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A Convenient Spectrophotometric Assay for the Determination of L-Ergothioneine in Blood

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1. A convenient spectrophotometric assay for the determination of L-ergothioneine in solution including deproteinized blood haemolysate was developed. 2. The method consists of deproteinization by heat precipitation and Cu^{2+}-catalysed oxidation of thiols such as glutathione and of L-ascorbic acid, both in alkaline media, and titration of L-ergothioneine (which is not oxidized under these conditions) by its virtually instantaneous reaction with 2,2'-dipyridyl disulphide at pH1. 3. This method and the results obtained with it for the analysis of human, horse, sheep and pig blood are compared with existing methods of L-ergothioneine analysis and the results obtained thereby.

L-Ergothioneine (I) (Scheme 1), the betaine of 2-mercapto-L-histidine, was first isolated from ergot, the fungus infection of rye grain, by Tanret (1909), and interest in this compound was greatly stimulated by its subsequent discovery in blood (Hunter & Eagles, 1925; Benedict *et al.*, 1926; Eagles & Johnson, 1927).

Despite a considerable amount of work on this compound [see Melville (1959) and Stowell (1961) for reviews] its function remains to be established and the question of its origin in the animal body to be resolved. The suggestion (Crossland *et al.*, 1966) that L-ergothioneine may be the unknown transmitter present in the neurons of the cerebellum ('cerebellar factor') has been criticized by Briggs (1972). Nevertheless, Briggs (1972) points out that the wide variation in the amounts of L-ergothioneine in different regions of the central nervous system may indicate that this compound has a role to play in certain areas of the system. It is notable in this connexion that L-ergothioneine can have excitatory effects on the neurons in the brain stem (Avanzino *et al.*, 1966).

The contradictory results that characterize some of the L-ergothioneine literature (see, e.g., Crossland *et al.*, 1966; and cf. Briggs, 1972) and the lack of significant progress in delineating the role of this compound probably derive in part from the lack of a convenient and specific assay for this compound in biological materials.

Of the many methods that have been reported for the assay of L-ergothioneine (see Melville, 1959; Stowell, 1961) the most satisfactory ones published to date are the modifications to the method of Hunter (1928, 1930) (see also Lawson *et al.*, 1951) reported by Melville & Lubschez (1953) and by Heath & Wildy (1956) and the method reported by Jocelyn (1958).

In the method of Hunter (1928, 1930), L-ergothioneine in a deproteinized solution is allowed to react with diazotized sulphanilic acid and the reaction mixture is subsequently made strongly alkaline. Spectrophotometric analysis of the resulting bluish-red product permits estimation of the L-ergothioneine concentration. When applied to analysis of biological materials such as blood, however, the Hunter reaction suffers from two serious drawbacks. Many other compounds, e.g. tyrosine and histidine, undergo the diazo-coupling reaction to provide coloured products. In addition other compounds present in blood or other tissues (e.g. L-cysteine, glutathione and purines) inhibit the development of the red colour in the coupling reaction (Melville & Lubschez, 1953; Baldridge & Lewis, 1953).

The most effective modifications of the Hunter method involve separation of the L-ergothioneine from the two types of compound that interfere with the estimation by prior chromatography on an alumina column (Melville & Lubschez, 1953; Heath & Wildy, 1956). As pointed out by Jocelyn (1958) these techniques are too lengthy for convenient general use.

The method of Jocelyn (1958) is based on the observation by Barger & Ewins (1911) that when L-ergothioneine is heated in 50% KOH solution at 100°C, trimethylamine is eliminated to provide thiolurocanic acid (II) (see Scheme 1).

In Jocelyn's (1958) method the trimethylamine released is transferred by aeration into a solution of

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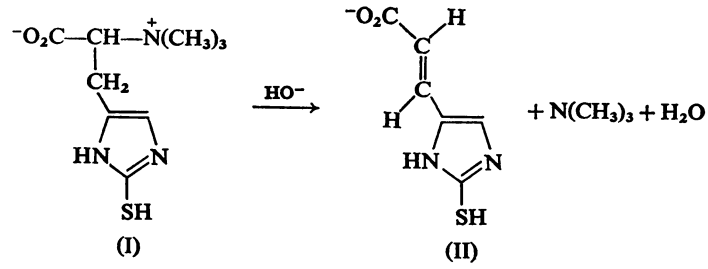

Scheme 1. *Basis of the Jocelyn* (1958) *method of determination of* L-*ergothioneine*

picric acid in benzene. The picrate thus formed is determined spectrophotometrically.

Although this method is not as laborious as those involving chromatographic procedures and does obviate the necessity for deproteinization, it is clearly not as convenient as a direct spectrophotometric assay. In addition, the report (Ackermann *et al.*, 1959) that hercynine (L-histidine betaine) is present in blood implies that L-ergothioneine concentrations estimated by the Jocelyn (1958) method may be too high, because hercynine also undergoes base-catalysed elimination of trimethylamine.

The present paper reports a relatively rapid and convenient direct spectrophotometric assay of L-ergothioneine. The assay is based on the resistance of L-ergothioneine to Cu^{2+}-catalysed oxidation in alkaline media and the very rapid reaction of L-ergothioneine with excess of 2,2'-dipyridyl disulphide (2-Py–S–S–2-Py) at pH1 to provide essentially stoicheiometric release of the chromophoric 2-thiopyridone (Py–2-SH). The remarkable reactivity of L-ergothioneine towards 2-Py–S–S–2-Py is discussed in the preceding paper (Carlsson *et al.*, 1974). It would be possible to use instead the rapid reaction of L-ergothioneine with 4,4'-dipyridyl disulphide (4-Py–S–S–4-Py) as a spectrophotometric titration. In this case, however, the titration would have to be carried out at pH6–8, where the equilibrium position favours the formation of 4-thiopyridone (Py–4-SH).

The assay here reported is perhaps best regarded as complimentary to that of Jocelyn (1958) because in the present state of study of L-ergothioneine in biological materials it would seem desirable to use at least two quite different assay methods that rely on different characteristics of the L-ergothioneine molecule.

Materials and Methods

Some materials and methods are described in the preceding paper (Carlsson *et al.*, 1974); others are described below.

Materials

GSH was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and L-ascorbic acid (AnalaR) from BDH Chemicals Ltd., Poole, Dorset, U.K.

Samples of defibrinated sheep and horse blood were purchased from Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, Kent, U.K. Defibrinated pig blood was obtained from Tissue Culture Services, Slough, Bucks., U.K., and citrated human blood was kindly supplied by the Department of Haematology, St. Bartholomew's Hospital, West Smithfield, London E.C.1, U.K.

Methods

Preparation of packed blood cells. Citrated or defibrinated whole blood was centrifuged for 30min at 1600*g* and the plasma removed. The blood cells were then washed with an equal volume of 0.9% NaCl solution and the centrifugation was repeated.

Haemolysation and deproteinization by heat precipitation at alkaline pH. Packed blood cells (1–3ml) were mixed with deionized water (6–10ml) and the pH was adjusted to 8 by addition of 1M-NaOH. After 20min the haemolysate was heated in a boiling-water bath for 3min. The resulting precipitate that had settled out was resuspended by stirring and the suspension was heated for a further 3min. Solid material was removed from the hot suspension by centrifugation for 10min in a bench centrifuge and the supernatant was then filtered through Whatman no. 1 filter paper. The volume of the clear filtrate was usually 2–5ml, depending on the initial volumes used.

The concentration of L-ergothioneine in the filtrate was sometimes increased by allowing some of the water to evaporate at 90–100°C. This technique proved useful in the analysis of human blood cells in which the L-ergothioneine content is relatively low (see the Results and Discussion section). In such cases, 8ml of packed cells was added to 24ml of water to yield 15ml of filtrate, which was then concentrated to about 3ml. This procedure does not

result in the destruction of L-ergothioneine as long as the solution is maintained at alkaline pH.

Deproteinization by treatment with trichloroacetic acid. This was performed as described by Melville *et al.* (1954) except that no reducing agent was added to the blood haemolysate before precipitation.

Recovery experiments. In the recovery experiments (see the Results and Discussion section) known amounts of L-ergothioneine were added to the blood haemolysate before the deproteinization.

Cu^{2+}-catalysed alkaline oxidation of contaminant thiols and/or L-ascorbic acid in solutions containing L-ergothioneine. The pH of the solution containing either L-ergothioneine or mixtures of L-ergothioneine and contaminant thiols and/or L-ascorbic acid was adjusted to pH9.5 with 1M-NaOH. To this solution was added 1mM-$CuSO_4$ solution (0.5ml/5ml of solution to be oxidized). The pH of the solution was maintained at 9.5 by the occasional addition of small volumes of 1M-NaOH and a stream of air was drawn through the reaction mixture at room temperature (approx. 26°C) for 1–2h.

Determination of L-ergothioneine. Solutions of L-ergothioneine of known concentrations were allowed to react with 2-Py–S–S–2-Py at pH approx. 1; the reaction volume was 3.6ml; 2.5ml of 2-Py–S–S–2-Py (1.5mM), 0.6ml of 1M-HCl and 0–0.5ml of H_2O were mixed in both sample and reference cells. After the Cary 16K spectrophotometer had been set to zero E_{343}, H_2O (up to 0.5ml) was added to the reference cell and the same volume of L-ergothioneine solution (to give a final concentration of up to 40μM) was added to the sample cell. The instantaneous increase in E_{343} was recorded. The results of the experiments were used to construct the calibration curve presented in the Results and Discussion section (Fig. 3).

Essentially the same method was used to determine the L-ergothioneine concentration in synthetic L-ergothioneine–GSH mixtures and in deproteinized and oxidized samples of blood haemolysate. In the latter case the ΔE_{343} value was corrected for the background extinction of the L-ergothioneine-containing sample.

The same procedure was used to obtain progress curves for the reactions of 2-Py–S–S–2-Py with L-ergothioneine, L-ergothioneine–GSH mixtures and with L-ascorbic acid at pH1.

Results and Discussion

Titration of L-ergothioneine by reaction with 2-Py–S–S–2-Py at pH1

Our finding, reported in the preceding paper (Carlsson *et al.*, 1974) that L-ergothioneine reacts very rapidly with 2-Py–S–S–2-Py at pH1 to provide the chromophoric Py–2-SH suggested that this reaction might provide the basis for a convenient assay for L-ergothioneine in biological materials such as blood. At pH1 the pH-dependent second-order rate constants for the reactions of 2-Py–S–S–2-Py with aliphatic thiols such as L-cysteine or GSH are small ($<200\,M^{-1}\cdot s^{-1}$) because these reactions involve attack on 2-Py–S–S–2-Py (albeit protonated) by thiolate ions (Brocklehurst & Little, 1972) and the fraction of an aliphatic thiol ($pK_a \geqslant 7$) reacting as thiolate ion at pH1 will be very small. In spite of these favourable factors, however, the determination of L-ergothioneine in the presence of excess of aliphatic thiols such as GSH and L-cysteine by reaction with 2-Py–S–S–2-Py is complicated by the thermodynamics of the system. Whereas the reactions of simple aliphatic thiols with a moderate excess of 2-Py–S–S–2-Py are essentially irreversible in neutral and acidic media (Brocklehurst & Little, 1973; Grassetti & Murray, 1967) the corresponding reaction of L-ergothioneine is markedly reversible (Carlsson *et al.*, 1974). To ensure that the reaction of L-ergothioneine with 2-Py–S–S–2-Py provides essentially stoicheiometric release of Py–2-SH it is necessary to use relatively high concentrations of 2-Py–S–S–2-Py. This is true even at pH1 where the equilibrium position is relatively favourable (Carlsson *et al.*, 1974). The consequence is that even though the reaction of L-ergothioneine under these conditions is virtually instantaneous, the rate of reaction of a thiol such as GSH is sufficiently high to make extrapolation of the second, relatively slow, phase of the progress curve (reaction of GSH) to zero time inaccurate, at least with conventional spectrophotometry. This is demonstrated in Fig. 1 (curves *a* and *b*) which shows the progress curves for the reaction of 2-Py–S–S–2-Py (1.04mM) with L-ergothioneine (28μM) and with a mixture of L-ergothioneine (28μM) and GSH (28μM). It might be possible to determine the stoicheiometry of the fast phase of such progress curves by rapid-reaction techniques and/or by decreasing the concentration of 2-Py–S–S–2-Py, although the concentrations of GSH in blood could be somewhat higher than that of L-ergothioneine. Concentration ranges recorded (Diem & Lentner, 1970) for the compounds are: L-ergothioneine, 5–40mg/litre of whole blood, 0.022–0.175mM; GSH, 586–816mg/litre of erythrocytes, 1.26–2.66mM.

Specific titration of L-ergothioneine: Cu^{2+}-catalysed alkaline oxidation of contaminant thiols or L-ascorbic acid followed by reaction of L-ergothioneine with 2-Py–S–S–2-Py at pH1

The possibility of using the L-ergothioneine–2-Py–S–S–2-Py reaction directly in the presence of other thiols was not investigated further because there exists a very effective method of rendering the

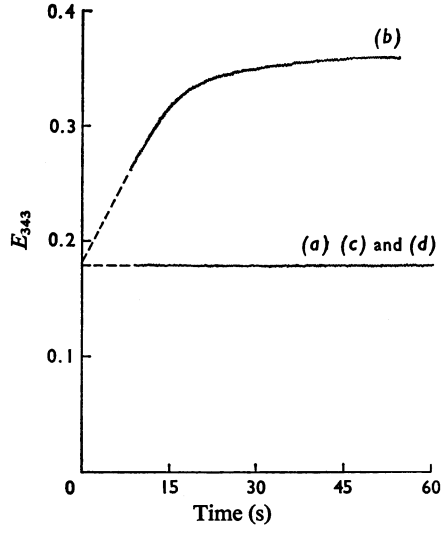

Fig. 1. *Progress curves for the reaction at pH 1 and 25.0°C of 2-Py–S–S–2-Py with various compounds*

2-Py–S–S–2-Py (1.04mM) was allowed to react with (*a*) L-ergothioneine (28μM), (*b*) a mixture of L-ergothioneine (28μM) and GSH (28μM) (*c*) a mixture as in (*b*) after Cu^{2+}-catalysed alkaline oxidation for 90min and (*d*) L-ergothioneine (28μM) after being subjected to alkaline oxidation as in (*c*).

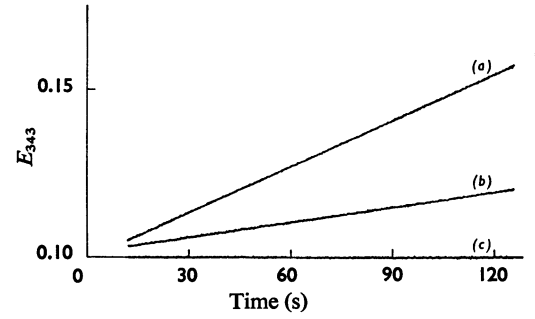

Fig. 2. *Progress curves for the reaction at pH 1 and 25.0°C of 2-Py–S–S–2-Py with various compounds*

2-Py–S–S–2-Py (1.04mM) was allowed to react with L-ascorbic acid (0.31mM) (*a*) before oxidation, (*b*) after Cu^{2+}-catalysed alkaline oxidation for 30min and (*c*) after Cu^{2+}-catalysed oxidation for 90min. Experimental details are given in the Materials and Methods section.

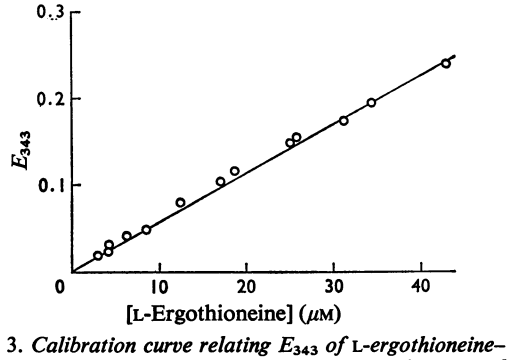

Fig. 3. *Calibration curve relating E_{343} of L-ergothioneine–2-Py–S–S–2-Py reaction mixtures at pH 1 to the original L-ergothioneine concentration*

Experimental details are given in the Materials and Methods section.

contaminant thiols unreactive without affecting the L-ergothioneine reactivity. This method derives from the stability of L-ergothioneine towards Cu^{2+}-catalysed oxidation in alkaline media (Heath & Toennies, 1958). Fig. 1 (curves *b* and *c*) demonstrates that when an equimolar mixture of L-ergothioneine and GSH (28μM in each compound) was subjected to aeration for 1.5h at pH9 in the presence of 0.1mM-Cu^{2+} all the GSH was oxidized whereas L-ergothioneine was unaffected. By aeration for up to 2h under these conditions it was possible to oxidize GSH completely in concentrations up to 10mM without detectable oxidation of the L-ergothioneine. Similar results were obtained with synthetic mixtures of L-cysteine and L-ergothioneine.

Two other reducing agents known to occur in blood are L-ascorbic acid (2.24–12.8mg/litre of whole blood, 13–73μM; Diem & Lentner, 1970) and D-glucose (60–100mg/litre, 1.95–3.3μM). D-Glucose does not react with 2-Py–S–S–2-Py, at least at pH1. L-Ascorbic acid reacts with 2-Py–S–S–2-Py at pH1 although at a rate that is very much lower than that of the corresponding reaction of L-ergothioneine under comparable conditions (see Fig. 2). In any case L-ascorbic acid is inactivated by the alkaline oxidation that inactivates aliphatic thiols (see above and Fig. 2).

Titration of L-ergothioneine in blood: general method

Heat-precipitation at alkaline pH was found to be a rapid and convenient method of obtaining protein-free samples of lysed blood cells. After removal of solid material by centrifugation and subsequent filtration the filtrate was subjected to Cu^{2+}-catalysed oxidation and subsequent reaction with 2-Py–S–S–2-Py as described in the Materials and Methods section. The concentration of L-ergothioneine was determined by using a calibration curve (Fig. 3). Fig. 3 demonstrates that values of E_{343} are linearly related to the original concentration of L-ergothioneine up to at least 40μM. When measuring the concentration of L-ergothioneine in blood the extinction changes at 343nm were corrected for the contribution to E_{343} from the pale-yellow filtrate.

To examine the effectiveness of this method of measurement various known amounts of L-ergothionine were added to samples of blood haemolysate and titrated with 2-Py–S–S–2-Py after deproteinization and Cu^{2+}-catalysed oxidation. Table 1 demonstrates the effectiveness of the method described above and also the unsuitability of effecting deproteinization by trichloroacetic acid treatment. The latter treatment results in the destruction of more than 90% of the added L-ergothioneine. This probably derives from oxidation of L-ergothioneine, which occurs relatively easily in acidic media, possibly catalysed by haem compounds (Melville *et al.*, 1954; Heath & Toennies, 1958; Jocelyn, 1958).

Oxidation of L-ergothioneine in samples of deproteinized blood haemolysate proceeds relatively rapidly at pH 1 even though under similar conditions oxidation of pure L-ergothioneine occurs only very slowly (see also Heath & Toennies, 1958).

Table 1. *Titration of* L-*ergothioneine added to human blood cells: typical results*

L-ergothioneine was determined by titration with 2-Py–S–S–2-Py at pH 1 after the Cu^{2+}-catalysed oxidation step as described under 'Methods'. The values are corrected for endogenous L-ergothioneine (11 μg/ml of packed cells).

Amount of L-ergothioneine added to 1 ml of packed blood cells (μg)	Amount of L-ergothioneine in the 1 ml of packed blood cells (μg)	Deproteinization method
14	11	Heat precipitation
24	23	Heat precipitation
66	66	Heat precipitation
89	84	Heat precipitation
100	<10	Trichloroacetic acid precipitation

Titration of L-*ergothioneine in blood: results*

The method of determination of L-ergothioneine described above and in the Materials and Methods section has been used to measure concentrations of L-ergothioneine in the blood of man, horse, pig and sheep. The relatively low concentrations of L-ergothioneine in human blood necessitated concentration of the deproteinized solution as described in the Materials and Methods section before spectrophotometric analysis. The results of these determinations are collected in Table 2 together with analogous results reported by other workers who used other methods of analysis.

In general the L-ergothioneine concentrations determined in the present work coincide with those at the lower end of the spectrum of results found with other methods of analysis.

Specificity of 2-Py–S–S–2-Py assay of L-*ergothioneine*

It was reported in the preceding paper (Carlsson *et al.*, 1974) that the rapid reaction of 2-Py–S–S–2-Py at pH 1 is exhibited not only by L-ergothioneine but also by other thiones that are made highly nucleophilic in their neutral forms by mesomeric electron release from a nitrogen atom linked either directly to the carbon atom of the thione grouping or remote from this atom but linked to it by a conjugated system.

Fortunately, compounds such as thiourea and thioacetamide that have been shown to exhibit this type of reactivity do not appear to be present in blood. If at any time they were shown to be present, however, L-ergothioneine could still be determined in their presence by carrying out the spectrophotometric titration with 2-Py–S–S–2-Py at pH values 6–7. In this range of pH the equilibrium position of the L-ergothioneine–2-Py–S–S–2-Py system is by far the

Table 2. L-*Ergothioneine concentrations in blood*

Values are expressed as mg/100 ml of whole blood; in the present work the L-ergothioneine content of packed blood cells was determined (see the text) and recalculated for purposes of comparison as mg/100 ml of whole blood by using a haematocrit value of 48%. In the present work the values represent the mean of three or four determinations on the same batch of blood; the accuracy was about ±15–20%; the three values for human blood refer to three different batches of blood.

	Method of determination					
			Modifications of the original Hunter method			
Species	Non-specific phosphotungstic acid method of Christman *et al.* (1944)	Method of Hunter (1928)	Lawson *et al.* (1950)	Melville *et al.* (1954)	Method of Jocelyn (1958)	Present work
Horse	12–21	—	—	—	—	10.4
Man	—	1.25–4.0	2.0–3.1	1.5–4.2	1.0–2.6	1.1, 2.9, 3.2
Pig	5.9–11.2	2.5–26.5	—	8.6	—	2.8
Sheep	3.8	—	—	—	—	3.1

most favourable of any of the aminothione–2-Py–S–S–2-Py systems studied (Carlsson *et al.*, 1974) and a concentration of 2-Py–S–S–2-Py could be used such that only reaction of L-ergothioneine was observed.

The only naturally occurring aminothiones that might be expected to react rapidly with 2-Py–S–S–2-Py at pH 1 are the 2-mercaptopyrimidine bases that occur rather rarely in tRNA species (Lipsett & Doctor, 1967; Scheit & Faerber, 1973); 2-Py–S–S–2-Py may be of use in the study of these compounds, but their reaction with it would be unlikely seriously to affect the determination of L-ergothioneine in tissue extracts, at least in blood.

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References

Ackermann, D., List, P. H. & Meussen, H. G. (1959) *Hoppe-Seyler's Z. Physiol. Chem.* **314**, 33–37

Avanzino, G. L., Bradley, P. B., Comis, S. D. & Wolstencroft, J. H. (1966) *Int. J. Neuropharmacol.* **5**, 331–333

Baldridge, R. C. & Lewis, H. B. (1953) *J. Biol. Chem.* **202**, 169–176

Barger, G. & Ewins, A. J. (1911) *J. Chem. Soc. London Trans.* 2336–2371

Benedict, S. R., Newton, E. B. & Behte, J. A. (1926) *J. Biol. Chem.* **67**, 267–277

Briggs, I. (1972) *J. Neurochem*, **19**, 27–35

Brocklehurst, K. & Little, G. (1972) *Biochem. J.* **128**, 471–474

Brocklehurst, K. & Little, G. (1973) *Biochem. J.* **133**, 67–80

Carlsson, J., Kierstan, M. P. J. & Brocklehurst, K. (1974) *Biochem. J.* **139**, 221–235

Christman, A. A., Foster, P. W. & Esterer, M. B. (1944) *J. Biol. Chem.* **155**, 161–171

Crossland, J., Mitchell, J. F. & Woodruff, G. N. (1966) *J. Physiol. (London)* **182**, 427–438

Diem, K. & Lentner, C. (1970) *Documenta Geigy, Scientific Tables*, 7th edn., J. R. Geigy S.A., Basle

Eagles, B. A. & Johnson, T. B. (1927) *J. Amer. Chem. Soc.* **49**, 575–580

Grassetti, D. R. & Murray, J. F. (1967) *Arch. Biochem. Biophys.* **119**, 41–49

Heath, H. & Toennies, G. (1958) *Biochem. J.* **68**, 204–210

Heath, H. & Wildy, J. (1956) *Biochem. J.* **64**, 612–620

Hunter, G. (1928) *Biochem. J.* **22**, 4–10

Hunter, G. (1930) *J. Chem. Soc. London* 2343–2376

Hunter, G. & Eagles, B. A. (1925) *J. Biol. Chem.* **65**, 623–642

Jocelyn, P. C. (1958) *Biochem. J.* **70**, 656–660

Lawson, A., Morley, H. V. & Woolf, L. I. (1950) *Biochem. J.* **47**, 513–518

Lawson, A., Morley, H. V. & Woolf, L. I. (1951) *Nature (London)* **167**, 82–83

Lipsett, M. N. & Doctor, B. P. (1967) *J. Biol. Chem.* **242**, 7072–4077

Melville, D. B. (1959) *Vitam. Horm. (New York)* **17**, 155–204

Melville, D. B. & Lubschez, R. (1953) *J. Biol. Chem.* **200**, 275–285

Melville, D. B., Horner, W. H. & Lubschez, R. (1954) *J. Biol. Chem.* **206**, 221–228

Scheit, K.-H. & Faerber, P. (1973) *Eur. J. Biochem.* **33**, 575–550

Stowell, E. C. (1961) in *Organic Sulphur Compounds* (Kharasch, N., ed.), vol. 1, pp. 462–490, Pergamon Press, Oxford, London, New York, Paris

Tanret, C. (1909) *C. R. Acad. Sci.* **149**, 222–224