REFERENCES

- Buffa, P., Lotspeich, W. D., Peters, R. A. & Wakelin, R. W. (1950). *Biochem. J.* **47**, xvii.
- Buffa, P. & Peters, R. A. (1949). *J. Physiol.* **110**, 488.
- Buffa, P., Peters, R. A. & Wakelin, R. W. (1951). *Biochem. J.* **48**, 467.
- Dickman, S. R. & Cloutier, A. R. (1950). *Arch. Biochem.* **25**, 229.
- Dickman, S. R. & Cloutier, A. R. (1951). *J. biol. Chem.* **188**, 379.
- Elliott, W. B. & Kalnitsky, G. (1950). *J. biol. Chem.* **186**, 487.
- Kandel, A., Johnson, L. B. & Chenoweth, M. B. (1951). *Fed. Proc.* **10**, 312.
- Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
- Lepage, G. A. & Mueller, G. C. (1949). *J. biol. Chem.* **180**, 975.
- Liébecq, C. & Peters, R. A. (1948). *J. Physiol.* **108**, 11 P.
- Lindenbaum, W., White, M. R. & Schubert, J. (1951). *J. biol. Chem.* **190**, 585.
- Lotspeich, W. D. & Peters, R. A. (1951). *Biochem. J.* **49**, 704.
- Lotspeich, W. D., Peters, R. A. & Wilson, T. H. (1951). *J. Physiol.* **115**, 24 P.
- Martius, C. (1949). *Liebigs Ann.* **561**, 227.
- Martius, C. & Lynen, F. (1950). *Advanc. Enzymol.* **10**, 198.
- Ochoa, S. (1948). *J. biol. Chem.* **174**, 133.
- Ogston, A. G. (1951). *Nature, Lond.*, **167**, 693.
- Potter, V. R. & Busch, H. (1950). *Fed. Proc.* **9**, 215.
- Pucher, G. W., Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.

Enzymic Transpeptidation Reactions Involving γ -Glutamyl Peptides and α -Amino-acyl Peptides

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(Received 30 July 1951)

In an earlier paper (Hanes, Hird & Isherwood, 1950) glutathione (γ -glutamylcysteinylglycine) was shown to react with various added amino-acids in the presence of an enzyme from kidney and other tissues to form new peptides containing glutamic acid. Circumstantial evidence suggested strongly that the new peptides formed in the system were γ -glutamyl peptides, and that the reaction consisted of the transfer of the γ -glutamyl group from its linkage in glutathione to linkage with the amino group of an 'acceptor' amino-acid.

Observations to be described in the present paper have confirmed this concept and the enzyme will be referred to as γ -glutamyl transpeptidase. The new peptides formed when glutathione reacts with phenylalanine and glycine have now been definitely identified as γ -glutamylphenylalanine and γ -glutamylglycine respectively. Furthermore, it has been shown that glutathione is by no means unique as a 'donor' of the γ -glutamyl group in this system; in fact, various synthetic γ -glutamyl peptides have been found to act readily as donors of the γ -glutamyl group to different acceptor amino-acids and peptides. The same new γ -glutamyl peptide can thus be formed in reactions between different γ -glutamyl donors with the same acceptor.

While the transpeptidation reactions resulting in redistribution of γ -glutamyl groups form a prominent feature in the systems investigated, it should be emphasized that slow, but ultimately complete, breakdown of all γ -glutamyl peptides takes place. Earlier work had established that extracts of kidney and certain other animal tissues contained enzymes which catalysed the breakdown of glutathione and oxidized glutathione in a process presumed to be hydrolytic in character (Woodward & Reinhardt, 1942; Schroeder, Munro & Weil, 1935; Schroeder & Woodward, 1937; Binkley & Nakamura, 1948). It is possible that the liberation of free glutamic acid from γ -glutamyl peptides may be due to hydrolysis catalysed by the same enzyme that catalyses the transfer of γ -glutamyl groups, but this question requires further study. It may be noted, however, that the appearance of such breakdown products may complicate the reaction, since amino-acids originating in this way (e.g. cysteine and glycine from glutathione) may act as acceptors of γ -glutamyl groups to form new dipeptides.

It appears that the γ -glutamyl transpeptidase is specific for γ -glutamyl peptides as distinct from the γ -glutamyl amide (glutamine). No transfer of γ -glutamyl group from glutamine to added amino-

acids, nor from γ -glutamyl peptides to ammonia, has yet been detected in experiments carried out to examine this possibility. On the other hand, it has been found that glutamine acts readily as an acceptor of the γ -glutamyl group (e.g. from glutathione) with the formation of γ -glutamylglutamine.

The γ -glutamyl transpeptidase is probably quite distinct from the so-called glutamo-transferases which catalyse the transfer of the γ -glutamyl group from glutamine to hydroxylamine with the formation of γ -glutamylhydroxamic acid. Enzymes of this type have been demonstrated in bacterial and mammalian preparations (Waelsch, Owades, Borek, Grossowicz & Schou, 1950; Grossowicz, Wainfain, Borek & Waelsch, 1950; Schou, Grossowicz, Abel & Waelsch, 1951) and in plant preparations by Stumpf & Loomis (1950) and Stumpf, Loomis & Michelson (1951).

In addition to reactions involving the special group of γ -glutamyl peptides, we referred in our earlier communication to the occurrence of transpeptidations involving normal α -amino-acyl peptides. As an example of this class of reaction, observations will be presented below on the action of an enzyme from cabbage leaves which catalyses the transfer of glycyl groups from glycyl peptides to various acceptor amino-acids, with the formation of new glycyl peptides. Preparations of the enzyme so far available catalyse hydrolysis of glycyl peptides, as well as transfer of the glycyl group, when a suitable acceptor is present; the hydrolysis rate is inhibited markedly by the presence of acceptor amino-acids, but in the end hydrolysis of both the original and newly formed glycyl peptides proceeds to virtual completion. A substantial body of evidence which has accumulated in recent months suggests that certain other proteolytic enzymes catalyse not only the hydrolysis of α -amino-acyl peptide (and ester) linkages appropriate to their specificity requirements, but also group transfer reactions of the type under consideration. While detailed reference to these recent findings will be reserved for the Discussion, it is obvious that the possibly widespread occurrence of transpeptidation reactions catalysed by the proteolytic group of enzymes is of considerable interest in connexion with the problem of protein synthesis.

EXPERIMENTAL

Preparation of enzymes

Preparation of γ -glutamyl transpeptidase from animal tissues. For most of the experiments the enzyme was prepared from sheep-kidney tissue. Active preparations have been obtained also from pig kidney, ox kidney and pancreas, and from lactating mammary-gland tissue of the cow. In general, the following type of preparation has been used: a homogenate of the tissue (e.g. 6 g. fresh tissue homogenized

with 10 ml. 0.05M-potassium phosphate, pH 7.4, containing 0.001M-Mg⁺⁺) is centrifuged for 15 min. at 3000 rev./min. and is then dialysed, with stirring, against 1 l. of buffer. In the case of the sheep-kidney enzyme, the activity is maintained with little loss when such extracts (or the whole kidney) are frozen and held at -20° for periods up to at least 5 months. The enzyme can also be prepared from acetone-extracted powders of kidney, and some degree of purification has been achieved by treatment of extracts with *n*-butanol (Morton, 1950).

Preparation from cabbage leaves of enzyme acting on glycyl peptides. These enzyme preparations were obtained from the juice pressed out through muslin, from finely pulped cabbage 'heads'. The crude juice was centrifuged for 10 min. at 3000 rev./min. and then dialysed exhaustively, i.e. for 24–48 hr. with stirring, against repeated changes of distilled water or dilute KCl. The dialysed solution was again centrifuged at 3000 rev./min., and brought to pH 6.8 by careful addition of 0.1N-NaOH. Such preparations, still cloudy, were used in many experiments, but somewhat purified preparations were also studied. Much impurity could be removed by adsorption on Ca₃(PO₄)₂ gel at pH 6.8, or by high-speed centrifuging; the clear extract so obtained could then be fractionated either with acetone in the cold or with (NH₄)₂SO₄.

Although about 20-fold purification of this enzyme has been achieved by these means, no separation has resulted of the capacities to promote hydrolysis of glycyl peptides and transfer of glycyl groups. It may be mentioned, however, that a capacity to hydrolyse leucyl peptides was eliminated from various of the purified preparations.

Substrates

The peptides used as donors of γ -glutamyl and glycyl groups in the respective transpeptidation systems, and the amino-acids added as acceptors, were selected so far as possible so as to ensure that the new peptides formed would be separable on the paper chromatogram from the other substances present in the reaction mixtures.

γ -Glutamyl peptides. In addition to glutathione (GSH) and oxidized glutathione (GSSG), five synthetic dipeptides were used as donors in the γ -glutamyl transpeptidase system: γ -L-glutamyl-L-phenylalanine, γ -L-glutamylglycine, γ -L-glutamyl-L-glutamic acid, γ -L-glutamyl-L-aspartic acid and γ -L-glutamyl-L-tyrosine. The first two peptides were synthesized by the method of Le Quesne & Young (1950), and we are indebted to Dr G. T. Young for the generous gift of specimens of the three last-mentioned peptides.

Glycyl peptides. The following synthetic glycyl peptides (obtained from Roche Products Ltd.) were used in studying the glycyl enzyme: glycylglycine, diglycylglycine, glycyl-L-leucine and glycyl-L-tryptophan.

Other peptides for comparisons. α -L-Glutamyl-L-phenylalanine and α -L-glutamylglycine were synthesized by the method of Simmonds, Tatum & Fruton (1947).

Reaction conditions

The normal conditions for following the action of the γ -glutamyl transpeptidase were as follows: the reaction mixture contained one-third of its volume of the enzyme solution, 0.05M-phosphate, pH 7.4, 0.001M-Mg⁺⁺, and 0.05M concentrations of the donor (γ -glutamyl peptide) and the acceptor (amino-acid).

In the case of the glycyl enzyme, the reaction mixture contained one-quarter or one-third of its volume of enzyme solution, and 0.05M concentrations of the donor (glycyl peptide) and the acceptor.

In both systems the reaction was stopped and protein precipitated by adding 2 vol. of warm ethanol. The mixture was then centrifuged warm, and the supernatant solution evaporated to dryness in a desiccator. The residue was taken up in water (usually 0.5 original volume, but occasionally 0.25) and examined chromatographically. As was mentioned in our earlier paper (Hanes *et al.* 1950) cysteine, and peptides containing cysteine, were coupled with *N*-ethyl maleimide at this stage (Friedmann, Marrian & Simon-Reuss, 1949), by taking up the residue in a solution containing a slight excess of *N*-ethyl maleimide, thereby overcoming the chromatographic difficulty presented by substances possessing free —SH groups.

peptidase system. The running time was normally about 20 hr., but for investigating certain slowly moving substances this was increased to 40 or even 70 hr. Under these conditions the fast running amino-acids showed typical forward running 'ghosts', but the peptides separated cleanly.

Our primary evidence for the formation of a new peptide, resulting from a group transfer reaction has been the appearance on the chromatogram of a new spot which fulfils the following conditions: (a) that it is absent initially, but appears and increases in amount as the reaction proceeds; (b) that it is not formed unless both donor and acceptor substances are present; (c) that it is distinct and separable not only from the donor and acceptor, but also from

Fig. 1. Chromatograms showing products of action of γ -glutamyl transpeptidase on various substrates as indicated. Abbreviations: Asp, aspartic acid; (CySH), cysteine; Glu, glutamic acid; GSH, glutathione; GSSG, oxidized glutathione; Gly, glycine; Leu, leucine; Try, tryptophan; Tyr, tyrosine; Phe, phenylalanine. 1, γ -Glu-Tyr; 2, γ -Glu-Tyr + Leu; 3, γ -Glu-Tyr + Gly; 4, GSH; 5, GSH + Phe; 6, GSSG; 7, GSSG + Phe; 8, γ -Glu-Asp; 9, γ -Glu-Asp + Phe; 10, γ -Glu-Glu + Phe; 11, γ -Glu-Glu; 12, γ -Glu-Glu; 13, γ -Glu-Glu + Try; 14, γ -Glu-Glu + Leu; 15, γ -Glu-Glu. The enzyme was from sheep kidney; reaction conditions, standard; period of reaction, 1 hr. Chromatography: paper, Whatman, no. 1, untreated; time of development chromatograms 1-11, 43 hr., 12-15, 20 hr.; solvent, *n*-propanol (80 vol.) + water (20 vol.).

Analytical techniques

Chromatography. The mixed peptides and amino-acids present in the samples of reaction mixtures were separated on a filter-paper chromatogram. Chromatograms were run at 20° with preliminary equilibration of the system before starting the running of the solvent, and with an extra pad of filter paper clipped to the bottom edge of each chromatogram, into which the solvent was allowed to flow. For most of the work the solvent used was *n*-propanol (80 vol.) + water (20 vol.) and the paper was Whatman no. 1. Phenol was used as solvent when the basic amino-acids histidine and arginine were used as acceptors in the γ -glutamyl trans-

possible breakdown products from these; and finally, (d) that its position is consistent with that expected for a peptide of the type in question, e.g. many peptides travel on the chromatogram at rates intermediate between the rates of the constituent amino-acids. Our chromatographic results so far are based solely on visual comparison of ninhydrin-staining spots; while estimates of quantities and rates are thus only rough and comparative, this primary chromatographic analysis has provided unequivocal evidence for the formation of new peptides in the enzymic systems. In key cases, the new peptides

have been isolated and investigated by the methods now to be described.

Isolation of new peptides from chromatograms. In our early experiments the new peptides were often contaminated with material extracted from the paper, which was apparently

number of chromatograms, and then after the chromatograms had been developed, to locate the desired substance by comparison with guide strips which had been sprayed with ninhydrin. Transverse strips of the paper chromatograms containing the desired substance were then eluted with water and the extract concentrated. The products from

Fig. 2. Chromatograms showing progress of action of glycyl enzyme from cabbage (*A*) on glycylglycine (chromatograms 16-19) and (*B*) on glycylglycine + phenylalanine (chromatograms 21-24). Chromatogram 20 was an identification mixture of glycylglycine, glycine and phenylalanine. The enzyme was prepared from cabbage, exhaustively dialysed and centrifuged 15 min. at 20000 *g*. Reaction conditions, standard. Periods of reaction, 0, 3, 6 and 21 hr., as indicated.

polypeptide in nature and which, on acid hydrolysis, yielded aspartic acid, glutamic acid, glycine and alanine. The contamination varied considerably with different batches of paper and for the final isolation of new peptides paper from a batch which was relatively free of peptide was used. The procedure used in the early experiments was to apply a close row of spots of the mixture along the 'starting line' of a

several papers were combined and run again on a narrow chromatogram so that the final area to be eluted was as small as possible. In this way contamination was reduced to negligible proportions.

Improvements to this earlier technique (which was used for the γ -glutamyl peptides) have resulted from: (*a*) the use of the thick Whatman no. 3 paper which permits the handling

of much larger quantities of material, (b) the development of a washing procedure for the paper and (c) the use of aqueous ethanol (e.g. 50%, v/v) for eluting the desired peptides. The combination of factors (b) and (c) eliminated completely contamination of the isolated peptides by amino-acid-containing material from the paper and, in the case of simple dipeptides, the preparations crystallize when the extract is concentrated. The product is normally dissolved in the minimum quantity of water, separated once more on a narrower chromatogram and re-isolated. In this later work the position of the materials on the developed chromatograms was detected by the use of the ninhydrin reagent

isolated glycylphenylalanine before and after hydrolysis is shown in Fig. 3. No detectable contamination was shown in 'control' extracts.

Modified conditions for isolation of 2:4-dinitrophenyl (DNP)-glycine. Preliminary attempts to identify the glycyl end group after hydrolysing the DNP-derivatives of glycyl peptides led to inconclusive results whether hydrolysis was carried out in 5N-HCl at 105° or in 12N-HCl at 37°. This was clearly due to the fact, already emphasized by Sanger (1945), that DNP-glycine is peculiarly liable to decomposition during hydrolysis. It was essential, therefore, to develop an improved procedure. Using DNP-glycylglycine as the

Fig. 3. Identification of the new peptide formed in reaction between glycylglycine and phenylalanine, catalysed by glycyl enzyme from cabbage. Abbreviations: Gly for glycine or glycyl, DNP for 2:4-dinitrophenol, DNP- for 2:4-dinitrophenyl, Phe for phenylalanine. The peptide was a crystalline specimen isolated from products of Reaction 26 (Table 1) and it is shown in chromatogram 27. The free peptide was hydrolysed in 6N-HCl at 105° for 5 hr.: chromatogram 26 shows production of glycine and phenylalanine only; chromatogram 25 was an identification mixture. The DNP-peptide was hydrolysed for 8 hr. at 100° in glacial acetic acid (9 vol.) + 60% (w/v) perchloric acid (1 vol.). Chromatographic examination of the DNP-products (chromatogram 31) showed the presence of DNP-glycine only; chromatograms 28–30 were DNP-glycine, DNP-phenylalanine and dinitrophenol, for identification. Examination of free amino-acids from splitting of DNP-peptide (chromatogram 34) showed phenylalanine only; chromatograms 32 and 33 were glycine and phenylalanine for identification.

described by Boissonnas (1950), which was applied as three rows of tiny spots down the two sides and the middle of the paper. After detection of the bands, and before elution of the peptide in 50% ethanol, the excess ninhydrin was removed by extraction of the paper with acetone.

The preliminary purification of the paper consists of the following sequence of washing treatments: N-Na₂CO₃, distilled water, 2N-acetic acid, distilled water, 0.1% (w/v) calcium acetate, distilled water, and 50% (v/v) aqueous ethanol. The washing was carried out on a 'block' of about forty sheets of paper by means of the apparatus described earlier (Hanes & Isherwood, 1949); each treatment was thorough, involving frequent changes of the washing liquid, and the whole process occupied about 5 days. This washed, calcium-treated paper (Whatman no. 3) had excellent chromatographic properties as may be seen from Fig. 2, and would operate with heavy loading; for example, glycyl-phenylalanine was isolated from a reaction mixture containing about 0.3M total peptides and amino-acids, this being spotted on the paper at the rate of 0.6 ml. (30 × 20 μl.) along the 20 cm. 'starting line'. A chromatogram of the

test material, a variety of acid hydrolytic treatments were studied; at the end of each treatment the DNP-products were isolated from the acid mixture, after the latter was diluted, by extracting with a mixture of 10% (v/v) *tert*-amyl alcohol in benzene. It is possible thus to examine separately the DNP-substituted products so extracted and the free amino-acids remaining in the aqueous acid phase (cf. p. 33). Chromatographic analysis under the conditions described by Blackburn & Lowther (1951) showed that extensive breakdown (with the formation of many spots on the chromatograms other than DNP-glycine and DNP-glycylglycine) occurred during treatments for varying periods under the following conditions: 6N-HCl (105°), 12N-HCl (37°), 5N-HNO₃ + urea (trace) (100°), and 6N-HClO₄ (100°).

Tests were then made with systems containing relatively little water, and these led to completely successful procedures, of which two may be mentioned: (i) DNP-glycylglycine dissolved in a mixture of 60% (w/v) HClO₄ (1 vol.) + glacial acetic acid (9 vol.) was heated at 100° for 8 hr. Hydrolysis was complete and no breakdown of the DNP-

glycine could be detected (a single spot corresponding to DNP-glycine occurred on the chromatogram). (ii) More rapid hydrolysis was achieved in a mixture of 90 % (w/v) formic acid (10 vol.) + acetic anhydride (5.5 vol., i.e. sufficient to combine with the water present in the formic acid) and 60 % (w/v) HClO_4 (1.5 vol.). The DNP-glycylglycine was hydrolysed completely in 2 hr. at 100° with no trace of decomposition. The formic acid reagent may prove more generally useful owing to its being a better solvent for proteins.

An example of the use of these reagents will be described in detail in connexion with the identification of enzymically synthesized glycylphenylalanine.

RESULTS

In Tables 1 and 2 are set out the various reactions involving the transfer of γ -glutamyl and glycyl groups which have now been shown to be catalysed by the two enzymes under study. For each reaction is shown: the donor peptide, the acceptor amino-acid (or peptide) and the new peptide formed by the transfer reaction, with an indication of the evidence upon which this identification rests. Other products formed in the different reactions are not tabulated; e.g. the amino moiety of the donor peptide, products of hydrolysis, or, in the case of glutathione, new peptides formed by transfer of γ -glutamyl group to products of hydrolysis.

Reactions involving transfer of the γ -glutamyl group

Donors and acceptors in the γ -glutamyl transpeptidase system. Evidence was given in our earlier communication (Hanes *et al.* 1950) which dealt

exclusively with glutathione, for the occurrence of Reactions 1–5, Table 1. The new peptide formed in Reaction 1 was identified provisionally as γ -glutamylphenylalanine on the basis of a preliminary investigation there described, and the general conception that the enzyme catalysed the transfer of γ -glutamyl groups from GSH to the various acceptor amino-acids was based on the study of these reactions.

Table 1 contains twenty additional examples of reactions of the same basic type, but in which other γ -glutamyl peptides act as donors. In support of the occurrence of each of these reactions we obtained primary chromatographic evidence fulfilling the conditions described above. Such evidence relating to any individual reaction could not establish conclusively its nature, but the consideration of these different reactions as a whole affords strong support for the interpretation advanced, besides establishing that the γ -glutamyl transpeptidase is by no means specific for GSH as donor peptide.

For example, in five of the reactions listed (nos. 1, 7, 8, 10 and 24), the new peptide formed had similar chromatographic properties to that provisionally identified from Reaction 1 as γ -glutamylphenylalanine. In all these cases phenylalanine had been added as the acceptor amino-acid, while in each case a different γ -glutamyl peptide had been added as donor. A further group of reactions in which the same new peptide (γ -glutamylglycine) was formed by interaction between different donors with the same acceptor (glycine) can be seen in nos. 5, 13, 18 and 23. Again, in Reactions 6, 9 and 25, γ -glutamylglutamine was formed by the transfer of the γ -

Table 1. *Transpeptidation Reactions. Transfer of γ -glutamyl groups catalysed by enzyme from kidney*

No.	Donor	Acceptor	New peptide	Fig.
1	Glutathione (GSH)	Phenylalanine	γ -Glutamylphenylalanine†	1 (5)
2	Glutathione (GSH)	Valine	γ -Glutamylvaline	—
3	Glutathione (GSH)	Leucine	γ -Glutamylleucine	—
4	Glutathione (GSH)	Glutamic acid	γ -Glutamylglutamic acid*	—
5	Glutathione (GSH)	Glycine	γ -Glutamylglycine*	—
6	Glutathione (GSH)	Glutamine	γ -Glutamylglutamine	—
7	Oxidized glutathione (GSSG)	Phenylalanine	γ -Glutamylphenylalanine*	1 (7)
8	γ -Glutamylaspartic acid	Phenylalanine	γ -Glutamylphenylalanine*	1 (9)
9	γ -Glutamylglutamic acid	Glutamine	γ -Glutamylglutamine	—
10	γ -Glutamylglutamic acid	Phenylalanine	γ -Glutamylphenylalanine*	1 (10)
11	γ -Glutamylglutamic acid	γ -Glutamylphenylalanine	γ -Glutamyl- γ -glutamylphenylalanine	1 (10)
12	γ -Glutamylglutamic acid	Cysteine	γ -Glutamylcysteine	—
13	γ -Glutamylglutamic acid	Glycine	γ -Glutamylglycine*	—
14	γ -Glutamylglutamic acid	Histidine	γ -Glutamylhistidine	—
15	γ -Glutamylglutamic acid	Tryptophan	γ -Glutamyltryptophan	1 (13)
16	γ -Glutamylglutamic acid	Leucine	γ -Glutamylleucine	1 (14)
17	γ -Glutamylglutamic acid	γ -Glutamylleucine	γ -Glutamyl- γ -glutamylleucine†	—
18	γ -Glutamyltyrosine	Glycine	γ -Glutamylglycine†	1 (3)
19	γ -Glutamyltyrosine	Leucine	γ -Glutamylleucine	1 (2)
20	γ -Glutamyltyrosine	γ -Glutamylleucine	γ -Glutamyl- γ -glutamylleucine	1 (2)
21	γ -Glutamyltyrosine	γ -Glutamyltyrosine	γ -Glutamyl- γ -glutamyltyrosine†	1 (3)
22	γ -Glutamylglycine	Cysteine	γ -Glutamylcysteine	—
23	γ -Glutamylphenylalanine	Glycine	γ -Glutamylglycine*	—
24	γ -Glutamylglycine	Phenylalanine	γ -Glutamylphenylalanine*	—
25	γ -Glutamylphenylalanine	Glutamine	γ -Glutamylglutamine	—

* Compared on chromatogram with authentic synthetic specimens.

† Extracted from the paper and examined.

glutamyl group to glutamine from a different donor in each case.

It is clear from these reactions that the nature of the new peptide which is formed is characteristic of the acceptor amino-acid, and is independent of the particular γ -glutamyl peptide which acts as donor—within the range of the seven γ -glutamyl peptides which have been available for testing and which were all effective as donors. These features are in harmony with the general conception of the reaction which has been developed, and with the earlier provisional identification of the new peptides as γ -glutamyl peptides and which has been confirmed by independent evidence to be presented below.

It is appropriate now to consider what further information is available regarding the donors and acceptors in the γ -glutamyl transpeptidase system. Of the available γ -glutamyl peptides, γ -glutamyl-glutamic acid offers the inherent advantage for the chromatographic separation of reaction mixtures that its breakdown can lead only to glutamic acid. This peptide was accordingly tested as a donor in conjunction with a wide range of amino-acids as potential acceptors. As indicated in Table 1 (nos. 10 and 12–16) γ -glutamylglutamic acid acted as donor to glutamine, phenylalanine, cysteine, glycine, histidine, tryptophan and leucine, with the formation of a different new peptide in each case. With proline and arginine, little reaction if any occurred. From these experiments with γ -glutamyl-glutamic acid as donor, as well as the more restricted observations with the other donors, it is clear that the enzyme is relatively non-specific as regards the amino-acid which acts as acceptor.

Particular interest attaches to Reactions 23 and 24 which illustrate the catalysis by the enzyme of reverse processes. Thus, in Reaction 23, the γ -glutamyl group is transferred from γ -glutamyl-phenylalanine (with the liberation of phenylalanine) to glycine as the acceptor; whereas the reverse reaction was demonstrated in Reaction 24. This aspect of reversibility, and particularly the determination of the equilibrium distribution of γ -glutamyl group between different acceptors, is an aspect of the system which invites early study by quantitative methods which are at present being developed.

A final general aspect which should be mentioned is that, although our attention has been directed mainly to the study of dipeptides formed in the reaction systems, there are strong indications that the process of enzymic transfer of γ -glutamyl groups leads to the formation of higher peptides. In Reactions 11, 17, 20 and 21, Table 1, there is presumptive evidence that tripeptides are formed by the transfer of γ -glutamyl groups from donors to existing γ -glutamyl dipeptides acting as acceptors. The observations relating to Reaction 21, to con-

sider the simplest example, are as follows: on incubating γ -glutamyltyrosine with the enzyme some liberation of glutamic acid and tyrosine occurs, but there is formed in addition a new substance which travels more slowly than glutamic acid (and considerably more slowly than γ -glutamyltyrosine); after isolation from the paper and acid hydrolysis this substance yields glutamic acid and tyrosine. Its position on the paper would be consistent with that to be expected for a diglutamyltyrosine peptide, and it seems probable that it may be γ -glutamyl- γ -glutamyltyrosine. An analogous peptide, which has been found to contain only glutamic acid and, phenylalanine, but which moves much more slowly than the dipeptide, is formed when γ -glutamyl-glutamic acid and phenylalanine are incubated with the enzyme. In this case the main reaction is no. 10, leading to the formation of γ -glutamyl-phenylalanine, but this peptide appears then to act as acceptor for a second γ -glutamyl group to form γ -glutamyl- γ -glutamylphenylalanine (Reaction 11). The corresponding analogue of leucine has been observed to form in two different reaction systems (nos. 17 and 20), providing further support for the presumed constitution of these peptides. While the clear characterization of these peptides requires the application of quantitative analytical methods, there is little doubt that they are more complex than dipeptides. It seems likely that continuation of the process of group transfer might lead to the formation of poly- γ -glutamyl peptides, a possibility which is of some interest in view of the reported natural occurrence of such substances.

Identification of γ -glutamyl peptides formed by group transfer

While much circumstantial evidence for the nature of the main reaction has been adduced, clear proof depends upon demonstrating that the new peptides formed are in fact γ -glutamyl peptides. This has been established in the case of two of the peptides, γ -glutamylphenylalanine and γ -glutamyl-glycine, which represent the new peptides found in nine out of the twenty-five reactions listed in Table 1. For reasons which will become clear, the demonstration in these cases renders it highly probable that the remaining new peptides formed in the other reactions are also γ -glutamyl peptides.

γ -Glutamylphenylalanine. The new peptide formed in Reaction 1 was isolated from chromatograms on Whatman no. 1 paper and freed from small amounts of contaminants by the procedure described. The chromatographic behaviour of the peptide after isolation was indistinguishable from that of the material as present in the original mixture from which it had been separated. Portions of the peptide on hydrolysis (6N-HCl 24 hr., 105°) yielded only glutamic acid and phenylalanine. End-group identification by the method of Sanger (1945) gave only DNP-glutamic acid.

A second specimen of the peptide was isolated by similar means from the products of Reaction 10; it was indistinguishable in chromatographic properties from the previous specimen and yielded only glutamic acid and phenylalanine on hydrolysis. Various observations made on this specimen demonstrated conclusively that it was γ -glutamylphenylalanine and not α -glutamylphenylalanine:

(i) It was compared chromatographically with synthetic specimens of the two peptides; these and the enzymically synthesized peptide could not be separated using the normal propanol/water solvent. However, advantage was taken of the considerable difference in the dissociation constants of the α - and γ -carboxyl groups of glutamic acid (which are free in the γ - and α -glutamyl peptides, respectively) to obtain clear differentiation in their behaviour. This occurs in systems of appropriate acidity—e.g. with butanol/acetic acid/water (20/10/40, v/v) or with propanol/acetic acid/water (80/10/10, v/v)—in which α -glutamylphenylalanine travels about twice as fast as the γ -glutamyl analogue. Under these conditions the enzymically synthesized peptide proved identical with γ -glutamylphenylalanine, and clearly separable from α -glutamylphenylalanine.

(ii) Another important difference between the γ - and α -glutamyl peptides is their rate of breakdown in the presence of the kidney enzyme preparations. When α -glutamylphenylalanine (0.05 M) was added to the enzyme system it was largely broken down within 2 min., and the reaction was complete when the next observation was made at 10 min. Under the same conditions γ -glutamylphenylalanine showed considerable stability, only a small portion being broken down after 60 min. incubation.

γ -Glutamylglycine. The peptide formed in Reaction 18 was isolated from chromatograms on Whatman no. 1 paper by the same procedure. On hydrolysis it yielded only glutamic acid and glycine. When compared chromatographically with synthetic specimens of α - and γ -glutamylglycine, using butanol/acetic acid/water (50/10/40, v/v) as solvent, the new peptide was indistinguishable from γ -glutamylglycine. Under these conditions the γ - and α -glutamylglycine are clearly separated.

Reactions involving transfer of the glycyl group

Donors and acceptors in the glycyl transpeptidase system. In Table 2 are shown nine examples of reactions which have been shown to be catalysed by the glycyl enzyme from cabbage; in each of these systems a new peptide was formed by reaction

between a glycyl peptide and an amino-acid. As in the case of the γ -glutamyl transpeptidase system, a consideration of the primary chromatographic evidence concerning these reactions as a whole provides strong circumstantial evidence regarding the basic nature of the reaction.

In Reactions 26, 32 and 33, in which phenylalanine was present as the acceptor, but in which the donors were different glycyl peptides, the same new peptide was formed and, as will be seen below, this was identified as glycylphenylalanine. In Reactions 26–29, in all of which glycylglycine was the donor but in which phenylalanine, leucine, tryptophan and methionine, respectively, were the acceptors, a different new peptide was formed in each case. No new peptide could be detected when alanine was added with glycylglycine. It is evident from these examples that the nature of the new peptide formed is determined by the acceptor amino-acid and is independent of the particular glycyl peptide acting as donor in the system. This provides a strong indication that the reaction consists of the transfer of the glycyl group from its linkage in the donor peptide to new linkage with the acceptor amino-acid.

All preparations of the glycyl enzyme catalysed the hydrolysis of the various glycyl peptides, as well as the transfer of the glycyl group when a suitable acceptor was present. A striking feature of this enzyme is the marked inhibition of hydrolysis which is brought about by the presence of acceptor amino-acids; this aspect requires quantitative study, but to consider a concrete case it appears from visual comparisons that the rate of hydrolysis of 0.05 M-glycylglycine is inhibited to the extent of about 50–75% by the presence of 0.05 M-phenylalanine.

The reversibility of the group transfer reactions is illustrated in Reactions 28 and 30 (transfer of glycyl group from glycine to tryptophan and reverse reaction); Reactions 27 and 31 (transfer from glycine to leucine, and reverse reaction); and Reactions 26 and 34 (transfer from glycine to phenylalanine, and the reverse). The last-mentioned case is of especial interest in that the donor peptide, glycylphenyl-

Table 2. *Transpeptidation reactions. Transfer of glycyl groups catalysed by enzyme from cabbage*

No.	Donor	Acceptor	New peptide	Fig.
26	Glycylglycine	Phenylalanine	Glycylphenylalanine*	2
27	Glycylglycine	Leucine	Glycylleucine†	—
28	Glycylglycine	Tryptophan‡	Glycyltryptophan†	—
29	Glycylglycine	Methionine	Glycylmethionine	—
30	Glycyltryptophan	Glycine	Glycylglycine†	—
31	Glycylleucine	Glycine	Glycylglycine†	—
32	Glycylleucine	Phenylalanine	Glycylphenylalanine	—
33	Glycylglycylglycine	Phenylalanine	Glycylphenylalanine	—
34	Glycylphenylalanine†	Glycine	Glycylglycine†	—

* Extracted from the paper and examined.

† Compared on chromatogram against authentic synthetic specimens.

‡ Enzymically synthesized from Reaction 26.

alanine, was part of a crystalline specimen which had been isolated from Reaction 26 as will be described below. It should be mentioned, that although glycylamide was hydrolysed slowly in the system, no peptide formation could be detected when glycylamide was incubated with either glycine or phenylalanine. Various of the more active preparations of the glycyl enzyme had no action on leucylglycine or leucyltryptophan when these were incubated either alone or with glycine.

Identification of glycylphenylalanine formed by group transfer. The glycyl transpeptidase was allowed to act for 20 hr. on 0.05 M-glycylglycine + 0.05 M-phenylalanine; by this time about one-half of the original glycylglycine had disappeared and the new peptide (Reaction 26) had slightly passed its maximum amount, as judged on the chromatogram. The reaction mixture was deproteinized with 66 % (v/v) hot ethanol, and the filtrate evaporated to about twice the original concentration. 4.8 ml. (equivalent to 55 mg. original glycylglycine) was spotted on eight Whatman no. 3 papers (on 20 cm. starting lines). The new peptide eluted after this first separation was again separated using two chromatograms, the eluate from which yielded 17.6 mg. of crystalline material. If the whole of the glycylglycine originally present had reacted to form glycylphenylalanine (Reaction 26), the yield would have been 92 mg.; it is clear, therefore, that at least 30–40 % of the glycylglycine which had disappeared had reacted to form the new peptide.

Several lines of inquiry led to the clear conclusion that the new peptide was glycylphenylalanine:

(a) *Hydrolysis.* Small amounts of the peptide were found on hydrolysis (either by heating in 6 N-HCl at 105° for 5 hr. or under the action of the glycyl enzyme under the usual conditions) to yield only glycine and phenylalanine (cf. Fig. 3), in approximately equivalent amounts so far as could be judged, and no other detectable products.

(b) *Action as glycyl donor.* When 5 mg. of the peptide (in 1.2 ml. final volume) were incubated with 0.05 M-glycine in presence of the glycyl enzyme, glycylglycine was formed as a new peptide (Reaction 34).

(c) *Examination of DNP-derivative.* To 4.8 mg. of the peptide dissolved in 0.2 ml. saturated NaHCO₃ was added 10 mg. 1:2:4-fluorodinitrobenzene dissolved in 0.5 ml. of ethanol. The mixture was shaken for 2 hr. The solution was concentrated *in vacuo* to remove ethanol and then acidified with HCl to pH 2.0. The precipitate was filtered, washed with water and dried. Examined chromatographically (Blackburn & Lowther, 1951) this derivative showed only one spot.

The resulting DNP-derivative was dissolved in 0.2 ml. of a mixture of glacial acetic acid (9 vol.) + 60 % (w/v) perchloric acid (1 vol.) and was heated for 8 hr. at 100° in a sealed capillary. It was then diluted with 2 ml. water and extracted three times by shaking with equal volumes of 10 % (v/v) *tert.*-amyl alcohol in benzene. DNP-derivatives were then recovered from the combined extracts for chromatographic examination (described later) by shaking with the minimum quantity of 2N-NaOH. Hydrolysis products remaining in the original aqueous acid mixture were recovered for chromatographic examination as follows: the mixture was neutralized to pH 3 with 2N-KOH to remove perchloric acid and the solution was evaporated to dryness *in vacuo* over solid NaOH. The residue was

thoroughly extracted with 50 % (v/v) aqueous ethanol to dissolve free amino-acids and the extracts were filtered from KClO₄ and evaporated to dryness. The product was examined chromatographically using propanol/water (80/20, v/v) as solvent. The chromatograms of the DNP-derivatives (Blackburn & Lowther, 1951) showed the presence of DNP-glycine only, there being no trace of any decomposition products nor of DNP-phenylalanine (Fig. 3). Chromatograms of the unsubstituted hydrolysis products showed the presence of phenylalanine; no trace of glycine was detectable (Fig. 3). It could be concluded that the product was glycylphenylalanine.

DISCUSSION

The experimental results presented above have established important characteristics of the two enzymes which have been referred to as γ -glutamyl transpeptidase and glycyl transpeptidase, respectively. Much remains to be done in determining more fully the specificity relations of these enzymes (when a fuller range of peptides is available) and in exploring the kinetics of the transfer reactions (when suitable quantitative methods are available). Systematic and quantitative work on the purification of both enzymes is needed also to determine whether, as seems likely, the same enzyme in each system catalyses the hydrolytic cleavage, as well as the transfer to an acceptor of the appropriate amino-acyl group.

It is to be noted that the transpeptidation reactions occurring in both enzymic systems are of the type which we have designated earlier as carboxyl transfer reactions, in which the carboxyl moiety of the donor peptide is transferred to new linkage with the free amino group of the acceptor (Hanes *et al.* 1950). So far no example is known of the alternative type of transpeptidation reaction (amino transfer) in which the amino moiety of the donor is transferred to new linkage with the free carboxyl group of the acceptor. The reactions involving the transfer of γ -glutamyl and glycyl groups may be represented by the following general equation, in which X.CO—symbolizes either [HOOC.CH(NH₂).CH₂.CH₂.CO—] or [CH₂(NH₂).CO—] and R₁ and R₂ symbolize side chain groups of amino-acids:

The equation represents the reversible transfer of the X.CO— group from linkage with one amino-acid with side chain R₁ to new linkage with the free amino group of a second amino-acid with side chain R₂. Both enzymes have been shown to exhibit rather wide specificity as regards the amino-acid (or peptide) forming the amino moiety of the donor, and the same is true as regards the acceptor.

Close correspondence in specificity range as regards acceptor on the one hand, and amino moiety

of the donor on the other, might be expected, since the acceptor for the reaction proceeding in one direction becomes the amino moiety of the donor for the reverse reaction. This aspect invites more extensive study, although available data are consistent with the expectation. For example, the γ -glutamyl transpeptidase catalyses the transfer reaction when phenylalanine, glycine or glutamic acid is present either as acceptor or as amino moiety of the donor; similarly, the glycyl transpeptidation reaction occurs when glycine, leucine, or phenylalanine is present in either role. It would be of interest to determine whether γ -glutamylproline and γ -glutamylarginine would prove unsuitable as donors in the γ -glutamyl system, and glycylalanine in the glycyl system since proline and arginine in the former, and alanine in the latter, appear not to act as acceptors of the respective groups.

Consideration of the wider implications of these reactions requires mention of the recent evidence indicating that, in addition to hydrolytic cleavage, some at least of the familiar proteolytic enzymes catalyse group transfer reactions as well. This possibility was suggested first by Bergmann & Fraenkel-Conrat (1937) in connexion with studies on enzymic formation of benzoylglycinanilide from benzoylglycinamide and aniline, under catalysis by papain. Fruton (1950) and Johnston, Mycek & Fruton (1950*a*) using N-labelled ammonia confirmed that papain catalyses the transfer of substituted (benzoyl, carbobenzyloxy) amino-acyl groups from one amide linkage to another. Johnston, Mycek & Fruton (1950*b*) demonstrated also a true transpeptidation reaction catalysed by chymotrypsin; it was shown in this case that the benzoyl-L-tyrosyl group from benzoyl-L-tyrosylglycinamide was transferred to glycineamide of which the amino-group was labelled. More recently, Fruton, Johnston & Fried (1951) showed that in reactions catalysed by chymotrypsin and papain, respectively, various substituted α -amino-acyl groups were transferred reversibly from amide to true peptide linkage. Brenner, Müller & Pfister (1950) demonstrated the formation of di- and tri-peptides resulting from the action of chymotrypsin upon certain esters of threonine and methionine; this interesting reaction would appear to exemplify enzymic transfer of α -amino-acyl groups from ester to peptide linkage. The latest example is that reported by Waley & Watson (1951) in which it appears that the transfer of lysyl group to lysine leads to the formation of lysyllysine during the action of a mixture of trypsin and chymotrypsin on lysyltyrosyllysine.

The early suggestion by Bersin & Köster (1935) regarding the mechanism of the catalytic action of papain (and the significance of the —SH group in connexion with this activity) is of considerable interest in connexion with the now recognized occurrence of transpeptidation reactions. Thus

these authors postulated, on the lines of Langenbeck's (1933) general theory of catalysis, that the hydrolysis of a peptide link takes place in two stages: the first stage consists in transfer of the acyl moiety of the peptide to an amino group on the enzyme adjacent to an —SH group, with the liberation of the amino moiety of the peptide; the second stage consists in reaction of the acyl-enzyme compound with water liberating free enzyme and the acyl moiety of the peptide. It is evident that this hypothesis deserves more detailed study in the present connexion, especially since it would appear to offer an explanation of catalysis of both hydrolytic and transpeptidation reactions by a single enzyme if it were assumed that the acyl-enzyme compound could react either with water or an acceptor amino compound. The possibility that proteolytic enzymes in general catalyse both types of reaction raises the interesting question as to whether such transpeptidations may not play an integral part in the continuous process of interchange and rearrangement which characterizes the amino-acid and protein metabolism of the cell.

Our investigations on transfer reactions involving the γ -glutamyl group arose from studies on glutathione which had as their object the elucidation of possible biochemical relationships between glutamine and glutathione, on the one hand, and between glutathione and more general processes of peptide synthesis, on the other. That various other γ -glutamyl peptides besides glutathione are able to act as donors of the γ -glutamyl group in the same transpeptidase system serves to focus attention even more sharply upon any special property exhibited by glutathione which might account for its apparently close association with processes of growth and protein synthesis (cf. Waelsch & Rittenberg, 1941). No more than tentative speculations on these questions can be advanced at present. One property of the tripeptide which might be suggested as being related to its biological importance is that the cysteinyl-glycine linkage in glutathione is not hydrolysed in the presence of the widespread peptidase which induces rapid hydrolysis of free cysteinylglycine (cf. Binkley & Nakamura, 1948). The presence of the γ -glutamyl group thus stabilizes this linkage, apparently by removing it from the specificity range of the cysteinylglycine enzyme. If, however, removal of the γ -glutamyl group occurs (either by transfer or by hydrolysis) the dipeptide becomes available for hydrolysis—and possibly also for participation in transpeptidation reactions. It is possible thus to envisage glutathione acting as a stabilized reserve of α -amino-acyl peptide links which become available for the synthesis of other peptides by transpeptidation when removal of the γ -glutamyl group occurs.

Such a conception of glutathione as a 'protected' primary peptide link from which other peptides

may arise secondarily by transpeptidation reactions throws emphasis upon the mechanism of biological synthesis of glutathione itself, and upon the important progress toward its elucidation which has resulted from the studies by Johnston & Bloch (1951) on the synthesis of glutathione in preparations from pigeon liver. The tripeptide is synthesized in this system from its constituent amino-acids in enzymic reactions which involve the breakdown of ATP. Detailed elucidation of the nature and sequence of the steps involved in this synthesis of glutathione may provide additional indications of its biochemical significance. In the meantime recent developments in this field give encouragement to the further exploration and testing of the speculative hypothesis outlined earlier (Hanes *et al.* 1950), which is basically similar to that advanced by Fruton (1950)—and again in a recent communication by Fruton *et al.* (1951). According to this conception the significance of transpeptidation reactions lies in their providing metabolic pathways whereby certain primary peptide linkages synthesized in exergonic processes (e.g. in reactions involving the breakdown of ATP) could give rise secondarily to other peptides; by the continuation of this process of group transfer, progressively longer chains could result, and these conceivably could contain amino-acid residues in a particular order reflecting the specificity of transpeptidase enzymes concerned.

SUMMARY

1. Two types of transpeptidation reaction, catalysed by different enzymes, are described. These

consist in the transfer of the γ -glutamyl and glycyl groups respectively, from linkage in a 'donor' peptide to linkage with the amino group of a suitable acceptor.

2. The γ -glutamyl transpeptidase from kidney tissue has been mainly studied. Reduced and oxidized glutathione, and five different synthetic γ -glutamyl dipeptides have been found to act as donors of the γ -glutamyl group in the system. Thus the enzyme is relatively non-specific as regards the amino moiety of the donor peptide, and the same is true as regards the acceptor amino-acid (or peptide).

3. The glycyl transpeptidase has been prepared from cabbage leaves. Various glycyl peptides have been shown to act as donors of the glycyl group and, as in the γ -glutamyl transpeptidase, the enzyme is relatively non-specific as regards the acceptor amino-acids.

4. While the group transfer reactions resulting in the redistribution of γ -glutamyl and glycyl groups, respectively, between donor peptides and various acceptors form a prominent feature of the two systems, hydrolysis proceeds simultaneously. In the glycyl system hydrolysis is markedly inhibited by the presence of suitable acceptors.

We wish to acknowledge the assistance of Mr F. C. Barrett and Mr E. T. Whitmore in the experimental work. One of us (F.J.R.H.) held a Sir John and Lady Higgins Research Scholarship (awarded by the University of Melbourne) when this work was begun. It has been continued under a research grant from the Agricultural Research Council. Support from these two sources is gratefully acknowledged. One of us (F.A.I.) has participated in this work as a member of the staff of the F.I.O. of D.S.I.R.

REFERENCES

- Bergmann, M. & Fraenkel-Conrat, H. (1937). *J. biol. Chem.* **119**, 707.
- Bersin, Th. & Köster, H. (1935). *Hoppe-Seyl. Z.* **233**, 59.
- Binkley, F. & Nakamura, K. (1948). *J. biol. Chem.* **173**, 411.
- Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
- Boissonnas, R. A. (1950). *Helv. chim. Acta*, **33**, 1966.
- Brenner, von M., Müller, H. R. & Pfister, R. W. (1950). *Helv. chim. Acta*, **33**, 568.
- Friedmann, E., Marrian, D. H. & Simon-Reuss, I. (1949). *Brit. J. Pharmacol.* **4**, 105.
- Fruton, J. S. (1950). *Yale J. Biol. Med.* **22**, 263.
- Fruton, J. S., Johnston, R. B. & Fried, M. (1951). *J. biol. Chem.* **190**, 39.
- Grossowicz, N., Wainfain, E., Borek, E. & Waelsch, H. (1950). *J. biol. Chem.* **187**, 111.
- Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Johnston, R. B. & Bloch, K. (1951). *J. biol. Chem.* **188**, 221.
- Johnston, R. B., Mycek, M. J. & Fruton, J. S. (1950 a). *J. biol. Chem.* **185**, 629.
- Johnston, R. B., Mycek, M. J. & Fruton, J. S. (1950 b). *J. biol. Chem.* **187**, 205.
- Langenbeck, W. (1933). *Ergeb. Enzymforsch.* **2**, 314. Leipzig Akademische Verlagsgesellschaft.
- Le Quesne, W. J. & Young, G. T. (1950). *J. chem. Soc.* p. 1959.
- Morton, R. K. (1950). *Nature, Lond.*, **166**, 1092.
- Sanger, F. (1945). *Biochem. J.* **39**, 507.
- Schou, M., Grossowicz, N., Abel, L. & Waelsch, H. (1951). *Nature, Lond.*, **167**, 818.
- Schroeder, E. F., Munro, M. P. & Weil, L. (1935). *J. biol. Chem.* **110**, 181.
- Schroeder, E. F. & Woodward, G. E. (1937). *J. biol. Chem.* **120**, 209.
- Simmonds, S., Tatum, E. L. & Fruton, J. S. (1947). *J. biol. Chem.* **169**, 91.
- Stumpf, P. K. & Loomis, W. D. (1950). *Arch. Biochem.* **25**, 451.
- Stumpf, P. K., Loomis, W. D. & Michelson, C. (1951). *Arch. Biochem.* **30**, 126.
- Waelsch, H., Owades, P., Borek, E., Grossowicz, N. & Schou, M. (1950). *Arch. Biochem.* **27**, 237.
- Waelsch, H. & Rittenberg, D. (1941). *J. biol. Chem.* **139**, 761.
- Waley, S. G. & Watson, J. (1951). *Nature, Lond.*, **167**, 360.
- Woodward, G. E. & Reinhardt, F. E. (1942). *J. biol. Chem.* **145**, 471.