Purification and General Properties of Glutamate Decarboxylase from *Clostridium perfringens*

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1. A seven-step procedure for preparing highly purified glutamate decarboxylase from *Clostridium perfringens* is described. 2. The homogeneity of the pure enzyme was established by sucrose-density-gradient centrifugation and starch-gel electrophoresis. 3. The isoelectric point of the pure enzyme is about pH 4.5 and the molecular weight is 290 000. 4. The pH optimum for activity is 4.7. The pure enzyme is specific for L-glutamate; β -hydroxyglutamate is decarboxylated at a lower rate. 5. Evidence is presented that each mol of enzyme contains 2 mol of firmly bound pyridoxal 5-phosphate. 6. Resolution does not occur at acid pH; by dilution with neutral or alkaline buffers the enzyme is inactivated and the coenzyme is released. 7. Reconstitution of active enzyme was obtained by protecting the apoenzyme with thiol compounds.

Bacterial glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) was previously purified from *Escherichia coli* by Shukuya & Schwert (1960a). They presented evidence that the enzyme contained firmly bound pyridoxal phosphate and calculated that 2 mol of coenzyme were present/mol of protein (Shukuya & Schwert, 1960b). Upon borohydride reduction of the same enzyme, Anderson & Wang Chang (1965) demonstrated the presence of ϵ -pyridoxyl lysine in the acid protein hydrolysate.

Independently, we purified glutamate decarboxylase from *Clostridium perfringens* and reported that the enzyme from this source was not activated by pyridoxal phosphate, although it showed spectral properties similar to glutamate decarboxylase from *E. coli* and to other pyridoxal phosphate-dependent enzymes. Treatment with sodium borohydride in acid medium both inactivated the enzyme and modified the characteristic properties of the absorbance spectrum (Cozzani, 1965).

These findings, and the absence of a prosthetic group in the histidine decarboxylase crystallized by Snell and co-workers from *Lactobacillus* 30a (Rosenthaler, Guirard, Chang & Snell, 1965), led Lawson & Quinn (1967) to reinvestigate the presence and binding of the coenzyme in glutamate decarboxylase and other amino acid decarboxylases from *E. coli*: such enzymes were resolved by dialysis and significant restoration of activity was obtained on addition of pyridoxal phosphate.

To elucidate the problem of the coenzyme of glutamate decarboxylase from *Cl. perfringens*,

a more exhaustive study of this enzyme was undertaken. The present paper reports data about a new purification procedure, homogeneity of the preparation and general properties of the enzyme.

Direct evidence that pyridoxal phosphate is the prosthetic group was obtained by quantitative determination of the cofactor liberated after protein denaturation. Resolution into apo- and coenzyme was not achieved on extensive dialysis at acid pH values. However, evidence is presented that in alkaline media part of the bound pyridoxal phosphate is liberated from the enzyme; reconstitution of the protein with synthetic pyridoxal phosphate can be obtained, provided that the apoenzyme is protected from inactivation which occurs after the prosthetic group is split off.

EXPERIMENTAL

Materials

Chemicals. Pyridoxal phosphate was a gift from Hoffmann-La Roche and Co. Ltd., Basle, Switzerland; pyridoxal hydrochloride was from Fluka A.-G., Buchs, Switzerland; monosodium L-glutamate was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.; other amino acids were obtained either from E. Merck A.-G., Darmstadt, Germany, or from Sigma Chemical Co., St Louis, Mo., U.S.A.; crystalline bovine serum albumin was from BDH (Chemicals) Ltd., Poole, Dorset, U.K. Sephadex G-100 and G-200 were products from Pharmacia Fine Chemicals, Uppsala, Sweden. Other reagents and buffer salts were reagent-grade materials. Glass-distilled water was used throughout.

Source of enzymes. *Cl. perfringens* (A.T.C.C. 8009) was used as the source material of glutamate decarboxylase. Cells were grown on thioglycollate broth (Difco) in Roux bottles standing at 37°C. After 16 h the cells were harvested by centrifugation and the sediment was washed twice with 0.9% NaCl. Apo-(aspartate-2-oxoglutarate transaminase) was prepared from brewer's yeast as described by Holzer & Gerlach (1963).

Catalase, alcohol dehydrogenase from yeast and malate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Equipment

Absorption spectra of the enzyme preparation were measured with a Unicam SP.800 spectrophotometer. For pH measurements a Metrohom Herisau E300B pH-meter was used.

Methods

Enzyme assays. Amino acid decarboxylase activities were determined by measuring the CO₂ released with the conventional manometric technique. The standard incubation mixture contained 6 mM-substrate dissolved in 1.5 ml of 0.2 M-pyridine-HCl buffer, pH 4.7, in the main compartment of the Warburg vessel, with enzyme preparation diluted in 0.3 ml of the same buffer in the side arm. The assays were run in duplicate with a control containing no substrate, at 37°C, with air as gas phase. Manometric readings were taken at 2 min intervals. Specific activity is expressed as μ l of CO₂ evolved/min per mg of protein.

Aspartate decarboxylase activity was determined by means of a manometric technique as described by Wilson & Kornberg (1963).

Glutaminase activity was determined by measuring the NH₃ formed from L-glutamine with Nessler's reagent.

Protein determination. Protein concentration of the enzyme preparations was measured by the spectrophotometric method described by Warburg & Christian (1942).

RESULTS

Purification of glutamate decarboxylase

(1) **Extraction.** To the sedimented cell-mass, suspended in an equal volume of cold water, 9 vol. of cold acetone (−15°C) was added with stirring. The suspension was filtered with suction on a Buchner funnel, washed with 1 vol. of ether and dried in air. The acetone-dried powder was suspended in water (15 g/200 ml) and was allowed to autolyse with stirring for 30–40 h at room temperature. Cell debris was removed by centrifugation and was found to be almost inactive.

The following purification steps were carried out at 0–4°C, with care being taken to minimise the time that the enzyme was kept in dilute solution.

(2) **Precipitation with manganese chloride.** To the extract was added 10% (v/v) of 1 M-manganese chloride. The mixture was immediately centrifuged

at 10000g for 10 min and the precipitate was discarded.

(3) **Ammonium sulphate precipitation.** The resultant supernatant was brought to 67% saturation with ammonium sulphate. The precipitate was collected by centrifugation and dissolved in a volume of 0.05 M-sodium acetate buffer, pH 4.8, corresponding to 10% of the original supernatant fluid.

(4) **Sephadex G-100 gel filtration.** The material from (3) (about 18 ml) was applied to a Sephadex G-100 gel column (2.6 cm × 60 cm) equilibrated with 0.05 M-sodium acetate buffer, pH 4.8, and elution was carried out with the same buffer. The flow rate was adjusted to 30 ml/h. Fractions (6 ml) were collected and assayed for protein and glutamate decarboxylase activity. The elution pattern is shown in Fig. 1(a). The active fractions were pooled and the protein was precipitated with ammonium sulphate (67% saturation).

(5) **First Sephadex G-200 gel filtration.** The precipitated proteins collected by centrifugation were redissolved in 5–6 ml of 0.05 M-sodium acetate

Fig. 1. Separation of glutamate decarboxylase from *Cl. perfringens* by Sephadex G-100 (a) and repeated G-200 [(b) and (c)] gel filtration. Each fraction [6 ml in (a) and 1.5 ml in (b) and (c)] was analysed for glutamate decarboxylase activity and for protein concentration. ●, glutamate decarboxylase activity; ○, protein concentration. Details of column separation and enzymic assays are described in the Experimental section.

buffer, pH 4.8, and passed down a Sephadex G-200 gel column (2cm × 45cm) equilibrated with the same buffer. Elution with this buffer was conducted at a flow rate of 12ml/h and 30 fractions (1.5ml) were collected. The decarboxylase appeared in fractions 10–16, corresponding to 15–24ml (Fig. 1b).

(6) *Second Sephadex G-200 gel filtration.* The active fractions were again pooled, precipitated with ammonium sulphate and redissolved in buffer as previously described. The Sephadex G-200 gel filtration was repeated under the same experimental conditions.

(7) *Concentration and pH precipitation.* The pooled active fractions (Fig. 1c) were frozen and reduced in volume under vacuum over silica gel. The pH of the concentrated preparation, which rose to 5.5–5.7, was adjusted to pH 4.6 with 0.1 M-acetic acid. By standing overnight at 3–4°C insoluble material was formed. The precipitate was discarded by centrifugation and the clear, pale yellow supernatant was retained. This solution (3–4ml containing 4–3mg of protein/ml) was stored in the cold-room for some weeks without appreciable loss of activity.

Characterization of the pure enzyme

Starch-gel electrophoresis. The purification of glutamate decarboxylase was checked by starch-gel electrophoresis in discontinuous buffer as described by Barrett, Friesen & Astwood (1962). Experiments for the determination of the isoelectric point were carried out by horizontal starch-gel electrophoresis essentially as described by Smithies (1955). The gels were stained with a 0.05% solution of Nigrosine in methanol-acetic acid-water (5:1:4, by vol.) and destained overnight by the same solvent.

Sucrose-density-gradient centrifugation. Linear

gradients of 5–20% (w/v) sucrose in 0.05 M-buffer were prepared in a volume of 4.7ml. The standard enzymes (alcohol dehydrogenase and catalase) and glutamate decarboxylase in volumes of 0.1ml were layered on to separate gradients buffered with sodium phosphate buffer, pH 7.2, and sodium acetate buffer pH 4.5, respectively. They were centrifuged for 16h at 4°C and 38000 rev./min in the Spinco model L centrifuge with SW39 rotor. From each tube 44 fractions were collected and assayed for enzyme activity and protein concentration. The molecular weight of glutamate decarboxylase was calculated as described by Martin & Ames (1961).

Determination of the coenzyme. The free pyridoxal phosphate liberated from glutamate decarboxylase at alkaline pH values was usually measured by the enzymic test described by Holzer & Gerlach (1963) [reactivation of apo-(aspartate-2-oxoglutarate aminotransferase) from brewer's yeast]. The samples were diluted with water to 0.05mg of protein/ml, kept for 5min at 100°C, cooled to room temperature and centrifuged; 0.4ml of supernatant was used for each determination. Assays were carried out as reported by Holzer & Gerlach (1963); the concentration of pyridoxal phosphate in each sample was calculated from a reference curve obtained under the experimental conditions described by the same authors. Occasionally the concentration of pyridoxal phosphate was measured from the molar extinction at 388nm in 0.1 M-sodium hydroxide as described by Peterson & Sober (1954).

Purification of glutamate decarboxylase. Table 1 shows a typical purification procedure. The active material from step (7) was found to be essentially homogeneous by the criteria of starch-gel electrophoresis in discontinuous buffer and sucrose-density-gradient-centrifugation. Fig. 2 shows that

Table 1. *Purification of glutamate decarboxylase from Cl. perfringens*

Summary of the effects of the purification steps in the preparation of glutamate decarboxylase from 15g of acetone-dried powder of *Cl. perfringens*, as described in the text.

Step	Total protein (mg)	10 ⁻⁵ × Total activity (μl of CO ₂ released/min)	10 ⁻² × Specific activity (μl of CO ₂ released/min per mg of protein)	Total yield (%)
(1) Extraction	7280	2.27	0.31	100
(2) Precipitation with MnCl ₂	2760	1.58	0.57	69
(3) (NH ₄) ₂ SO ₄ precipitation	1289	1.27	0.99	56
(4) Sephadex G-100 gel filtration	240	0.99	4.14	44
(5) First Sephadex G-200 gel filtration	46	0.53	11.64	24
(6) Second Sephadex G-200 gel filtration	17	0.36	21.18	16
(7) Concentration and pH precipitation	12	0.28	23.80	12

only a single band is given by glutamate decarboxylase under conditions where crystalline serum albumin is resolved into three components. After sucrose-density-gradient centrifugation one symmetrical protein peak with constant specific glutamate decarboxylase activity was observed (Fig. 3a).

Molecular weight and isoelectric point. A molecular weight of $290\,000 \pm 12\,000$ was calculated for glutamate decarboxylase by comparison of its sedimentation behaviour in sucrose gradients with

that of catalase and alcohol dehydrogenase (Fig. 3b). An isoelectric point at pH 4.5 was found for glutamate decarboxylase by starch-gel electrophoresis in 0.1 M-acetate buffers (Table 2).

Optimum pH and substrate specificity. Enzymic activity as a function of pH is shown in Fig. 4. The optimum pH value occurs at approximately 4.7 in 0.1 M-pyridine-HCl buffer.

The purified preparation from step (7) decarboxylated only L-glutamate among the following amino acids tested: glycine, α - and β -alanine, aspartate, asparagine, glutamine, valine, serine, threonine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, histidine, cysteine, methionine, arginine, lysine. No carbon dioxide was formed from D-glutamate. β -Hydroxyglutamate was decarboxylated at a rate which was estimated to be about 6% of that of the true substrate.

Both intact cells and acetone-dried-powder extracts of *Cl. perfringens* display glutaminase, which is responsible for the apparent decarboxylation of L-glutamine (Krebs, 1948), and aspartate decarboxylase activities (Meister, Sober & Tice, 1951). The former enzyme was separated from glutamate decarboxylase by Sephadex G-100 gel filtration and the latter activity was lost during step (5).

Prosthetic group. The absorption spectra of pure glutamate decarboxylase at various pH values are reported in Fig. 5. In the range 300–450 nm the spectra exhibit complementary maxima at 333 and 410 nm when the pH is varied from 3.8 to 6.5. At alkaline pH values, however, the absorption maximum is shifted toward 388 nm, indicating that

Fig. 2. Starch-gel electrophoresis patterns of crystalline bovine serum albumin (a) and glutamate decarboxylase from *Cl. perfringens* (b). Experiments were performed in discontinuous buffer as described by Barrett *et al.* (1962) at 25–30 V/cm and 30 mA for 2 h at 3–4°C.

Fig. 3. (a) Distribution of glutamate decarboxylase activity, ●, and protein concentration, ○, following sucrose-density-gradient centrifugation of purified glutamate decarboxylase from *Cl. perfringens*. Δ, Plot of enzyme activity/protein concentration. (b) Comparative distribution of glutamate decarboxylase, ●, (0.55 mg), catalase, ▲, (0.2 mg), and yeast alcohol dehydrogenase, ■, (0.3 mg) activities after sucrose-density-gradient centrifugation. Details of experimental procedure are given in the text.

Table 2. *Electrophoretic mobilities of glutamate decarboxylase from Cl. perfringens at different pH values*

The purified protein (0.07mg) was subjected to horizontal starch-gel electrophoresis in 0.1M-acetate buffer at 20–30 V/cm and 20mA for 6h in the cold-room. The pH values reported are those measured electrometrically at 20°C.

pH	Distance moved from origin (mm)
3.8	–15
4.2	–5
4.4	–1
4.6	+3
4.8	+7
5.0	+12
5.3	+15
5.5	+22

Fig. 4. Effect of pH on reaction velocity of glutamate decarboxylase from *Cl. perfringens*. ●, 0.2M-Glycine-HCl buffer; ○, 0.2M-pyridine-HCl buffer. The pH values reported are those measured electrometrically at 20°C.

pyridoxal phosphate becomes dissociated from the apoenzyme. In 0.1M-sodium hydroxide a solution of pure glutamate decarboxylase containing 2.2mg of protein/ml gave an extinction value of 0.105 at 388nm. Assuming that the ϵ_{388} for free pyridoxal phosphate in 0.1M-sodium hydroxide is 6600 (Peterson & Sober, 1954), from the estimated molecular weight of 2.9×10^5 it was calculated that 2.1mol of pyridoxal phosphate was released by 1mol of enzyme.

The free pyridoxal phosphate liberated from the apoenzyme in 0.1M-alkali was further estimated by quantitative reactivation of apo-(aspartate-2-oxoglutarate aminotransferase) by method described by Holzer & Gerlach (1963). By comparison with the standard curve obtained with synthetic pyridoxal phosphate, it was calculated that $1.38 \mu\text{g}$ of pyridoxal phosphate was released by 1mg of protein, corresponding to 1.7mol of coenzyme/

Fig. 5. Spectra of purified glutamate decarboxylase at various pH values. Enzyme concentration was 2.2mg/ml. Spectra were measured in 0.1M-sodium acetate buffer in the range pH3.8–5.6, in 0.1M-sodium phosphate buffer in the range pH6.0–8.0 and in 0.1M-sodium carbonate buffer at pH9.0 and in 0.1M-NaOH.

290 000g of enzyme. From the above results it was tentatively concluded that 2mol of pyridoxal phosphate is bound/mol of glutamate decarboxylase.

Binding of pyridoxal phosphate to the protein. Resolution of glutamate decarboxylase could not be demonstrated in the pH range 3.5–6.0. After repeated gel filtration and prolonged dialysis (48h) neither appreciable reactivation by pyridoxal phosphate nor significant change of spectral properties were observed.

However, rapid inactivation was observed following dialysis or dilution of the enzyme with neutral or alkaline buffers. The presence of free pyridoxal phosphate in the dilution mixture was checked by the method of Holzer & Gerlach (1963), and related to the loss of enzymic activity. Results reported in Fig. 6 indicate that significant correspondence occurs between raising of pyridoxal phosphate concentration and decrease of glutamate decarboxylase activity. Inactivation was not reversed by re-acidification or by addition of the coenzyme. However, appropriate concentrations of pyridoxal phosphate in the dilution mixtures protected glutamate decarboxylase from inactivation (Table 3). Similar effects were observed with pyridoxal hydrochloride, reduced glutathione or mercaptoethanol. The initial activity was completely restored by addition of pyridoxal phosphate when the enzyme was diluted in the presence of reduced glutathione.

DISCUSSION

The specific activity of the purified glutamate decarboxylase from *Cl. perfringens* is in good agreement with the data reported by Shukuya &

Schwert (1960a) and Strausbauch & Fischer (1967) for glutamate decarboxylase from *E. coli*. The molecular weight, determined by sucrose-density-

gradient centrifugation, agrees well with the value reported by the former authors, but is significantly lower than that found by the latter.

The pH optimum for activity of glutamate decarboxylase from *Cl. perfringens* in pyridine-HCl buffer is 0.9 pH units higher than that for the enzyme from *E. coli*. Acetate buffer, which is a competitive inhibitor of bacterial glutamate decarboxylase, shifts the pH-activity curve of the enzyme from *Cl. perfringens* towards the acid range (Cozzani, 1965), while the reverse seems to be true for the enzyme from *E. coli* (Shukuya & Schwert, 1960a). The presence of pyridoxal phosphate as coenzyme in bacterial glutamate decarboxylases has been a controversial problem. Since the early work of Taylor & Gale (1945) on bacterial amino acid decarboxylases the question arose whether histidine decarboxylase and glutamate decarboxylase were pyridoxal phosphate-dependent enzymes. In fact, highly purified preparations of histidine decarboxylase from *Lactobacillus* 30a (Rosenthaler *et al.* 1965) and from *Micrococcus* sp. n (Semina & Mardashev, 1965) were found neither to contain pyridoxal phosphate nor to be affected by this cofactor. On the other hand pyridoxal phosphate was shown to be present in the molecule of glutamate decarboxylase from different strains of *E. coli*, although different pyridoxal phosphate/protein molar ratios were reported (Shukuya & Schwert, 1960b; Strausbauch & Fischer, 1967). Moreover, partial resolution by dialysis and restoration by pyridoxal phosphate was obtained (Lawson & Quinn, 1967).

We reported earlier that glutamic decarboxylase purified from *Cl. perfringens* was not activated by pyridoxal phosphate, although it showed spectral properties similar to glutamate decarboxylase from *E. coli* and to other pyridoxal phosphate-dependent enzymes (Cozzani, 1965). The maxima of the

Fig. 6. Inactivation of glutamate decarboxylase and release of pyridoxal phosphate by dilution with neutral or alkaline buffers. After dilution of glutamate decarboxylase with 0.05M-sodium acetate buffers, ●, with 0.05M-tris-acetate buffer, ■, and with 0.05M-sodium carbonate buffer, ▲, % of initial activity was calculated by taking as 100% the activity of the sample diluted at pH 4.6. In all experiments 0.02 ml of stock glutamate decarboxylase solution (containing 2.2 mg of protein/ml) was diluted to 0.4 ml with buffer at the pH values indicated. After 15 min of incubation at 20°C, 0.1 ml of each sample was withdrawn for the determination of the residual activity; the remaining 0.3 ml was used to estimate pyridoxal phosphate concentration, ○, by the method of Holzer & Gerlach (1963), as described in the text.

Table 3. Effects of pyridoxal phosphate, pyridoxal hydrochloride and thiol compounds on inactivation of glutamate decarboxylase diluted in 0.02M-tris-hydrochloric acid buffer, pH 7.4

A portion (0.01 ml) of purified glutamate decarboxylase (2.2 mg of protein/ml) was diluted to 0.2 ml with the initial dilution medium and kept at 20°C. After 3 h the addition indicated was made. A volume corresponding to 0.01 mg of protein was then used for glutamate decarboxylase assay under the standard conditions. The activity of 0.01 mg of stock concentrated solution of glutamate decarboxylase was taken as 100%.

Initial dilution medium	Addition after 3 h	Initial activity remaining after 3 h (%)
Buffer	None	22
Buffer	0.5 mM-Pyridoxal phosphate	25
Buffer + 0.5 mM-pyridoxal phosphate	None	82
Buffer + 0.5 mM-pyridoxal hydrochloride	None	46
Buffer + 10 mM-GSH	None	56
Buffer + 10 mM-GSH	0.5 mM-Pyridoxal phosphate	98
Buffer + 10 mM-mercaptoethanol	None	42
Buffer + 10 mM-mercaptoethanol	0.5 mM-Pyridoxal phosphate	68

absorption spectrum reported in Fig. 5, the characteristic shifts with pH and the absorption maximum in 0.1M-alkali strongly support the presence of pyridoxal phosphate. Even more compelling conclusions can be drawn from the data obtained with the method of Holzer & Gerlach (1963) which is both specific for pyridoxal phosphate and highly sensitive. Further evidence that pyridoxal phosphate is present in the molecule of glutamate decarboxylase from *Cl. perfringens* is given by detection and quantitative determination of the coenzyme liberated by dilution of glutamate decarboxylase with neutral or alkaline buffers.

The failure to resolve the enzyme at acidic pH values can be explained by the particularly firm binding of the structure absorbing at 410nm, which is presumably a Schiff base where the hydrogen of the phenolic hydroxyl of pyridoxal phosphate is hydrogen-bonded to the imino nitrogen of the Schiff base (Metzler, 1957).

On the other hand the structure absorbing at 333nm appears to be less stable, and presumably in equilibrium with the aldehyde form of pyridoxal phosphate, since above pH 6 both inactivation and liberation of the coenzyme takes place (Fig. 6). On these grounds it seems unlikely that this structure results from addition of a group on the protein surface across the imine bond, as suggested for the binding of pyridoxal phosphate to the protein moiety in phosphorylase b (Kent, Krebs & Fischer, 1958) and in serum albumin (Dempsey & Christensen, 1962). In the case of glutamate decarboxylase the hypsochromic shift connected with the raising of pH could be better explained by disruption of the hydrogen-bonded chelate ring after ionization of the phenolic hydroxyl group.

Inactivation of glutamate decarboxylase on dilution in neutral or alkaline buffers and protection exerted by excess of pyridoxal phosphate can be explained by the assumption that the apoenzyme undergoes irreversible denaturation after the splitting-off of the cofactor. Since restoration of activity does not occur by addition of pyridoxal phosphate after inactivation, a structural change may be supposed that lowers the affinity of the protein for the prosthetic group. Protection by thiol compounds and reactivation by pyridoxal

phosphate of the enzyme protected with reduced glutathione strongly suggest that thiol groups of the apoenzyme are involved in the modification related to the splitting-off of the cofactor. Preliminary results, by titration with *p*-chloromercuribenzoate, have shown the presence of reactive thiol groups in the molecule of glutamate decarboxylase from *Cl. perfringens* in 8M-urea buffered with 0.1M-sodium acetate, pH 4.6 (I. Cozzani & C. Santoni, unpublished work).

REFERENCES

- Anderson, J. A. & Wang Chang, H. F. (1965). *Archs Biochem. Biophys.* **110**, 346.
Barrett, R. J., Friesen, H. & Astwood, E. B. (1962). *J. biol. Chem.* **237**, 432.
Cozzani, I. (1965). *Ital. J. Biochem.* **14**, 363.
Dempsey, W. B. & Christensen, H. N. (1962). *J. biol. Chem.* **237**, 1113.
Holzer, H. & Gerlach, U. (1963). In *Methods in Enzymatic Analysis*, p. 606. Ed. by Bergmeyer, H. U. London and New York: Verlag Chemie and Academic Press.
Kent, A. B., Krebs, E. G. & Fischer, E. H. (1958). *J. biol. Chem.* **232**, 549.
Krebs, H. A. (1948). *Biochem. J.* **43**, 51.
Lawson, A. & Quinn, A. G. (1967). *Biochem. J.* **105**, 483.
Martin, R. G. & Ames, B. N. (1961). *J. biol. Chem.* **236**, 1372.
Meister, A., Sober, H. A. & Tice, S. V. (1951). *J. biol. Chem.* **189**, 591.
Metzler, D. E. (1957). *J. Am. chem. Soc.* **79**, 485.
Peterson, E. A. & Sober, H. A. (1954). *J. Am. chem. Soc.* **76**, 169.
Rosenthaler, J., Guirard, B. M., Chang, G. W. & Snell, E. E. (1965). *Proc. natn Acad. Sci. U.S.A.* **54**, 152.
Semina, L. A. & Mardashev, S. R. (1965). *Biokhimiya*, **30**, 100.
Shukuya, R. & Schwert, G. W. (1960a). *J. biol. Chem.* **235**, 1649.
Shukuya, R. & Schwert, G. W. (1960b). *J. biol. Chem.* **235**, 1653.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Strausbauch, P. H. & Fischer, E. H. (1967). *Biochem. biophys. Res. Commun.* **28**, 525.
Taylor, E. S. & Gale, E. F. (1945). *Biochem. J.* **39**, 52.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.
Wilson, E. M. & Kornberg, H. L. (1963). *Biochem. J.* **88**, 578.