhibition and, in addition, indicate its involvement in activity in agreement with previous observations that the enzyme is also inactivated by $HgCl_2$ and *N*-ethylmaleimide (Weitzman, 1966*b*).

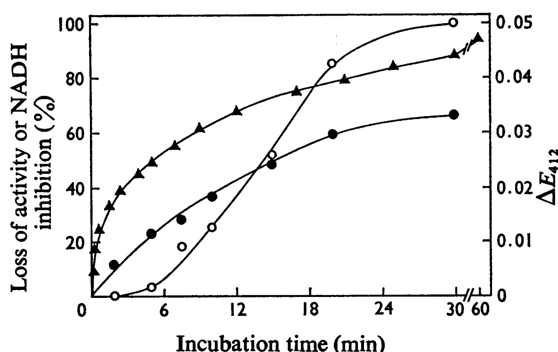

Fig. 11. *Effects of 5,5′-dithiobis-(2-nitrobenzoate) on the activity and NADH inhibition of citrate synthase*

Treatment with 0.1 mM-5,5′-dithiobis-(2-nitrobenzoate) was carried out as described in the text. ●, Loss of enzyme activity; ○, loss of inhibition by 0.2 mM-NADH; ▲, change in E_{412}. No further change in E_{412} was observed beyond 60 min and this maximum value of 0.047 corresponds to 2.06 thiol groups/monomer (mol.wt. 57000).

The sensitivity to rapid inactivation by 5,5′-dithiobis-(2-nitrobenzoate) was observed even after 10min of photo-oxidation, suggesting that the highly reactive thiol group is not photo-oxidized. It is probable that the rapid photo-oxidation of other amino acids at the active site causes a protective quenching effect similar to that observed for the tyrosine residues. Comparable observations have been made with other enzymes. Thus pig pancreatic lipase possesses a thiol group that is only very slowly photo-oxidized yet is readily available to modification by 5,5′-dithiobis-(2-nitrobenzoate) (Sémériva *et al.*, 1971). On photo-oxidation of ox heart lactate dehydrogenase, cysteine oxidation begins only after 40% of the initial enzyme activity has been lost; up to this stage the rapid photo-oxidation of histidine, methionine and tryptophan exert a quenching effect on both the cysteine and tyrosine groups (Millar & Schwert, 1963).

It is interesting that modification of enzyme thiol groups by 5,5′-dithiobis-(2-nitrobenzoate) or by

Table 4. *Summary of amino acid residues implicated in the catalytic and regulatory properties of E. coli citrate synthase*

Function	Amino acid residue	Modification
Catalytic activity	Histidine	Photo-oxidation; diethyl pyrocarbonate
	Tryptophan	2-Hydroxy-5-nitrobenzyl bromide
	Cysteine	5,5′-Dithiobis-(2-nitrobenzoate)
Regulation		
NADH	Cysteine	Photo-oxidation; 5,5′-dithiobis-(2-nitrobenzoate)
α-Oxoglutarate	Histidine	Photo-oxidation

photo-oxidation leads to different functional changes. It is possible that the two modification procedures attack different thiol groups. On the other hand, the thiol group reacting slowly with 5,5′-dithiobis-(2-nitrobenzoate) may be the same as that susceptible to photo-oxidation; whereas both treatments result in desensitization to NADH, loss of catalytic activity observed only on treatment with 5,5′-dithiobis-(2-nitrobenzoate) may reflect prior modification of the more reactive thiol.

The participation of cysteine in enzyme activity indicated by our results and those of Wright & Sanwal (1971) is in direct contrast to the conclusion of Faloona & Srere (1969) that thiol groups are probably not involved in the activity. However, the experiments of the latter workers were done in buffers of relatively high ionic strength which are known to protect the enzyme against inactivation by 5,5′-dithiobis-(2-nitrobenzoate) (Weitzman, 1966*b*).

In conclusion, Table 4 summarizes the results of our studies and indicates the amino acid residues implicated in the catalytic and regulatory properties of *E. coli* citrate synthase. It should be emphasized that the involvement of the residues in enzymic activity does not necessarily imply their participation in the actual catalytic process. It may be that their role is concerned with substrate binding or maintaining the correct enzyme conformation. Similar considerations apply to those residues implicated in the regulatory properties. More detailed studies of the effects of the various modifications of the enzyme would be required to distinguish between these possibilities.

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References

Atkin, G. E. & Ferdinand, W. (1970) *Anal. Biochem.* **38** 313–329

Atkin, G. E. & Ferdinand, W. (1971) *J. Chromatogr.* **62**, 373–381

Barlow, G. H. & Margoliash, E. (1969) *Biochim. Biophys. Acta* **188**, 159–161

Barman, T. E. (1972) *Biochim. Biophys. Acta* **257**, 297–313

Barman, T. E. & Koshland, D. E. (1967) *J. Biol. Chem.* **242**, 5771–5776

Benassi, C. A., Scoffone, E., Galiazzo, G. & Jori, G. (1967) *Photochem. Photobiol.* **6**, 857–866

Bencze, W. L. & Schmid, K. (1957) *Anal. Chem.* **29**, 1193–1196

Buckel, W. & Eggerer, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, S1367–S1376

Calvin, M. (1954) in *Glutathione: A Symposium* (Colowick, S. P., Lazarow, A., Racker, E., Schwartz, D. R., Stadtman, E. & Welsch, H., eds.), p. 9, Academic Press, New York

Cann, J. R. & Goad, W. B. (1968) *Ann. N.Y. Acad. Sci.* **151**, 638–649

Chrambach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967) *Anal. Biochem.* **20**, 150–154

Cohen, R. & Mire, M. (1971) *Eur. J. Biochem.* **23**, 267–275

Coulson, A. F. W. & Yonetani, T. (1972) *Eur. J. Biochem.* **26**, 125–131

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427

Elödi, P. (1968) in *Biochemical Evolution and Homologous Enzymes* (Thoai, N. V. & Roche, J., eds.), pp. 105–118, Gordon and Breach, New York

Faloona, G. R. & Srere, P. A. (1969) *Biochemistry* **8**, 4497–4503

Ford-Hutchinson, A. W. & Perkins, D. J. (1972) *Eur. J. Biochem.* **25**, 415–419

Fowlks, W. L. (1959) *J. Invest. Dermatol.* **32**, 233

Groudinsky, O. (1971) *Eur. J. Biochem.* **18**, 480–484

Horton, H. R. & Koshland, D. E. (1965) *J. Amer. Chem. Soc.* **87**, 1126–1132

Isherwood, F. A. (1959) *Biochem. Soc. Symp.* **17**, 3–16

Koshland, D. E., Karkhanis, Y. D. & Latham, H. G. (1964) *J. Amer. Chem. Soc.* **86**, 1448–1450

Layne, E. (1957) *Methods Enzymol.* **3**, 447–454

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Martinez-Carrion, M., Turano, C., Riva, F. & Farella, P. (1967) *J. Biol. Chem.* **242**, 1426–1430

Martinez-Carrion, M., Kuczenski, R., Tiemeier, D. C. & Peterson, D. L. (1970) *J. Biol. Chem.* **245**, 799–805

Melchior, W. B. & Fahrney, D. (1970) *Biochemistry* **9**, 251–257

Millar, D. B. S. & Schwert, G. W. (1963) *J. Biol. Chem.* **238**, 3249–3255

Mühlrad, A., Hegyi, G. & Tóth, G. (1967) *Acta Biochim. Biophys.* **2**, 19–29

Mühlrad, A., Hegyi, G. & Horanyi, M. (1969) *Biochim. Biophys. Acta* **181**, 184–190

Ovádi, J., Libor, S. & Elödi, P. (1967) *Acta Biochim. Biophys.* **2**, 455–458

Poulos, T. L. & Price, P. A. (1971) *J. Biol. Chem.* **246**, 4041–4045

Ray, W. J. & Koshland, D. E. (1962) *J. Biol. Chem.* **237**, 2493–2505

Sémériva, M., Dufour, C. & Desnuelle, P. (1971) *Biochemistry* **10**, 2143–2149

Srere, P. A., Brazil, H. & Gonen, L. (1963) *Acta Chem. Scand.* **17**, S129–S134

Vodrážka, Z. (1959) *Chem. Listy* **53**, 829–843

Vodrážka, Z., Čejka, J. & Salák, J. (1961) *Biochim. Biophys. Acta* **52**, 342–348

Wagner-Romero, F., Convit, J., Bernt, E. & Nelböck-Hochstetter, M. (1966) *Biochem. Z.* **346**, 167–170

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Weil, L. (1965) *Arch. Biochem. Biophys.* **110**, 57–68

Weil, L. & Buchert, A. R. (1951) *Arch. Biochem. Biophys.* **34**, 1–15

Weil, L. & Maher, J. (1950) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **9**, 244
Weil, L. & Seibles, T. S. (1955) *Arch. Biochem. Biophys.* **54**, 368–377
Weil, L., Gordon, W. G. & Buchert, A. R. (1951) *Arch. Biochem. Biophys.* **33**, 90–109
Weil, L., Seibles, T. S. & Herskovits, T. T. (1965) *Arch. Biochem. Biophys.* **111**, 308–320
Weitzman, P. D. J. (1966*a*) *Biochim. Biophys. Acta* **128**, 213–215
Weitzman, P. D. J. (1966*b*) *Biochem. J.* **101**, 44C–45C
Weitzman, P. D. J. (1969) *Methods Enzymol.* **13**, 22–25
Weitzman, P. D. J. & Dunmore, P. (1969) *FEBS Lett.* **3**, 265–267
Weitzman, P. D. J. & Jones, D. (1968) *Nature (London)* **219**, 270–272
Westhead, E. W. (1965) *Biochemistry* **4**, 2139–2144
Wright, J. A. & Sanwal, B. D. (1971) *J. Biol. Chem.* **246**, 1689–1699
Wright, J. A., Maeba, P. & Sanwal, B. D. (1967) *Biochem. Biophys. Res. Commun.* **29**, 34–38