group was noted when the reduction was carried out with a 50-mole excess of thioglycolic acid but with a 100-mole excess the treated albumin had 1.2 mole of sulfhydryl group. With a 200-mole excess, the treated albumin had 3.0 mole of sulfhydryl group and also contained 16% polymer.

The treated albumins were converted to mercurial derivatives. Figure 3 was obtained with albumin treated with 100-mole excess of thioglycolic acid, and Fig. 4 was obtained with albumin treated with 200-mole excess of thioglycolic acid. The band with a K of 1 present in the untreated sample had disappeared, but a new band had appeared with a K of 1.6 to 2.0. Material isolated from the region with a K of 1.6 to 2.0 was estimated to be composed of 70% monomer, 14% dimer and 16% polymer.

The albumin recovered from the main bands in Fig. 3 and 4 was combined and redistributed to give the patterns in Fig. 5. Here the small band with a K of 1 reappeared because of the tendency of the mercury derivative to dissociate as discussed above.

When the albumin was treated with thioglycolate at pH 9.0, it showed 4.7 mole of sulfhydryl group The distribution of the mercurial derivative of this sample gave the pattern shown in Fig. 6. Based on the albumin band (K = 1.3) in the distribution pattern, it was calculated that about 15% of the albumin in the reduction mixture was the unaltered albumin but 50% had formed insoluble polymers.

The reduction of the disulfide bonds of the albumin could conceivably occur by two distinct pathways, (a) the susceptible disulfide linkages of all the albumin molecules cleaved one at a time with essentially the same rates or (b) the initial cleavage of one disulfide linkage of an albumin which could then lead to the explosive cleavage of the remaining susceptible linkages present in the same molecule. In the above reduction experiments, the albumin concentration was held at a constant value but the thioglycolate concentrations and pH of the solutions were varied. In all cases, significant amounts of apparently unchanged albumin could be recovered from the reaction mixture. While a detailed analysis of the products was not possible due to formation of polymers, the results suggest that reaction pathway (b) is favored and that cleavage of the first linkage of the albumin introduced new labilities to the protein structure. Another possible reaction with the thioglycolate treatment would be in the formation and equilibration of albumin isomer(s) due to the migration of the sulfhydryl group in the chain. This is suggested by the sulfhydryl group value of one mole per mole of albumin isolated from thioglycolate treated sample in Fig. 5, as compared with the value of approximately 0.9 from untreated albumins in Figs. 1 and 2. Such reaction products would not have been differentiated if the new isomers had K values similar to that of the original albumin. In the case of human plasma albumin as described in the following paper,[22] there seemed to be definite evidence for this type of interchange.

The reduction experiments support the belief that the band with a K of 1 and the shoulder on the right edge of the main band were products of sulfhydryl-disulfide reactions of the albumin, thus indicating that the observed broadness of the albumin bands did not result from some slow disorganization of the secondary structure by the organic solvents. This observation was fully substantiated by the measurements of the rotatory dispersion and sedimentation rate of the recovered albumin which indicates that it had retained its native secondary structure, shape and molecular size. In terms of countercurrent distribution, those observations implied the possibility of fractionation of many proteins in these near-critical point, high-salt content solvent systems if their reactive functional sites could be blocked. It perhaps should be stressed that the sulfhydryl-disulfide groups are not the only sensitive sites in proteins, since chymotrypsinogen, a protein containing no detectable sulfhydryl group, was rapidly denatured in the present solvent system.[3]

Acknowledgments.—The authors are grateful to Mrs. J. O'Brien and Mr. O. Griffith for their technical assistance.

(22) T. P. King, D. A. Yphantis and L. C. Craig, THIS JOURNAL, **82**, 3355 (1960).

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE, NEW YORK, N. Y.]

Distribution Studies with Human Plasma Albumin[1]

BY T. P. KING, D. A. YPHANTIS AND L. C. CRAIG

RECEIVED DECEMBER 11, 1959

Dissolution of human plasma albumin in a solvent system composed of ethanol, 1-propanol, water and ammonium sulfate leads to the formation of a mixture of dimer and monomer. The two fractions can be separated by countercurrent distribution in this solvent system. The dimer was shown to be formed through an oxidative coupling of two molecules of mercaptalbumin.

Introduction

In the preceding paper[2] concerning the distribution of crystalline bovine plasma albumin, it was reported that the albumin slowly underwent dimer and polymer formation when dissolved in a solvent system of ethanol, n-propanol, water and ammonium sulfate and that the reaction rate was diminished by the presence of sodium caprylate in the solvent system. The present paper will deal with the fractionation of human albumin in the same solvent system.

(1) This investigation was supported in part by a research grant, A-2493 B.B.C., from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

(2) T. P. King, D. A. Yphantis and L. C. Craig, THIS JOURNAL, **82**, 3350 (1960).

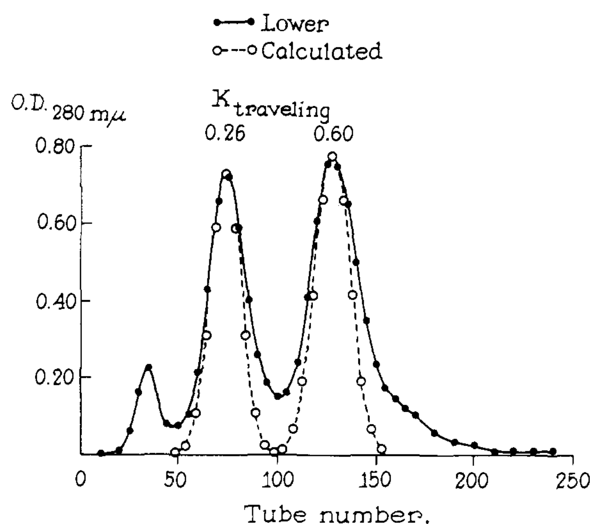


Fig. 1.—Distribution of human plasma albumin, 342 transfers.

In contrast to crystalline bovine plasma albumin, a portion of the human plasma albumin was found to undergo rapid dimerization in the solvent system, and sodium caprylate had no effect on the rate or extent of the reaction in this case. Upon distribution, an effective separation of the monomeric and dimeric albumins was easily realized due to the large difference in their partition coefficient values (K).

The formation of human plasma albumin dimer has been reported on two previous occasions.[3] Cohn, Hughes and Weare[3a] noted that in the process of removing adsorbed lipids from albumin by extraction with methanol or ethanol about 30% of the albumin was converted to the dimer. Straessle[3b] found that the treatment of the mercury dimer of mercaptalbumin with iodine effected a 60% conversion to the dimer.

Experimental

The human mercaptalbumin and an albumin preparation with a low sulfhydryl content which was a residue from the fractionation of mercaptalbumin from Fraction V were gifts of Dr. J. L. Oncley. The mercaptalbumin sample had 0.90 ± 0.04 mole of sulfhydryl group by amperometric titration and ultracentrifugal analysis revealed its composition as 91 ± 3% monomer and 8 ± 3% dimer. The fraction with a low sulfhydryl content had 0.20 ± 0.02 mole of sulfhydryl group. Sulfhydryl group determinations, ultracentrifugal analyses and rotatory dispersions were obtained as in the preceding paper.

Fatty acid analyses were made by the method of Gordon.[4] The lyophilized albumin (30 mg.) was extracted with 15 ml. of a mixture of equal parts of iso-octane and glacial acetic acid for 30 minutes at room temperature with occasional trituration. After decantation, the mixed solvent was washed with three 10-ml. portions of 0.1 N sulfuric acid. A 3-ml. aliquot of the iso-octane extract plus 1 ml. of 0.001% phenol red in 80% ethanol was then titrated under a fine stream of nitrogen gas with 0.01 N sodium hydroxide, using a microburet. With appropriate blank determinations the recovery was generally 95% for an amount of stearic acid similar to the acid content found in the albumin.

Human plasma albumin was prepared by ammonium sulfate precipitation between 0.55 and 0.75 saturation. Blood from a single donor was withdrawn in a heparinized syringe. After removal of the cells by centrifugation at 5°, the plasma was adjusted to 0.55 saturation by the addition of saturated ammonium sulfate solution. After 1 hr., the precipitated globulins were removed, and the supernatant was adjusted to 0.75 saturation by the addition of solid ammonium sulfate. After 3 hr., the albumin precipitate was collected and dissolved in twice its volume of 0.1 N acetate buffer (pH 5.2). The ammonium sulfate precipitation was repeated, and finally the albumin precipitate was placed in a cellophane sac and dialyzed against distilled water at 5° for 40 hr. Some precipitation took place, but the yellow supernatant was passed through a column (26 × 1.6 cm.) of mixed bed resin (Amberlite MB-1) at a flow rate of 35 ml. per hour. After lyophilization of the eluate, 1.85 g. of albumin was obtained from 57 ml. of plasma. The sample was found to contain 0.39 ± 0.02 mole of sulfhydryl group, 1.1 ± 0.1 mole of bound fatty acid and 95 ± 3% monomer ($S_{20,w}$ = 4.40 ± 0.08 S.).

(3) (a) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947). (b) R. Straessle, *ibid.*, **76**, 3138 (1954).

(4) R. S. Gordon, Jr., *J. Clin. Invest.*, **36**, 810 (1957).

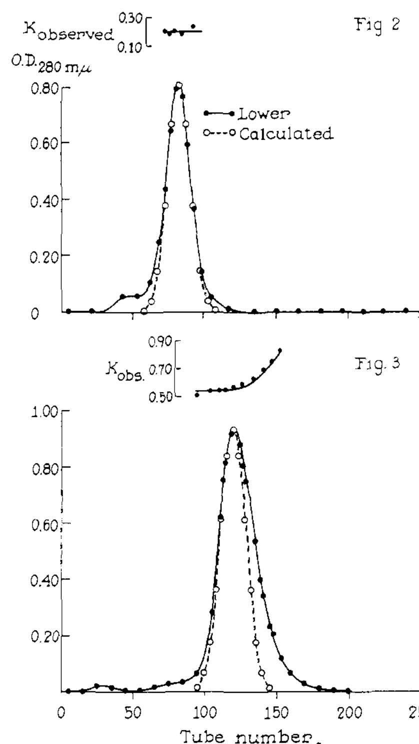
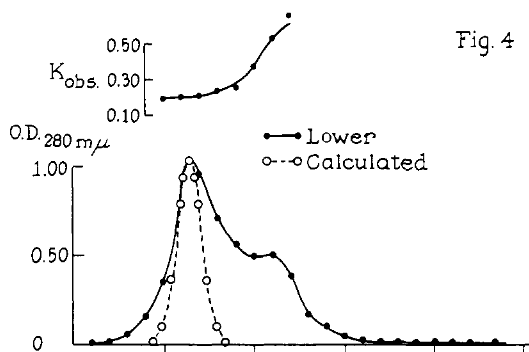


Fig. 2.—Redistribution of K 0.20 fraction, 402 transfers.
Fig. 3.—Redistribution of K 0.57 fraction, 334 transfers.

The distributions were carried out at 25° in a 250-tube automatic machine of 2-ml. capacity. The composition of solvent system and the procedure for the recovery of the protein from the solvent system were the same as described in the preceding paper.

The ammonium sulfate precipitated albumin gave the distribution pattern shown in Fig. 1 at 342 transfers. The sample (300 mg.) was dissolved in 24 ml. of each of the upper and the lower phases of the solvent system following the procedure described in the preceding paper. The solution was allowed to stand for 24 hr. at 25°. A small amount of precipitate formed and was removed before placing the solution in the first twelve tubes of the distribution train. The adsorbed yellow pigment traveling with a K of 5.3 is not shown in the pattern. From tubes 60 to 90, 70 mg. of the K 0.20 fraction was recovered, and from tubes 116 to 142, 150 mg. of the K 0.57 fraction was recovered.

The K 0.20 fraction had no sulfhydryl group, 0.1 ± 0.1 mole of bound fatty acid and was composed of 96 ± 3% dimer ($S_{20,w}$ = 6.23 ± 0.12 S. and $D_{20,w}$ = 4.31 ± 0.08 F.) and 4 ± 3% heavy component. By the equilibrium sedimentation method in ultrashort columns, its molecular weight was estimated to be 137,000. Similar estimation carried out in 8 M urea at pH 7.8 gave a value of 210,000, assuming that the partial specific volume in 8 M urea was the same as that in water, 0.734. Analyses of the K 0.57 fraction showed 0.03 ± 0.03 mole of sulfhydryl group, 0.1 ±



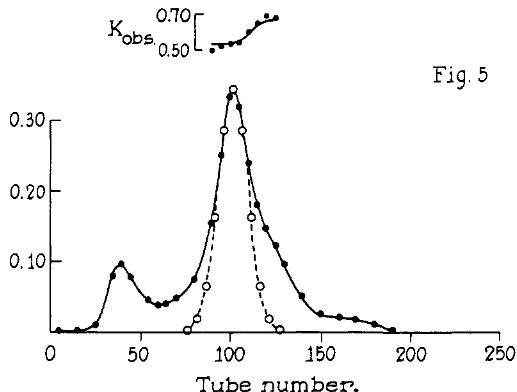
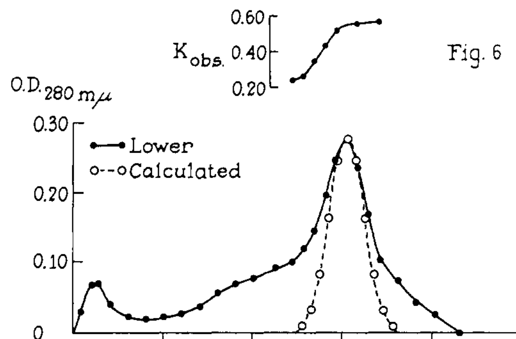


Fig. 4.—Distribution of human mercaptalbumin, 270 transfers.

Fig. 5.—Distribution of human non-mercaptalbumin, 297 transfers.



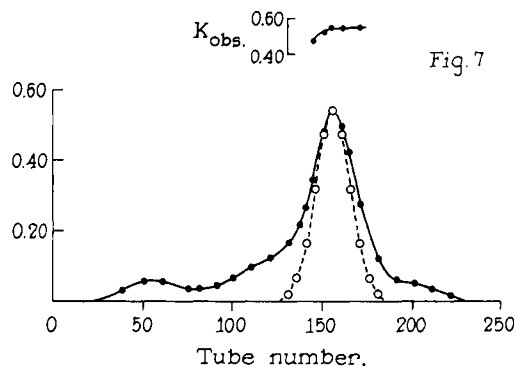


Fig. 6.—Distribution of the thioglycolate treated K 0.20 fraction, 402 transfers.

Fig. 7.—Distribution of the thoglycolate treated K 0.57 fraction, 402 transfers.

0.1 mole of bound fatty acid and 95 ± 3% monomer ($S_{20,w}$ = 4.30 ± 0.08 S. and $D_{20,w}$ = 6.14 ± 0.12 F.) and 5 ± 3% dimer. Both fractions had identical rotatory dispersion constants and the same specific optical rotation: $[\alpha]^{27°}D$ = −62 ± 2°.

When redistributed to 402 transfers, the K 0.20 fraction (105 mg.) gave the pattern in Fig. 2, and redistribution of the K 0.57 fraction (210 mg.) to 334 transfers gave the pattern in Fig. 3.

Figure 4 was obtained with 297 mg. of human mercaptalbumin at 270 transfers. Figure 5 was obtained at 297 transfers with 126 mg. of the albumin fraction with a low sulfhydryl content.

Thioglycolate treatment was carried out at 0° in an aqueous solution (10 ml.) containing albumin (5 μmole), thioglycolic acid (1000 μmole) and sodium bicarbonate (1030 μmole). The pH of the solution was between 5.4 and 5.7. After 20 hr., the solution was passed through a column (20 × 0.9 cm.) of mixed bed ion exchange resin at a flow rate of 25 ml. per hour, and the cloudy eluate was lyophilized to give 90–95% recovery of the albumin.

Treatment of the K 0.20 fraction gave a product with 2.0 ± 0.1 mole of sulfhydryl group and containing 73 ± 3% monomer ($S_{20,w}$ = 4.30 ± 0.08 S.), 10 ± 3% dimer and 16 ± 3% heavy component. Treatment of the K 0.57 fraction gave a product with 1.6 ± 0.1 mole of sulfhydryl group and containing 79 ± 3% monomer ($S_{20,w}$ = 4.30 ± 0.08 S.), 10 ± 3% dimer and 10 ± 3% heavy component. Treatment of the mercaptalbumin sample gave a product with 1.6 ± 0.1 mole sulfhydryl group and containing 91 ± 3% monomer and 8 ± 3% dimer.

Distributions patterns in Figs. 6, 7 and 8 were those of the thioglycolate treated albumins in the order described above and the amounts used were 100, 166 and 220 mg., respectively. From tubes 151 to 175 in Fig. 8, 85 mg. was recovered which showed 0.44 ± 0.05 mole of sulfhydryl group and 83 ± 3% monomer and 17 ± 3% dimer.

Discussion

The human plasma albumin prepared by ammonium sulfate precipitation was found to be composed of 95% monomer. When distributed to 342 transfers in the present solvent system, two major bands with K 0.20 ± 0.02 and K 0.57 ± 0.02 were found as illustrated in Fig. 1. These two bands were also obtained with Fraction V albumin. The fraction with K 0.20 did not give any titratable sulfhydryl group and in the ultracentrifuge it behaved as a dimeric albumin, its estimated molecular weight being 137,000. The fraction with K 0.57 was the non-mercaptalbumin monomer.

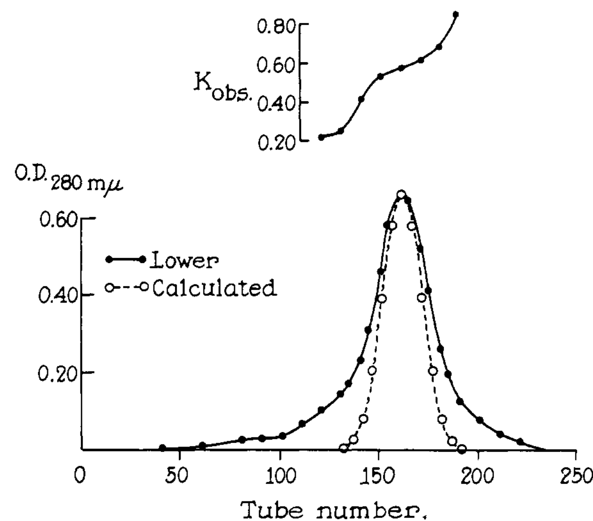


Fig. 8.—Distribution of the thioglycolate treated mercaptalbumin, 402 transfers.

When the dimer fraction was redistributed as in Fig. 2, an excellent agreement between the experimental curve and that calculated for a single solute was obtained.

The redistribution of the fraction with K 0.57 in Fig. 3 clearly did not satisfy the criteria required of a single solute. The broadness of the experimental curve suggested the presence of a number of components in non-mercaptalbumin. This broadening effect did not result from the adsorption of fatty acids since none was found before redistribution. All the albumin samples isolated by distribution proved to be free of fatty acid. It is of interest to note, that to prepare fatty acid free albumin Goodman[5] found it necessary to extract the albumin with a mixture of iso-octane and acetic acid.

Human plasma albumin is known to contain only about one half mole of sulfhydryl group per mole of protein, and the fraction with the sulfhydryl group had been isolated by Hughes[6] through crystallization as the mercuric salt dimer. As the amount of dimer formed in Fig. 1 was reasonably correlated to the amount of mercaptalbumin in the sample, 39% as determined by sulfhydryl group titration, it seemed likely in the present case that the dimer was formed through an oxidative union of two molecules of mercaptalbumin.

The following facts support this supposition. The dimer had no titratable sulfhydryl group and it was not dissociated in 8 M urea. Distribution of the mercaptalbumin gave mainly the band with K 0.20 in Fig. 4, thus proving that the dimer was not formed through the union of a mercaptalbumin molecule and a non-mercaptalbumin molecule. Distribution of the albumin with the low sulfhydryl content, the residual fraction from the mercaptalbumin crystallization process, gave the pattern in Fig. 5. Treatment of the albumin sample with p-chloromercuribenzoate prior to the distribution gave only one band with a K value of 0.57, and after recovery and removal of the mercurial group, the dimer was formed on redistribution.

The results described here have shown definitely the presence of two kinds of albumin in human plasma, mercaptalbumin and non-mercaptalbumin. These were differentiated on the basis that the mercaptalbumin portion was readily oxidized to a dimeric albumin, though the mechanism of this oxidation is not known. It is likely that in this solvent environment the conformation of the mercaptalbumin took a shape to permit the oxidative union of two molecules by the dissolved oxygen. The rate of this dimerization reaction was rapid, and the reaction was essentially complete after 24 hr. If the distribution was started shortly after dissolution of the sample in the solvent system, an incomplete separation of the monomer and dimer bands was usually obtained.

Schmid[7] has reported the isolation of an "electrophoretically homogeneous" human plasma albumin from Fraction VI. Upon distribution of a sample of this albumin provided by Dr. Schmid, only one broad band with a K of 0.57 was obtained, similar to that in Fig. 3.

The dimer fraction could be cleaved by treatment with thioglycolate at pH 5 to yield the monomeric form of albumin as the main product, indicating that the linkage in the dimer molecule was a disulfide bond. Although the reduced albumin contained 2.0 moles of sulfhydryl group, it is believed that the major portion was a mercaptalbumin without the cleavage of any internal disulfide bonds, based on experience with the thioglycolate treated bovine plasma albumin.[2] Distribution of this regenerated mercaptalbumin (Fig. 5) did not show the band with K 0.20; therefore, it seems probable that the sulfhydryl group reappearing in the course of thioglycolate treatment is not the same as the original.

Similar treatment was carried out with the K 0.57 fraction, and the treated material had 1.6 moles of sulhydryl group. On distribution in Fig. 6, it also did not show the band with K 0.2.

Katchalski, Benjamin and Gross[8] have studied the treatment of mercaptalbumin and Fraction V albumin with thioglycolate. They found that treatment between pH 5 to 7 gave only one mole of sulfhydryl group. In the present study, the reaction conditions were nearly identical to those employed by these authors, but the treated albumin samples were usually found to contain one to two moles of sulfhydryl group.

In the presence of thioglycolate, a possible reaction with albumin would be the intramolecular sulfhydryl–disulfide exchange. If there exist different types of mercaptalbumins, it should then be possible to equilibrate the different types of treatment with thioglycolic acid. This was the case with the treated mercaptalbumin, as it has less tendency to form the band with K 0.20 upon distribution in Fig. 8, but upon isolation the fraction with K 0.57 was found to contain 17% dimer and about 0.4 mole of sulfhydryl group.

In the case of the cleavage of the dimer by thioglycolate, it might be reasoned that the direct cleavage of the disulfide linkage connecting the two large albumin spheres would be improbable due to steric hinderance. Probably the cleavage of the dimer was initiated through an attack on a more accessible disulfide bond by thioglycolate and the linkage joining the monomer units was finally broken by a series of sulfhydryl–disulfide exchanges.

The results described here suggest that the two kinds of albumin possess the same amino acid content, perhaps also sequence, and that they differ only in the nature of the sulfhydryl group. This suggestion would be in line with the observed N- and C-terminal amino acid homogeneity of human plasma albumin[9,10] and with the results of our amino acid analysis of the monomer and dimer fractions. The questions which remain to be answered are how the sulfhydryl group is masked in non-mercaptalbumin and whether the sulfhydryl

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group occupies the same position of the peptide chain in both mercaptalbumin and non-mercaptalbumin.

Acknowledgments.—The authors are grateful to Mrs. J. O'Brien and Mr. O. Griffith for their technical assistance.

[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

t-Butyl Esters of Amino Acids and Peptides and their Use in Peptide Synthesis[1]

BY GEORGE W. ANDERSON AND FRANCIS M. CALLAHAN

RECEIVED NOVEMBER 30, 1959

The synthesis of *t*-butyl esters of amino acids and peptides and their use in peptide synthesis is described. The most convenient method was the acid-catalyzed reaction of isobutylene with benzyloxycarbonylamino acids or peptides followed by catalytic hydrogenation to produce the basic esters. Another general method entailed the use of silver salts and *t*-butyl iodide. *t*-Butyl esters are particularly useful in peptide synthesis in that the ester group may be removed by acid catalysis and thus side reactions encountered in alkaline hydrolysis may be avoided. Other advantages arise from the stability of amino acid or peptide esters as free bases, particularly in allowing their storage and use as such. Comparative stabilities to ethyl esters are reported in several examples. The synthesis of several *t*-butyl peptides is reported as well as selective removal of this ester group or of amine-protecting groups when both are present.

In recent years naturally occurring peptides with high biological activities in such diverse fields as antibiotics, bacterial growth factors, hormones, smooth muscle stimulants and pain-producing substances have been isolated. Synthesis of such peptides has not kept up with structure determination largely because present methods are time-consuming and frequently give poor yields.[2] As part of an investigation of synthetic methods, the work reported here was directed toward improvements of carboxyl-protecting groups.

It has been found that *t*-butyl esters of amino acids and peptides have advantages over the customarily used methyl and ethyl esters. Specifically, they are much more stable as the free bases to self-condensation reactions (Table IV) and thus may usually be stored and used as such. This avoids the customary neutralization of hydrohalide salts with bases such as triethylamine during a peptide synthesis, a complicating procedure which adds to the danger of racemization in sensitive cases as well.[3,4] Perhaps more important, the many side reactions of saponification of peptide esters[2] can be avoided since the *t*-butyl group is readily removed by acid catalysis under mild conditions. In comparison to benzyl esters, *t*-butyl esters are much more readily removed by acid catalysis and are not affected by hydrogenation in the presence of palladium or platinum.

Two general procedures for the synthesis of amino acid and peptide *t*-butyl esters have been investigated. In both an amine-protecting group which was subsequently removed was used. In spite of the extra steps necessary it seems likely that the utility of *t*-butyl esters will make this worth while.

The first method entailed the reaction of silver salts of acylamino acids or acylpeptides with *t*-butyl iodide (example 1 in Experimental and Table I). Although this is straightforward, the second method is simpler, gives better yields and is preferable for large-scale reactions. In the latter, acylamino acids or acylpeptides were treated with a large excess of isobutylene in the presence of sulfuric acid or *p*-toluenesulfonic acid as catalyst. The products were isolated by treatment with aqueous alkali (example 2 and Table I). The preferred amine-protecting group in this investigation was benzyloxycarbonyl (carbobenzoxy) but others were used (see experimental).

The benzyloxycarbonyl group was removed by hydrogenation and phosphite salts were prepared for purification and characterization. Since phosphorous acid is weak, danger of cleavage of the *t*-butyl group was avoided (example 3 and Table II). In some cases, phosphite salts were not isolated, but were converted to the free bases (example 4 and Table III).

Several peptides were synthesized from amino acid *t*-butyl esters by the tetraethyl pyrophosphite,[3] dicyclohexylcarbodiimide[5] and *p*-nitrophenyl ester[6] procedures.

Since commonly used amine-protecting groups have varying degrees of sensitivity to removal by acids, selective or simultaneous removal with *t*-butyl esters is possible. Thus refluxing with *p*-toluenesulfonic acid in benzene was used to remove selectively *t*-butyl esters in the presence of benzyloxycarbonyl or trifluoroacetyl groups (examples 6 and 8B). A stronger acid, hydrogen bromide in glacial acetic acid, does not affect phthaloyl groups (example 11) or trifluoroacetyl groups at 10° (example 8A), but it will simultaneously remove benzyloxycarbonyl or *t*-butyloxycarbonyl groups along with *t*-butyl esters (example 15).

The trifluoroacetyl group can be selectively removed from trifluoroacetylpeptide *t*-butyl esters

(1) Presented in part at the 133rd Meeting of the American Chemical Society at San Francisco, Calif., April, 1958. During preparation of this manuscript, a brief note on amino acid *t*-butyl esters appeared (R. W. Roeske, *Chemistry & Industry*, Sept. 5, 1959).

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