Studies in the Enzymology of Glutathione Metabolism in Human Erythrocytes

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Spectrophotometric assay methods are described for glutathione synthetase, γ -glutamylcysteine synthetase and γ -glutamyl transpeptidase of erythrocytes. The contents of these enzymes in normal human erythrocytes are reported. Erythrocyte glutathione synthetase is inhibited by ADP; this inhibition is competitive with respect to ATP. γ -Glutamylcysteine synthetase is subject to feedback inhibition by GSH, and is also inhibited by NADH, and to a lesser extent by NAD⁺ and NADPH. This enzyme is irreversibly inactivated by cysteamine.

Although the precise role of GSH in many tissues is still obscure, in the erythrocyte it forms, with glutathione reductase, an essential component of the enzyme system that is responsible for the protection of haemoglobin from oxidation. According to Mills (1957) the glutathione peroxidase system of erythrocytes can remove hydrogen peroxide that may be present in concentrations too low to activate catalase. Further, Jacob & Jandl (1966) have shown that GSH, with its associated oxidoreduction system, is involved in the regulation of the activity of the hexose monophosphate pathway in erythrocytes.

The observations of Eldjarn, Bremer & Børresen (1962) and of Horejsi & Mircevovala (1964), that neither GSSG nor GSH penetrates the erythrocyte membrane, made it clear that erythrocyte GSH must be synthesized *in situ*. Since the discovery (Dimant, Landsberg & London, 1954) that the GSH of human erythrocytes is in a dynamic state, with a half-life of about 4 days, there have been many studies of the synthesis of erythrocyte GSH. Kasbekar & Sreenivasan (1959) demonstrated a definite, though small, synthesis by a colorimetric (alloxan '305') procedure. Another technique used involved measuring the amount of labelled glycine incorporated into GSH; in this case it was difficult to differentiate between synthesis *de novo* and enzyme-catalysed exchange of the terminal glycine residue of the tripeptide. Boivin & Galand (1965), using [³⁵S]cysteine and [U-¹⁴C]glutamate, followed the synthesis both of γ -glutamylcysteine and of GSH from the constituent amino acids, and concluded that the erythrocytes of patients with congenital GSH deficiency of the erythrocyte were lacking in GSH synthetase but not in γ -glutamylcysteine synthetase.

The present study describes observations on the two synthetases obtained by the use of improved spectrophotometric assay techniques, and also considers GSH degradation in the erythrocyte, which hitherto has received scant attention.

MATERIALS

GSH, GSSG and cysteamine were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.), and DTNB* was from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). Glyoxylic acid and DL-homocysteine were purchased from Koch-Light Ltd. (Colnbrook, Bucks.); ATP, ADP, sodium 3-phosphoglycerate and nicotinamide nucleotides were from Boehringer Corp. (London) Ltd. (London, W. 5). Triton X-100 was obtained from Charles Lennig and Co. (Great Britain) Ltd. (London, W.C. 1). γ -Glutamylcysteine was prepared by the method of Gutcho & Laufer (1954); the product contained 9% of unchanged GSH but no detectable cysteine. Other chemicals were obtained from British Drug Houses Ltd. (Poole, Dorset), Hopkin and Williams Ltd. (Chadwell Heath, Essex), or May and Baker Ltd. (Dagenham, Essex), A.R. grades being used where available.

Human blood was obtained by cubital venepuncture, and heparin was used as anticoagulant. The blood was centrifuged at 350g for 10 min., the plasma and buffy layer were discarded, and the erythrocyte fraction was washed in aq. 0.9% NaCl. Erythrocytes were lysed by freezing, stored at -40°, and thawed immediately before use.

EXPERIMENTAL

Assay of γ -glutamyl transpeptidase (EC 2.3.2.-). Modern colorimetric methods for the determination of this enzyme that depend upon the hydrolysis of synthetic substrates cannot be used with erythrocytes because of the high background absorption by haemoglobin. Consequently a modification of the method of Ball, Revel & Cooper (1956)

* Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

was adopted. To 1 ml. of haemolysate (from approx. 8×10^9 erythrocytes) were added 0.85 ml. of 0.1M-triethanolamine-HCl buffer, pH 8.6, 0.05 ml. of 0.2M-GSH and 0.1 ml. of 1.0M-alanine. The mixture, at 0°, was gassed with O₂-free argon for 10 min. and then incubated, with shaking, for 60 min. at 37° in a tightly stoppered flask. Immediately before and after the incubation, samples (0.1 ml.) were removed and added to tubes containing 3.9 ml. of ice-cold 2% sulphosalicylic acid. After standing for 20 min. at 0° these tubes were centrifuged at 3000g for 10 min. at 4°. Portions (1.0 ml.) of the supernatants were assayed for GSH by the method of Ball (1966). In this procedure both cysteine and cysteinylglycine are removed as thiazolidine derivatives, and consequently the loss in extinction of the final sample relative to the initial sample gives a measure of the degradation of GSH during the incubation. A blank flask containing 1 ml. of aq. 0.9% NaCl in place of the haemolysate was also assayed. Extinctions were read on a Unicam SP.500 spectrophotometer, or, where the changes measured were small, on a Hilger-Gilford reaction-kinetics spectrophotometer with scale expansion.

Assay of glutathione synthetase (EC 6.3.2.3). Assay conditions were based on the method of Snoke & Bloch (1955) modified as follows: 0.3 ml. of haemolysate (representing approx. 2×10^9 erythrocytes) was added to 2.0 ml. of ice-cold 0.1M-triethanolamine-HCl buffer, pH 8.3, and further additions were made of 0.4 ml. of 1.0M-KCl, 0.4 ml. of 0.1M-MgSO₄, 0.06 ml. of 1.0M-KCN, 0.06 ml. of 1.0M-glycine, 0.04 ml. of 3-phosphoglycerate, 0.25 ml. of 40 mM- γ -glutamylcysteine and 0.04 ml. of 0.3M-ATP. The total volume was adjusted to 4.0 ml. with water. The mixture, still at 0°, was gassed with O₂-free argon for 10 min. and then agitated at 37° for 45 min. in a tightly-stoppered flask. Immediately before and after the incubation, samples (1 ml.) were removed and added to tubes containing 2 ml. of 25% (w/v) metaphosphoric acid and 7 ml. of water at 0°. After standing for 20 min. at 0° these tubes were centrifuged at 3000g for 10 min. and the supernatants were then electrolytically reduced by the method of Dohan & Woodward (1939). Portions (1 ml.) of the reduced supernatants were assayed for GSH by the alloxan '305' method of Patterson & Lazarow (1955). γ -Glutamylcysteine also reacts with alloxan to give a product that absorbs at 305 m μ , and the molar extinction coefficient of this complex is equal to 16% of that given by GSH. Since γ -glutamylcysteine was removed stoichiometrically as GSH was synthesized, the overall increment in E_{305} was divided by 0.84 to give a measure of the GSH synthesized.

Assay of γ -glutamylcysteine synthetase (EC 6.3.2.2). Activity of this enzyme was measured by determining the increase in γ -glutamylcysteine during an incubation at 37°. γ -Glutamylcysteine can be assayed in presence of cysteine by the method used by Ball (1966) for GSH, but modifications and extensive precautions are necessary in the present system, as cysteine is present in about 100-fold excess.

When solutions of cysteine and γ -glutamylcysteine are incubated at 60° and pH 6.8 for 5 min. in the presence of glyoxylate the following equilibria are established.

Fig. 1. Reaction of DTNB with cysteine and γ -glutamylcysteine after preincubation with glyoxylate. A, Buffer blank; B, cysteine (2.3 mM); C, γ -glutamylcysteine (0.023 mM); D, cysteine (2.3 mM) + γ -glutamylcysteine (0.023 mM). The appropriate substance in 1M-potassium phosphate buffer, pH 6.8 (2.5 ml.), containing also ascorbic acid (1 g./l.) and glyoxylic acid (3 g./l.), was taken, and 0.5 ml. of 3.8 mM-DTNB in 1.0M-potassium phosphate buffer, pH 6.8, was added at zero time. The reaction was followed at 25°.

If the mixture is then cooled to 25°, and DTNB is added, free cysteine and γ -glutamylcysteine are removed as mixed disulphide, and the consequent shift in the equilibrium causes dissociation of both the thiazolidine and the hemimercaptal. However, the equilibrium of reaction (1) lies much further to the right than that of reaction (2), and therefore formation of the yellow reduction product of DTNB proceeds much more rapidly from the hemimercaptal than from the thiazolidine. It was thus possible to adjust the glyoxylate concentration so that virtually complete recovery of the dipeptide was obtained on addition of DTNB, whereas the rate of thiazolidine breakdown remained low. Fig. 1 shows typical progress curves of colour development, as followed on a Hilger-Gilford reaction-kinetics spectrophotometer, at 25°. Recoveries of γ -glutamylcysteine in the final procedure as described below were 92–97%. The course of colour development was also influenced by reduction due to the ascorbic acid present (see Fig. 1A).

In the course of the γ -glutamylcysteine synthetase assay the proportion of cysteine converted into dipeptide was small, and its contribution to E_{412} was therefore effectively identical in initial and final samples. Thus, provided that the γ -glutamylcysteine assay in initial and final samples was rigidly standardized, in spite of the apparently cumbersome nature of the method, a very accurate result could be obtained. The actual procedure adopted was as follows. (Composition of the assay mixture is discussed in the Results section.) Portions (1.5 ml.) of assay mixture were taken immediately before and after the 37° incubation, and each was added to 2.5 ml. of ice-cold 3.2% (w/v) sulphosalicylic acid in centrifuge tubes. After standing for 20 min. at 0° these tubes were centrifuged at 3000g for 10 min. Supernatants were electrolytically reduced by the method of Dohan & Woodward (1939), and portions (1 ml.) were added to tubes containing 4 ml. of 1.0M-potassium phosphate

buffer, pH 6.8, with 5 mM-EDTA, 1.0 g. of ascorbic acid/l., and 3.0 g. of glyoxylic acid monohydrate/l. These tubes were incubated at 60° for exactly 5 min., rapidly cooled in ice, and then equilibrated at 25°. To each tube was then added 0.5 ml. of 3.8 mM-DTNB in 1.0 M-potassium phosphate, pH 6.8. E_{412} was read exactly 7 min. after addition of the DTNB in a 1 cm.-light-path cell on a Unicam SP. 500 spectrophotometer. Results were measured against a γ -glutamylcysteine standard.

RESULTS

Degradation of GSH by haemolysates. By using the assay method described above, the activity (mean $\pm$ S.E.M.) of γ -glutamyl transpeptidase in seven samples was $6.4 \pm 0.9 \mu\text{moles}$ of GSH degraded/min./ 10^9 erythrocytes. As appreciable activity was found in the absence of any amino acid acceptor, one haemolysate was dialysed for 16 hr. at 4° against 200 vol. of 0.05 M-potassium phosphate, pH 7.2. This treatment eliminated the activity in the absence of acceptor. Dialysis did not result in any appreciable loss of activity when acceptor was present. These results, together with the effects of replacing alanine by equimolar amounts of various other acceptors, are summarized in Table 1. Under the assay conditions employed degradative activity was shown to be proportional to the amount of haemolysate used, up to 3×10^9 erythrocytes/ml., and to the time of incubation, up to 100 min.

Glutathione synthetase in haemolysates. Activity was proportional to haemolysate concentration, up to 10^9 erythrocytes/ml., and to incubation time, up to 100 min. The assay mixture contained 3-phosphoglycerate to regenerate ATP from ADP. If the 3-phosphoglycerate was omitted, activity fell off sharply after the first 30 min. of incubation. Since Snoke & Bloch (1955) showed that the glutathione synthetase of liver is inhibited by ADP, the effect of ADP on the erythrocyte enzyme was measured. In this experiment, 3-phosphoglycerate was omitted from the assay mixtures. The results are summarized in Fig. 2. ADP is a potent inhibitor, and the degree of inhibition by a given concentration of ADP depends on the ATP concentration. The reciprocal plot (Fig. 3) is compatible with a competitive mechanism of inhibition (Dixon & Webb, 1964).

When assayed by the method described, a series of eight normal erythrocyte samples gave an activity (mean $\pm$ S.E.M.) of $21.0 \pm 0.7 \mu\text{moles}$ of GSH synthesized/min./ 10^9 erythrocytes.

γ -Glutamylcysteine synthetase in haemolysates. Preliminary experiments were carried out with lysate from 2×10^9 erythrocytes, made up to 4.0 ml. in buffer containing cysteine (120 μmoles), sodium glutamate (120 μmoles), ATP (12 μmoles), potassium chloride (600 μmoles) and magnesium sulphate

Table 1. *GSH breakdown by erythrocytes in the presence of various acceptors*

The acceptors were all at 50 mM. Activities are expressed as percentages of the activity found when L-glutamine was used as acceptor.

	Acceptor	Relative activity (%)
Undialysed haemolysate	None	22
	L-Alanine	62
Dialysed haemolysate	None	0
	L-Glutamine	100
	L-Methionine	76
	L-Alanine	61
	Glycylglycine	51
	Glycine	40

Fig. 2. Effect of ADP on the activity of glutathione synthetase. O, 1.0 mM-ATP; ●, 3.0 mM-ATP. The incubation time was 30 min.; other conditions were as described in the text.

Fig. 3. Reciprocal plot of the effect of ADP on glutathione synthetase. Experimental details were as for Fig. 2.

(40 μmoles). The mixture was gassed with argon for 10 min. and then incubated with shaking at 37°. Samples were taken before and after this incubation

and assayed for γ -glutamylcysteine by the method described in the Experimental section. Fig. 4 illustrates the variation in activity with pH. Subsequent experiments were carried out in 0.05M-triethanolamine-hydrochloric acid buffer at pH 7.5. Under these conditions activity was proportional to incubation time, up to 180 min., and to amount of haemolysate taken, up to 10^9 erythrocytes. The optimum concentration of ATP in this system was about 3 mM (Fig. 5). The true optimum is probably lower than this, but erythrocyte stroma contains a very active adenosine triphosphatase. Fig. 5 also shows reaction rate plotted against cysteine concentration; the apparent K_m was about 3×10^{-4} M. The loss in activity in the presence of supramaximal

Fig. 4. Variation in the activity of erythrocyte γ -glutamylcysteine synthetase with pH. The buffers were: 0.1M-potassium phosphate at pH 7.2 and below, and 0.05M-triethanolamine-HCl at pH 7.5 and above. Other assay conditions were as described in the text.

Fig. 5. Variation in the activity of γ -glutamylcysteine synthetase with substrate concentrations. Δ , Concn. of ATP varied; $\circ$, concn. of cysteine varied; $\bullet$, concn. of glutamate varied. All conditions except the variable under study were optimum (see the text).

cysteine concentrations is at present unexplained. The variation in rate with glutamate concentration followed normal saturation kinetics; the apparent K_m was about 2.4×10^{-3} M.

The addition of the detergent Triton X-100 (0.125%, v/v) to the assay system caused a rather variable degree of activation, and probably because it solubilized the stroma, enabled generally more repeatable results to be obtained. In earlier studies of the overall synthesis of GSH in intact erythrocytes (Koj, 1962), it was claimed that synthesis was enhanced by the addition of glucose. In the present system, addition of comparable concentrations of glucose had no effect; the effect of glucose in whole erythrocytes probably reflected the fact that the glucose acts as an energy source. In view of an earlier report (Mandel & Bloch, 1955) that the liver enzyme is inhibited by ADP, the effect of ADP was examined in the present system. No significant inhibition was found even with ADP concentrations as high as 3 mM.

For all further assays of this enzyme, the procedure was as follows. To 2.0 ml. of 0.1M-triethanolamine-hydrochloric acid buffer, pH 7.5, containing Triton X-100 (0.25%, v/v), 0.3M-potassium chloride and 0.02M-magnesium sulphate were added 0.2 ml. of haemolysate, 0.08 ml. of 1.0M-glutamate, 0.04 ml. of 1.0M-cysteine, and 0.04 ml. of 0.3M-ATP. The mixture was gassed at 0° for 10 min. and thereafter incubated at 37° for 45 min. Initial and final samples were assayed for γ -glutamylcysteine as described above.

When measured by this method, the activity (mean $\pm$ S.E.M.) of a series of eight normal erythrocyte samples was 17.9 ± 1.5 m-moles of γ -glutamylcysteine synthesized/min./ 10^9 erythrocytes.

DL-Homocysteine (20 mM) could be substituted for cysteine; the reaction rate was about 9% of that obtained with cysteine.

GSH, but not GSSG, inhibited γ -glutamylcysteine synthetase at physiological concentrations (Table 2). That this decrease in the increment of E_{412} is not caused by degradation of the added GSH was

Table 2. Inhibition of γ -glutamylcysteine synthetase by GSH

Assay conditions were as described in the text. Activities are quoted as percentages of the activity of the control sample. The concentration of glutamate was 15 mM.

Sample no.	Concn. of GSH (mM)	Concn. of GSSG (mM)	Relative activity (%)
1 (Control)	—	—	100
2	1	—	85
3	2	—	71
4	3	—	65
5	—	2	96
6	—	3	92

Fig. 6. Inhibition of erythrocyte γ -glutamylcysteine synthetase by nicotinamide nucleotides. $\square$, Nicotinic acid; $\blacktriangle$, NADP $^{+}$; $\triangle$, NADPH; $\circ$, NAD $^{+}$; $\bullet$, NADH. Assay conditions were optimum (see the text).

Table 3. Inhibition of γ -glutamylcysteine synthetase by NADH and NAD $^{+}$

Assay conditions were as described in the text. Activities are expressed as percentages of the maximum value.

(a)	Concn. of ATP (mM)	Concn. of NADH (mM)	Relative activity (%)
	3	0	100
	3	0.5	86
	3	1.0	77
	3	3.0	57
	1	0	84
	1	0.5	78
	1	1.0	63
	1	2.0	55

(b)	Concn. of ATP (mM)	Concn. of NAD $^{+}$ (mM)	Relative activity (%)
	3	1.0	97
	3	3.0	73
	3	5.0	59
	1	0.5	69
	1	1.0	68
	1	2.0	66

Table 4. Inhibition of γ -glutamylcysteine synthetase by cysteamine

Assay conditions were as described in the text. Activities are expressed as percentages of the maximum value.

Concn. of cysteamine (mM)	Relative activity (%)
0	100
2	68
5	42
10	38
30	35

shown by a control experiment in which cysteine was omitted from the incubation mixture; appreciable GSH breakdown did not occur under these conditions. A threefold decrease in the glutamate concentration did not significantly alter the proportionate inhibition at a given GSH concentration.

NADH, and to a smaller extent NAD $^{+}$ and NADPH, also inhibited γ -glutamylcysteine synthetase at low concentration. NADP $^{+}$ and nicotinic acid did not give appreciable inhibition (Fig. 6). If the effect of NADH was examined at a lower ATP concentration, the proportion of activity inhibited at a given NADH concentration was not materially different (Table 3a). These results rule out the possibility that NADH inhibits by competing for an ATP-binding site. The result of a similar experiment with NAD $^{+}$ (Table 3b) was more difficult to interpret; again, however, simple competitive inhibition was ruled out.

Finally, the presence of cysteamine (2-mercaptoethylamine) in the incubation mixture was observed to cause appreciable inhibition (Table 4). That the inhibitory effects of NADH and cysteamine were not simply artifacts caused by interference with the DTNB colour reaction was proved by the observation that recoveries of added γ -glutamylcysteine (2.5 mM) from incubation mixtures containing 10 mM-cysteamine and 5 mM-NADH were 100% and 98% respectively.

In an attempt to clarify the nature of the inhibitory effects of NADH and cysteamine on γ -glutamylcysteine synthetase, haemolysates were incubated for 45 min. at 37° in 0.05 M-triethanolamine-hydrochloric acid buffer, pH 7.5, containing, respectively, 3 mM-NADH and 4 mM-cysteamine. A control flask containing lysate and buffer only was similarly preincubated. The incubation mixtures were dialysed for 12 hr. at 4° against 1000 vol. of 0.01 M-potassium phosphate buffer, pH 7.0. The haemolysates were then assayed in the normal way and the activities compared with those of samples that had been dialysed, but not preincubated, and to which cysteamine or NADH was added during assay (Table 5). It was found that preincubation in the presence of 3 mM-NADH caused no greater loss of activity than preincubation in its absence, but preincubation with cysteamine resulted in an irreversible partial loss of activity.

DISCUSSION

The study of GSH synthesis by the erythrocyte has, in the past, been concerned almost exclusively with overall synthesis, rather than with the separate activities of the two synthetases. Boivin & Galand (1965) were able to compare the rates of the two enzymes by measuring the incorporation of [14 C]glutamate and [14 C]glycine into the dipeptide

Table 5. *Effects of cysteamine and NADH on the activity of γ -glutamylcysteine synthetase*

Preincubation and assay were as described in the text. Activities are expressed as percentages of the control value.

Sample no.	Preincubation	Additive during preincubation	Additive during assay	Relative activity (%)
1 (Control)	—	—	—	100
2	—	—	Cysteamine (4 mM)	55
3	—	—	NADH (3 mM)	60
4	+	—	—	86
5	+	Cysteamine (4 mM)	—	52
6	+	NADH (3 mM)	—	87

and tripeptide, which were isolated and counted as cupromercaptides, but they did not calculate absolute values for the enzyme activities. The present study showed that the potential GSH-synthetic capacity of erythrocytes is very high: under optimum conditions an erythrocyte could, at 37°, synthesize its entire content of GSH in about 10 min. [Earlier estimates, e.g. that of Koj (1962), were of the order of hours, rather than minutes.] The activity of glutathione synthetase is slightly greater than that of γ -glutamylcysteine synthetase; this, together with the fact that free γ -glutamylcysteine is never found in normal erythrocytes, suggests that γ -glutamylcysteine formation is the rate-limiting stage of GSH biosynthesis. Unlike the other substrates of GSH synthesis (ATP, glutamate and glycine) free cysteine (or cystine) is not found in normal human erythrocytes. The availability of cysteine may therefore be the most important single rate-limiting factor; in this connexion the rather low K_m of γ -glutamylcysteine synthetase for cysteine (about 3×10^{-4} M) is noteworthy.

Erythrocyte glutathione synthetase appears to resemble the liver enzyme in its sensitivity to inhibition by ADP: this was found to be competitive with ATP. Erythrocyte γ -glutamylcysteine synthetase, however, differs from the liver enzyme in that it is not inhibited by ADP.

The feedback inhibition of γ -glutamylcysteine synthetase by GSH at physiological concentrations (2 mM) by about one-third may be an important metabolic regulatory mechanism; this finding agrees with the observation of Boivin & Galand (1965) that the activity of γ -glutamylcysteine synthetase in patients with congenital GSH deficiency of the erythrocyte was rather higher than the activity in erythrocytes of normal donors.

The interest in the effects of nicotinamide nucleotides on the activity of γ -glutamylcysteine synthetase was prompted by speculation that alterations in the contents of GSH and NAD⁺ in the blood cells of patients suffering from haemato-

logical disease may be correlated (Silber, Gabrio & Huennekens, 1962; Harrap & Speed, 1964; Ehrhart & Scheffel, 1966). The NADH effect appears to be reversible, but non-competitive. These inhibitions are difficult to interpret, and need to be followed up by work on purified enzyme preparations, rather than, as the present work, on crude haemolysates.

The observation of Révész & Modig (1965), that addition of cysteamine to ELD ascites cells growing in culture resulted in increased cellular content of GSH, suggested that cysteamine might activate GSH synthesis. However, the present work showed that cysteamine irreversibly inactivates γ -glutamylcysteine synthetase; it thus seems more likely that the explanation considered by Révész & Modig (1965) is correct: that the cysteamine releases GSH from protein, to which it is bound by mixed disulphide bonds. This tendency of cysteamine to react with protein disulphide groups may possibly account for the inactivation of γ -glutamylcysteine synthetase by cysteamine: loss of activity may be the result of the cleavage of a disulphide group that is necessary for retention of the tertiary configuration of the enzyme.

The activity of γ -glutamyl transpeptidase in erythrocytes is low in comparison with the activity of the synthetic enzymes. According to Cliffe & Waley (1961), γ -glutamyl transpeptidase of liver is a nuclear enzyme; if this is also the case for cell's of the haemopoietic system the low GSH-degradative activity found in mature erythrocytes may represent enzyme that originates from the erythroblast nucleus.

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REFERENCES

- Ball, C. R. (1966). *Biochem. Pharmacol.* **15**, 809.
- Ball, E. G., Revel, J. P. & Cooper, O. (1956). *J. biol. Chem.* **129**, 393.
- Boivin, P. & Galand, C. (1965). *Nouv. Rev. franç. Hémat.* **5**, 707.
- Cliffe, E. E. & Waley, S. G. (1961). *Biochem. J.* **79**, 118.
- Dimant, E., Landsberg, E. & London, I. M. (1954). *J. biol. Chem.* **213**, 769.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 329. London: Longmans, Green and Co. Ltd.
- Dohan, J. S. & Woodward, G. E. (1939). *J. biol. Chem.* **129**, 393.
- Ehrhart, H. & Scheffel, G. (1966). *Klin. Wschr.* **44**, 307.
- Eldjarn, L., Bremer, J. & Børresen, H. C. (1962). *Biochem. J.* **82**, 192.
- Gutcho, M. & Laufer, L. (1954). In *Glutathione*, p. 79. Ed. by Colowick, S. P., Lazarow, A., Racker, E., Schwarz, D. R., Stadtman, E. & Waelsch, H., New York: Academic Press Inc.
- Harrap, K. R. & Speed, D. E. M. (1964). *Brit. J. Cancer*, **18**, 809.
- Horejsi, J. & Mircevova, L. (1964). *Acta biochim. pol.* **11**, 107.
- Jacob, H. S. & Jandl, J. H. (1966). *J. biol. Chem.* **241**, 4243.
- Kasbekar, D. K. & Sreenivasan, A. (1959). *Biochem. J.* **72**, 389.
- Koj, A. (1962). *Acta biochim. pol.* **9**, 11.
- Mandeles, S. & Bloch, K. (1955). *J. biol. Chem.* **214**, 639.
- Mills, G. C. (1957). *J. biol. Chem.* **229**, 189.
- Patterson, J. W. & Lazarow, A. (1955). *Meth. biochem. anal.* **2**, 259.
- Révész, L. & Modig, H. (1965). *Nature, Lond.*, **207**, 430.
- Silber, R., Gabrio, B. W. & Huennekens, F. M. (1962). *J. clin. Invest.* **41**, 230.
- Snoke, J. E. & Bloch, K. (1955). In *Methods in Enzymology*, vol. 2, p. 342. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.