which recombine to form the appropriate cyclic compounds. Both their work and the present study, in which the observed labeling patterns are compatible, demonstrate the need for intermediates that do not equilibrate with symmetrical products of glycolysis or the Krebs cycle. From pyruvate, this is more easily envisioned, since randomization or interaction with glycolysis products does not occur extensively; from glucose, the possible pathway is less clear. C_4-acid formation through $C_3 + C_1$ condensation is favored by pyruvate (as distinguished from acetate,[8,9]) and the failure of tyrosine to be formed from C^{14}-bicarbonate in the absence of pyruvate or other substrate[16] may reflect the previously observed importance of the C_3-C_1 condensation reaction in yeast and its dependence upon pyruvate.[8]

The conversion of acetate to tyrosine may also proceed through the C_4-acids, although quantitative agreement between observed and expected values is less satisfactory than in the pyruvate sample. The high activity of the carboxyl group, as well as the high ring activity at C_{2+4+6}, confirm the intramolecular distribution reported by Baddiley, *et al.*,[4] as far as the present degradation studies were carried out. It may thus be assumed that C_4 of the ring contains most of the activity designated in Table I as C_{2+4+6}. This would be expected if oxalacetate were the precursor of tyrosine, since aspartic acid derived from acetate in this yeast contained C^{14} only in the carboxyl groups.[8] It would also account for the absence of isotope in C_1 of tyrosine. The deviation between expected and observed values occurs at C_{3+5}; the activity of these atoms should equal the activity of C_4. An inspection of the C^{14} distribution obtained by Baddiley, *et al.*[4] (using $C^{13}H_3C^{14}OOH$ as substrate), reveals a similar observed deficiency of radioactivity at C_{3+5}, although the C^{13} distribution agrees well with expectations based on the foregoing scheme.

The present results, as well as others,[12,15,17] suggest that the citric acid cycle does not participate directly in the formation of tyrosine from pyruvate, acetate or glucose, although the over-all effect of Krebs cycle intermediates is to increase tyrosine synthesis by yeast.[18] Studies are in progress to relate further the behavior of glucose and pyruvate in these transformations.

(16) L. Levy and M. J. Coon, *J. Biol. Chem.*, **192**, 807 (1951).

(17) C. Gilvarg and K. Bloch, *ibid.*, **193**, 339 (1951).

(18) A. Kleinzeller and G. Kubie, *Chem. Listy*, **46**, 106 (1952).

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[CONTRIBUTION FROM THE CHEMOTHERAPY DIVISION, STAMFORD RESEACH LABORATORIES, AMERICAN CYANAMID COMPANY]

The Preparation of Optically-active Peptides Using Mixed Carbonic–Carboxylic Acid Anhydrides

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The study of racemization by this method of synthesis has been continued. Using N-carbobenzoxyamino acid or peptide–isobutylcarbonic acid anhydrides and amino acid or peptide esters, L-lysyl-L-valyl-L-phenylalanylglycine was prepared by two routes and shown to have almost identical properties in each case. Therefore, very little, if any, racemization occurred. When these mixed anhydrides in anhydrous solution were caused to react with the sodium salts of amino acids (or peptides) in aqueous solution no racemization was observed if the mixed anhydride was formed from an optically-active *N-carbobenzoxy*-amino acid. Partial racemization was observed, however, if the mixed anhydride was formed from an optically-active *N-acyl*amino acid, *i.e.*, an optically-active N-carbobenzoxy dipeptide, etc.

In a preliminary report[1] it was demonstrated that, in the reaction employing mixed carbonic–carboxylic acid anhydrides for the formation of carbobenzoxy peptide esters, racemization can be avoided or greatly minimized by the proper choice of reaction solvents and conditions. This investigation has now been extended to the preparation of the tetrapeptide L-lysyl-L-valyl-L-phenylalanylglycine and to a study of the reaction of mixed carbonic–carboxylic acid anhydrides with salts of amino acids or peptides under aqueous conditions. Interest in the above-mentioned tetrapeptide was initiated by the announcement[2] that the sequence on the amino end of the single polypeptide chain of lysozyme is lysylvalylphenylalanylglycyl-, and it was hoped that a direct comparison of a synthetic material with the natural product might be made. This has now been accomplished through the cooperation of Dr. Walter A. Schroeder of the California Institute of Technology, who compared the synthetic material prepared here with his natural product in the form of their dinitrophenyl (DNP) derivatives, and found them to be essentially identical.[3] No conclusions concerning the optical identity of the individual amino acids could be drawn, however.

For comparison of optical purity, the derivatized tetrapeptide was prepared by two routes. In the first of these, dicarbobenzoxy-L-lysyl-L-valine was prepared by saponificaton of its ethyl ester and coupled through its mixed isobutylcarbonate anhydride with ethyl L-phenylalanylglycinate to give the dicarbobenzoxy tetrapeptide ester in 54% yield m.p. 199–200°, $[\alpha]^{24}D$ $-21.4 \pm 0.6°$ (*c* 2, glacial acetic acid).

By the second route carbobenzoxy-L-valine was first condensed with ethyl L-phenylalanylglycinate to give the tripeptide derivative, which was then hydrogenated to ethyl L-valyl-L-phenylalanylglycinate. This was coupled with dicarbobenzoxy-L-lysine by the standard method[1] to give ethyl dicar-

(1) J. R. Vaughan, Jr., THIS JOURNAL, **74**, 6137 (1952).

(2) W. A. Schroeder, *ibid.*, **74**, 281 (1952).

(3) W. A. Schroeder, *ibid.*, **74**, 5118 (1952).

bobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycinate in 70% yield and having $[\alpha]^{25}D$ −21.2 ± 0.5° (*c* 2, glacial acetic acid) but having a melting point of only 190–192°. All attempts to raise this melting point to the value obtained above were unsuccessful. This experience is similar to that observed previously[1] with the tripeptide derivative, ethyl carbobenzoxyglycyl-L-phenylalanylglycinate, and may result from contamination of the product by the carbamate that would be formed by attack of the peptide ester amino group at the alkylcarbonate portion of the mixed anhydride molecule.

The two tetrapeptide derivatives were converted separately to the free tetrapeptides by first removing the carbobenzoxy groups, using hydrogen bromide in glacial acetic acid according to the method of Anderson, *et al.*[4] Water was then added to the solution and heating continued to effect hydrolysis of the ester groups. The hydrobromides so obtained were neutralized with lithium carbonate and precipitated with ethanol as 1:1 neutral salts with lithium bromide containing 1 molecule of alcohol of crystallization.[5] To eliminate the possibility that the compounds were esters containing 1 molecule of water of hydration ethyl L-lysyl-L-valyl-L-phenylalanylglycinate was also prepared and, although not obtained completely pure, was shown to be an entirely different substance. The tetrapeptide–lithium bromide salt from the high-melting dicarbobenzoxy ester derivative had m.p. 223–225° dec. and $[\alpha]^{24}D$ −8.3 ± 0.3° (*c* 2, 0.1 *N* HCl); that from the low-melting derivative melted with decomposition above 225° and had $[\alpha]^{24}D$ −8.9 ± 0.3° (*c* 2, 0.1 *N* HCl). There seems, therefore, to be a significant difference between the two materials. The sample submitted to Dr. Schroeder was of the salt melting at 223–225° dec.

Biological testing of the peptide in these laboratories by Mr. G. S. Redin showed it to be devoid of antibacterial activity in concentrations as high as 100 mg. %.[6]

Attention was next focused on the procedure in which a mixed carbobenzoxyamino acid or peptide–alkylcarbonic acid anhydride is prepared in anhydrous solution (tetrahydrofuran) and is caused to react with an amino acid or peptide salt in aqueous solution. The dipeptide reaction was examined first and, as an example, dicarbobenzoxy-L-lysyl-L-valine was prepared by this procedure and found to be identical with the material prepared by saponification of the corresponding ethyl ester. When the method was applied to the preparation of higher peptides, however, some racemization occurred. Thus, in the preparation of carbobenzoxyglycyl-L-phenylalanylglycine from the isobutyl carbonate anhydride of carbobenzoxyglycyl-L-phenylalanine in tetrahydrofuran solution and the sodium salt of glycine in aqueous solution, the product isolated by simple crystallization had $[\alpha]^{22}D$ −9.8° (ethanol). The product isolated by countercurrent distribution followed by crystallization, however, had $[\alpha]^{22}D$ −13.9° (ethanol), which compares favorably with the value $[\alpha]^{24}D$ −14.2° (ethanol) obtained on a sample prepared by saponification of the corresponding "optically-pure" ethyl ester.

The method was also applied to the preparation of a tetrapeptide derivative. Thus, in the preparation of dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycine from the isobutyl carbonate anhydride of dicarbobenzoxy-L-lysyl-L-valine in tetrahydrofuran solution and the sodium salt of L-phenylalanylglycine in aqueous solution, the product isolated by simple crystallization had $[\alpha]^{24}D$ −20.7° (acetic acid). Again, this compares with the value of $[\alpha]^{24}D$ −22.0° (acetic acid) obtained from a sample prepared by the acid hydrolysis of the corresponding ester (assumed to be optically pure). This procedure, therefore, appears to be perfectly satisfactory for the preparation of carbobenzoxy dipeptides, but should be used with reservation in the case of higher peptide derivatives.

Experimental[7]

Ethyl L-Valinate Hydrochloride.—A 14.0-g. (0.091 mole) sample of L-valine hydrochloride was suspended in 100 cc. of ethanol and the solution was saturated with hydrogen chloride. No attempt was made to cool the solution during this time. At the end of 1 hour saturation was complete and the reaction mixture was then heated for an additional hour under reflux, concentrated and the residue dried by azeotropic distillation of the water with benzene. Two crystallizations of this material from benzene–petroleum ether followed by one crystallization from chloroform gave 13.5 g. (82%) of pure product, m.p. 102–104°, $[\alpha]^{24}D$ +6.7 ± 0.5° (*c* 2, water).

Anal. Calcd. for $C_7H_{15}NO_2 \cdot HCl$: C, 46.28; H, 8.88; N, 7.71. Found: C, 46.28; H, 8.88; N, 7.75.

Ethyl Dicarbobenzoxy-L-lysyl-L-valinate.—A solution of 8.28 g. (0.02 mole) of dicarbobenzoxy-L-lysine (oil)[8] and 2.04 g. (0.02 mole) of triethylamine in 50 cc. of toluene was cooled to −5° and 2.74 g. (0.02 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature a second solution of 0.02 mole of ethyl L-valinate (prepared from 3.65 g. (0.02 mole) of the hydrochloride and 2.04 g. (0.02 mole) of triethylamine) in 50 cc. of chloroform was added with stirring. The reaction mixture was then heated rapidly to the point of reflux and immediately recooled and washed with dilute sodium bicarbonate solution. The organic phase was separated, dried briefly over anhydrous sodium sulfate and concentrated to about 20 cc. in a stream of air. Dilution with petroleum ether (100 cc.) gave a cloudy solution from which the product separated as colorless crystals on overnight standing; yield 9.35 g. (86%), m.p. 104–107°.

Recrystallization of this product from a mixture of 25 cc. of ethyl acetate and 75 cc. of petroleum ether gave 8.80 g. (81%) of pure product as colorless crystals melting at 109–110°, $[\alpha]^{23}D$ −17.4 ± 0.4° (*c* 2.9, ethanol).

Anal. Calcd. for $C_{29}H_{39}N_3O_7$: C, 64.30; H, 7.26; N, 7.76. Found: C, 64.44; H, 7.17; N, 7.53.

Dicarbobenzoxy-L-lysyl-L-valine. A. Preparation by Direct Synthesis.—A solution of 8.28 g. (0.02 mole) of dicarbobenzoxy-L-lysine (oil)[8] and 2.02 g. (0.02 mole) of triethylamine in 40 cc. of tetrahydrofuran was cooled to −5° and 2.74 g. (0.02 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature a solution of 2.34 g. (0.02 mole) of L-valine ($[\alpha]^{24}D$ +27.2 ± 0.5° (*c* 2, 6 *N* HCl)) in 20 cc. of 1 *N* sodium hydroxide was added

(4) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, **74**, 5309 (1952).

(5) Salts of this type are reported in detail by P. Pfeiffer (*Z. angew. Chem.*, **36**, 137 (1923)) but appear to have received little attention in recent years.

(6) The compound was tested *in vitro* by a blood-agar dilution spot plate test against strains of β-hemolytic *Streptococcus, pneumococcus* type I, *Klebsiella pneumoniae* type A, *Aerobacter aerogenes, Staphylococcus aureus, E. typhi, Salmonella schottmulleri, E. coli* and *Pasteurella multocida.*

(7) All melting points were taken on a Fisher–Johns block and are corrected. The analyses reported were determined in these laboratories under the direction of Dr. J. A. Kuck.

(8) Prepared according to M. Bergmann, *et al.*, *J. Biol. Chem.*, **111** 249 (1935). Found: $[\alpha]^{24}D$ −3.8 ± 0.2° (*c* 5.4, ethanol).

with good stirring. The reaction mixture was allowed to warm to room temperature during 30 minutes and acidified with 25 cc. of 1 *N* hydrochloric acid. The oil product that separated was extracted into two 100-cc. portions of ether which were combined and concentrated to a light oil residue. Crystallization was induced by dissolving this oil in 90 cc. of hot benzene and seeding the solution with a small sample of material from another preparation. On allowing the solution to cool slowly, the product separated as colorless needle clusters; wt. 7.31 g. (71%), m.p. 70–80°. Two recrystallizations from 250-cc. portions of benzene gave 6.68 g. (65%) of pure product as colorless needles, m.p. 70–73°, $[\alpha]^{22}D$ −7.0 ± 0.4° (*c* 2.4, ethanol).

Anal. Calcd. for $C_{27}H_{35}N_3O_7$: C, 63.14; H, 6.68; N, 8.18. Found: C, 63.00; H, 6.84; N, 8.26.

B. Preparation by Saponification of the Ethyl Ester.—A 5.30-g. (0.01 mole) sample of ethyl dicarbobenzoxy-L-lysyl-L-valinate was dissolved in 100 cc. of ethanol, and 20 cc. (2 equivalents) of 1 *N* sodium hydroxide added. After 2 hours at room temperature the solution was acidified with 25 cc. of 1 *N* hydrochloric acid and concentrated under an air stream to give a white, sticky oil. The oil was washed with 10 cc. of water and then redissolved in 50 cc. of saturated sodium bicarbonate solution. The solution was extracted with ether and the product was reprecipitated by acidification of the aqueous layer with hydrochloric acid. For crystallization, the oily product was taken up in 200 cc. of warm benzene, dried briefly over anhydrous sodium sulfate and the solution then concentrated to about 100 cc. on a steam-bath and allowed to cool. The pure product separated slowly as a solid mass of crystalline needles; yield 4.25 g. (85%), m.p. 70–73°, $[\alpha]^{23}D$ −6.4 ± 0.4° [*c* 2.8, ethanol).

Carbobenzoxy-L-valine.—A solution of 11.7 g. (0.1 mole) of L-valine, $[\alpha]^{22}D$ +24 ± 0.3° (*c* 3.9, 1 *N* HCl), in 50 cc. (0.1 mole) of 2 *N* sodium hydroxide was cooled to 0° and 15.6 cc. (0.11 mole) of benzyl chlorocarbonate and 22 cc. (0.11 mole) of 5 *N* sodium hydroxide were added dropwise and simultaneously with good stirring over a period of 8 minutes. After an additional 45 minutes, stirring and cooling, the solution was twice extracted with ether and acidified to congo red with hydrochloric acid to precipitate the product as a white oil. This was extracted into ether and the solution dried over sodium sulfate. A sample of this solution was set aside and allowed to evaporate spontaneously at room temperature. After 2 days, seed crystals had formed in the sample, and on scratching rapid crystallization occurred to give 4.3 g. (17%) of product, m.p. 58–60°.

The main portion of the reaction product was extracted into sodium bicarbonate solution, reprecipitated with acid and back-extracted into ether. The process was then repeated. On seeding the final ether solution with the crystals obtained above, the remainder of the product crystallized out; wt. 18.0 g. (72%), m.p. 57–59°.

The two crops were combined and recrystallized twice from ethyl acetate–petroleum ether to give 11.6 g. (46%) of product melting at 59–61° and having zero rotation in ethanol. The literature[9] gives m.p. 64–65° and $[\alpha]^{20}D$ +4° (*c* 2.9, ethanol).

Since the literature reports a positive rotation for this material, a sample was catalytically hydrogenated in the presence of palladium black[10] back to the original L-valine. This product gave $[\alpha]^{24}D$ +24 ± 2° (*c* 2.2, 1 *N* HCl), which is identical with that of the starting material. The carbobenzoxyvaline obtained, therefore, is the L-isomer.

Ethyl Carbobenzoxy-L-valyl-L-phenylalanylglycinate.—A solution of 3.77 g. (0.015 mole) of carbobenzoxy-L-valine and 1.53 g. (0.015 mole) of triethylamine in 50 cc. of toluene was cooled to −5° and 2.05 g. (0.015 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature, a second solution of 0.015 mole of ethyl L-phenylalanylglycinate (prepared from 4.97 g. of the hydrobromide[1] and 1.53 g. of triethylamine) in 50 cc. of cold chloroform was added with stirring and the reaction mixture was then warmed rapidly to the point of reflux and immediately recooled. The product separated very rapidly as a colorless solid during this process. After cooling, the product was filtered off, washed with petroleum ether, dried and rewashed with saturated sodium bicarbonate solution and with water. The still damp solid was recrystallized from 100 cc. of ethanol to give 5.60 g. (77%) of product as colorless crystals melting at 189–190°. Recrystallization from 130 cc. of ethanol gave 4.70 g. (65%) of pure product melting at 191.5–192°, $[\alpha]^{24}D$ −22.5 ± 0.4° (*c* 2.1, glacial acetic acid).

Anal. Calcd. for $C_{26}H_{33}N_3O_6$: C, 64.58; H, 6.88; N, 8.69. Found: C, 64.63; H, 6.92; N, 8.89.

Ethyl L-Valyl-L-phenylalanylglycinate Hydrobromide.—A 4.84-g. (0.01 mole) sample of ethyl carbobenzoxy-L-valyl-L-phenylalanylglycinate was suspended in 250 cc. of ethanol and 1 cc. of glacial acetic acid and about 200 mg. of palladium black catalyst[10] added. The mixture was then warmed to 40° in a water-bath while hydrogen was bubbled in. After about 2 hours all of the material had dissolved and carbon dioxide evolution had ceased.

The solution was filtered to remove catalyst, acidified with one equivalent of alcoholic hydrogen bromide and concentrated by vacuum distillation to about 50 cc. This was rapidly diluted to about 400 cc. with ether to give a clear solution from which the product crystallized as colorless needles after several minutes; wt. 3.95 g. (92%), m.p. 210–211°, $[\alpha]^{25}D$ +29.2 ± 0.5° (*c* 2, water). Recrystallization did not change the melting point.

Anal. Calcd. for $C_{18}H_{27}N_3O_4 \cdot HBr$: C, 50.24; H, 6.56; N, 9.77. Found: C, 50.36; H, 6.71; N, 9.52.

Ethyl Dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycinate. A. By Coupling Dipeptide Derivatives.—A solution of 2.50 g. (0.005 mole) of dicarbobenzoxy-L-lysyl-L-valine and 0.50 g. (0.005 mole) of triethylamine in 30 cc. of tetrahydrofuran was cooled to −5° and 0.68 g. (0.005 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature a suspension of 1.66 g. (0.005 mole) of ethyl L-phenylalanylglycinate hydrobromide[1] in 20 cc. of dioxane containing 0.50 g. (0.005 mole) of triethylamine was added with good stirring. Since the peptide ester hydrobromide was not completely soluble in the dioxane–tetrahydrofuran solution, the reaction mixture was stirred cold for about 5 minutes and then allowed to warm to room temperature during 30 minutes. Dilute sodium bicarbonate (250 cc. of 1% solution) was then added. On standing, the product separated slowly as a colorless, crystalline powder, wt. 2.75 g. (74%), m.p. 175–180°. After two recrystallizations from 175-cc. portions of ethanol, 2.00 g. (54%) of pure material was isolated as a colorless solid, m.p. 199–200°, $[\alpha]^{24}D$ −21.4 ± 0.6° (*c* 2, glacial acetic acid).

Anal. Calcd. for $C_{40}H_{51}N_5O_9$: C, 64.41; H, 6.89; N, 9.39. Found: C, 64.37; H, 6.78; N, 9.36.

B. By Coupling Amino Acid and Tripeptide Derivatives.—A solution of 2.90 g. (0.007 mole) of dicarbobenzoxy-L-lysine (oil)[8] and 0.70 g. (0.007 mole) of triethylamine in 100 cc. of toluene was cooled to −5° and 0.96 g. (0.007 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature, a second solution of 3.00 g. (0.007 mole) of ethyl L-valyl-L-phenylalanylglycinate hydrobromide and 0.70 g. (0.007 mole) of triethylamine in 20 cc. of chloroform was added with good stirring. The reaction mixture was then heated rapidly to reflux and immediately cooled to crystallize the product. This was washed with water and with petroleum ether to give 4.30 g. (83%) of material melting at 189–192°. Recrystallization of this from 200 cc. of ethanol gave 3.65 g. (70%) of product as a colorless solid melting at 190–192°, $[\alpha]^{25}D$ −21.2 ± 0.5° (*c* 2, glacial acetic acid). Repeated recrystallization of this material did not change its optical rotation or melting point.

Anal. Calcd. for $C_{40}H_{51}N_5O_9$: C, 64.41; H, 6.89; N, 9.39. Found: C, 64.39; H, 6.92; N, 9.65.

L-Lysyl-L-valyl-L-phenylalanylglycine Lithium Bromide Monoethanolate.—A 0.75-g. (0.001 mole) sample of ethyl dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycinate (m.p. 199–200°) was placed in 6 cc. (50% excess) of 1 *N* hydrogen bromide in glacial acetic acid and the mixture heated for 3 minutes on the steam-bath. During this time the material dissolved with evolution of carbon dioxide. Water (2 cc.) was then added to the solution and heating was continued for 45 minutes. The solution was cooled, diluted with 40–50 cc. of ether and set aside in the cold to allow for the separation of an aqueous phase. The ether phase was decanted and the aqueous residue was washed with two 50-cc. portions of fresh ether.

To obtain the free peptide, 74 mg. (0.001 mole) of lithium

(9) R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

(10) R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, **54**, 128 (1921).

carbonate plus 1 cc. of water was added. After carbon dioxide evolution had ceased, the solution was adjusted to neutrality by the addition of several extra milligrams of lithium carbonate and diluted with 50 cc. of alcohol to precipitate a colorless, crystalline solid, wt. 0.35 g., m.p. 223–225° dec. with darkening above 205°. For recrystallization, the material was dissolved in 1.5 cc. of water and the solution filtered and diluted with 40 cc. of alcohol to cause separation of colorless micro-crystals; wt. 0.30 g., m.p. 223–225° dec. with darkening above 215°, $[\alpha]^{24}D$ −8.3 ± 0.3° (*c* 2, 1 *N* HCl). The analysis indicates that the material is the tetrapeptide neutral lithium bromide salt monoalcoholate.

Anal. Calcd. for $C_{22}H_{35}N_5O_5 \cdot LiBr \cdot C_2H_5OH$: C, 49.49; H, 7.10; N, 12.02. Found: C, 49.91; H, 7.16; N, 12.04.

A paper chromatogram in butanol:water:acetic acid (5:4:1) showed that more than 95% of the material had an R_f value of 0.44 with a trace of fast running material at R_f 0.50.

Repetition of this experiment using a sample of the 190–192° melting derivative gave 170 mg. of the peptide salt which melts with decomposition above 225° and darkens above 205°, $[\alpha]^{22}D$ −8.9 ± 0.3° (*c* 2, 1 *N* HCl). Paper chromatography gave essentially identical results with a main spot at R_f 0.45 and a trace spot at 0.51.

Ethyl L-Lysyl-L-valyl-L-phenylalanylglycinate Dihydrobromide.—A 0.75-g. (0.001 mole) sample of ethyl dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycinate (m.p. 190–192°, $[\alpha]^{25}D$ −21.2 ± 0.5° (*c* 2, glacial acetic acid)) was placed in 5 cc. (25% excess) of 1 *N* hydrogen bromide in glacial acetic acid and the mixture heated for 3 minutes on the steam-bath. During this time the solid dissolved with evolution of carbon dioxide. The hot solution was then diluted with 25 cc. of ether to precipitate the product as an amorphous solid. After cooling, this was filtered off, redissolved in 40 cc. of hot ethanol (Darco) and the solution was diluted hot with ether until it just failed to turn cloudy. On standing, a crystalline product separated, wt. 0.55 g. (86%), m.p. about 160–170°. This product was again crystallized from alcohol–ether as above and then twice more from 10- and 15-cc. portions of alcohol–benzene (1:1) to give 0.15 g. (24%) of a colorless, crystalline product melting at 216–218° with a preliminary transition at 182–185°. The material analyzes for a hemihydrate.

Anal. Calcd. for $C_{24}H_{39}N_5O_5 \cdot 2HBr \cdot 1/2H_2O$: C, 44.45; H, 6.53; N, 10.80. Found: C, 44.41; H, 6.47; N, 10.27.

Ethyl L-Lysyl-L-valyl-L-phenylalanylglycinate.—A 114-mg. (0.000225 mole) sample of ethyl L-lysyl-L-valyl-L-phenylalanylglycinate dihydrobromide was dissolved in 2 cc. of water and 16.5 mg. (0.000225 mole) of lithium carbonate added. There was no particular evidence of carbon dioxide evolution and the lithium carbonate did not dissolve until the mixture was warmed on the steam-bath. The solution was filtered hot and diluted with 40 cc. of ethanol. On cooling, only a trace of amorphous solid separated. This was removed and the solution was concentrated in an air stream to give a white, solid residue which was washed with water and dried. The material gave a strong lavender biuret test, was insoluble in water or dilute alkali, although it dissolved slowly in alkali after standing for 10–15 minutes, but was very soluble in dilute hydrochloric acid and ethanol. A silver nitrate test for halogen was negative. These properties, therefore, are those expected for the desired tetrapeptide ester and are quite different from those observed for the tetrapeptide lithium bromide monoethanolates. These latter compounds are soluble in water, insoluble in ethanol and give both positive biuret and halogen tests.

Carbobenzoxyglycyl-L-phenylalanylglycine. A. By Direct Synthesis.—A solution of 3.56 g. (0.01 mole) of carbobenzoxyglycyl-L-phenylalanine[11] (m.p. 124–126°, $[\alpha]^{22}D$ +39.0° (*c* 5, ethanol) and 1.02 g. (0.01 mole) of triethylamine in 25 cc. of tetrahydrofuran was cooled to −5° and 1.37 g. (0.001 mole) of isobutyl chlorocarbonate added with stirring. After 10 minutes at this temperature, a second solution of 0.75 g. (0.01 mole) of glycine in 10 cc. (1 equivalent) of 1 *N* sodium hydroxide was added with stirring. The reaction mixture was stirred at room temperature for 10 minutes and then acidified with 1 *N* hydrochloric acid and concentrated almost to dryness in an air stream. The residue was placed in the first four tubes of a 200-tube countercurrent distribution machine and distributed between ethyl acetate–1 *M* phosphate buffer (0.75 *M* KH_2PO_4 + 0.25 *M* K_2HPO_4) as described by Kenner and Stedman.[12] After 190 transfers the product was separated into two materials which by observation (emulsification on shaking) were concentrated in tubes 51–98 and 118–137. The buffer phases from tubes 51–98 were combined and acidified with hydrochloric acid to give 1.60 g. (39%) of the tripeptide derivative as colorless crystals, m.p. 160–161°, $[\alpha]^{22}D$ −10.4° (*c* 2.7, ethanol). The dipeptide derivative was recovered from tubes 118–137 in the same manner, wt. 0.15 g. (4%), m.p. 161–163°, $[\alpha]^{22}D$ +5.0° (*c* 2, ethanol). The published values[12] for the L- and DL-isomers of these two compounds are: Z-Gly-Phe-OH (DL), m.p. 162°; Z-Gly-Phe-OH (L), m.p. 124–126°, $[\alpha]^{22}D$ +39° (ethanol); Z-Gly-Phe-Gly-OH (DL), m.p. 141–142°; Z-Gly-Phe-Gly-OH (L), m.p. 155–157° $[\alpha]^{22}D$ −15.7° (ethanol).

On recrystallization from ethyl acetate the tripeptide derivative gave a product melting at 163–164° and having $[\alpha]^{22}D$ −13.9° (*c* 2.4, ethanol).

A similar experiment on the same scale in which an attempt was made to purify the product by recrystallization alone gave a material melting at 146–151° (15%) and having $[\alpha]^{22}D$ −9.8° (*c* 2.9, ethanol).

B. **By Saponification of the Ethyl Ester.**—A 5.0-g. (0.011 mole) sample of ethyl carbobenzoxyglycyl-L-phenylalanylglycinate was dissolved in a mixture of 40 cc. of ethanol and 14 cc. of 1 *N* sodium hydroxide (25% excess) and the solution allowed to stand at room temperature for 1.25 hours. The solution was then acidified with 1 *N* hydrochloric acid and concentrated in an air stream to give a colorless, crystalline residue. This was recrystallized three times from ethyl acetate–ethyl ether to give 0.9 g. (20%) of colorless, crystalline product melting at 152–155°, $[\alpha]^{24}D$ −14.2 ± 0.4° (*c* 2, ethanol).

Benzyl Carbobenzoxy-L-phenylalanylglycinate.—Ammonia gas was bubbled into a suspension of 36.6 g. (0.113 mole) of benzyl glycinate–benzenesulfonate[13] in 300 cc. of ether for 1 hour and the mixture filtered to remove precipitated ammonium benzenesulfonate. The filtrate was concentrated under vacuum to remove the ether and excess ammonia and the oil residue was then redissolved in 30 cc. of toluene and the solution filtered and cooled until used.

Meanwhile, a second solution of 22.4 g. (0.075 mole) of carbobenzoxy-L-phenylalanine and 7.6 g. (0.075 mole) of triethylamine in 120 cc. of toluene was cooled to −5° and 10.3 g. (0.075 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature, the solution of benzyl glycinate (50% excess) prepared above was added with stirring. The reaction mixture was then heated rapidly to the point of reflux and immediately recooled. The product separated out during this process as a heavy white precipitate which was filtered off, washed with sodium bicarbonate and dried, wt. 25.2 g. (75%), m.p. 128–136°. Recrystallization from ethyl acetate (250 cc.)–petroleum ether followed by crystallization from 850 cc. of 95% ethanol gave 16.2 g. (48%) of pure product as colorless crystals melting at 135.5–137.5°, $[\alpha]^{24}D$ −9.2 ± 0.2° (*c* 2, glacial acetic acid).

Anal. Calcd. for $C_{26}H_{26}N_2O_5$: C, 69.94; H, 5.87; N, 6.28. Found: C, 69.89; H, 6.08; N, 6.45.

L-Phenylalanylglycine Monohydrate.—A 4.10-g. (0.009 mole) sample of benzyl carbobenzoxy-L-phenylalanylglycinate was dissolved in 50 cc. of glacial acetic acid and approximately 200 mg. of palladium black catalyst and 2 cc. of water added. The reaction mixture was then shaken under 50 lb./in.^{2} of hydrogen pressure at room temperature for 1 hour. The catalyst was filtered off and the filtrate concentrated under high vacuum to a colorless sirup. On redissolving this in 5 cc. of water and diluting the solution with 100 cc. of ethanol, the product separated slowly as colorless micro-crystalline needles, wt. 1.70 g. (71%), m.p. 250° dec., $[\alpha]^{22}D$ +95.6 ± 0.5° (*c* 2.1, water). The literature[14] gives m.p. 259.5–260.5° and $[\alpha]^{23}D$ +84.4° (*c* 20, water).

Dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycine. A. By Direct Synthesis.—A solution of 3.50 g. (0.007 mole) of dicarbobenzoxy-L-lysyl-L-valine and 0.70 g. (0.007 mole)

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of triethylamine in 15 cc. of tetrahydrofuran was cooled to −5° and 1.0 g. (0.007 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature a solution of 1.67 g. (0.007 mole) of L-phenylalanylglycine monohydrate in 7 cc. of 1 *N* sodium hydroxide was added with stirring and the reaction mixture was allowed to warm to room temperature during 30 minutes. On extraction of this solution with ether, the sodium salt of the product separated immediately as a colorless, amorphous solid. This was filtered off, washed with water, resuspended in dilute hydrochloric acid and stirred for several hours at room temperature to convert the salt to the free acid. The product was again filtered off, washed with water and crystallized from a mixture of 25 cc. of glacial acetic acid and 10 cc. of water; wt. 1.45 g. (29%), m.p. about 185–188° (indefinite), $[\alpha]^{24}D$ −20.7 ± 0.5° (*c* 2.1, glacial acetic acid).

Anal. Calcd. for $C_{38}H_{47}N_5O_9$: C, 63.58; H, 6.60; N, 9.76. Found: C, 63.13; H, 6.48; N, 9.47.

B. Acid Hydrolysis of the Ethyl Ester.—A 0.30-g. (0.0004 mole) sample of ethyl dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycinate (m.p. 199–200°) was dissolved in 30 cc. of dioxane containing 2 cc. of 1 *N* hydrochloric acid (0.002 mole) and the solution was heated on the steam-bath for 1 hour and then concentrated to dryness in an air stream. The colorless, solid residue was recrystallized from dilute acetic acid and twice from alcohol–water to give 0.21 g. (73%) of pure material melting at 173–175°, $[\alpha]^{24}D$ −22.0 ± 0.4° (*c* 2.4, glacial acetic acid).

Anal. Calcd. for $C_{38}H_{47}N_5O_9$: C, 63.58; H, 6.60; N, 9.76. Found: C, 63.29; H, 6.62; N, 9.55.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Statistical and All-or-None Binding of Alkylbenzenesulfonate by Albumins[1,2]

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The binding of commercial sodium dodecylbenzenesulfonate (SDBS) by bovine plasma albumin (A) and by ovalbumin (O) has been studied by equilibrium dialysis and electrophoresis. The combination of the two techniques permits further insight into the reaction than can be gained from a study by either method alone. Three regions can be distinguished in the binding curve of A. In the first (region A) the binding is essentially statistical with a limit of about twelve ions per molecule. In region B the reaction is all-or-none giving rise to a complex containing about 48 ions (approximately one per two cationic residues of the protein). In region C the binding is again of a statistical character, no upper limit being attained under the conditions employed. In the case of O the first statistical region is absent, an all-or-none reaction taking place with the binding of about 43 ions per molecule (one per cationic group). This suggests that O is more labile than A and is denatured by the first ions bound. A partial interpretation of the binding curves is undertaken in spite of the heterogeneity of the detergent. It is concluded that the free concentration of true detergent in equilibrium with the all-or-none complex is well below the critical concentration for micelle formation. It is further shown that the presence of micelles is not necessary for the all-or-none reaction (denaturation).

In recent years many investigations have been published on the interaction of proteins with ions. Of particular interest are the surface active ions which produce many diverse effects on proteins and biological systems, such as protein denaturation, dispersion and precipitation.

It is well known that proteins exhibit markedly strong affinity for long-chain alkyl sulfates and alkyl aryl sulfonates. Disagreement arises, however, as to the number of anions bound and also as to the nature of the binding process. Perhaps the most striking feature of the combination in the case of native proteins is the all-or-none character, first demonstrated in the case of ovalbumin by Lundgren, *et al.*,[3] and shown also for serum albumin by Putnam and Neurath.[4] Thus, in the electrophoretic pattern of protein–detergent mixtures of appropriate composition two distinct components are observed rather than the single broad boundary, or possibly a number of poorly resolved boundaries, which would be expected if combination were stepwise. In their classical study Lundgren and coworkers demonstrated that this all-or-none characteristic is absent if denatured ovalbumin is used.

More recently Karush and Sonenberg,[5] in studies at detergent concentration below the critical micelle level, found that binding obeys essentially a statistical law but felt it necessary to invoke the idea of heterogeneous binding sites.

It thus seemed important to reinvestigate the combination over a broad range of detergent concentration covering both the regions studied by Karush and Sonenberg and by the other workers. In this paper are presented results on the interaction of both serum albumin and ovalbumin with dodecylbenzenesulfonate. The results are in substantial agreement with those of all the abovementioned authors but lead to a different interpretation from that given by Karush and Sonenberg.

Experimental

Materials.—Crystalline bovine plasma albumin (hereafter called A) was obtained through the courtesy of Armour and Company. Ovalbumin (O) was prepared in the cold room (1–3°) from fresh egg white by a modification of the procedure of Sørensen and Høyrup.[6] Crude O was recrystallized thrice from ammonium sulfate at its isoelectric point, dialyzed free of salt against distilled water, lyophilized to dryness and kept in the cold room.

Santomerse No. 3, principally sodium dodecylbenzenesulfonate (hereafter called SDBS), was supplied by the Monsanto Chemical Company and freed of inorganic salts by dispersion in 95% ethanol. The dried SDBS was kept in an air-tight bottle. Its apparent number-average molecular weight was 354, as determined by Parr bomb sulfur analysis, the theoretical value being 348.

Partial purification of the commercial detergent was achieved by either continuous dialysis or partial precipita-

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(2) Taken in part from a thesis presented by Jen Tsi Yang in partial fulfillment of the requirements for the degree Doctor of Philosophy, Iowa State College, 1952. Presented at the 122nd meeting of the American Chemical Society, Atlantic City, N. J., September, 1952.

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