Acidic Peptides of the Lens

5. *S*-SULPHOGLUTATHIONE*

By S. G. WALEY

Nuffield Laboratory of Ophthalmology, University of Oxford

(Received 9 June 1958)

The occurrence in lens extracts of acidic peptides, apparently derived from glutathione, was described in the first paper of this series (Waley, 1956). These compounds were isolated by electrophoresis; they migrated rapidly towards the anode at pH 4 (the 'fast-acid' fraction). A major constituent of this fraction (called 1α4) was shown to have glutamic acid as the *N*-terminal amino acid of a tripeptide, which gave also cystine and glycine on hydrolysis. This paper describes further work leading to the identification of this derivative as the thiosulphate related to glutathionine; following the suggestion of Swan (1957), the thiosulphate will be called *S*-sulphoglutathione. The main analytical technique employed has been ion-exchange chromatography; *S*-sulphoglutathione is the most acidic ninhydrin-positive compound in lens extracts and can be readily isolated by using a buffer of high ionic strength so that all the other compounds are barely retarded.

EXPERIMENTAL

Analytical techniques

Separation of peptides from proteins. Previously (Waley, 1956), dialysis in alkaline solution was used to remove high-molecular-weight compounds; the pH was maintained at a high value so as to minimize enzymic reactions. Such conditions, however, are likely to lead to oxidation. Therefore two other methods of removing proteins have also been used. One is heat-precipitation at pH 5.3 (Hunter, 1957) and the other is precipitation with trichloroacetic acid.

Fractionation of acidic compounds. Anion-exchange chromatography has been used to separate these strongly acidic compounds. As the earlier work had shown that the 'fast-acid' fraction contained nucleotides, chromatographic methods were similar to those developed for nucleotides (Hurlbert, Schmitz, Brumm & Potter, 1954).

In these methods an anion-exchange resin (Dowex 1 formate) is used, and the acidic species are eluted by a gradually increasing concentration of formic acid or ammonium formate (gradient elution, Alm, Williams & Tiselius, 1952). It is inconvenient to use ammonium formate as the eluent for ninhydrin-positive compounds, but the formic acid system proved quite suitable and was used in one of the experiments described below. As an example of the resolving power of this system, it may be mentioned that the sulphonic acid (GSO_3H) was well

separated from *S*-sulphoglutathione (GSSO_3H), although these two compounds have similar electrophoretic mobilities. There is, however, one disadvantage of the formic acid system: the pH of each of the fractions has to be individually adjusted before carrying out the ninhydrin reaction. The use of a pyridine formate buffer (described below) overcomes this disadvantage: this system is well suited for the isolation of *S*-sulphoglutathione formed enzymically from *S*-sulphocysteine; moreover, pyridine formate buffers, being more volatile than ammonium formate ones, can be removed by addition of water and evaporation at the pressure attained by a water pump. The separation of a synthetic mixture of *S*-sulphoglutathione and *S*-sulphocysteine is shown in Fig. 1.

Materials

***S*-Sulphoglutathione.** This compound was prepared by the reaction of oxidized glutathione (GSSG) with excess of Na_2SO_3 (Clarke, 1932). Auto-oxidation of glutathione (GSH) to the disulphide was accomplished by exposing it (1.23 g.) in 0.4*N*-NaOH (10 ml.) containing CHCl_3 (to

Fig. 1. Chromatography of a mixture of synthetic *S*-sulphoglutathione (7.9 μ moles) and *S*-sulphocysteine (8.8 μ moles) on Dowex 1 formate column (28 cm. $\times$ 1.2 cm.). Elution was with *m*-pyridine formate, pH 5.4; the eluate was collected in 3 ml. fractions.

* Part 4: Cliffe & Waley (1958).

prevent bacterial growth) and a trace of FeSO_4 , to the air for 48 hr.; as the oxidation proceeds, the red colour fades. The disulphide thus obtained gave only one ninhydrin-positive spot on paper chromatography in the solvent described below. The use of hydrogen peroxide (Pirie, 1931; Schöberl & Hornung, 1938) tends to give material contaminated with a little of the sulphonic acid (GSO_3H).

The reaction of GSSG with excess of Na_2SO_3 was carried out under conditions such that the GSH first formed would be re-oxidized to GSSG. This facilitates isolation of the product. The pH must be kept close to pH 7, as at higher pH values the equilibrium is unfavourable (Cecil & McPhee, 1955), and at lower ones the reaction (and auto-oxidation) is too slow.

The solution of GSSG obtained above was treated with $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (5 g.), and acetic acid added to lower the pH to 6.9 and O_2 bubbled through for 24 hr. Further $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (5 g.) was added and the passage of O_2 continued for 48 hr. The solution was treated with a cation-exchange resin [Amberlite IR-120 (H), British Drug Houses Ltd.] (7 g.) and freed from SO_2 by passing N_2 through. Barium acetate (25%, w/v; 20 ml.) was added, and barium sulphate centrifuged down and ethanol (75 ml.) added to the supernatant. The barium salt of S-sulphogluthathione was collected and dried in air (yield 0.88 g., 37%); $[\alpha]_D^{20} + 44^\circ$ (c, 1.3 in water) (Found: C, 20.1; H, 3.8; N, 6.9; S, 10.5; Ba, 22.7. $\text{C}_{10}\text{H}_{15}\text{O}_9\text{N}_3\text{S}_2\text{Ba}_4\text{H}_2\text{O}$ requires C, 20.2; H, 3.9; N, 7.1; S, 10.8; Ba, 23.1%).

The absence of GSSG from the product can be checked by paper chromatography, or by paper electrophoresis at pH 4. The paper chromatography was carried out with butanol-acetic acid-water-pyridine (30:6:24:20, by vol.) as solvent on no. 4 paper; in a 16 hr. run, GSSG moves about 3 cm. and GSSO_3H about 9 cm. The solvent runs off the end of the paper, so R_f values are not quoted, but an available compound which travels the same distance as GSSO_3H is aspartic acid.

Earlier preparations of GSSO_3H were contaminated with GSSG and, on occasions, with GSO_3H . Ion-exchange chromatography, under the conditions described below ('heat-precipitation experiment') was used to isolate pure GSSO_3H from these mixtures.

RESULTS

Alkaline-dialysis experiment

Calf lenses (30 g.) were added to cooled triethylamine formate buffer (prepared from 12.5 ml. of triethylamine, 2.25 g. of 90% formic acid and 90 ml. of water), and macerated and dialysed as described previously (Waley, 1956; experiment L. II). The diffusate was concentrated to a syrup, and extracted with CHCl_3 and dried *in vacuo*. The residue was dissolved in water (25 ml.) and fractionated on a column 1.2 cm. $\times$ 28 cm. long of Dowex 1 formate. The resin (Dow Chemical Co., Michigan; 4% cross-linked, 200–400 mesh, 440 g., moist chloride form) had been converted into the formate form and purified as follows. It was washed with water by decantation until the supernatant was clear, and then transferred to a Büchner funnel and washed with 3M-sodium formate (4 l.), and then

made into a column in a separating funnel and washed with 90% formic acid (1 l.). Finally the resin was washed free from acid with water. A portion (about 30 ml.) of the resin was added to 60 ml. of m-pyridine formate buffer, pH 5.4 [42 ml. of 90% formic acid and 162 ml. of purified pyridine and water to 1 l.; the pyridine was purified by distillation under N_2 after adding Br_2 (2 ml./l.)], and de-gassed at the water pump and the column packed; the buffer used for elution was also de-gassed.

The solution of the extract was brought to pH 5 with N-NaOH and then added to the column during 3 hr. The column was eluted overnight with the m-pyridine formate buffer at a rate of 12 ml./hr.; fractions of 3 ml. were collected. Portions (1 ml.) of the even-numbered tubes were taken for ninhydrin estimation; the modification of Rosen (1957) of the method of Yemm & Cocking (1955) was used. The high concentration of formate in the buffer used for elution prevents the adsorption of any but the most strongly acidic species; thus glutamic acid, glutathione and ophthalmic acid are not retained by the column and form part of the large peak in Fig. 2. The contents of two sets of tubes, 31–39 and 48–58, were concentrated, and water added and removed by distillation several times to remove most of the buffer. The materials were then examined by paper chromatography in butanol-acetic acid-water-pyridine and by paper electrophoresis at pH 4 (Fig. 3; the apparatus of Cliffe & Waley, 1958, was used).

The second fraction (tubes 48–58) showed only one ninhydrin-positive spot, which travelled at the same rate as authentic GSSO_3H in paper chromatography and paper electrophoresis; comparison of

Fig. 2. Chromatography of diffusate obtained by dialysis of an extract of calf lenses (30 g.). Chromatography was carried out as described for Fig. 1.

Figs. 1 and 2 shows that this substance also emerged from the ion-exchange column at the position expected for GSSO_3H . The amount of this substance (assuming it to be GSSO_3H), calculated from the area of the peak in Fig. 2, was $2.5\ \mu\text{moles}$; this corresponds to a concentration of $10\ \mu\text{moles}/100\ \text{g. of calf lens}$.

The ninhydrin-positive material in tubes 31–39 seems to be labile, as after evaporation only weak and ill-defined spots were observed when the residue was examined by paper electrophoresis.

Heat-precipitation experiment

Thirty calf lenses were macerated with water (120 ml.); 1% acetic acid (24 ml.) was added to lower the pH to 5.1, and the mixture was poured into centrifuge tubes, and heated with stirring at 90° for 4 min., and cooled and the protein centrifuged down. The supernatant was treated with Hyflo Supercel (Johns-Manville) and filtered. The precipitate was extracted with 0.03% acetic acid (120 ml.), and the extract clarified as before. The extracts were passed through a column (1.2 cm. $\times$ 15 cm. long) of Dowex 1 formate (10% cross-linked), equilibrated with the pyridine formate buffer diluted 1:10 (pH 5.5). The elution was then carried out with a linear gradient between the 0.1M-pyridine formate (110 ml.) in the mixing vessel, and 4M-formic acid (110 ml.) in the reservoir. The two vessels (volume 1 l.) were connected (at the bottom) by a short length of capillary tubing, and the mixing vessel was stirred by a magnetic stirrer. Titration against standard alkali confirmed that the concentration of formic acid in the eluate increased approximately linearly. The eluate was collected in 2 ml. fractions; after 77 fractions, the mixing vessel was replenished with 3M-formic acid (100 ml.) and the reservoir with 4M-formic acid (100 ml.). The tubes were analysed by spotting on paper and developing with ninhydrin; after the first 20 tubes, there was ninhydrin-positive material in tubes 105–125; the peak at tube 118 was close to that previously obtained with authentic GSSO_3H . The contents of tubes 105–125 were concentrated, and the presence of material which was chromatographically homogeneous and chromatographically indistinguishable from GSSO_3H was confirmed.

The bulk of the material in water (0.1 ml.) containing barium acetate (0.7 mg.) was diluted with ethanol (0.3 ml.), and the barium salt centrifuged down and used in the comparisons of the reactions of the material from the lens with those of authentic GSSO_3H (see below).

The amount of GSSO_3H obtained in this experiment was not measured, but it was of the same order as that obtained in the preceding experiment; in a repetition of the heat-precipitation experiment, however, much less GSSO_3H was isolated.

Trichloroacetic acid experiment

In this experiment the bulk of the lens proteins were removed by precipitation with cold 8% (w/v) trichloroacetic acid, and the supernatant was extracted with ether, and brought to pH 6 and freed from residual protein by ultrafiltration under pressure through a dialysis sac. The fractionation was essentially similar to that described in the heat-precipitation experiment. *S*-Sulphogluthione was isolated, in amount corresponding to approx. $3\ \mu\text{moles}/100\ \text{g. of calf lens}$.

In another experiment of this type, similar except that the ultrafiltration step was omitted, no GSSO_3H could be detected. The variations in the amount of GSSO_3H isolated are discussed below.

Enzymic synthesis of S-sulphogluthione

Dialysed calf-lens extracts were used as a source of enzymes, and *S*-sulphocysteine (Clarke, 1932), glutamic acid and glycine as substrates, under the conditions previously described (Cliffe & Waley, 1958). I am indebted to Mr E. E. Cliffe for carrying out the incubation. The incubation mixture contained adenosine triphosphate (ATP) (2.5 mM), 3-phosphoglyceric acid (22 mM), KCl (42 mM), MgSO_4 (10 mM), potassium phosphate buffer, pH 6.4 (6 mM), L-glutamic acid (8 mM), *S*-sulphocysteine (8 mM) and glycine (8 mM). The total volume of the solution was 20 ml. and it contained 15 ml. of dialysed calf-lens preparation. The reaction was carried out at pH 6.5 at 37° for 24 hr.; a portion (15 ml.) of the solution was treated with hot ethanol (38 ml.) and the protein centrifuged down. The supernatant was concentrated to 2 ml., and adsorbed on a 1.2 cm. $\times$ 28 cm. long column of Dowex 1 formate (4% cross-linked) equilibrated with M-pyridine formate and eluted with this buffer overnight, fractions (2.8 ml.) being collected every 15 min. The tubes in the position of *S*-sulphogluthione were ninhydrin-positive, and concentration of the material in these tubes and analysis by paper chromatography and paper electrophoresis confirmed that *S*-sulphogluthione had been formed by the enzymic reaction.

Reaction of S-sulphogluthione with iodine

Iodine (1.1 mg.) in ethanol (0.5 ml.) was added to the barium salt of *S*-sulphogluthione (4.7 mg.) in water (0.5 ml.) and the mixture kept at 100° for 10 min. Analysis of the reaction was by paper electrophoresis at pH 4 and showed that the usual conversion of an alkyl thiosulphate into the disulphide (here GSSG) took place. The conversion was incomplete; addition of more iodine and further heating for 5 min. at 100° caused further oxidation of the GSSG to GSO_3H . When a specimen (about 0.2 mg.) of the barium salt obtained from

calf lens (heat-precipitation experiment) in water (0.02 ml.) was heated with iodine (0.04 mg.) in ethanol (0.02 ml.) for 10 min. at 100°, GSSG was again formed.

Reaction of S-sulphoglutathione with cysteine

Cysteine hydrochloride hydrate (2.9 mg.) in 0.05N-NaOH (0.5 ml.) was added to the barium salt of *S*-sulphoglutathione (4.5 mg.) in water (0.5 ml.); a similar experiment (on a smaller scale) was carried out with the barium salt from the lens. The reaction mixtures were analysed by paper electrophoresis at pH 4 (Fig. 3). Both samples of barium salts gave a product whose mobility is consistent with its formulation as the mixed disulphide (CyS-SG) from cysteine (CySH) and GSH. In addition, spots in the positions of GSH and GSSG can be seen; these probably arise from the decomposition of the mixed disulphide (Swan, 1957).

Decomposition of S-sulphoglutathione in lens extracts

Several experiments were carried out to see if *S*-sulphoglutathione, added to lens extracts, was stable or not. Two kinds of extract were used. In the first, calf lenses were ground with their own weight of water, and the dispersion (referred to as 1:1 extract) was used directly, or diluted; in the second this dispersion was dialysed for 48 hr. and centrifuged. In each case the reaction mixture, consisting of 0.055M-barium salt of GSSO₃H (20 μl.) and the lens extract (60 μl.), was kept at room temperature for 50 min. The pH was then

brought to 5, and the protein removed by centrifuging after heat denaturation (100° for 4 min.). Portions (5 μl.) of the supernatant were examined by paper electrophoresis at pH 4 to see if the GSSO₃H was still present.

S-Sulphoglutathione was stable in the dialysed lens extract, and in a diluted lens extract (the 1:1 extract diluted ten times). But when the 1:1 extract was neither diluted nor dialysed the *S*-sulphoglutathione decomposed; this result was obtained repeatedly, but not invariably.

On some occasions the *S*-sulphoglutathione was stable. The variable recovery of GSSO₃H added to lens extracts may be the cause of the variability in the amount of GSSO₃H isolated from lens extracts.

DISCUSSION

Evidence for the presence of S-sulphoglutathione in lens extracts

That the strongly acidic peptide ('1α4') previously encountered in lens extracts was closely related to glutathione was suggested by hydrolysis to the amino acids of glutathione (Waley, 1956). The strongly acidic group was, however, unidentified. It has now been shown that this compound does not contain phosphorus, so the possibility that the compound was a nucleotide peptide may be discounted. The strongly acidic group was therefore probably a sulphonic acid, but its location in the glutathione must be such that the only amino acids liberated on hydrolysis are those derived from glutathione. The only simple way of accommodating these facts is by replacing the thiol hydrogen of GSH by the sulphonic acid (SO₃H) radical, so that the compound is the thiosulphate, GSSO₃H; the thiosulphate group in these compounds is known to be unstable to acid hydrolysis.

Clarke (1932) described the preparation of a compound believed to be this thiosulphate, although it could not be purified; he used the reaction:

It has now been found that the *S*-sulphoglutathione can be characterized as a barium salt; in earlier experiments, the thiosulphate could be purified by ion-exchange chromatography to separate it from GSH or GSSG, but if oxygen is passed through the reaction mixture the GSH formed is re-oxidized and reacts further.

This synthetic sample of *S*-sulphoglutathione was compared with the material obtained from the lens extracts. There were no differences on paper chromatography, paper electrophoresis and ion-exchange chromatography. A direct comparison of X-ray-powder photographs was impossible as the barium salts are not sufficiently crystalline to give

Fig. 3. Paper electrophoresis of synthetic *S*-sulphoglutathione, the lens specimen and the products of their reaction with cysteine. Electrophoresis was at pH 4 on no. 52 paper for 4 hr. at 8 v/cm.

a characteristic diagram. Fortunately, however, thiosulphates undergo several characteristic reactions which confirm the nature of the material from the lens. Thus heating a solution of a thiosulphate with iodine gives the disulphide, a reaction often used preparatively. Another reaction (which proceeds rapidly at room temperature in neutral solutions) is the reaction with thiols (Swan, 1957; Schöberl & Bauer, 1957), and *S*-sulphogluthathione reacted with excess of cysteine (Fig. 3) to give a compound which is presumably the mixed disulphide formed by the following reaction:

In both these reactions, the material in the lens extracts behaved in the same way as the synthetic *S*-sulphogluthathione. It is therefore concluded that the lens extracts do contain *S*-sulphogluthathione, and it will now be considered whether the substance is formed in the course of the extraction and fractionation.

Is S-sulphogluthathione an artifact?

It is an obvious possibility that *S*-sulphogluthathione might be formed in some way from glutathione; there is only about one-hundredth as much of the thiosulphate as there is of glutathione in the lens extract. *S*-Sulphogluthathione was first isolated from extracts which had, during the dialysis, been exposed to a high pH value for several days. In subsequent experiments, however, it was isolated from extracts from which the proteins had been removed by precipitation with trichloroacetic acid, or by heating for a short time at pH 5. These conditions are clearly less likely to lead to oxidative changes. Moreover, if the thiosulphate is being formed from glutathione during the course of isolation, the only known reaction by which this could happen would be the reaction of sulphite with GSSG (reaction 1). As the apparent equilibrium constant of reaction (1) is of the order of unity at pH 7, concentrations of glutathione, *S*-sulphogluthathione and sulphite at equilibrium could be 10, 0.1 and 1 mM respectively (the concentration of glutathione in calf lens is about 10 mM and the concentration of *S*-sulphogluthathione is about 0.1 mM). It is not known whether the lens contains sulphite, but even if a value for the concentration of sulphite were available, it would not indicate whether the sulphite was a cause of the presence of *S*-sulphogluthathione (reaction 1 proceeding to the right) or a consequence of it (reaction 1 proceeding to the left). Moreover, glutathione reductase is present in the lens (van Heyningen & Pirie, 1953); in the presence of reduced triphosphopyridine nucleotide (TPNH), this enzyme catalyses the apparently irreversible reduction of GSSG. Thus this system will displace the

equilibrium of reaction (1) to the left, and is probably the cause of the decomposition of GSSO_3H by lens extracts:

The rate of decomposition of GSSO_3H will be affected by many factors, including the concentration of TPNH and the activity of glutathione reductase. It was observed above that GSSO_3H was usually, but not invariably, decomposed by lens extracts, and it has been pointed out that lens extracts sometimes show no glutathione reductase activity (van Heyningen & Pirie, 1953); the isolation of *S*-sulphogluthathione from lens extracts was also sometimes unsuccessful. It seems possible that these observations are consequences of the reactions mentioned.

It should be noted that the products of reaction (3) as written above include sulphite and triphosphopyridine nucleotide (TPN^+). If these reacted according to reaction (4):

then the overall reaction would be:

Whether reaction (4) takes place depends partly on how rapid alternative processes for the oxidation of sulphite are. It is particularly interesting that the oxidation of sulphite in mammalian tissues is accelerated by thioctic acid (RSSR') probably via

the alkyl thiosulphate, $\text{RSH R'SSO}_3\text{H}$, with hypoxanthine as cofactor, (Handler & Fridovich, 1956). This process may thus be formally similar to the one discussed above, except that GSSG is replaced by thioctic acid and TPN by hypoxanthine.

Although it has been assumed implicitly that the only thiol groups that GSSO_3H will react with are those of glutathione, it is, of course, possible that GSSO_3H will react with the thiol groups of proteins which, in the lens, outnumber the glutathione thiol groups. This reaction would give a mixed disulphide derived from the protein and glutathione. It is not known to what extent this reaction occurs.

Thus the situation is too complex to decide whether the *S*-sulphogluthathione in lens extracts arises from GSSG and sulphite by reaction (1); but it seems at least as likely that the concentration of GSSO_3H in the cell is far greater than it is in the extracts.

It should also be considered whether the *S*-sulphogluthathione could arise by any other reactions, enzymic or otherwise. There are no other non-enzymic reactions known to convert GSH into

GSSO₃H. There is, however, a possible enzymic route to *S*-sulphoglutathione. Several analogues of glutathione are synthesized from their constituent amino acids by lens extracts (Cliffe & Waley, 1958), so the synthesis of GSSO₃H from glutamic acid, *S*-sulphocysteine and glycine was investigated. It was, in fact, found that *S*-sulphoglutathione is built up; but unfortunately this answer is equivocal. The GSSO₃H could be formed by direct reactions of the same type as lead to glutathione (omitting ATP):

but equally well the following indirect mechanism may hold:

There is no evidence that *S*-sulphocysteine is present in the lens, so this enzymic reaction does not provide information about how the *S*-sulphoglutathione in lens extracts is formed.

Possible metabolic role of S-sulphoglutathione

If *S*-sulphoglutathione is present in lens cells, then the oxidation of TPNH to TPN⁺ shown in reaction (3) could be important, especially in the carbohydrate metabolism of the lens. The suggestion that GSSG, by regenerating TPN, plays a part in the pentose phosphate cycle has been put forward by Kinoshita & Masurat (1957) and has been discussed recently elsewhere (Waley, 1958). The 'missing step' in the earlier scheme was the conversion of GSH into GSSG; if GSSO₃H is present, then (by reaction 3) this difficulty is overcome; but then the 'missing step' (i.e. step for which there is no evidence about whether it occurs in the cell, and if so, how) becomes the formation of GSSO₃H. An enzymic mechanism for the formation of GSSO₃H from precursors known to be present in the lens would go some way towards establishing this scheme.

SUMMARY

1. A derivative of glutathione has been isolated from calf-lens extracts.

2. This derivative has been identified as the thiosulphate (GSSO₃H) corresponding to glutathione (GSH); the identification is based on comparison of the physical and chemical properties of the material from the lens with those of synthetic GSSO₃H.

3. Lens extracts catalyse the synthesis of GSSO₃H from glutamic acid, the thiosulphate (CySSO₃H) derived from cysteine and glycine.

4. The isolation of GSSO₃H from lens extracts was accomplished by ion-exchange chromatography; elution was effected with pyridine formate buffers, which are volatile and do not react with ninhydrin.

I should like to thank my colleagues at this Laboratory for advice and encouragement, Mrs Little and Mrs Davies for the collection of calves' eyes, the staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their co-operation, the National Council to Combat Blindness, Inc., New York, for a 'Fight for Sight' grant-in-aid for chromatographic equipment and The Distillers Co. Ltd. for a generous gift of glutathione.

REFERENCES

- Alm, R. S., Williams, R. J. P. & Tiselius, A. (1952). *Acta chem. scand.* **6**, 826.
- Cecil, R. & McPhee, J. R. (1955). *Biochem. J.* **60**, 496.
- Clarke, H. T. (1932). *J. biol. Chem.* **97**, 235.
- Cliffe, E. E. & Waley, S. G. (1958). *Biochem. J.* **69**, 649.
- Handler, P. & Fridovich, I. (1956). In *Colloque sur la Biochimie du Soufre*, p. 83. Paris: Centre National de la Recherche Scientifique.
- Hunter, G. (1957). *J. clin. Path.* **10**, 161.
- Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, van R. (1954). *J. biol. Chem.* **209**, 23.
- Kinoshita, J. H. & Masurat, T. (1957). *Arch. Ophthalm., Chicago*, **57**, 266.
- Pirie, N. W. (1931). *Biochem. J.* **25**, 1565.
- Rosen, H. (1957). *Arch. Biochem. Biophys.* **67**, 10.
- Schöberl, A. & Bauer, G. (1957). *Angew. Chem.* **69**, 478.
- Schöberl, A. & Hornung, T. (1938). *Liebigs Ann.* **534**, 210.
- Swan, J. M. (1957). *Nature, Lond.*, **180**, 643.
- van Heyningen, R. & Pirie, A. (1953). *Biochem. J.* **53**, 436.
- Waley, S. G. (1956). *Biochem. J.* **64**, 715.
- Waley, S. G. (1958). *Symp. biochem. Soc.* no. 17 ('Glutathione') (in the Press).
- Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.