264. *Peptides. Part VI.* Further Studies of the Synthesis of Peptides through Anhydrides of Sulphuric Acid.*

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Various improvements in the method described previously [1] are recorded. The extent of racemisation during the condensation of benzyloxycarbonylglycyl-L-alanine with L-phenylalanylglycine has been studied by separating any DL-diastereoisomer from the main product. In this instance racemisation can be very nearly excluded if the coupling is carried out in aqueous dimethylformamide buffered with magnesium carbonate. No racemisation could be detected when the ethyl ester of L-phenylalanylglycine was acylated in anhydrous dimethylformamide.

Part I of this series [1] contained a method for condensing the carboxyl group of an *N*-acylamino-acid or -peptide with the amino-group of another amino-acid or peptide. The three steps of the process are preparation of an anhydrous dimethylformamide solution of a salt of the carboxylic acid, reaction between the salt and a solution of sulphur trioxide in dimethylformamide, and coupling of the resultant mixed anhydride † (I) with the amino-compound. We now report closer examinations of each step and of the retention of configuration during the whole process.

The earlier work was carried out with potassium salts or, when these were insufficiently soluble, with trimethylphenylammonium salts. During the drying of the dimethylformamide solutions by fractional vacuum-distillation at about 50° there is some decomposition of the latter salts to dimethylaniline and the methyl ester of the carboxylic acid. We have not tried other quaternary ammonium salts which would be less prone to this side-reaction, because the lithium salts have proved perfectly satisfactory. Even when crystallisation has occurred, the lithium salts have been sufficiently soluble for convenient drying and reaction with the sulphur trioxide solution. It is noteworthy that the hitherto uncrystallised benzyloxycarbonyl-L-leucine gives a crystalline lithium salt.

A drawback of the original method was its need of anhydrous sulphur trioxide. However, sulphur trioxide prepared simply by distillation of oleum gives equally good results if the co-ordination compound with dimethylformamide ($^-SO_3\cdot O\cdot CH{:}NMe_2^+$) is allowed to crystallise at −40° before being redissolved in pure dimethylformamide. The solution is unaffected by storage at −40° for two months, but decomposes slowly at 0°. It gives 95—97% yields in the condensation of toluene-*p*-sulphonylglycine with *cyclo*hexylamine, but the *cyclo*hexylamides of benzyloxycarbonyl-glycine, -glycylglycine, and -glycylphenylalanine are formed in no more than 88% yield. We attribute this difference to

$$NH_2R' + \underset{(I)}{R\cdot CO\cdot O\cdot SO_3^-} \longrightarrow \begin{cases} \rightarrow R\cdot CO\cdot NHR' \\ \rightarrow {}^-SO_3\cdot NHR' \ (II) \end{cases}$$

competitive formation of the sulphamate (II); although there is no close analogy, this assumption is in line with the results of work with mixed carboxylic anhydrides.[3] Similar or slightly lower yields were obtained when the esters of amino-acids or peptides were used instead of *cyclo*hexylamine; in these instances an equivalent amount of triethylamine was also added in order to neutralise the liberated lithium hydrogen sulphate.

An unusual feature of the original method was that coupling of the mixed anhydrides

* Part V, *J.*, 1956, 371.

† The acylating reagent might be the cation $R\cdot CO\cdot O\cdot CH{:}NMe_2^+$, derived by ejection of sulphate ion instead of dimethylformamide from the sulphur trioxide–dimethylformamide complex. We regard the mixed anhydride as more likely in analogy with the known acetylsulphuric acid [2] and the numerous peptide syntheses involving mixed anhydrides, but an attempt to isolate a mixed anhydride from the dimethylformamide solution was unsuccessful.

[1] Kenner and Stedman, *J.*, 1952, 2069.
[2] Van Peski, *Rec. Trav. chim.*, 1921, **40**, 103.
[3] Wieland and Stimming, *Annalen*, 1953, **579**, 97.

(I) with amines was carried out in aqueous solution; this permitted the acylation of free amino-acids or peptides, instead of their esters, and consequent simplification of the synthesis. The acyl sulphates (I) are fortunately not only soluble in water but also surprisingly stable to hydrolysis by water and, to a smaller extent, hydroxyl ions; the half-life of lithium benzyloxycarbonylglycyl sulphate was estimated to be 10 hr. at pH 6·7 and 1 hr. at pH 9. In selecting a pH for the coupling, a balance has to be struck between the advantages of a low value in avoiding hydroxyl ions and of a high one in liberating the amino-groups from the ammonium form. In reactions between trimethylphenylammonium toluene-*p*-sulphonylglycyl sulphate and phenylalanine or phenylalanylglycine, the optimum pH appears to be near the p*K* of the ammonium ions. The yields fall off more sharply above the optimum pH than below it and they are higher, particularly at the lower pH's, with the dipeptide (p*K* 7·71)[4] than with the amino-acid (p*K* 9·13).[4]* We refrain from detailed discussion of these results for three reasons: the reactions were carried out under preparative conditions, and neither the composition of the medium nor the pH was constant; trimethylphenylammonium toluene-*p*-sulphonylglycyl sulphate is not a typical anhydride, for, as already mentioned, it gives higher yields under anhydrous conditions than do other acyl sulphates; the pH must in any case be kept below the optimum value in order to avoid serious racemisation (see next paragraph). A generally convenient and satisfactory technique is to add the dimethylformamide solution of the anhydride (I) to an aqueous solution of the amino-compound, buffered to about pH 6·8 with powdered magnesium carbonate. Our earlier work was done with much cruder technique and an unsatisfactory coupling of benzyloxycarbonyl-L-phenylalanine with glycine was recorded;[1] no such difficulty was encountered during a repetition under better controlled conditions. Holley and Holley[6] have shown that the long known[7] benzyloxycarbonyl-L-phenylalanine, m. p. 128°, has a neutralisation equivalent of 370 instead of 299, and they obtained an oil with an equivalent of 310. By counter-current distribution we have isolated material with m. p. 87° and equivalent 298.

Racemisation of the asymmetric centre next to the active carbonyl group is possible during any peptide synthesis of the mixed anhydride type, but the risk is very small for the anhydrides of the benzyloxycarbonyl, toluene-*p*-sulphonyl, or phthaloyl derivatives of amino-acids. The condensation of benzyloxycarbonylglycyl-L-phenylalanine with glycine was examined in our earlier work[1] and it was satisfactory. Re-investigation confirmed this when the pH of the coupling was controlled with phenol-red as indicator between 7·4 and 8·5, but there was much racemisation during control with phenolphthalein. These results were obtained by determining the optical rotation of the crude tripeptide derivative, because crystallisation could have removed some of the D-isomer. A better method was needed for a closer study of the phenomenon. Enzymic tests[8] could have been used, but it seemed simpler to arrange that racemisation would produce a diastereoisomer, separable from the main product. A suitable example was found in the coupling of benzyloxycarbonylglycylalanine with phenylalanylglycine. The mixture of tetrapeptide derivatives produced from the two DL-components was resolved by an extended counter-current distribution between ethyl acetate and a phosphate buffer, and the proportions in the mixture could, of course, be calculated accurately from the distribution curve.†

* An explanation of this difference is provided by the accurate investigation[5] of the analogous reaction between carbon disulphide and amino-acids or peptides. The weaker bases have, as expected, lower specific rates of reaction, but this effect is more than counterbalanced by the larger concentrations of free amino-groups.

† A spectrophotometric method, based on the iodate–iodide reaction, was developed for estimating the concentration of acid in the ethyl acetate. This was convenient for following the progress of a distribution, because negligible amounts of solution were removed for analysis. The more reliable method of weighing the residues after evaporation was used when precision was needed.

[4] Cohn and Edsall, "Proteins, Amino Acids and Peptides," Reinhold, New York, 1943, p. 84.
[5] Léonis, *Compt. rend. Trav. Lab. Carlsberg*, 1949, **26**, 315.
[6] Holley and Holley, *J. Amer. Chem. Soc.*, 1952, **74**, 3074.
[7] Bergmann, Zervas, Rinke, and Schleich, *Z. physiol. Chem.*, 1934, **224**, 36.
[8] Cf. Greenstein, *Adv. Protein Chem.*, 1954, **9**, 187.

It is worth noting that three parts of the LL-DD racemate were produced for every two parts of the LD-DL racemate. For the actual test runs assuredly pure samples of the two L-reagents were required. Small amounts of benzyloxycarbonylglycyl-DL-alanine (m. p. 183°) are easily detected in the L-isomer (m. p. 133°). The purity of our L-phenylalanylglycine was checked by solubility analysis [9] since there is disagreement in the literature [10] about its optical rotation. To our disappointment, coupling of the two L-reagents at about pH 8 gave one part of DL-tetrapeptide derivative for every six parts of the LL-form, although the amount of racemisation during the preparation of benzyloxycarbonylglycyl-L-phenylalanylglycine under similar conditions must have been smaller. However, when the pH was kept below 6·8 by powdered magnesium carbonate, the amount of DL-diastereoisomer was not detectable from the distribution curve; the minute amount of material recovered from the position expected for the DL-diastereoisomer must have been a mixture for it had a rotation of about −18° instead of +15°. It must have been mainly LL (rotation −31°) and less than 1% of the product can have been the DL-form. The difference between the behaviours of these two coupling reactions emphasises the danger of generalising without wide experience, but it is reasonable to expect an acceptably small amount of racemisation during most other couplings buffered with magnesium carbonate. The risk of racemisation will probably be greater when the coupling reaction is slower and the life of the anhydride is longer.

Racemisation through removal of a proton from the α-position of a mixed anhydride should be hindered by the absence of water. In fact we have been unable to detect it at all during the reactions, in anhydrous dimethylformamide, of the sulphuric anhydrides of benzyloxycarbonylglycyl-L-phenylalanine and benzyloxycarbonylglycyl-L-alanine with the ethyl esters of glycine and L-phenylalanylglycine respectively. Anderson, Blodinger, and Welcher [11] have shown that quite small amounts of benzyloxycarbonylglycyl-DL-phenylalanylglycine ethyl ester crystallise from ethanolic solution in preference to the L-isomer, but only the latter crystallised in our experiment. The second example is regarded by us as a stricter test both because, judged from our experience of the couplings in aqueous solution, the chance of racemisation is greater and the efficiency of detection, through alkaline hydrolysis followed by counter-current distribution, is superior.

Most of our work has been done with peptides derived from simple amino-acids, but some miscellaneous experiments with cystine, *S*-benzylcysteine, serine, tyrosine, and tryptophan are also recorded. These show that the phenolic hydroxyl group of tyrosine needs protection when it is part of the mixed anhydride, but the indole nucleus of tryptophan is unaffected.

Our conclusions are that the sulphuric anhydride method is an efficient means of acylating the esters of amino-acids and peptides in anhydrous dimethylformamide; it has the advantage over most other methods of the mixed-anhydride type that the by-product, in this instance a sulphamic acid, can be extracted easily. The method is also useful for acylating the amino-acids and peptides themselves in aqueous media buffered with magnesium carbonate, but there is a greater, although by no means excessive, risk of racemisation under these conditions; the merit of this more direct route has been discussed elsewhere.[1, 12]

Experimental

M. p.s are corrected. Analytical samples were dried for 6 hr. at 80°/1 mm. over phosphoric oxide. Evaporations were carried out under reduced pressure.

Spectrophotometric Estimation of Microgram Quantities of Carboxylic Acids Dissolved in Ethyl Acetate.—Potassium iodide (5 g.; "AnalaR") and potassium iodate (0·1 g.; "AnalaR")

[9] Mader, "Organic Analysis," Interscience Pub., Inc., New York, 1954, Vol. II, p. 253; Williams and Young, *J.*, 1951, 1745.

[10] Fischer and Schoeller, *Annalen*, 1907, **357**, 19; Sheehan, Chapman, and Roth, *J. Amer. Chem. Soc.*, 1952, **74**, 3825; Vaughan and Eichler, *ibid.*, 1953, **75**, 5559.

[11] Anderson, Blodinger, and Welcher, *J. Amer. Chem. Soc.*, 1952, **74**, 5309.

[12] Kenner, *Chem. Soc. Special Publ.*, No. 2, 1955, p. 103.

were dissolved in water (100 c.c.), which had been distilled in glass and freed from carbon dioxide by having had nitrogen slowly bubbled through it for not less than ½ hr. An ample quantity of this solution, which kept better in bulk, was made immediately before a series of estimations. The optical density of 2·5 c.c. of the solution, which was below 0·2, was measured in a 1 cm. cuvette at 355 mμ,[13] and then an accurately measured volume (between 0·01 and 0·15 c.c.) of the ethyl acetate solution was added to the cuvette. The solution was agitated for 15 sec. with a square-headed glass stirrer before the optical density was measured again. A calibration run with benzyloxycarbonylglycine showed the expected linear relation between the increase in optical density (ΔD) and volume of added solution with $\Delta D = 1$ corresponding to 0·425 microequivalent of acid. During a series of determinations the cuvette was not washed out but the syringe was flooded twice with the solution before the aliquot sample was ejected. Ethyl acetate which had been washed with sodium hydrogen carbonate solution and distilled gave a negligible ΔD, but after several hours, particularly during counter-current distribution, acetic acid could be detected.

Counter-current Distribution.—The partition coefficients varied slightly in different runs, because the room was not thermostatically controlled. Unless otherwise specified, the solvent system was ethyl acetate (fractionally distilled) and M-phosphate buffer, made from the appropriate amounts of KH_2PO_4 and K_2HPO_4. Before use the two phases were shaken together at the temperature of distribution and, as an additional surety of equilibrium, solute was not added to the first tube. Instead, the solute was dissolved in ethyl acetate and added to the second tube (tube 0) and sometimes two or four more. For all the long distributions a fully automatic glass 100-tube apparatus holding 20·5 c.c. of each phase was used; the settling time was variable, 5 min. being usual. At the end the peptide derivatives were driven into the ethyl acetate by addition of either syrupy phosphoric acid (1 c.c. for every 20 c.c. of buffer) or concentrated hydrochloric acid [for every 20 c.c. of buffer containing $(10 - x)$ parts of KH_2PO_4 to x of K_2HPO_4, $(3 + x)/6$ c.c.]. A second equal volume of ethyl acetate was used to complete the extraction. The bulk of the ethyl acetate was evaporated and the remainder was pipetted from any salts which crystallised. The remaining solvent was evaporated and crystallisation of the peptide derivative was encouraged. Finally, dry air was blown through the flask until its weight was constant.

Calculation of Counter-current Distribution Curves.—The partition coefficient, K, was derived from the position of the peak in tube $r_{max.}$ and the relation $K = (r_{max.} + 0{\cdot}5)/(n - r_{max.} + 0{\cdot}5)$, where n is the number of transfers and the tubes are numbered from 0 to n with appropriate additions for recycling distributions. For large numbers of transfers the amount of material Y, in a tube lying at a distance x from tube $r_{max.}$, which contained Y_0, was calculated from $Y/Y_0 = \exp(-x^2/p)$, where $p = 2nK/(K + 1)^2$. When the material had originally been added to the first three tubes, the values of Y calculated as above for single-tube filling were multiplied by $(1 - 2/3p + 4x^2/3p^2)$. For five-tube filling the factor was $(1 - 2/p + 1{\cdot}6/p^2 + 4x^2/p^2 - 4{\cdot}8x^2/p^3)$. When the spectrophotometric estimation was applied to a long distribution, a small base-line reading, caused by acetic acid, was deducted from the values of ΔD.

Anhydrous Dimethylformamide.—This was the residue after traces of moisture had been stripped, by fractionation at 15 mm. through a 6″ column packed with steel gauze,[14] from commercial material which had been fractionally distilled after being mixed with benzene.

Dimethylformamide Solution of Sulphur Trioxide–Dimethylformamide Complex.—Oleum (100 c.c. of 60%) was heated by a mantle at 110° under a 6 × 1″ column packed with Fenske glass helices, while nitrogen was bubbled through it. The receiver was cooled in ice and guarded with a tube of phosphoric oxide. Distillation was stopped when about 30 g. of sulphur trioxide had collected, and most of this was then distilled by gentle warming into a bulb fitted with a tail-joint. The bulb was then immediately attached to a three-necked flask, fitted with a stirrer and guard-tube and containing 85 c.c. of anhydrous dimethylformamide. The flask was cooled to −15° and the bulb was warmed gently so that sulphur trioxide distilled over the surface of the dimethylformamide. Like the previous transfer, this was interrupted when oily droplets were seen in the residue; about 21 g. of sulphur trioxide were added. The flask was kept for 2 hr. between −40° and −60° before the yellow liquor was sucked off through a sintered-glass filter-stick. The colourless crystals were washed with anhydrous dimethylformamide (10 c.c.) before being dissolved in 200 c.c. The solution (approx. M) was kept at −40° in a flask, the

[13] Cf. Custer and Natelson, *Analyt. Chem.*, 1949, **21**, 1005.
[14] Dixon, *J. Soc. Chem. Ind.*, 1949, **68**, 88.

stopper of which was surrounded by air dried with phosphoric oxide. The strength of this solution was determined by titration with alkali and its quality by the yield of toluene-*p*-sulphonylglycine *cyclo*hexylamide, which was between 93 and 97%. Storage for one month at −5° caused 3% drop in the yield, but deterioration at −40° was considerably slower.

Preparation of the Mixed Anhydrides.—The exact volume of sulphur trioxide solution, required according to the alkaline titration, was pipetted into the ice-cooled solution of the carboxylate salt.[15] Occasionally this had crystallised during the drying, but it quickly dissolved in presence of the sulphur trioxide solution.

In several runs about 5% of *toluene*-p-*sulphonylglycine methyl ester*, m. p. 90° (Found: C, 49·5; H, 4·9; N, 6·0. $C_{10}H_{13}O_4NS$ requires C, 49·4; H, 5·4; N, 5·8%), identified by comparison with material prepared with diazomethane, was produced from the trimethylphenylammonium salt of toluene-*p*-sulphonylglycine. Decomposition of the salt of phthaloyl-DL-phenylalanine was more rapid and 78% of the *methyl ester*, m. p. 87—88° (Found: C, 70·3; H, 4·6; N, 4·5. $C_{18}H_{15}O_4N$ requires C, 69·9; H, 4·9; N, 4·5%), was produced in 20 hr.

Toluene-p-*sulphonylglycine* cyclo*Hexylamide.*—*cyclo*Hexylamine (3 c.c., 25 mmoles) was added to a solution of the lithium, potassium, or trimethylphenylammonium salt of toluene-*p*-sulphonylglycylsulphuric acid (2 mmoles) in dimethylformamide (30 c.c.) at 0°. The solution was kept at 20° for 30 min. before evaporation. The residue was partitioned between ethyl acetate (40 c.c.) and 3N-sulphuric acid, which was extracted twice more with ethyl acetate. The combined ethyl acetate extracts (120 c.c.) were washed with saturated sodium hydrogen carbonate solution (3 × 10 c.c.) and water (10 c.c.). The aqueous washings were re-extracted with ethyl acetate (40 c.c.) before being acidified with hydrochloric acid and extracted with ethyl acetate (5 × 40 c.c.). Crystalline toluene-*p*-sulphonylglycine was recovered by evaporation and was dried to constant weight in a current of air. Similarly, the *cyclo*hexylamide (*ca.* 95%) was obtained from the earlier extracts. The total weight of air-dried materials corresponded to *ca.* 2·02 mmoles. The cyclo*hexylamide* was recrystallised from benzene–light petroleum (b. p. 40—60°) and had m. p. 113·5—115° (Found: C, 58·0; H, 7·2; N, 8·7. $C_{15}H_{22}O_3N_2S$ requires C, 58·0; H, 7·2; N, 9·0%).

Benzyloxycarbonylglycylglycine cyclo*Hexylamide.*—Prepared in the same way as the foregoing compound (yield 88%; 13% of starting material recovered), this *compound* recrystallised from methanol in needles, m. p. 173° (Found: C, 62·3; H, 7·3; N, 12·1. $C_{18}H_{25}O_4N_3$ requires C, 62·2; H, 7·3; N, 12·1%). Benzyloxycarbonylglycine *cyclo*hexylamide[16] was prepared likewise.

Benzyloxycarbonylglycyl-DL-*phenylalanine* cyclo*Hexylamide.*—This preparation was like the foregoing ones except that considerably more ethyl acetate was needed to dissolve the cyclo*hexylamide*, which, recrystallised from ethyl acetate, had m. p. 189° (Found: C, 68·6; H, 7·2; N, 9·6. $C_{25}H_{31}O_4N_3$ requires C, 68·6; H, 7·1; N, 9·6%).

General Procedures of Coupling the Mixed Anhydrides with Amines.—(a) *In water buffered with magnesium carbonate.* The ice-cooled dimethylformamide solution of the mixed anhydride (5—10 c.c. per mmole) and then magnesium carbonate (0·2 g. for each mmole) were added to a stirred solution of the amino-acid or peptide (equivalent to the anhydride) in a convenient volume of water. Stirring was continued for 15 hr. while the pH rose rapidly to 6·7 and finally to about 7·5. The excess of carbonate was dissolved with hydrochloric acid (pH 5), and the solvents were then evaporated. The peptide derivative was obtained by thorough partition between ethyl acetate and 3N-sulphuric acid, followed by evaporation of the ethyl acetate.

(b) *In dilute sodium hydroxide.* An aqueous solution of the amino-acid or peptide together with an indicator was magnetically stirred. An ice-cooled solution of the mixed anhydride in dimethylformamide was forced during *ca.* 15 min. by pressure of dry nitrogen to drop on to the surface of the aqueous solution, while N-sodium hydroxide was added so as to maintain the indicator colour (matched with a standard). The tip of the delivery tube was kept in an atmosphere of dry nitrogen. The mixture was stirred for 15 min. further. It was then brought to pH 5 and worked up as in (a). As the dimethylformamide content of the solution increased, the pH tended to rise and the approximate extent of this rise was determined with a glass electrode in a blank experiment with glycine. The indicators used and the respective initial and approximate final pH's were bromocresol-purple (6·0—6·05), bromothymol-blue (7·0—8·2),

[15] Kenner and Stedman, *J.*, 1952, 2072.
[16] Clayton, Kenner, and Sheppard, *J.*, 1956, 375.

phenol-red (7·5—8·5), *m*-cresol-purple (8·0—9·0), thymol-blue (8·7—10·0), phenolphthalein (9·0—*ca.* 12·0), and thymolphthalein (10·0—*ca.* 12·3).

(c) *In anhydrous dimethylformamide.* Triethylamine (2 equivs.) was added to a solution of either the hydrochloride or the hydrobromide of the amino-compound in anhydrous dimethylformamide. This mixture was added to the ice-cooled mixed anhydride, and the whole was kept at 20° for 1 hr. A little water was added and the pH was brought to 5 with N-sulphuric acid. The solvents were evaporated and the residue was partitioned between ethyl acetate and 3N-sulphuric acid. The neutral product and the recovered acid were then isolated in the usual way (see above). They accounted for all the anhydride used.

Benzyloxycarbonylglycylglycine.—Method (b) with phenolphthalein yielded 81% of material, m. p. 178°. Most of the product remained undissolved in the ethyl acetate and sulphuric acid.

Benzyloxycarbonylglycylglycine Ethyl Ester.—Method (c) yielded 85% and m. p. 77·5° after crystallisation from benzene–light petroleum (b. p. 60—80°).

Benzyloxycarbonylglycylglycylglycine.—Method (b) with thymol-blue yielded 75% of material, which had been recrystallised from water and had m. p. 196°. Most of the crude product crystallised directly from the acid and ethyl acetate, and the remainder was recovered by butan-1-ol extraction of the acidic liquors (total 84%).

Benzyloxycarbonylglycyl-L-alanine.—Method (b) with phenol-red gave 87% of product and 13% of benzyloxycarbonylglycine, separated by a 96-transfer distribution with 0·9M-KH_2PO_4/0·1M-K_2HPO_4 and recovered from tubes 10—45 and 48—70 respectively. The peak corresponding to the product had a steep leading edge and broad summit; three 20·5 c.c. tubes had been filled originally. The product was recrystallised from ethyl acetate and had m. p. 133°, $[\alpha]_D^{19}$ −9·5° (±0·2°) (*c* 4·4 in EtOH). This compound is evidently dimorphic.[17]

Method (a) yielded 61% of material, m. p. 124—126°, without counter-current distribution.

Benzyloxycarbonylglycyl-DL-alanine.—Method (b) with phenolphthalein gave 75% of material, m. p. 181° raised to 183° by recrystallisation from methanol. Most of this crystallised from the ethyl acetate and acid.

Benzyloxycarbonyl-L-leucylglycine.—Method (b) with thymol-blue yielded 64% of material, m. p. 118°, isolated from tubes 3—6 of a 10-transfer distribution with 0·5M-KH_2PO_4/0·5M-K_2HPO_4.

Benzyloxycarbonyl-L-leucylglycine Ethyl Ester.—Method (c) yielded 85% of ester, m. p. 103—104°.

Benzyloxycarbonyl-DL-leucylglycine Ethyl Ester.—Method (c) yielded 87% of *ester*, which was recrystallised from aqueous ethanol and had m. p. 91° (Found, in material dried at 40°: C, 61·9; H, 7·4; N, 8·0. $C_{18}H_{26}O_5N_2$ requires C, 61·7; H, 7·5; N, 8·0%).

Benzyloxycarbonylglycyl-DL-leucylglycine.—Hydrogen was bubbled for 3 hr. through a stirred solution of the foregoing ester (6·24 g., 20 mmoles) in ethanol (100 c.c.) and concentrated hydrochloric acid (2 c.c.) containing a mixture of platinum and palladium oxide catalysts. Evaporation of the liquor gave an oil, which was dried by repeated evaporation with anhydrous dimethylformamide and then coupled with benzyloxycarbonylglycine (20 mmoles) by method (c). The neutral product (5·7 g.) did not crystallise. It was treated with dioxan (50 c.c.) and N-sodium hydroxide (15 c.c.) for 5 hr. at 20°. The acidic product of this hydrolysis was subjected to a 9-transfer distribution with 0·8M-KH_2PO_4/0·2M-K_2HPO_4. The *benzyloxycarbonyltripeptide* (4·22 g., 60% overall yield) was recovered from tubes 0—5 and, recrystallised from aqueous ethanol, had m. p. 161° (Found: C, 57·2; H, 6·7; N, 11·1. $C_{18}H_{25}O_6N_3$ requires C, 57·0; H, 6·6; N, 11·1%). Subsequent preparations could be crystallised without counter-current distribution.

Benzyloxycarbonylglycyl-L-leucylglycine Ethyl Ester.—Benzyloxycarbonyl-L-leucylglycine ethyl ester was hydrogenated and then coupled with benzyloxycarbonylglycine in the same fashion as in the DL-series. The yield was 78% of product, having m. p. 109° [18] after recrystallisation from aqueous ethanol.

Benzyloxycarbonyl-L-phenylalanine.—Material prepared in the ordinary way was recrystallised from benzene and had m. p. 122—124°, $[\alpha]_D^{14}$ +4·8° (±0·2°) (*c* 4·4 in acetic acid), neutralisation equivalent 358, in agreement with published data.[6,7] An 8-transfer distribution with 0·075M-KH_2PO_4/0·425M-K_2HPO_4 showed a peak with K 1·81. From tubes 3—7 65% of crystalline material, very soluble in benzene were obtained. Recrystallised from ethyl acetate,

[17] Erlanger and Brand, *J. Amer. Chem. Soc.*, 1951, **73**, 3509.
[18] Simmonds, Harris, and Fruton, *J. Biol. Chem.*, 1950, **188**, 259.

this had m. p. 87°, $[\alpha]_D^{18}$ +5·3° (±0·2°) (*c* 6·6 in acetic acid), neutralisation equivalent 298. Later runs gave similar material without counter-current distribution.

*Benzyloxycarbonyl-*DL*-phenylalanine.*—The behaviour of this substance somewhat resembled that of the L-isomer. Various m. p.s between 98° and 162° and high neutralisation equivalents were obtained until counter-current distribution yielded material with m. p. 100·5° after recrystallisation from carbon tetrachloride, equivalent 295.

*Benzyloxycarbonyl-*L*-phenylalanylglycine.*—Method (b) with phenol-red gave an oil, which was subjected to 358 transfers with 0·5M-KH_2PO_4/0·5M-K_2HPO_4 using the single-withdrawal method on the 100-tube machine. The first material withdrawn was recovered benzyloxycarbonyl-L-phenylalanine (8·5%) (*K* 4·0). The product was spread out in a broad band. Owing to a mechanical defect, 15% of this was lost, but the total yield was estimated to be 92%. The dipeptide derivative was recrystallised from ethyl acetate and had m. p. 154°, $[\alpha]_D^{18}$ −10·2° (±0·2°) (*c* 4·3 in acetic acid).[19, 1]

*Benzyloxycarbonyl-*L*-phenylalanylglycine Ethyl Ester.*—Method (c) yielded 83% of the dipeptide derivative, which was recrystallised from ethyl acetate (2 parts)–light petroleum (b. p. 60—80°) (3 parts) and had m. p. 107·5—108·5°, $[\alpha]_D^{16}$ −19·2° (±0·2°) (*c* 4·1 in EtOH).[20]

L-*Phenylalanylglycine.*—A solution of benzyloxycarbonyl-L-phenylalanylglycine (4·95 g.) in methanol (68 c.c.) and acetic acid (0·68 c.c.) was stirred with palladous oxide catalyst (0·6 g.) while hydrogen was bubbled through it. After 3 hr. no more carbon dioxide could be detected and the filtered solution was evaporated. The dipeptide, which crystallised during the evaporation, was lyophilised (3·24 g.) and then recrystallised twice from water, methanol, or aqueous acetone. The *dihydrate* had $[\alpha]_D^{20}$ +100·0° (±0·3°) (*c* 2·4 in H_2O) (Found, in material equilibrated with the atmosphere: C, 50·7; H, 7·2; N, 10·7. $C_{11}H_{14}O_3N_2,2H_2O$ requires C, 51·1; H, 7·0; N, 10·8%). Drying over phosphoric oxide at 130°/2 mm. for 8 hr. apparently formed some dioxopiperazine, which sublimed[21] (Found, in the residue: C, 60·9; H, 6·2; N, 13·3. Calc. for $C_{11}H_{14}O_3N_2$: C, 59·5; H, 6·4; N, 12·6%). Mixtures of the dihydrate with water (*ca.* 0·02 c.c.) in the proportions of 2, 4, and 6 g. to 10 g. of water were sealed in 4 mm. glass tubes and kept at 21·0° for 3 days. The refractive indices of the solutions were then 1·3648, 1·3650, and 1·3645 respectively.

A sample prepared by the method of Sheehan, Chapman, and Roth[10] from phthaloyl-L-phenylalanylglycine [m. p. 186—187°, $[\alpha]_D^{20}$ −140° (± 0·2°) (*c* 1·7 in EtOH)] had $[\alpha]_D^{17}$ +85·5° (±0·2°) (*c* 2 in H_2O), but this was raised to 100·4° by two recrystallisations from aqueous acetone.

Hydrobromide of L*-Phenylalanylglycine Ethyl Ester.*—After hydrogenation of benzyloxycarbonyl-L-phenylalanylglycine ethyl ester as in the foregoing experiment, one equivalent of ethanolic hydrogen bromide was added and the hydrobromide, obtained by evaporation, was recrystallised from ethanol–light petroleum (b. p. 60—80°) in large plates, m. p. 134—135°, $[\alpha]_D^{16}$ +40·4° (±0·2°) (*c* 4·2 in H_2O).[11]

*Phthaloyl-*DL*-phenylalanylglycine.*—A solution of phthaloyl-DL-phenylalanyl chloride (12·7 g.)[22] in dioxan (180 c.c.) was added during 30 min. to a mixture of glycine (10·6 g.), magnesium oxide (7·3 g.), and water (400 c.c.), which was stirred at 4°. After having been kept for 20 min. at 20°, the solution was acidified with 3N-sulphuric acid (55 c.c.) and thoroughly extracted with ethyl acetate, from which 9·9 g. (62% from phthaloyl-DL-phenylalanine) of *phthaloyl-*DL*-phenylalanylglycine*, m. p. 128—133° (Found: C, 64·8; H, 4·7; N, 7·9. $C_{19}H_{16}O_5N_2$ requires C, 64·8; H, 4·6; N, 8·0%), was obtained by concentration. The substance was dimorphic; the other *form* had m. p. 163·5—167° (Found: C, 64·3; H, 4·3; N, 8·3%) after recrystallisation from aqueous ethanol, and its crystals repelled each other violently when ground in a mortar. Crystals were obtained for the first time by a 10-transfer distribution with 0·65M-KH_2PO_4/0·35M-K_2HPO_4. The distribution curve corresponded to the presence of 79% of the dipeptide derivative (*K* 1·17), 19·5% of phthaloyl-DL-phenylalanine (*K* 5·66), and 1·5% of an unidentified material (*K* 0·013), probably phthaloyl-DL-phenylalanylglycine.

DL*-Phenylalanylglycine.*—A solution of phthaloyl-DL-phenylalanylglycine (3·52 g.), phenylhydrazine (4·92 c.c.), and triethylamine (0·98 c.c.) was boiled[23] for 2 hr.

[19] Behrens, Doherty, and Bergmann, *J. Biol. Chem.*, 1940, **136**, 63.
[20] Anderson and Young, *J. Amer. Chem. Soc.*, 1952, **74**, 5307.
[21] Cf. Gross and Grodsky, *ibid.*, 1955, **77**, 1678.
[22] Sheehan and Frank, *ibid.*, 1949, **71**, 1856.
[23] Cf. Schumann and Boissonas, *Helv. Chim. Acta*, 1952, **35**, 2235.

Ethyl methyl ketone (25 c.c.) was added, and the solution was boiled for 15 min. further. Acetic acid (4·27 c.c.) was added to the cooled solution, and the dipeptide (1·59 g., 72%) separated. When it was recrystallised from water, large plates or needles were formed (Found, in each form: N, 12·5. Calc. for $C_{11}H_{14}O_3N_2$: N, 12·6%).

Toluene-p-*sulphonylglycyl*-L-*phenylalanine*.—This *compound* was obtained by method (b) in a series of runs, and it recrystallised from ethyl acetate–light petroleum (b. p. 40—60°) in needles, m. p. 160·5°, $[\alpha]_D^{16}$ +50·3° (± 0·8°) (*c* 2·55 in EtOH) (Found: C, 57·5; H, 5·3; N, 7·4. $C_{18}H_{20}O_5N_2S$ requires C, 57·4; H, 5·4; N, 7·4%). It is insoluble in 10% aqueous ethanol and hence the yields could be determined by dissolving the acidic product (2 mmole scale) in ethanol (5 c.c.) and adding this solution dropwise with shaking to water (50 c.c.). After the mixture had been stored for 3 hr. at 0°, the pure product, m. p. 160·5°, was collected. The efficiency of this method was confirmed by a 10-transfer distribution with 0·7M-KH_2PO_4/0·3M-K_2HPO_4 of the product of pH 9; the distribution curve showed the presence of 1·72 mmoles of the dipeptide derivative (*K* 1·65) and 0·23 mmole of toluene-*p*-sulphonylglycine (*K* 0·124). The yields obtained with the various initial pH's of coupling were: 6, 75; 7, 78; 7·5, 81; 8, 82; 8·7, 86; 9, 86; 10, 83%.

Toluene-p-*sulphonylglycyl*-DL-*phenylalanine*.—This *product*, m. p. 144—145° (Found: C, 57·4; H, 5·2; N, 7·2%), was also obtained by method (b) in a series of runs, and it was isolated by counter-current distribution. Runs were not done below pH 8 because DL-phenylalanine was too insoluble.

Toluene-p-*sulphonylglycyl*-DL-*phenylalanylglycine*.—This *compound* was obtained from DL-phenylalanylglycine by method (b) in a series of runs, and recrystallised from ethanol in needles, m. p. 192° (Found: C, 55·6; H, 5·6; N, 9·7. $C_{20}H_{23}O_6N_3S$ requires C, 55·4; H, 5·4; N, 9·7%). The yields were estimated from the weight of the acidic material, which was crystalline although it was a mixture of product and toluene-*p*-sulphonylglycine. The product of the run (2 mmoles scale) at pH 6 was also subjected to a 10-transfer distribution with 0·81M-KH_2PO_4/0·19M-K_2HPO_4, which showed the presence of 1·77 mmoles of product (*K* 0·97) and 0·15 mmole of toluene-*p*-sulphonylglycine (*K* 0·27). Runs at various pH's gave the following respective yields: 6, 87; 7, 88; 7·5, 90; 8, 91; 8·7, 92; 9, 88; 10, 75%.

Benzyloxycarbonylglycyl-L-*phenylalanine*.—The product from method (a) (20 mmoles scale) was distributed for 15 transfers with 0·7M-KH_2PO_4/0·3M-K_2HPO_4 (100 c.c. tubes). The distribution curve corresponded to an 86% yield (*K* 1·49) with 15% recovery of benzyloxycarbonylglycine (*K* 0·29). The dipeptide derivative, recrystallised from chloroform, had m. p. 126°, $[\alpha]_D^{14}$ +41·9° (±0·2°) (*c* 2·5 in EtOH)[1].

Benzyloxycarbonylglycyl-L-*phenylalanylglycine*.—The product obtained from benzyloxycarbonylglycyl-L-phenylalanine (2 mmoles) by method (b) with initial pH 7·4, was distributed for 96 transfers with 0·75M-KH_2PO_4/0·25M-K_2HPO_4. The optical rotations of the whole of the material contained by tubes 37—40 and 67—70 (*i.e.*, at the two peaks) were $[\alpha]_D^{15}$ +41·6° (±0·1°) (*c* 1·4 in EtOH) and $[\alpha]_D^{15}$ −12·7° (±0·1°) (*c* 2·0 in EtOH) respectively. A redetermination of the rotation of the pure tripeptide derivative,[1] recrystallised from ethyl acetate and having m. p. 162·5—163°, gave $[\alpha]_D^{21}$ −14·6° (±0·3°) (*c* 1·3 in EtOH). The yields of tripeptide derivative (*K* 0·697) and starting material (*K* 2·46) recovered from tubes 22—54 and 56—84 respectively were 83% and 13%.

Benzyloxycarbonylglycyl-L-*phenylalanylglycine Ethyl Ester*.—Method (c) gave 81% and 19% of the benzyloxycarbonylglycyl-L-phenylalanine was recovered. A solution of the ester (0·150 g.) in warm ethanol (7·5 c.c.) was stored at 0° and seeded with the racemic ester. No crystals formed in the first hour. After 3 days the crystals were collected and washed with ethanol (2 × 0·5 c.c.), which left 0·093 g., m. p. 119·5—120°, $[\alpha]_D^{20}$ −12·0° (±0·2°) (*c* 2·0 in EtOH).[11]

Benzyloxycarbonylglycyl-L-*alanyl*-L-*phenylalanylglycine and the* DL-*Isomer*.—(*a*) Benzyloxycarbonylglycyl-L-alanine (3 mmoles) and L-phenylalanylglycine (3 mmoles) were coupled by method (b) at initial pH 7·4. The mixed products (0·605 g., 42% previously [12] incorrectly reported as 60%; tubes 33—65) and the starting material (0·510 g., 61%; tubes 0—32) were separated by a 96-transfer distribution (3-tube filling) with 0·9M-KH_2PO_4/0·1M-K_2HPO_4. The tetrapeptide derivatives were then subjected to 95 transfers (3-tube filling) with 0·85M-KH_2PO_4/0·15M-K_2HPO_4; the first 20 tubes were then emptied and refilled with equilibrated solvents before recycling distribution was continued for in all 900 transfers. The contents of the tubes were combined in groups of ten and worked up. The distribution curve has already

been published (Fig. 1 of ref. 12). The *benzyloxycarbonylglycyl-L-alanyl-L-phenylalanylglycine* from tubes 88—99 and 0—17 recrystallised from methanol in needles, m. p. 175°, $[\alpha]_D^{21}$ −31·7° (±0·3°) (*c* 2·5 in 2-methoxyethanol) (Found: C, 59·3; H, 5·7; N, 11·6. $C_{34}H_{38}O_7N_4$ requires C, 59·5; H, 5·8; N, 11·6%). The DL-*diastereoisomer* from tubes 38—67 recrystallised from ethyl acetate in needles, m. p. 177·5°, $[\alpha]_D^{21}$ +14·9° (±0·4°) (*c* 1·9 in 2-methoxyethanol) (Found: C, 59·8; H, 5·7; N, 11·8%).

(*b*) During a similar preparation by method (a), the pH rose on addition of the magnesium carbonate from 5·3 to 6·5 in 2 min., and to 6·9 in 10 min. The first distribution separated the tetrapeptide derivative (*K* 1·02; 78%) from the starting material (*K* 0·197; 23%), and the second distribution was carried out for 847 transfers (5-tube filling). The distribution curve was very similar in shape and correspondence between the experimental points and the theoretical curve to that obtained for the following preparation. The material from the peak (tubes 0—9; *K* 0·560) had specific rotation −31°, whereas that from the trough (tubes 50—59) had specific rotation −18°(±4°) and consequently contained less than 30% of the DL-diastereoisomer, which therefore made up about 0·3% of the total product.

(*c*) Method (c) (3 mmole scale) gave 71% of amorphous benzyloxycarbonyltetrapeptide ester and 30% of benzyloxycarbonylglycyl-L-alanine. A solution of the former in methanol (10 c.c.) was mixed with N-sodium hydroxide (2·26 c.c.) and kept at 20° for 40 min. N-Sulphuric acid (2·26 c.c.) was added and the solvents were evaporated. The residue was partitioned between 3N-sulphuric acid (60 c.c.) and ethyl acetate (200 c.c.), which was then extracted with sodium hydrogen carbonate solution (5 × 20 c.c.) and water (10 c.c.). Only 0·021 g. of oil remained in the ethyl acetate, and the crystalline acid (0·986 g., 71% overall) was subjected to a 900-transfer distribution (5-tube filling); the results have already been published (Fig. 2 of ref. 12; the tube numbers should read 20, 40, 60, 80, 0, 20 from left to right). The material from the trough (tubes 62—71) had specific rotation −33° (±1°).

Bisbenzyloxycarbonyl-L-cystinylbisglycine Diethyl Ester.—This substance [24] was prepared in 81% yield by method (c) and had m. p. 162° after recrystallisation from ethyl acetate.

Acetyl-S-benzyl-L-cysteinylglycine Ethyl Ester.—This *ester* was prepared from acetyl-*S*-benzyl-L-cysteine [25] in 73% yield by method (c) and had m. p. 119° after recrystallisation from ethyl acetate (Found: C, 57·3; H, 6·5; N, 8·4. $C_{16}H_{22}O_4N_2S$ requires C, 56·8; H, 6·6; N, 8·3%).

Benzoylglycyl-DL-serine Methyl Ester [Mrs. B. F. NESBITT].—This *ester* was prepared from hippuric acid in 70% yield by method (c) and had m. p. 89—90° after recrystallisation from ethanol–light petroleum (b. p. 60—80°) (Found: C, 52·6; H, 6·2; N, 9·5. $C_{13}H_{16}O_5N_2,H_2O$ requires C, 52·3; H, 6·1; N, 9·4%).

Benzyloxycarbonyl-L-alanyl-L-tyrosine [Dr. R. J. STEDMAN].—Benzyloxycarbonyl-L-alanine (3 mmoles) and L-tyrosine ethyl ester (3 mmoles) were coupled by method (c); the neutral and the acidic fraction both crystallised in needles and their weights corresponded to a yield of 87% and a recovery of 16%. The ester was dissolved directly in N-sodium hydroxide (5·5 c.c.) by shaking and the solution was kept at 20° for 30 min. The acidic product was separated from a very small neutral fraction, and then crystallised from ethyl acetate–light petroleum (b. p. 40—60°). The overall yield of benzyloxycarbonyldipeptide,[26] m. p. 149—151°, was 66%.

An attempt to couple this substance with glycine ethyl ester by method (c) gave a negligible neutral fraction.

Benzyloxycarbonyl-L-alanyl-O-acetyl-L-tyrosylglycine Ethyl Ester.—Acetic anhydride (0·103 c.c.) was added to a solution of benzyloxycarbonyl-L-alanyl-L-tyrosine (0·338 g.) in N-sodium hydroxide (2·19 c.c.) at 0°. Ice was added and the mixture was left for 15 min. Two drops of aqueous ammonia (*d* 0·88) were added, and after 15 min. further the solution was acidified with dilute hydrochloric acid. The precipitated acetyl derivative (0·352 g.) was collected and recrystallised from ethyl acetate, having m. p. 145·5—146·5° depressed to 140—145° by the starting material.

The acetyl derivative (0·310 g.) was coupled with glycine ethyl ester by method (c), which yielded 62% of neutral powder. The *tripeptide derivative* recrystallised from ethanol as a waxy solid, m. p. 178—178·5° (Found: C, 60·7; H, 6·0; N, 8·5. $C_{36}H_{31}O_8N_3$ requires C, 60·8; H, 6·1; N, 8·2%).

[24] Bergmann and Zervas, *Ber.*, 1932, **65**, 1196.
[25] Du Vigneaud, Wood, and Irish, *J. Biol. Chem.*, 1939, **129**, 173.
[26] Bergmann and Fruton, *ibid.*, 1942, **145**, 251.

Benzyloxycarbonyl-L-tryptophyl-L-alanine.—The product from method (b) with initial pH 7·4 was distributed for 98 transfers with 0·3M-KH_2PO_4/0·7M-K_2HPO_4; the distribution curve corresponded to the presence of 78% of product (K 2·77) and 22% of starting material (K 8·21). The dipeptide derivative,[27] was recrystallised from chlorobenzene, and had m. p. 155°, $[\alpha]_D^{21}$ −13·2° (±0·3°) (c 4 in EtOH).

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[27] Smith, *J. Biol. Chem.*, 1948, **175**, 45.