chromatographic development detected with tetra-azotized di-*o*-anisidine.

2. The technique is applicable to a variety of thiols, but is not yet suitable for alkyl thiols.

3. Quantities of thiols as low as 0.3 μ m-mole were detected, and under optimum conditions 0.1 μ m-mole was visualized under ultraviolet light. The sensitivity of the method is therefore superior to the *N*-ethylmaleimide detection of thiols or the ninhydrin detection of 2-amino acids.

4. A sample application of the method is presented.

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The Synthesis of some Peptides Containing Glutamic Acid

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For use in experiments concerning the mechanism of the assimilation of glutamic acid by *Staphylococcus aureus* (Rowlands, Gale, Folkes & Marrian, 1957), a number of peptides containing glutamic acid have been prepared, of which L-valyl-L-glutamic acid, L-valyl- α -L-glutamyl-L-valine, L-leucyl- α -L-glutamyl-L-leucine and α -L-glutamyl- α -L-glutamyl-L-glutamic acid are new. α -L-Glutamyl-L-alanine has been prepared by an alternative route.

For the synthesis of L-valyl- α -L-glutamyl-L-valine, benzyloxycarbonyl-L-valine was converted into the mixed anhydride, with ethyl chloroformate in toluene solution, and this solution was allowed to react with an aqueous solution of the sodium salt of α -L-glutamyl-L-valine; the coupling product crystallized immediately, and hydrogenation gave the tripeptide. An analogous procedure succeeded in the preparation of L-leucyl- α -L-glutamyl-L-leucine. For the synthesis of α -L-glutamyl- α -L-glutamyl-L-glutamic acid, benzyloxycarbonyl-L-glutamic anhydride was allowed to react with diethyl methyl α -L-glutamyl-L-glutamate; the isomeric products were separated by fractional extraction with aqueous sodium carbonate, and the α -isomer was hydrolysed and then hydrogenated to the tripeptide. The synthesis of L-valyl-L-glutamic acid requires no comment. α -L-Glutamyl-L-alanine was prepared in

the normal fashion from benzyloxycarbonyl-L-glutamic anhydride (e.g. Le Quesne & Young, 1950a); its synthesis by a different route has since been reported by Sachs & Brand (1953).

The preparation of the γ -glutamyl peptides used in the work reported in the succeeding paper has been described previously (Le Quesne & Young, 1950b; Rowlands & Young, 1952), but recently Sachs & Brand (1954) have reported that the intermediate benzyloxycarbonyl- γ -L-glutamyl azide may isomerize during coupling, to yield both α and γ products. We have therefore examined our original samples by paper chromatography with high concentrations of peptide and a mixture of *n*-butanol-acetic acid-water, as the mobile phase, and by comparison with samples to which known quantities of the α -isomer have been added we have estimated the approximate amount of the latter. The γ -L-glutamyl-glycine, -L-valine, -L-leucine, -L-tyrosine and -glycylglycine showed only traces (less than 0.2%) or none of the α -isomer. The γ -L-glutamyl-L-alanine was estimated to contain less than 0.5% and γ -L-glutamyl-L-glutamic acid less than 2% of the α -isomer, but the sample of γ -L-glutamyl-L-aspartic acid contained considerably more. In the synthesis of γ -L-glutamyl-L-glutamic acid only one of the intermediates after coupling

had been obtained crystalline, and in the synthesis of γ -L-glutamyl-L-aspartic acid both intermediates had remained as syrups which had evidently retained the α -isomer. It seemed possible that the benzyl-oxycarbonyl- γ -L-glutamylhydrazide used might still have contained some α -isomer; γ -ethyl benzyl-oxycarbonyl-L-glutamate was therefore recrystallized until hydrogenation gave a solution in which none of the α -ethyl isomer could be detected by paper chromatography; from this material, the hydrazide was prepared and recrystallized until hydrogenation gave a solution in which none of the α -hydrazide could be detected by paper chromatography. The benzyl-oxycarbonyl- γ -L-glutamylhydrazide so obtained was converted into the azide and coupled with L-alanine methyl ester and with diethyl L-glutamate. Without any purification, the products were saponified and hydrogenated, and the resulting solutions were examined by paper chromatography; in each case α -peptide was detected. Similarly, coupling of the azide with L-alanine benzyl ester gave a crude product which, after hydrogenation, was shown by paper chromatography to contain α -isomer. We therefore confirm the conclusion of Sachs & Brand (1954) that some isomerization of the γ -azide occurs, but when the subsequent products can be recrystallized this route would appear still to be useful for the preparation of γ -glutamyl peptides. γ -L-Glutamyl-L-glutamic acid has subsequently been prepared chromatographically pure by this method (Waley, 1955) and it is of interest that Abraham & Newton (1954) have prepared D-8-amino-8-carboxyvaleryl-glycine by this route; no isomeric peptide was found in the recrystallized product.

More recently we have attempted the synthesis of γ -L-glutamyl-L-aspartic acid by the method of Rudinger (1954) and of Swan & du Vigneaud (1954), in which toluene-*p*-sulphonyl-5-oxopyrrolidine-2-carboxylic acid is allowed to react with an amino ester. However, even under vigorous conditions diethyl L-aspartate gave a product which fractionation showed to be chiefly unchanged lactam, together with a small amount of coupling product (Winn & Young, unpublished work). Rudinger found that more drastic conditions were required for tyrosine ethyl ester than for glycine ethyl ester, and where such hindrance occurs the competitive formation of diketopiperazines is likely to cause reduced yields.

The determination of the partition coefficients of the peptides between *n*-butanol and water at 25° is described in the following paper.

EXPERIMENTAL

Melting points are uncorrected. The solvents used in paper chromatography were: *n*-butanol-acetic acid-water (62:12:26, by vol.), and phenol saturated with water. In

the latter case the chromatography tank contained also 0.1% NH_3 soln. Before analysis, samples were dried over P_2O_5 at 15 mm. at 61° or 100° according to their melting points, but the results indicate that in some cases non-stoichiometric water remained.

Benzyl-oxycarbonyl-L-valyl- α -L-glutamyl-L-valine. Benzyl-oxycarbonyl-L-valine (1.37 g.) and triethylamine (0.55 g.) were dissolved in toluene (20 ml.) and cooled to -5°. Ethyl chloroformate (0.59 g.) was added and the mixture was left in the freezing mixture for 5 min. α -L-Glutamyl-L-valine (1.34 g.) in *N*-NaOH (11 ml.) was then added and the mixture was stirred vigorously for 1 hr. at -5°. The freezing mixture was then removed and stirring continued for a further 2 hr. The aqueous phase was separated, washed with ether, and acidified with 5*N*-HCl. The product crystallized immediately, and after being kept at 0° overnight it was filtered off, dried, and twice recrystallized from ethyl acetate-light petroleum. This gave *benzyl-oxycarbonyl-L-valyl- α -L-glutamyl-L-valine*, m.p. 245°, with previous sintering (1.5 g.; yield 58%). (Found: C, 56.5; H, 6.7; N, 9.1. $\text{C}_{23}\text{H}_{35}\text{O}_8\text{N}_3, \frac{1}{2}\text{H}_2\text{O}$ requires C, 56.6; H, 7.0; N, 8.6%.)

L-Valyl- α -L-glutamyl-L-valine. Benzyl-oxycarbonyl-L-valyl- α -L-glutamyl-L-valine (0.8 g.) was hydrogenated in the usual way in methanolic solution in the presence of palladium black, reduction taking 5-6 hr. The product readily solidified and was washed with ethanol and recrystallized from aqueous acetone. The *L-valyl- α -L-glutamyl-L-valine* (0.47 g.; yield 82%) had m.p. 225° with previous sintering; $[\alpha]_D^{19} - 16.6^\circ$ (c, 2.2 in 0.5*N*-HCl). (Found: C, 47.7; H, 7.7; N, 10.6. $\text{C}_{15}\text{H}_{27}\text{O}_8\text{N}_3, 2\text{H}_2\text{O}$ requires C, 47.3; H, 8.1; N, 11.0%.) R_F : in butanol-acetic acid-water, 0.56; in phenol-water, 0.69.

Benzyl-oxycarbonyl-L-leucyl- α -L-glutamyl-L-leucine. Benzyl-oxycarbonyl-L-leucine (1.7 g.) and triethylamine (0.65 g.) were dissolved in toluene (25 ml.) and cooled in a freezing mixture to -5°. Ethyl chloroformate (0.7 g.) was added, and then, after 5 min., α -L-glutamyl-L-leucine (1.67 g.) in *N*-NaOH (12.9 ml.) The mixture was stirred vigorously for 3 hr. whilst coming to room temperature. The aqueous phase was separated, washed with ether and acidified with 5*N*-HCl. The product solidified immediately and had m.p. 101-103°. It was twice recrystallized from acetone-light petroleum, giving *benzyl-oxycarbonyl-L-leucyl- α -L-glutamyl-L-leucine*, m.p. 105° (1.6 g.; yield 43%). (Found: C, 58.5; H, 7.7; N, 8.3. $\text{C}_{25}\text{H}_{37}\text{O}_8\text{N}_3, \frac{1}{2}\text{H}_2\text{O}$ requires C, 58.1; H, 7.4; N, 8.1%.)

L-Leucyl- α -L-glutamyl-L-leucine. Benzyl-oxycarbonyl-L-leucyl- α -L-glutamyl-L-leucine (1.0 g.) was hydrogenated in the usual manner in aqueous methanol, reduction being complete in 3 hr. The product solidified immediately on evaporation, and was recrystallized from aqueous acetone, giving *L-leucyl- α -L-glutamyl-L-leucine* (0.55 g.; yield 75%), m.p. 228°; $[\alpha]_D^{19} - 26.8^\circ$ (c, 1.4 in 0.5*N*-HCl). (Found: C, 51.8; H, 8.8; N, 10.3. $\text{C}_{17}\text{H}_{31}\text{O}_8\text{N}_3, \text{H}_2\text{O}$ requires C, 52.2; H, 8.4; N, 10.7%.) R_F : in butanol-acetic acid-water, 0.75; in phenol-water, 0.76.

Diethyl N-(benzyl-oxycarbonyl- α -L-glutamyl)-O-(methyl- α -L-glutamyl)-L-glutamate. Diethyl benzyl-oxycarbonyl- α -L-glutamyl- α -L-glutamate (Bergmann & Zervas, 1932) in ethyl acetate was converted into the methyl ester by the action of diazomethane in ether at 0°, and the product (7.5 g., m.p. 79-81°, after crystallization from ethanol-ether) was hydrogenated in a mixture of methanol (75 ml.) and *N*-HCl (20 ml.). After 2 hr. the theoretical quantity of carbon dioxide had been evolved. The solution was filtered

and evaporated to dryness *in vacuo* at room temperature, and the syrupy residue was evaporated several times with methanol to remove excess HCl. It was then coupled with benzyloxycarbonyl-L-glutamic anhydride as follows.

A solution of the hydrochloride (6.6 g.) and KHCO_3 (11 g.) in water (100 ml.), covered with ethyl acetate (100 ml.), was stirred mechanically at room temperature while a solution of benzyloxycarbonyl-L-glutamic anhydride (4.2 g.) in ethyl acetate (50 ml.) was added during 5–10 min.; stirring was continued for 5 hr. and the mixture was left overnight at 5°. The aqueous layer was separated, acidified with 5N-HCl and left for 1 hr. at 0°, when the solid material was filtered off and dried. It had m.p. 128–138° (3.9 g.; yield 41%). The solid was dissolved in ethyl acetate (50 ml.) and successively extracted with 20 ml. portions of 0.04N- Na_2CO_3 . By refractionation of the later fractions and subsequent recrystallization from ethyl acetate–light petroleum diethyl *N*-(benzyloxycarbonyl- α -L-glutamyl)-*O*-(methyl- α -L-glutamyl)-L-glutamate was obtained (0.8 g.), m.p. 147–148°, $[\alpha]_D^{25} -33.3^\circ$ (c, 1.5 in ethanol). (Found: C, 54.7; H, 6.0; N, 7.0. $\text{C}_{23}\text{H}_{30}\text{O}_{12}\text{N}_3$ requires C, 55.2; H, 6.4; N, 6.9%.) This material was saponified without further recrystallization.

Benzyloxycarbonyl- α -L-glutamyl- α -L-glutamyl-L-glutamic acid. Diethyl methyl benzyloxycarbonyl- α -L-glutamyl- α -L-glutamyl-L-glutamate (1.7 g.) was dissolved in N-NaOH (14 ml.) and allowed to stand for 2 hr. After acidification of the solution with 5N-HCl an oil was precipitated which crystallized during several days. Filtration, drying, and recrystallization from acetone yielded benzyloxycarbonyl- α -L-glutamyl- α -L-glutamyl-L-glutamic acid (1.2 g.; yield 82%) of m.p. 163–165°, $[\alpha]_D^{25} -17.1^\circ$ (c, 1.3 in ethanol). (Found: C, 49.7; H, 5.8; N, 7.7. $\text{C}_{23}\text{H}_{28}\text{O}_{12}\text{N}_3 \cdot \text{H}_2\text{O}$ requires C, 49.6; H, 5.6; N, 7.5%.)

α -L-Glutamyl- α -L-glutamyl-L-glutamic acid. Benzyloxycarbonyl- α -L-glutamyl- α -L-glutamyl-L-glutamic acid (1.0 g.) in aqueous methanol containing a drop of acetic acid was hydrogenated in the usual way. After 3 hr. the theoretical quantity of carbon dioxide had been evolved. The solution was filtered and evaporated to dryness and the residue allowed to solidify under acetone, when it had m.p. 184–190°. It was twice reprecipitated from water with ethanol, giving α -L-glutamyl- α -L-glutamyl-L-glutamic acid (0.55 g.; yield 74%), m.p. 190–192°, $[\alpha]_D^{25} -7.2^\circ$ (c, 1.44 in water). (Found: C, 44.7; H, 5.4; N, 10.5. $\text{C}_{15}\text{H}_{23}\text{O}_{10}\text{N}_3$ requires C, 44.4; H, 5.7; N, 10.4%.) R_F : in butanol–acetic acid–water, 0.24; in phenol–water, 0.03.

Benzyloxycarbonyl-L-valyl-L-glutamic acid. Benzyloxycarbonyl-L-valine (1.5 g.) was dissolved in ether (20 ml.) at 0° and PCl_5 (1.5 g.) was added, with stirring. After 15 min. a further amount of ether (10 ml.) was added, and the solution was quickly washed twice with ice–water (30 ml.). After drying (Na_2SO_4) briefly, the ether layer was mixed with a solution of diethyl L-glutamate hydrochloride (1.4 g.) and triethylamine (1.3 g.) in ethyl acetate (15 ml.). Next morning the solution was washed with dil. HCl, water, 5% KHCO_3 , and finally water; it was then dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The syrupy product failed to crystallize after being scratched and set aside for some days under light petroleum. It was dissolved in acetone (18 ml.) and added to N-NaOH (18 ml.) and left for 2 hr. at room temperature. The acetone was then removed by evaporation *in vacuo* below 30°, and the solution was acidified and extracted with several 10 ml. portions of ethyl

acetate, the combined extracts being dried (Na_2SO_4) and evaporated *in vacuo*. The product crystallized after standing under light petroleum for some days. Several recrystallizations from ethyl acetate–light petroleum gave benzyloxycarbonyl-L-valyl-L-glutamic acid (1.1 g.; yield 49%), m.p. 105–106°. (Found: C, 56.9; H, 6.5; N, 7.4. $\text{C}_{18}\text{H}_{24}\text{O}_7\text{N}_2$ requires C, 56.8; H, 6.5; N, 7.4%.)

L-Valyl-L-glutamic acid. Benzyloxycarbonyl-L-valyl-L-glutamic acid (0.5 g.) was hydrogenated in the usual fashion, the evolution of carbon dioxide being complete after 7–8 hr. and several renewals of the catalyst. On evaporation of the filtered solution and recrystallization of the solid product from ethanol–water, L-valyl-L-glutamic acid was obtained (0.3 g.; yield 86%), m.p. 190–194°, $[\alpha]_D^{25} +3.2^\circ$ (c, 1.5 in water). R_F : in butanol–acetic acid–water, 0.54; in phenol–water, 0.47. (Found: C, 49.1; H, 7.7; N, 11.2. $\text{C}_{10}\text{H}_{18}\text{O}_6\text{N}_2$ requires C, 48.8; H, 7.3; N, 11.4%.)

Benzyloxycarbonyl- α -L-glutamyl-L-alanine ethyl ester. A solution of L-alanine ethyl ester hydrochloride (4.6 g.) and KHCO_3 (8.3 g.) in water (50 ml.), covered with ethyl acetate (50 ml.), was stirred mechanically while a solution of benzyloxycarbonyl-L-glutamic anhydride (7.2 g.) in ethyl acetate (50 ml.) was added gradually during 2–3 min.; stirring was continued for 3 hr. The aqueous layer was then separated, washed with ether and acidified with 5N-HCl. The solid product was filtered off and dried. For the separation of the isomers it was dissolved in ethyl acetate (100 ml.) and extracted with 25 ml. portions of 1% Na_2CO_3 , giving 10 fractions in all. The later fractions crystallized readily, the tenth having the highest m.p. (147–149°). The middle fractions were refractionated, combined with the last and recrystallized from ethyl acetate–light petroleum. This gave benzyloxycarbonyl- α -L-glutamyl-L-alanine ethyl ester (2.1 g.; yield 20%), m.p. 149–150°. (Found: C, 56.8; H, 6.3; N, 7.4. $\text{C}_{18}\text{H}_{24}\text{O}_7\text{N}_2$ requires C, 56.8; H, 6.5; N, 7.4%.)

Benzyloxycarbonyl- α -L-glutamyl-L-alanine. Benzyloxycarbonyl- α -L-glutamyl-L-alanine ethyl ester (0.6 g.) was dissolved in N-NaOH (4.8 ml.) and left for 2 hr. at room temperature. The solution was acidified with 5N-HCl and crystals separated on scratching. This gave benzyloxycarbonyl- α -L-glutamyl-L-alanine (0.5 g.; yield 90%), m.p. 176–177°, raised by recrystallization from acetone–ether to 178–179°. (Found: C, 54.4; H, 5.7; N, 8.1. $\text{C}_{16}\text{H}_{20}\text{O}_7\text{N}_2$ requires C, 54.5; H, 5.7; N, 8.0%.)

α -L-Glutamyl-L-alanine. Benzyloxycarbonyl- α -L-glutamyl-L-alanine (0.4 g.) was hydrogenated in the usual fashion. The product was recrystallized from aqueous ethanol, giving α -L-glutamyl-L-alanine (0.21 g.; yield 84%), m.p. 190°, $[\alpha]_D^{25} +10.1^\circ$ (c, 1.4 in water). (Found: C, 43.7; H, 6.4; N, 12.8. Calc. for $\text{C}_8\text{H}_{14}\text{O}_5\text{N}_2$: C, 44.0; H, 6.4; N, 12.8%.) R_F : in butanol–acetic acid–water, 0.33; in phenol–water, 0.34.

Detection of α product from benzyloxycarbonyl- γ -L-glutamylhydrazide. The γ -ethyl benzyloxycarbonyl-L-glutamate used had m.p. 86°, and on hydrogenation gave a solution showing a single component when chromatographed on paper with butanol–acetic acid–water, which effects a good separation of α - and γ -ethyl glutamates. The hydrazide prepared from this ester was recrystallized from aqueous ethanol and finally from acetone–light petroleum, when it had m.p. 180°. On hydrogenation it gave a solution which showed a single component when chromatographed on paper with butanol–acetic acid–water, which separates α -

and γ -glutamylhydrazides effectively. The pure hydrazide was converted into the azide in chloroform solution and coupled in one experiment with L-alanine methyl ester and in another with diethyl L-glutamate, according to the procedure of Le Quesne & Young (1950*b*). The products were saponified with *N*-NaOH, and after acidification the acids were extracted into ethyl acetate, which, after drying (Na_2SO_4), was removed under reduced pressure. The crude residues were hydrogenated in the normal fashion, with palladium black as catalyst, in the presence of acetic acid. The crude products (10% solutions in water) were examined by paper chromatography, with butanol-acetic acid-water as solvent; in both cases α -peptide was detected. A further portion of the pure hydrazide was coupled with L-alanine benzyl ester under the conditions of Sachs & Brand (1954); hydrogenation of the crude product gave material which, without purification, was examined by paper chromatography as above, and again α -isomer was detected.

SUMMARY

1. For use in experiments concerning the mechanism of the assimilation of glutamic acid by *Staphylococcus aureus* (Rowlands *et al.* 1957), the following new peptides have been synthesized: L-valyl-L-glutamic acid, L-valyl- α -L-glutamyl-L-valine, L-leucyl- α -L-glutamyl-L-leucine, α -L-glutamyl- α -L-glutamyl-L-glutamic acid. α -L-Glutamyl-L-alanine has been prepared by an alternative route.

2. When γ -glutamyl peptides are synthesized through benzyloxycarbonyl- γ -L-glutamyl azide some α products may occur in the crude material (as found by Sachs & Brand, 1954), but subsequent recrystallization will usually eliminate the α -isomer.

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The Assimilation of Amino Acids by Bacteria

23. ACCUMULATION OF FREE GLUTAMIC ACID WITHIN *STAPHYLOCOCCUS AUREUS* INCUBATED WITH DERIVATIVES OF GLUTAMIC ACID*

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Gram-positive bacteria concentrate certain amino acids in the free state in the internal medium of the cells (Gale, 1947, 1953; Taylor, 1947). The passage of amino acids across the cell wall and associated structures may be by a process of diffusion, as with lysine (Gale, 1947; Najjar & Gale, 1950), or by an active process involving exergonic metabolism on the part of the cell, as with glutamic acid (Gale, 1947). If washed suspensions of *Staphylococcus*

aureus (*Micrococcus pyogenes* var. *aureus*) are incubated with glucose and glutamic acid, the amino acid enters the cell and becomes concentrated therein, the ratio of internal to external glutamic acid becoming as high as 350–400:1 when the external concentration is small. The accumulation is prevented by the presence of inhibitors of fermentation, 'uncoupling agents', 8-hydroxyquinoline, or by growth of the cells in the presence of penicillin for a short time before harvesting (Gale, 1949, 1951; Gale & Taylor, 1947). Free amino acids accumulate

* Part 22: Creaser (1956).