mixture was diluted with water. The acidic and neutral materials were separated by the usual extraction procedures. Where the solvent was miscible with water, the reaction mixture was acidified with acetic acid; most of the solvent was then removed under reduced pressure. The residue was processed as described above.

The neutral fraction was chromatographed on alumina (Fisher). Elution with benzene yielded the O-cyanoethylated product (I), mp 104–106°, which was identified by mixture melting point. The acidic material was chromatographed on silica gel (Davison). Unchanged 2-naphthol was eluted with benzene; elution with ether–benzene gave the C-cyanoethylation product (II), mp 140–142°, characterized by mixture melting point. The two cyanoethylation products together with the recovered naphthol accounted for 85–95% of the 2-naphtol employed.[16]

B. Sodium 2-Naphthoxide.—These reactions were carried out as described above in part A except that appropriate quantities of sodium 2-naphthoxide[9d] were substituted for the 2-naphthol and/or base. (1) The equimolar, 24-hr reaction (solvent, yields) showed benzene, 0% I, 70% II; tetrahydrofuran, 1% I, 20% II; acetonitrile, 0% I, 23% II; *t*-butyl alcohol, 0% I, 50% II; water, 0% I, 0% II; (2) catalytic, 24-hr reaction, benzene, 17% I, 5% II; *t*-butyl alcohol, 4% I, 33% II; and, (3) equimolar with added naphthol, 2-hr reaction, benzene, 19% I, 52% II.

Isomerization of β-(2-Naphthoxy)propionitrile (I).[8]—A mixture of 5.0 g (0.025 mole) of the β-cyanoethyl ether (I) and 1.0 g (0.025 mole) of sodium hydroxide in 50 ml of benzene was refluxed for 2 hr under nitrogen. The heterogeneous reaction mixture was diluted with water, and separated into neutral and acidic fractions. Chromatography of the fractions as described above gave the following yields: unchanged I, 10%; II, 4%; 2-naphthol, 84%. The reaction was repeated several times: maximum yield of II, 15%. The reaction time was extended to 24 hr: yield of unchanged I, 5%; II, 35%; 2-naphthol, 54% (average of two runs).

The reaction was also repeated for 24 hr in isopropyl alcohol (yields II, 22%; 2-naphthol, 77%), diglyme (99% naphthol), and methanol (93% naphthol).

Registry No.—I, 14233-72-8; II, 14233-73-9; 2-naphthol, 135-19-3.

(16) Evidence for the formation of any significant amounts of biscyanoethylated naphthol was absent.

The Controlled Synthesis of Peptides in Aqueous Medium. III. Use of Leuchs' Anhydrides in the Synthesis of Dipeptides. Mechanism and Control of Side Reactions[1]

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The successful use of N-carboxy-α-amino acid anhydrides (NCA's) in the controlled synthesis of optically pure dipeptides in aqueous medium is described. High yields of dipeptides were obtained by adding the crystalline NCA directly to an aqueous solution of an amino acid at 0–2° and generally at pH 10.2 with rapid mixing of reactants, thus minimizing side reactions. In the case of relatively insoluble NCA's, rapid mixing enhances the rate of dissolution of the NCA and thereby reduces the opportunity for carbamate exchange, which leads to overreaction (formation of tripeptide) and to inactivation of the starting amino acid. With the more soluble NCA of alanine, rapid mixing was shown to reduce greatly the amount of overreaction brought about by oligomerization of the NCA *via* its anion. The control of pH is important because carbamate stability increases with pH, but at a pH above 10.5 hydrolysis of the NCA becomes an important side reaction and the formation of the NCA anion is increased. This anion is believed to be responsible for the formation of hydantoic acids, a side reaction not previously observed under these conditions. Low temperature favors the desired reaction over side reactions partly because of the change in K_w with temperature. With careful control of reaction conditions, the NCA method permits the rapid synthesis of optically pure peptides with the use of a minimum number of protecting groups. Dipeptides are generally formed in about 90% yield. Examples are given for the isolation of the dipeptides by a variety of methods. ^{14}C-Labeled amino acids were frequently employed for the radiochemical analysis of reaction mixtures.

N-Carboxy-α-amino acid anhydrides (Leuchs' anhydrides, NCA's) have been used widely[2] in polymerization reactions in anhydrous media for the synthesis of homopeptides and the random synthesis of heteropeptides. On the other hand, NCA's have been reported not to possess general utility in the controlled synthesis of heteropeptides.[3,4] About 40 years ago, Wessely described the use of N-carboxy-N-phenylglycine anhydride in the preparation of N-phenylglycylglycine and of N-phenylglycyl-L-tyrosine ethyl ester.[5a] One year later,[5b] the condensation of N-carboxy-DL-phenylalanine anhydride with glycine and with glycylglycine was reported. More recently this NCA was used in the synthesis of phenylalanylphenylalanine.[6] In these experiments the NCA was dissolved in a water-immiscible solvent and allowed to react with an aqueous solution of the amino acid or peptide. In 1957, Bartlett and his collaborators,[7,8] investigating the potential of the method, undertook a study of the reactions of the NCA's of glycine, alanine, α-aminoisobutyric acid, and phenylalanine with aqueous solutions of amino acids, glycylglycine, and triglycine. They concluded that the reaction cannot be controlled adequately to provide a useful general method for peptide synthesis in water.

(1) (a) A preliminary report dealing with certain aspects of this work has appeared: R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob, and R. Hirschmann, *J. Am. Chem. Soc.*, **88**, 3163 (1966); (b) E. F. Schoenewaldt, R. G. Denkewalter, H. Joshua, R. G. Strachan, H. Schwam, D. F. Veber, W. J. Paleveda, Jr., and R. Hirschmann, presented in part at the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966.

(2) For recent reviews, see V. V. Korshak, S. V. Rogozhin, V. A. Davankov, Yu A. Davidovich, and T. A. Makarova, *Russ. Chem. Rev.*, 329 (1965). M. Szwarc, *Advan. Polymer Sci.*, **4**, 1 (1965).

(3) M. Brenner and W. Hofer, *Helv. Chim. Acta*, **44**, 1798 (1961).

(4) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1961, p 860, and M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, Inc., New York, N. Y., 1966, provide recent reviews.

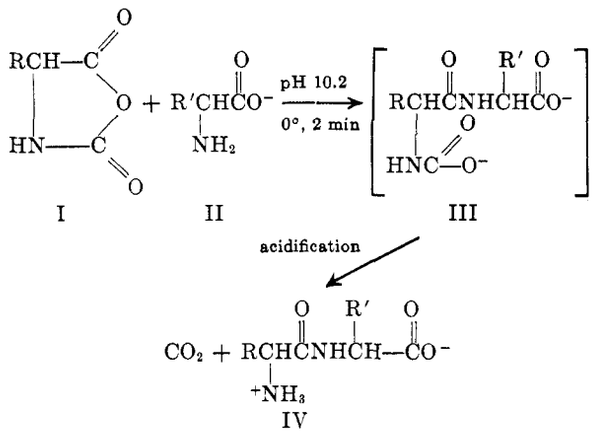
(5) (a) F. Wessely, *Z. Physiol. Chem.*, **146**, 72 (1925); (b) F. Sigmund and F. Wesseley, *ibid.*, **147**, 91 (1926).

(6) F. Wessely, K. Schogl, and G. Korger, *Monatsh. Chem.*, **82**, 672 (1951).

(7) P. D. Bartlett and R. H. Jones, *J. Am. Chem. Soc.*, **79**, 2153 (1957).

(8) P. D. Bartlett and D. C. Dittmer, *ibid.*, **79**, 2159 (1957).

SCHEME I



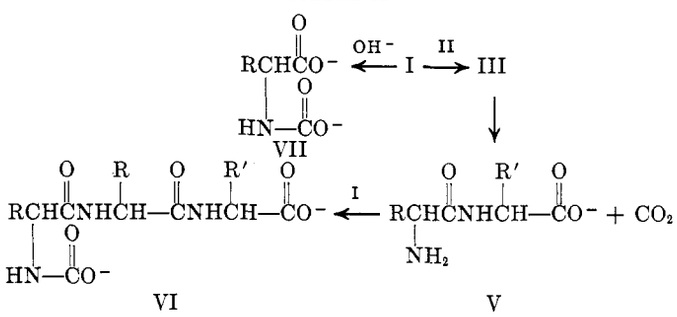
I II III IV

wanted side reactions as a function of the experimental conditions. Subsequent publications will describe the preparation of novel NCA's[1] and the application of the method to the sequential synthesis of higher peptides without isolation of intermediates.

Our method for the synthesis of a dipeptide is depicted in Scheme I. The crystalline NCA is added with rapid mixing to a solution of an amino acid anion at 0° at pH 10.2. After 2 min the resulting bisanion of the dipeptide carbanate III is smoothly decarboxylated by acidification to give the unprotected dipeptide.

Bartlett and Jones[7] were primarily concerned about two side reactions which can occur in the preparation of dipeptides in dilute base by the NCA method: (1) the premature loss of carbon dioxide from the dipeptide carbamate III leading to the formation of dipeptide

SCHEME II



VII VI V

In consonance with this view, Grant and Alburn[9] suggested in a recent paper that controlled peptide synthesis in water by the NCA method is useful only when the reacting amine is a much weaker base than the amine resulting from the synthesis, as in the preparation of alanyl-6-aminopenicillanic acid from 6-aminopencillanic acid and the NCA of alanine in water at pH 5. Substantially the same argument had been advanced earlier by Brenner and Hofer.[3]

Greater success had been attained in controlling the reaction of amino acid esters with NCA's in anhydrous media.[10] The most successful application of the NCA method was developed by Bailey,[11] who allowed amino acid and peptide esters to condense with NCA's at very low temperatures in organic solvents.[12] Even Bailey's procedure has been utilized only in rare instances.[13-15]

In this paper we describe the preparation of dipeptides by the condensation, in water, of known NCA's with representative amino acids and we discuss unanion V (Scheme II) which can compete with II for NCA to give the tripeptide carbamate VI (we refer to the unwanted formation of VI as overreaction) and (2) the hydrolysis of NCA by hydroxyl ion to yield the amino acid carbamate VII (Scheme II). Bartlett, *et al.*,[7,8] determined yields of the desired dipeptide by measuring the evolution of carbon dioxide from the crude reaction mixture in the Van Slyke ninhydrin assay. In this assay carbon dioxide is evolved from α-amino acids but not from peptides and thus this procedure distinguishes between amino acids and peptides but it does not distinguish between unchanged amino acid (II) and amino acid formed *via* hydrolysis of NCA (→ VII) nor between di- and higher peptides. Consequently the product yield could not be precisely determined. For the preparation of glycylglycine at pH 10.61, the yield was calculated to be either 96 or 83%. The lower figures were thought more likely to represent the true yield. In the formation of phenylalanylglycine,[8] the yield was found to be consistently below 80%. It was concluded,[7,8] therefore, that the product carbamate III was not sufficiently stable and that the desired reaction was not fast enough (relative to "a set of protonation or carboxylation equilibria")[8] to permit an adequate control of the reaction.

Although we observed additional side reactions which can be detrimental to the yield, we have found that a narrow range of experimental conditions exists in which the NCA's can be generally and usefully employed in aqueous solution in the synthesis of peptides. These conditions require careful control of certain reaction parameters.

Requirements for Control of Reaction. 1. Rapid Mixing.—We have found rapid mixing to be very important for adequate control of the reaction. With conventional stirring, the rate of dissolution of

(9) N. H. Grant and H. E. Alburn, *J. Am. Chem. Soc.*, **86**, 3870 (1964).
(10) M. Hunt and V. du Vigneaud, *J. Biol. Chem.*, **124**, 699 (1938).
(11) J. Leggett Bailey, *J. Chem. Soc.*, 3461 (1950).
(12) In view of the results of Bailey[11] and of Bartlett,[7,8] we studied at first the use of NCA's at low temperature in mixed solvent systems under a variety of conditions. Treatment of glycine with 2 equiv of the NCA of alanine in the presence of 3 mmoles of triethylamine in aqueous DMF at −40° for 5 min afforded, after decomposition of excess NCA with dimethylamine, an 88% yield of crystalline L-alanylglycine. A single recrystallization gave the analytically pure product with a recovery of 81%. When this method was applied to the preparation of other simple dipeptides or of tripeptides, the results were unsatisfactory. For instance, the NCA of phenylalanine reacted only very slowly with glycine under these conditions and this was also true of the reaction of the NCA of alanine with either leucine or with alanylglycine. Better results were obtained in the preparation of phenylalanylglycine at −20°, but even at this temperature the preparation of phenylalanylalanine gave a poor yield. Attempts to find a procedure of broader utility by changing temperature, molar ratios of reactants, or the base used to stabilize the product carbamate were unsuccessful.
(13) J. Rudinger and F. Sorm, *Coll. Czech. Chem. Commun.*, **16**, 214 (1951).
(14) J. Honzyl and J. Rudinger, *ibid.*, **20**, 1190 (1955).
(15) W. Langenback and P. Kresse, *J. Prakt. Chem.*, **4**, 261 (1955).

the NCA of phenylalanine was too slow to permit us to take full advantage of the very rapid rate of the condensation reaction. We found that the opportunity for the unwanted carbamate exchange[7,8] (eq 1 and 2)

$$(H_3O)^+ + \text{III} \rightleftharpoons \text{V} + CO_2 \quad (1)$$

$$CO_2 + \text{II} \rightleftharpoons \text{carbamate of II} + (H_3O)^+ \quad (2)$$

was minimized, however, when the reaction was carried out in a Waring Blendor which provides a greater rate of dissolution of the NCA. Suppression of the carbamate exchange by rapid mixing reduces the opportunity for overreaction and it achieves a reduction in the inactivation of II (eq 2) by carbon dioxide. The inactivation of starting material will be discussed in greater detail below.

Addition of a solution of the NCA in a water-miscible solvent might be expected to be a satisfactory substitute for high-speed mixing. However, we have consistently found that the use of solutions of the NCA in even small amounts of solvents such as acetone and dioxane gave lower yields than the introduction of an NCA as a solid.

High-speed mixing was found to be important also with more soluble NCA's although for a different reason. Figure 1 dramatically demonstrates the effect of mixing on the control attainable in the preparation of alanylphenylalanine. It is known that the stability of amino acid or peptide carbamates in water is increased by raising the pH of the solution. As expected, therefore, more of the overreacted product (L-alanyl-L-alanyl-L-phenylalanine) was formed at pH 9 than at pH 10 both by high-speed (Blendor) and by conventional stirring. Thus with conventional mixing, small amounts of even L-alanyl-L-alanyl-L-alanyl-L-phenylalanine were detectable by tlc in the pH 9 reactions, but only a faint trace was seen at pH 10.0. Unexpectedly, however, we observed an *increase* in the formation of L-alanyl-L-alanyl-L-alanyl-L-phenylalanine when the *magnetically* stirred reaction was carried out at a still higher pH (10.5), and at pH 11 we detected not only a further increase in the amount of the latter, but also a significant amount of the next higher homolog.

Carbamate instability, which had been discussed by Bartlett,[7] does not explain an increase in overreaction at pH 10.5 and 11. We believe that this tendency toward overreaction at high pH in the magnetically stirred reactions can be explained by assuming that abstraction of the N proton from the NCA by base can occur under aqueous conditions and that oligomerization of NCA, propagated *via* the NCA anion B,[16] leads to C and F and ultimately to the tetrapeptide G (Scheme III). If an N-carboxy anhydride such as A or C (Scheme III, R = CH_3) reacts with the NCA anion B (polymerization) rather than with the amino acid (D) (peptide synthesis) primarily because of a local depletion of D and because of a high concentration of NCA at the site of the dissolving crystal of the latter, then high-speed mixing should inhibit the polymerization of the NCA as well as reduce the extent of overreaction. This proved indeed to be the case. As may be seen in Figure 1, we were able to prevent tetrapeptide formation at pH 10.5 by using a Waring Blendor. Even at pH 11, the amounts of tri- and tetrapeptides were strikingly

(16) Recent studies by M. Goodman and J. Hutchison (*J. Am. Chem. Soc.*, **87**, 3524 (1965); **88**, 3627 (1966)) have shown convincingly that in anhydrous media the propagation step in a strong base initiated polymerization involves an NCA anion.

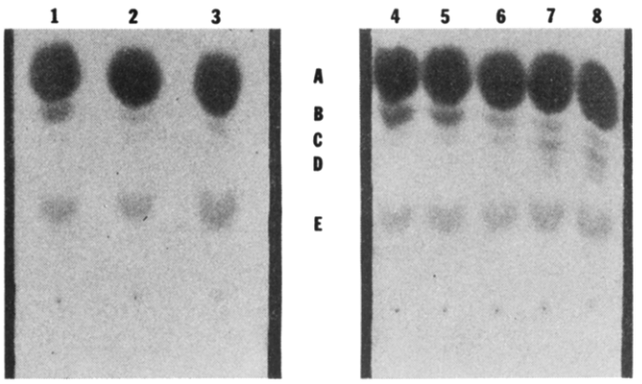
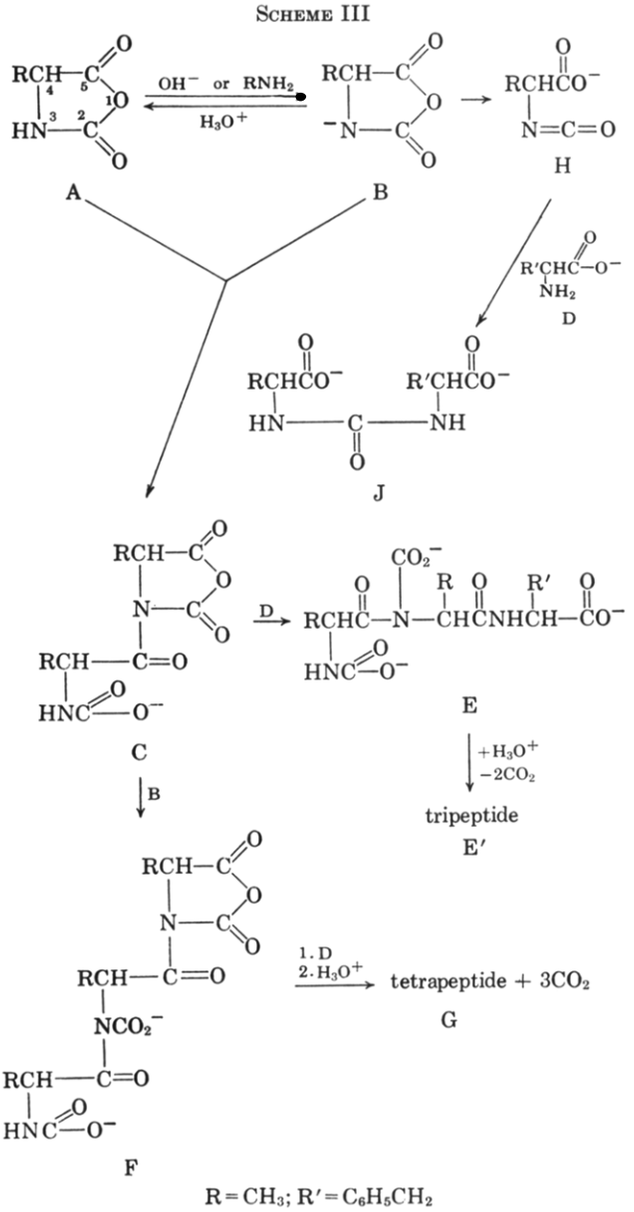


Figure 1.—Reaction of phenylalanine with the NCA of alanine: runs 1–3, pH 9.0, 10, 11.0, respectively, Waring Blendor; runs 4–8, pH 9.0, 9.5, 10.0, 10.5, 11.0, respectively, magnetic stirring; A, phe + ala-phe; B, ala-ala-phe; C, ala-ala-ala-phe; D, ala-ala-ala-ala-phe; E, ala.

SCHEME III



diminished by comparison with the reactions which had been magnetically stirred. At pH 10, in a Blendor, very little overreaction occurred. A quantitative study (Blendor), using radioactive phenylalanine, indicated the formation of only about 3% of L-alanyl-L-alanyl-L-

phenylalanine and <1% of the tetrapeptide at pH 10.2. We conclude therefore that high-speed mixing minimizes the formation of C (Scheme III, R = CH_3) and that overreaction is thereby reduced. For these reasons we believe that high-speed mixing provides added control in NCA peptide synthesis both with relatively soluble and relatively insoluble anhydrides.

The condensation of an amino acid with a 5% excess of NCA left about 3% of the original amino acid. It was generally not possible to achieve complete disappearance of the amino acids by increasing the molar excess of the NCA. We postulate that the remaining amino acid had become inactivated by conversion to the amino acid carbamate. In accord with this view, it was possible to bring about the condensation of the remaining amino acid with NCA only after lowering the pH, sweeping with nitrogen, and readjusting the pH to 10.2 prior to the addition of more NCA, a procedure which would have decarboxylated amino acid carbamates.[17] The inactivating carbon dioxide may arise from carbamate exchange between the amino acid and either the peptide carbamate (eq 1 and 2) or the carbamate VII (Scheme II) derived from the NCA itself.[18]

It is possible, however, that dimerization or oligomerization[19] of NCA *via* the anion B (Scheme III) may also be a source of the inactivating carbon dioxide. We obtained evidence for this by comparing the NCA of proline, which cannot form an N anion, with the NCA of alanine at a pH high enough (12.5) to ensure the stability of carbamates such as III or VII (Scheme II) (but not of the carbamates of amides[20a] which are present in E or F (Scheme III)). It was found that phenylalanine could be caused to react completely with the NCA of proline at this pH, as long as a sufficiently large excess of NCA (four- to fivefold) was employed, to compensate for the great extent of hydrolysis of the NCA brought about by the high hydroxyl ion concentration. On the other hand, 4–5% of amino acid remained when the phenylalanine was allowed to react even with a tenfold excess of the NCA of alanine under otherwise comparable conditions. Since proline carbamate is less stable then alanine carbamate at a given pH, these results strongly suggest oligomerization of the NCA of alanine, and not decarboxylation of alanine carbamate, to be the source of the inactivating carbon dioxide at high pH.[20b]

In summary, high-speed mixing minimizes side reactions caused by slow dissolution of some NCA's and by polymerization of others. Either event can lead to overreaction and to inactivation of the amino acid.

2. Control of pH.—The control of pH during the condensation step is also critical. The importance of pH control has already been briefly referred to above and the effect of changes in pH is illustrated in Figure 1. We have found it convenient to carry out the condensation reactions in 0.4 *M* borate buffer (5–10 ml/millimole of amino acid) in the presence of an electrode which responds rapidly to changes in pH. The pH was easily maintained during the condensation reactions within 0.05–0.1 units by the addition of aqueous alkali. We now generally carry out reactions at pH 10.2 (0°) which is optimal for most reactions because of the following considerations. Carbamate stability (which prevents overreaction) is favored by high pH. On the other hand, a pH above 10.5, for reactions involving amino acids, or above 10.2 for reactions involving peptides, is not desirable because at higher pH the increased hydroxyl ion concentration increases hydrolysis at the expense of aminolysis and it also increases the formation of the NCA anion. High concentrations of the latter can bring about NCA oligomerization.

The preferred pH (10.2–10.5 for amino acids, 10.2 for peptides) represents therefore the pH at which overreaction—either *via* acid-catalyzed decarboxylation of product carbamates or *via* base-catalyzed oligomerization of the NCA—is minimized. This agrees with the findings of Bartlett and his associates,[7,8] who obtained higher yields (Van Slyke ninhydrin assay) at pH 10 than at pH 9 or 11.

An additional side reaction makes it undesirable to carry out condensation reactions at a pH above 10.2. We have obtained evidence for the formation of hydantoic acids (J, Scheme III). The formation of these compounds from NCA's under these weakly basic conditions in aqueous medium has not, to our knowledge, been previously reported. On the other hand, the formation of urethans and urea derivatives from the reaction of NCA's with alkoxide anions and with amines under anhydrous conditions has been known for some time.[21] Formally these products may be thought to arise from the addition of the nucleophile to carbon atom 2 (A, Scheme III) of the NCA. More recent evidence[2,22] has strongly suggested that these anomalous products are formed *via* the isocyanate derived from the NCA anion. We believe that hydantoic acid formation under our experimental conditions arises largely, if not exclusively, *via* the isocyanate (H, Scheme III) for the following reasons.

(1) Hydantoic acid formation was found to increase with pH. In the formation of phenylalanylarginine we obtained 1.3% of the hydantoic acid at pH 9.5, 2.8% at pH 10, 5.6% at pH 10.5, and 22% at pH 12. These results are to be expected if urea formation arises as shown in Scheme III. On the other hand, we see no reason why high pH should favor the direct attack of the nucleophile on carbon 2 of the NCA.

(2) We have seen no evidence for hydantoic acid formation with the NCA of proline which cannot form an isocyanate. The treatment of radioactive proline

(17) This operation has, of course, no practical utility, since acidification not only reactivated the residual amino acid, but also effected decarboxylation of the peptide carbamates.

(18) Since the rate of decarboxylation of a carbamate should be related to its basicity, peptide carbamates are expected to be more stable than amino acid carbamates.

(19) The polymerization of NCA's under aqueous alkaline conditions has been reported;[6] see, *e.g.*, F. Wessely, *Z. Physiol. Chem.*, **146**, 72 (1925); R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 727 (1953).

(20) (a) Amide carbamates can be envisaged as decarboxylating *via* direct production of a resonance stabilized amide anion, a process which would be independent of pH. (b) That no inactivation occurred when the NCA of proline was used at high pH was borne out also by a study of the reaction of this NCA with phenylalaninamide; this amide, conveniently prepared from the NCA, was chosen because it is considerably less reactive than phenylalanine and had given only an 83% yield of prolylphenylalaninamide at pH 10.2 with a 10% excess of NCA (radiochemical analysis). With the NCA of proline at pH 12.5, only 4.4% of unchanged phenylalaninamide remained after the addition of 10 equiv of NCA and only 2–3% after the addition of 15 equiv. It may be assumed that a still larger excess of the NCA of proline would have further reduced the amount of residual amide, indicating absence of inactivation with this NCA at high pH.

(21) M. Sela and A. Berger, *ibid.*, **75**, 6350 (1963); A. Berger, M. Sela, and E. Katchalski, *Anal. Chem.*, **25**, 1554 (1953).

(22) K. D. Kopple, *J. Am. Chem. Soc.*, **79**, 6442 (1957).

with the NCA of phenylalanine at pH 13.5 resulted in the formation of the hydantoic acid as the major product (72% yield). In striking contrast, none of this hydantoic acid was formed by the reaction of the NCA of proline with ^{14}C-phenylalanine at this pH.

(3) It has recently been shown by Dittert and by Christensen[23] that the mechanism of the alkaline hydrolysis of *p*-nitrophenyl N-methylcarbamate involves abstraction of the N–H proton and isocyanate formation, rather than nucleophilic attack upon the carbamate carbonyl group.

Hydantoic acid ("urea") formation is not immediately apparent by tlc, since these side products do not give a positive ninhydrin test nor are they detectable in the Van Slyke CO_2 assay employed by Bartlett and Jones.[8] Urea formation could, however, be readily demonstrated through the use of radioactive amino acids or NCA's as described above because the hydantoic acids derived from difunctional amino acids are dibasic acids which separated fron the dipeptides on tlc or on electrophoresis at alkaline pH. The hydantoic acid derived from proline and phenylalanine NCA was obtained as a crystalline solid. We also prepared a symmetrical hydantoic acid by allowing the NCA of phenylalanine to react with ^{14}C-phenylalanine at pH 13. The resulting crystalline hydantoic acid was obtained in about 60% yield. It was characterized by elemental analysis, equivalent weight determination, and conversion to the hydantoin. The hydantoic acid also exhibited the expected spectral characteristics and its specific activity was consistent with the mechanism of Scheme III (formation of J). We have also isolated the urea by-product from the reaction of the NCA of phenylalanine with arginine. This hydantoic acid could not be obtained crystalline, but the spectral characteristics, electrophoretic behavior, and Spinco amino acid analysis were consistent with the assigned structure. Isocyanate formation[24,25] constitutes, therefore, an additional reason why the reactions should not be carried out at pH >10.5 even though carbamate stability increased with pH.[26]

3. Temperature Control.—Low temperatures (0–2°) constitute another requirement for the successful use of NCA's in peptide synthesis. It was already recognized by Siegfried[27a] in 1905 that amino acid carbamates are stable in water at 0° but that they lose carbon dioxide at room temperature. Bailey,[11] using anhydrous solvents, effected condensation reactions well below 0°. Low temperature minimizes carbamate exchange and it also favors the aminolysis reaction relative to the competitive hydrolysis of the NCA and to the competitive formation of NCA anion, partly because, at a given pH, the hydroxyl ion concentration is lower at 0° than at room temperature by a factor of about 10 owing to a change in K_w, whereas the concentration of the amino acid anion is reduced by a much smaller factor.[27b] Since the Waring Blendor generates appreciable heat, external cooling is required to maintain the temperature between 0 and 2°. Addition of ice to the reaction mixture is less satisfactory for controlling temperature, because the ice brings about dilution of the reaction mixture. It is apparent that at given pH, dilution decreases the concentration of the amino acid but not that of hydroxyl ions.

Synthesis of Dipeptides.—When the above optimal conditions are employed, the NCA method permits the rapid formation of dipeptides in good yield. The extraordinary rapidity of the method—which is particularly useful in the sequential preparation of higher peptides without isolation of intermediates[1]—results partly from the great reactivity of the anhydride but primarily from the fact that in the NCA the amino protecting group is incorporated into the acid activating group. After the condensation reaction is completed, the protecting group may be removed almost instantly by acidification.

An important feature of the use of NCA's in the controlled synthesis of peptides is the lack of racemization observed with these reagents. Diastereoisomeric dipeptides are often separable by tlc. (A striking example is the difference in the R_f of L-leucyl-L-phenylalanine and of D-leucyl-L-phenylalanine.) In none of our reactions has tlc revealed any evidence of racemization. The same conclusion was reached from numerous cleavage experiments with leucine aminopeptidase. In addition, absence of racemization was demonstrated on the measurements of tritium incorporation during peptide syntheses. The results, which have been briefly presented,[1] will be discussed fully in a separate paper.

An important advantage of the aqueous reaction medium was found to be the fact that, with the exception of the ϵ-amino group of lysine and the sulfhydryl group of cysteine, polyfunctional amino acids used as the nucleophile did not require protection of the third functionality. Thus the guanidino group of arginine was adequately protected by protonation at pH 10, and the heterocyclic nitrogen of histidine, tryptophan, the hydroxyl group of serine, threonine, and tyrosine as well as the ω-carboxy groups of aspartic and glutamic acids, and the amide groups of asparagine and glutamine did not require protection. In the preparation of higher peptides, it may prove to be advisable to protect the phenolic hydroxyl of tyrosine.

When this work was begun, NCA's had been described which were useful for the addition of 13 of the 20 important amino acids. Three of these NCA's contained protecting groups for additional functional groups: the NCA's of ϵ-Cbx-lysine, S-benzylcysteine, and im-N-benzylhistidine. We have used these 13 NCA's and the 20 amino acids in the synthesis of peptides. In a separate paper[1] we will show that no protection is required for the third functionality of the NCA's of aspartic and glutamic acids, asparagine, glutamine, and arginine. Finally, contrary to a widely held view, we have found NCA's, with the exception of

(23) L. W. Dittert, Ph.D., Dissertation, University of Wisconsin, 1961; *Dissertation Abstr.*, **22**, 1837 (1961); I. Christensen, *Acta Chem. Scand.*, **18**, 904 (1964); see also M. L. Bender and R. B. Homer, *J. Org. Chem.*, **30**, 3975 (1965).

(24) The amino acid formed by "hydrolysis of the NCA" may, of course, also arise *via* the isocyanate, especially at higher pH.

(25) We have found that the NCA of glycine is atypical in that it has an unusual propensity for urea formation. For example, in the reaction of phenylalanine with the NCA of glycine at pH 10.2, crude reaction mixtures contained more than 20% of the urea. The extent of urea formation in the reaction of peptides with this NCA has been found to be variable. In striking contrast to the NCA of glycine, the NCA of alanine gave no urea with ^{14}C-labeled phenylalanine at pH 10.2.

(26) It should be pointed out that the appearance of small amounts of urea in a sequential synthesis of the higher peptides is not particularly troublesome since it cannot react with NCA in a subsequent step and is readily separated from the desired ultimate product.

(27) (a) M. Siegfried, *Z. Physiol. Chem.*, **44**, 85 (1905); (b) see ref 4, Vol. I, Chapter 4.

TABLE I

DIPEPTIDES PREPARED BY THE NCA METHOD IN ADDITION TO THOSE DESCRIBED IN THE EXPERIMENTAL SECTION

	Glycine	Alanine	Valine	Leucine	Isoleucine	Methionine	Phenylalanine	Tyrosine	Tryptophane	Proline	ε-Cbz-lysine	S-Bz-cysteine	Histidine	Serine	Threonine	Aspartic Acid	Glutamic Acid	Asparagine	Glutamine	Arginine
Glycyl-[c]							>98					>98								
Alanyl-	>98[a]	*		*	*		>96	*	*	*			*		*	*	*	*	*	*
Valyl-	>98	*					98													
Leucyl-				>98			*													*
Isoleucyl-	>98								96											
Methionyl-	*[b]	*																		
Phenylalanyl-	>98			*	*		*					>98	90	>98						>98
Tyrosyl-	>98	96										93	87	88						88
Tryptophanyl-	>98			>98		93														
Prolyl-			96				>98			>98										*
ε-Cbz-lysyl-	96						90				70									
S-Bz-cysteinyl-	93	90					93					>95			96					
im-N-Bz-histidyl-							96							96						

[a] Numbers denote "disappearance yields" (see text). [b] Asterisks indicate that the disappearance yield was not determined. [c] See ref 25.

those of proline and glycine, perfectly stable for several months when stored in a Dry Ice chest with protection from moisture.

We have relied extensively on thin layer chromatography, electrophoresis, and paper-strip chromatography to determine reaction yields and to identify by-products. We found it convenient to estimate the amount of starting material, present at the end of the reaction, by matching the intensity of the ninhydrin color in the starting material zone after tlc against that of suitable standards. Excellent agreement was obtained when the values thus obtained were compared with those derived from the radiochemical analysis of reactions involving ^{14}C-labeled amino acids. Using a 5–10% excess of the NCA, the yields based on the disappearance of starting amino acid were often of the order of 97% in the synthesis of dipeptides. In view of the fact that overreaction and hydantoic acid formation do occur, it is clear that these "disappearance yields" represent the upper limit of the true yield. Nevertheless, we believe that all reactions have given adequate yields and that dipeptides are generally formed in about 90% yield. The only exception appears to be the NCA of glycine.[25] It has been possible to carry out the reactions on a 200-mmole scale and a further scale-up should be readily feasible.

In addition to the representative syntheses described in the Experimental Section, we have carried out many additional condensation reactions in which the syntheses were evaluated by paper-strip or thin layer chromatography, but the products were not isolated. In these cases the structure of the product was confirmed by comparison of the R_f with that of an authentic specimen. These syntheses are summarized in Table I in order to provide an indication of the scope of the reaction. When disappearance yields had been determined, these are given in the table. An asterisk is used for those syntheses for which the disappearance yield was not determined.

Isolation of Products.—Some dipeptide zwitterions are relatively insoluble in water and such compounds often began to crystallize during the acidification of the reaction mixture. When this was the case, the product could be removed by filtration, often in pure crystalline form, a few minutes after the beginning of the experiment. A variety of other procedures was employed to effect separation of water-soluble peptides from inorganic compounds and these are illustrated in the Experimental Section. It was, for example, often possible to obtain the dipeptides substantially free of alkali sulfate and other inorganic material by extracting the lyophilized reaction mixture with methanol. The dipeptides could generally be obtained in pure form by crystallization of the methanol extracts. In one instance, a dipeptide was purified by countercurrent distributions. Alternatively, peptides composed of at least one aromatic amino acid could be separated from inorganic and nonaromatic material through the use of a carbon column which retards the aromatic compound. It was nearly always possible to effect separation of the inorganic compounds by absorbing the dipeptides on a suitable ion-exchange resin. The dipeptides could then be eluted with a solution of aqueous ammonium hydroxide. In the syntheses of a few water-soluble dipeptides, the pH was controlled titrimetrically by the con-

TABLE II

EXPERIMENTAL DETAILS FOR THE CONDENSATION STEPS

Compound	Amino acid, mmoles	Vol, ml	Borate buffer, *M*	Excess of NCA, %	pH range	Disappearance yield, %
L-Valyl-L-serine	6	40	1.0	5	10.2–10.3	98
L-Leucyl-L-valine	4	40	1.0	5	10–10.2	
L-Alanyl-L-leucine	4	200	0.5	20	10[e]	
L-Phenylalanyl-L-leucine	8	20	0.45	5	10.4–10.5	
Glycyl-L-phenylalanine	5	45	1.0	10	10.2	
L-Tyrosyl-L-serine	10	125	1.0	5	10	
L-Alanyl-L-serine[a]	5	250	0.5	0	10[e]	
L-Tryptophanyl-L-leucine	2.82	25	0.5	40	10.8[f]	99
L-Prolyl-L-phenylalanine	10	350	0.5	20[d]	10.2[c]	
L-Isoleucyl-ε-*t*-Boc-L-lysine L-Phenylalanyl-L-arginine[b]	4	40	1.0	10	10.15–10.2	98
L-Methionyl-L-tyrosine	2	20	1.0	10	10.2–10.6	80
L-Alanyl-L-phenylalanine	20	200	1.0	10	10.0	98
L-Isoleucyl-L-tryptophan	10	100	1.0	5	10.1–10.2	97
L-Valyl-L-histidine	2[c]	20	1.0	5	10.1–10.2	95
L-Leucyl-L-phenylalanine	5	25	0.45	5	10.1–10.5	

[a] Magnetic stirring. [b] See experimental details below. [c] The histidine contained 47 μcuries of the ^{14}C-labeled amino acid. [d] NCA dissolved in 20 ml of acetone. [e] No base was added during the condensation reaction. [f] Three-minute blending.

current addition of NCA and of an aqueous solution of barium hydroxide in the absence of buffer. In this case, acidification with sulfuric acid permitted the simultaneous decarboxylation of the carbamates and the precipitation of barium as the sulfate.

It should be noted that the study described herein is primarily concerned with the elucidation of the mechanism of the side reactions and the development of conditions which minimize side reactions. Generally we did not attempt to find optimal procedures for the isolation of the product dipeptides. The NCA method is particularly useful in the sequential synthesis of higher peptides, which reduces the number of the required isolation steps. The syntheses of higher peptides will be the subject of a manuscript which is in preparation.

Experimental Section

Peptide Synthesis. General Method.—Reactions were carried out in a metallic Waring Blendor which had been modified to permit external cooling of the reaction mixture. The Blendor was fitted with a thermometer and with an A. H. Thomas combination electrode, no. 4858-L60 (0.25-in. diameter, 6 in. long). It was considered desirable but not essential to protect the alkaline solution of the amino acid from carbon dioxide with an atmosphere of nitrogen. With the relatively soluble NCA's (proline, glycine, and alanine), we had initially carried out condensation reactions in magnetically or mechanically stirred beakers or round-bottomed flasks. However, we now prefer to use the Blendor in all reactions. Amino acids were dissolved in an approximately 0.4 *M* solution of sodium borate (pH 10) or in a 1 *M* solution of potassium borate at the same pH. Alternatively, the amino acid was dissolved in a 0.45 *M* solution of boric acid and the pH was then adjusted with a 50% aqueous solution of sodium hydroxide in an atmosphere of nitrogen. About 5–10 ml of buffer was used per mmole of amino acid. The pH meter (Leeds & Northrup, Model 7401) was standardized at room temperature, but the final adjustment of the pH of the solution of the amino acid to 10.2 was carried out at 0° just prior to the addition of the NCA. One drop of capryl alcohol was generally added to minimize foaming and a 0.1-ml aliquot was withdrawn for the preparation of appropriate dilution standards for the estimation of the disappearance of starting material. The anhydride was then added with vigorous blending, generally all at once or over a period of 15–30 seconds. Alkali was added either above or below the surface of the reaction mixture from a microliter syringe as required to maintain the pH at 10.2. The concentration of the alkali in the microliter syringe varied with the scale of the experiment. For syntheses on a 2-mmole scale, 5 *N* KOH was a satisfactory titrant, whereas on a 20–40-mmole scale a saturated solution of alkali was added from a pipet. The temperature of the reaction mixture was maintained between 0 and 2° during the reaction by external cooling. The reactions were generally completed within less than 2 min as judged by the results and by the cessation of base requirements. Longer periods were required with the NCA of tryptophan. The reaction volume was recorded at the end of the condensation step and an aliquot was withdrawn for evaluation of the experiment by chromatography (generally tlc) and sometimes by electrophoresis.

Some of the syntheses described below were carried out before optimal conditions had been worked out. Therefore these reactions may differ in certain details from the general procedure outlined above.

Table II lists the experimental details for the syntheses of those dipeptides which have been isolated. The isolation and characterization of these crystalline compounds is described below. The products moved as a single component in at least one tlc system which was known to separate the dipeptide from the component amino acids and other relevant impurities.

Valylserine.—The pH of the solution was adjusted to 4.5 with concentrated sulfuric acid and the solution was freeze dried. The residue was extracted four times with 50-ml portions of methanol. The solvent was distilled *in vacuo* and the residue was dissolved in 20 ml of water and crystallized by the addition of 100 ml of ethanol. The product (450 mg) was removed by filtration and the mother liquor was treated with an additional 75 ml of ethanol to afford a total of 800 mg (65% isolated yield) of dipeptide, $[\alpha]_{589}$ +33.7° (*c* 1, water).

Anal. Calcd for $C_8H_{16}N_2O_4$: C, 47.05; H, 7.90; N, 13.72. Found: C, 46.78; H, 7.78; N, 13.68.

Leucylvaline.—The solution was acidified to pH 3.5 and freeze dried; the residual solid was extracted with seven 35-ml portions of methanol. The solution was concentrated on a steam bath in a current of nitrogen to the cloud point. Addition of 200 ml of ethyl ether gave 700 mg (67% yield) of product, $[\alpha]_{589}$ +17.5° (*c* 1, 1 *N* HCl).

Anal. Calcd for $C_{11}H_{22}O_3N_2 \cdot CH_3OH$: C, 54.94; H, 9.99. Found: C, 54.92; H, 9.55.

After acid hydrolysis, the amino acid ratio was $Leu_{1.00}$ $Val_{0.98}$.

Alanylleucine.—The pH of the solution was adjusted to 5.0 with 2.5 *N* hydrochloric acid and the peptide was adsorbed on a Dowex 50-W ion-exchange column (100–200 mesh) on the acid cycle. The column was washed with 100 ml of water at a flow rate of 4 ml/min and the product was eluted with an aqueous solution of triethylamine (pH 11.5). The fractions giving a positive ninhydrin test were combined and freeze dried. The residue was dissolved in 50 ml of hot methanol and the solution was filtered and concentrated to a volume of 20 ml. Slow addition of ether gave 520 mg of dipeptide (65% isolated yield), $[\alpha]_{589}$ −17.1° (*c* 1.9, water) (lit.[28a] $[\alpha]_D$ −16.8°, (*c* 5, water)).

(28) (a) W. J. Polgase and E. L. Smith, *J. Am. Chem. Soc.*, **71**, 3081 (1949); (b) R. W. Holley and A. D. Holley, *ibid.*, **74**, 3069 (1952).

Phenylalanylleucine.[28b]—Tlc (in butanol–acetic acid–water (10:1:3)) showed the product (R_f 0.62) to be completely free of the isomeric DL dipeptide (R_f 0.46). Traces of insolubles were removed by filtration and the filtrate was acidified to pH 5.5 with a 50% solution of sulfuric acid to give 1.76 g of dipeptide as a hydrate which was single spot by tlc in the above system and in ethyl acetate–pyridine–acetic acid–water (10:5:1:3). The residual water was distilled with methanol to afford 1.56 g of product.

Anal. Calcd for $C_{15}H_{22}N_2O_3$: C, 64.72; H, 7.97; N, 10.06. Found: C, 64.32; H, 7.77; N, 9.91.

After acid hydrolysis, the amino acid ratio was $Phe_{1.03}Leu_{1.00}$. The filtrate from the original precipitation was freeze dried and the residue was extracted with a mixture of chloroform–methanol–18% aqueous ammonium hydroxide (60:30:10). The solvents were removed *in vacuo* and the residue was redissolved in 30 ml of the same solvent system, adsorbed on 200 g of a "dry column"[29] of washed silica gel H. The column was developed with the same solvent system and 24-ml fractions were collected. Fractions 23–26 afforded an additional 0.163 g of pure crystalline dipeptide, bringing the total isolated yield to 78%.

Glycylphenylalanine.—The pH was adjusted to 3.0 with concentrated sulfuric acid and the peptide was adsorbed on 55 ml of carbon (Pittsburgh, OL grade) at a flow rate of 0.5 ml/min. The column was washed with six volumes of water and the product was eluted with a solution containing 10% acetic acid in 50% aqueous acetone. The solvents were removed *in vacuo* and water was added. The water was evaporated *in vacuo* and this operation was repeated twice. The residue was redissolved in water and ethanol was added to the cloud point. The crystalline product was removed by filtration and washed with ethanol and then with ether to give the desired dipeptide, mp 260–264°. One further recrystallization raised the melting point to 262–265°, $[\alpha]_{589}$ +40.8° (*c* 2.5, water) (lit.[30] $[\alpha]_D$ +41.5° (*c* 2, water).

Anal. Calcd for $C_{11}H_{14}O_3N_2$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.74; H, 6.28; N, 12.79.

In a similar experiment, but using ^{14}C-labeled phenylalanine, electrophoretic analysis (for details of method, see preparation of L-phenylalanyl-L-arginine at three different pH's) indicated the presence of 23% of the hydantoic acid by-product in the crude reaction mixture.

Tyrosylserine.—The reaction mixture (pH 6) was diluted fourfold with water and adsorbed on 125 ml of carbon (Pittsburgh, OL grade) at a flow rate of 5 ml/min. The column was eluted with about 750 ml of water (flow rate 10 ml/min) to remove serine and inorganic salts. The product was eluted (flow rate 5 ml/min) with a mixture of water–acetic acid–acetone (47.5:5:47.5). The bulk of the product was in six fractions of 125 ml each. The effluents were combined and the solvent was removed *in vacuo*. The crystalline residue (1.37 g, 51.2% yield) was dissolved in boiling water (21.6 mg/ml), insolubles were removed by filtration, and the resulting solution was gradually diluted with 1.5 volumes of ethanol to give a 30% yield of the dipeptide, dec pt 260°, $[\alpha]^{24}_{589}$ + 26.8° (*c* 2.0, 1 *N* HCl). The reported value[31] is $[\alpha]^{24}_D$ +24.2 ± 0.5° (*c* 1.6, HCl).

Anal. Calcd for $C_{12}H_{16}N_2O_5$: C, 53.70; H, 6.00; N, 10.50. Found: C, 54.07; H, 6.20; N, 10.26.

The compound was completely cleaved by leucine aminopeptidase (LAP). The amino acid ratio after acid hydrolysis was $Tyr_{1.00}Ser_{0.99}$ (Spinco).

Alanylserine.—The pH of the solution was adjusted to 5.5 with 2.5 *N* hydrochloric acid. The peptide was adsorbed on Dowex resin 50-X2 (H^+ cycle) and eluted with an aqueous solution of triethylamine. The effluents containing product were freeze dried to give 558 mg of dipeptide. The product was dissolved in water and the solution was treated slowly with ethanol (17.5 parts/part of water) and finally with 2.5 parts of ether to give a 43% yield of pure product, mp 208–210°, $[\alpha]^{24}_{589}$ +11.2° (*c* 1.97, water) (lit.[32] $[\alpha]^{23}_D$ +11.5° (*c* 2.0, water)).

Anal. Calcd for $C_6H_{12}N_2O_4$: C, 40.90; H, 6.87; N, 15.90. Found: C, 41.60; H, 6.75; N, 16.04.

Tryptophanylleucine.—The product precipitated at pH 3.5 to give 0.38 g (42.5% yield). Successive recrystallizations from aqueous ethyl alcohol and from aqueous methyl alcohol gave 0.273 g of peptide (30.5% isolated yield), $[\alpha]^{25}_{589}$ +14.3° (*c* 1.0, water) (lit.[33] +18° (*c* 1.0, water).

Anal. Calcd for $C_{17}H_{23}N_3O_3$: C, 64.33; H, 7.30. Found: C, 64.46; H, 7.16.

The amino acid ratio after acid hydrolysis was $Trp_{0.98}Leu_{1.00}$ (Spinco) for Leu (Trp from alkaline hydrolysis).

Prolylphenylalanine.—The solution was adjusted to pH 5 with sulfuric acid and the crystalline dipeptide was filtered, washed, and dried. There was obtained 2.31 (89% of theory) of crystalline L-propyl-L-phenylalanine, single spot by tlc in a butanol–acetic acid–water system (4:1:5 upper phase) and in isoamyl alcohol–pyridine–water (7:7:6). An analytical sample, mp 236–237.5°, was prepared by recrystallization from water, $[\alpha]^{22}_D$ −41.2 (*c* 2, 6 *N* HCl) (lit.[34] $[\alpha]_D$ −41.7° (*c* 1, 6 *N* HCl)).

Anal. Calcd for $C_{14}H_{18}N_2O_3 \cdot {}^1/_2H_2O$: C, 61.97; H, 7.06; N, 10.32. Found: C, 62.12; H, 7.00; N, 10.29.

The isolation of the anhydrous material is described below.

Isoleucyl-ε-*t*-Boc-lysine.—The pH of the solution was brought to 5.0 by the addition of concentrated sulfuric acid. A small amount of insolubles was separated by filtration and the filtrate was freeze dried. The residue was extracted with methanol and the solvent was removed *in vacuo*. The residue (1.190 g) was dissolved in 15 ml of a solution of chloroform–methanol–18% aqueous ammonium hydroxide (50:40:10) and adsorbed on 185 g of a "dry column"[29] of silica gel H which had been prewashed with this solvent system. The product was eluted with the same solvent system to afford 0.67 g (46% isolated yield) of dipeptide, single spot by tlc in the same solvent system. A sample was crystallized from ethanol to give $[\alpha]^{25}_{589}$ +6.7° (2%, 0.1 *N* NaOH).

Anal. Calcd for $C_{17}H_{35}N_3O_5$: N, 11.70. Found: N, 11.91.

After acid hydrolysis, a Spinco amino acid analysis gave $Ile_{1.03}Lys_{1.00}$.

Phenylalanylarginine.—An aqueous solution (200 ml) of 4.35 g (25 mmoles) of L-arginine free base, brought to pH 10.5 with sulfuric acid, was treated in portions with 5.0 g (26.3 mmoles) of L-phenylalanine NCA in a Waring Blendor with addition of saturated barium hydroxide solution as required to maintain that pH. After the reaction was complete, the pH was lowered to 4.5 with concentrated sulfuric acid, the mixture was filtered through Supercel, and the filtrate was freeze dried. The white solid residue was dissolved in 50 ml of water, the pH was readjusted to 4.5, and the product was crystallized by the slow addition of 500 ml of methanol to give 4.25 g (40% of theory) of dipeptide bisulfate. Additional product (total 8.25 g, 80% of theory) was obtained by silica gel chromatography of the mother liquor using *n*-propyl alcohol–ammonium hydroxide–water (7:2:1) as eluent. The peptide was completely cleaved by LAP.

Anal. Calcd for $C_{15}H_{23}N_5O_3 \cdot H_2SO_4$: C, 43.00; H, 6.01; N, 16.70; S, 7.64; mol wt, 419. Found: C, 43.50; H, 6.13; N, 16.14; S, 7.29; equiv wt (base titration), 418.

A Spinco amino acid analysis gave $Phe_{1.00}Arg_{1.00}$. The acetate salt has been described as an amorphous powder.[35]

Methionyltyrosine.—The pH was adjusted to 5 with concentrated sulfuric acid and the solution was adsorbed on a 25-ml carbon column. The column was washed with approximately 10 column volumes of water and the product was eluted with acetic acid–50% aqueous acetone (1:3). The solvent was removed *in vacuo* to give 284 mg (45.5% yield) of pure dipeptide. Crystallizations from aqueous ethanol gave an analytical sample, mp 259–265° dec (lit.[36] 260–266°).

Anal. Calcd for $C_{14}H_{20}N_2O_4S$: C, 53.83; H, 6.45; N, 8.97. Found: C, 54.23; H, 6.67; N, 9.34.

Alanylphenylalanine.—The pH of the reaction mixture was adjusted to 5.0. The product was then adsorbed on 150 ml of Pittsburgh OL granular carbon and the carbon was washed free of salts and of alanine with 1 l. of water. The product was then eluted with a 20% solution of acetic acid in aqueous acetone

(29) The "dry column" technique of chromatography is, in essence, preparative thin layer chromatography. As developed by Mr. T. Beesley and Dr. T. Jacob, it has proved extremely useful in the separation and purification of peptides. Details will be published in a separate publication. See also B. Loev and K. M. Snader, *Chem. Ind.* (London), 15 (1965).

(30) R. Rao Krishnarau, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).

(31) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).

(32) J. I. Harris and J. S. Fruton, *J. Biol. Chem.*, **191**, 143 (1951).

(33) M. Wilcheck and A. Patchornik, *J. Org. Chem.*, **28**, 1874 (1963).

(34) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).

(35) K. Hofmann, W. D. Peckham, and A. Rheiner, *ibid.*, **78**, 238 (1956).

(36) C. A. Dekker, S. P. Taylor, and J. S. Fruton, *J. Biol. Chem.*, **180**, 155 (1949).

(1:1) and the eluate was evaporated to a heavy oil. The oil was crystallized from an aqueous solution by the addition of acetone to give 1.87 g of pure dipeptide. A second crop (1.0 g) brought the yield to 64%. A separate experiment, using ^{14}C-labeled alanylphenylalanine, showed that the recovery of the dipeptide from the carbon column by the above procedure was only 91%, bringing the corrected yield of the reaction to 70%. Furthermore the mother liquors from the second crop of the reaction were shown by tlc to contain significant amounts of additional dipeptide which could, however, not be obtained pure by direct crystallization. In a comparable experiment with ^{14}C-phenylalanine, it was shown by electrophoretic analysis (see glycylphenylalanine) that no hydantoic acid had been formed and that the dipeptide had been formed in about 90% yield. An analytical specimen was obtained from ethanol, $[\alpha]^{25}_{589}$ +17° (*c* 2, 1 *N* HCl).

Anal. Calcd for $C_{12}H_{16}O_3N_2$: C, 60.99; H, 6.83; N, 11.86; equiv wt, 236. Found: C, 60.42; H, 6.55; N, 11.24; equiv wt (base titration), 236.

Isoleucyltryptophan.—The pH of the reaction mixture was adjusted to 4.5 with 6 *N* sulfuric acid. The mixture was allowed to stand at room temperature for 36 hr. About 1.21 g (38% yield) of crystalline dipeptide contaminated by trace amounts of tryptophan and of isoleucine was removed by filtration. The filtrate was freeze dried and the methanol extracts were combined with the crystalline dipeptide and purified by countercurrent distribution (18 plates, *n*-butyl alcohol–acetic acid–water, 5:1:5) to give, after crystallization from methanol, a 40% yield of dipeptide, mp 144–145°, $[\alpha]^{25}_{589}$ +23.4° (*c* 2.05, methanol). Cleavage of the dipeptide with LAP left no unchanged dipeptide and gave the ratio of amino acids as $Ile_{0.99}Trp_{1.00}$. The compound readily formed a monohydrate. On drying the compound at 120°, it was converted into the *diketopiperazine* which gave a negative ninhydrin test and a positive Ehrlich test.

Valylhistidine.—The pH was lowered to 5 with 6 *N* sulfuric acid and the solution was freeze dried. The residue was extracted three times with 50-ml portions of a solution of chloroform–methanol–18% aqueous ammonium hydroxide (50:40:10). The solvents were evaporated *in vacuo*. The residue was redissolved in 25 ml of the chloroform, methanol, and aqueous ammonium solvent system (60:30:10) and adsorbed on 220 g of a "dry column"[29] of silica gel H. The column was developed with the same solvent system and 22-ml cuts were collected. Fractions 28–38 contained 30 μcuries of pure dipeptide and fractions 39–41 eluted 7.1 μcuries of dipeptide contaminated by small amounts of valine. The solvents were evaporated and the latter cuts were dissolved in 25 ml of water and charged to 25 ml of IRC-84 (acetate cycle). The valine contaminant was eluted with 4 volumes of water and the pure dipeptide with 7 volumes of a 50% solution of aqueous acetic acid to afford an additional 4.6 μcuries of product or a total yield of 74%. The product could be completely hydrolyzed by LAP and it gave the expected amino acid analysis after acid hydrolysis of $Val_{0.97}His_{1.00}$. The dipeptide was crystallized from aqueous methanol to afford a hygroscopic solid, $[\alpha]^{28}_{589}$ +44° (*c* 4.2, water) (lit.[37] $[\alpha]^{24}_{D}$ +46° (*c* 4.2, water)).

Leucylphenylalanine.[38]—Small amounts of insolubles were removed by filtration and the filtrate was acidified to pH 5.6 with sulfuric acid. The crude product was purified *via* its sodium salt to afford 1.01 g of single-spot dipeptide which was freed of residual water essentially as described for phenylalanylleucine. Further purification of the original filtrate on silica gel H brought the yield of dipeptide to 1.145 g (82%). After acid hydrolysis, a Spinco amino acid analysis gave $Leu_{1.00}Phe_{1.02}$. The compound was completely cleaved by LAP.

Preparation of L-Phenylalanyl-^{14}C-L-arginine at pH 9.5, 10.0, and 10.5.—An arginine stock solution was prepared from 4.350 g (25 mmoles) of L-arginine free base and ^{14}C-L-arginine (uniformly labeled, 0.50 μcurie, 0.392 mg in 5.0 ml of 0.01 *N* hydrochloric acid) and brought to a volume of 250 ml with a saturated solution of boric acid.

The reaction was carried out in a Waring Blendor at three different pH's according to the standard procedure using 20 ml of the arginine stock solution (2.0 mmoles, 40 μcuries) and 1 drop of capryl alcohol with a 10% excess (420 mg) of the NCA of L-phenylalanine. The pH was maintained between the stated limits by the addition of a solution of 5 *N* aqueous potassium hydroxide. After allowing the reactions to proceed at 0–2° for 2 min, the solutions were diluted to a volume of 50 ml by the addition of water. Electrophoresis of 10-μl. samples of the diluted reaction mixture on S & S No. 598 paper in a solution 0.01 *N* in hydrochloric acid and 0.1 *N* in potassium chloride at a voltage gradient of 11.3 v/cm over a 3-hr period revealed four radioactive peaks when scanned by a recording count rate meter–strip scanner: hydantoic acid (11 cm), phenylalanylphenylalanylarginine (17.5 cm), phenylalanylarginine (20.4 cm), and arginine (23.3 cm). The electrophoresis strips were cut into sections and burned by the Schöniger combustion method.[39] The resulting carbon disoxide was adsorbed with Hyamine and counted in a liquid scintillation counter. The results are given in Table III. The data agree well with those obtained from electrophoresis at pH 9.5. At the latter pH the di- and tripeptides appeared as a single peak. The value for residual arginine in the pH 10.5 reaction was estimated to be 2–3% by the ninhydrin color intensity by tlc, in excellent agreement with the radiochemical measurement.

TABLE III

EFFECT OF pH ON THE COURSE OF THE REACTION OF ARGININE WITH THE NCA OF PHENYLALANINE

Product	% of products of condensation reaction at pH 9.5	10.0	10.5
Arginine	5.4	3.5	3.2
Phenylalanylarginine	86.5	89.2	89.2
Phenylalanylphenylalanylarginine	6.8	4.0	1.9
Hydantoic acid	1.3	2.8	5.6

N-(α-Carboxyphenethylcarbamoyl)arginine, the Hydantoic Acid Derived from L-Arginine and L-Phenylalanine (J, Scheme III, R = $C_6H_5CH_2$; R′ = NH=C(NH$_2$)NH(CH$_3$)$_3$).—By the method described above, but at pH 11.9–12.05 in 0.1 *M* phosphate buffer, 2.0 mmoles of ^{14}C-L-arginine was treated with 2.2 mmoles of L-phenylalanine NCA. The reaction mixture was analyzed by electrophoresis at pH 2 and by tlc in an *n*-propyl alcohol–water–concentrated ammonia system (80:16:4). The electrophoretic strip was scanned by a recording count rate–meter strip scanner. The pertinent areas of the tlc were scraped off and counted in a liquid scintillation counter. All radioactive spots were Sakaguchi positive; arginine and phenylalanylarginine spots were ninhydrin positive. The results obtained are tabulated in Table IV.

TABLE IV

PRODUCTS FROM THE REACTION OF ARGININE WITH THE NCA OF PHENYLALANINE AT pH 12

Product	Tlc, %	Electrophoresis, %
Arginine	22.3	22.2
Phenylalanyl arginine	18.9	19.3
Urea	58.8	58.5

The reaction mixture was fractionated on a dry silica gel column (46 × 7 cm i.d.) using *n*-propyl alcohol–water–concentrated ammonia (80:16:4) as eluent. Of the 20-ml cuts, fractions 41–75, containing the urea and phenylalanyl arginine, were combined and evaporated to dryness. The noncrystalline urea was obtained as electrophoretically single-spot material by extraction from water at pH 3.3 with *n*-butyl alcohol.

N-(α-Carboxyphenethylcarbamoyl)phenylalanine. (Phenylalanylphenylalanylurea) (J, Scheme III, R = R′ = $C_6H_5CH_2$).—L-Phenylalanine (1.734 g, 10.5 mmoles) in 50 ml of a saturated solution of boric acid was adjusted to pH 13 with a solution of 45% aqueous potassium hydroxide and treated, at 0–2° with vigorous blending, with 2.206 g (11.55 mmoles) of the NCA of L-phenylalanine. The pH was maintained at 13 ± 0.5 by addition of a 45% solution of potassium hydroxide as required. The reaction mixture was filtered and acidified with a concentrated solution of hydrochloric acid to pH 3. A white solid separated.

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The mixture was extracted with 250 ml of ethyl acetate. The extract was washed with water and dried over magnesium sulfate, and the solvent was removed *in vacuo*, leaving an oily residue. The crude product was dissolved in ethanol, filtered, and crystallized by addition of water. The product was filtered and air dried to yield 2.34 g (62.6% yield) of N-(α-carboxyphenethylcarbamoyl)phenylalanine, mp 187–187.5°. An analytical sample, mp 188–188.5°, was prepared by recrystallization from ethyl acetate–*n*-hexane: $[\alpha]^{25}_{589}$ +67° (*c* 1, ethanol), λ_{max}^{EtOH} 253, 258, 264 mμ (ε 335, 407, 309).

Anal. Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N, 7.86; equiv wt, 178. Found: C, 63.67; H, 5.54; N, 7.85; equiv wt (base titration), 177.

In another preparation, carried out as above but in which ^{14}C-L-phenylalanine (16,600 counts/min/μmole) was allowed to react with unlabeled phenylalanine NCA, urea having a specific activity of 16,600 counts/min/μmole was obtained. This product had the same mobility, in electrophoresis at pH 7, as the acidic component of a reaction carried out at pH 10.5.

α,4-Dibenzyl-2,5-dioxo-1-imidazolineacetic Acid.—The phenylalanylphenylalanineurea (0.27 g) was converted to the hydantoin by heating under reflux for 1 hr with 5 ml of concentrated aqueous hydrochloric acid. The reaction mixture containing precipitated solid was extracted with ethyl acetate, the extract was washed with water, dried over magnesium sulfate, and evaporated to dryness *in vacuo* to yield 0.233 g (91% of theory) of the crude hydantoin. An analytical sample was prepared by recrystallization from dilute acetic acid. The product melted at 212–214° (lit. 212°,[40] 205.5° [41]); $[\alpha]^{25}_{589}$ −207.5° (*c* 0.85, ethanol) (lit.[41] $[\alpha]^{25}_{D}$ −210.7° (ethanol)); λ_{max}^{EtOH} 253, 258, 264 mμ (ε 375, 450, 338).

Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.45; H, 5.36; N, 8.28; equiv wt, 338. Found: C, 67.72; H, 5.27; N, 8.52; equiv wt, 336.

Effect of Mixing on the Reaction of NCA Alanine with Phenylalanine.—By the general method described above, L-phenylalanine (2.0 mmoles) in 20 ml of 1 *M* potassium borate buffer was treated with 1.9 mmoles of the NCA of L-alanine at various pH's in either a magnetically stirred beaker or in a Waring Blendor. An insufficient amount of NCA was used to ensure that overreaction was proceeding in the presence of starting material. The solutions were diluted to 25 ml. The reaction mixtures were examined by tlc (upper phase of the butanol–acetic acid–water system (4:1:5)). Figure 1 shows the ninhydrin-developed tlc plates of these reaction mixtures, after spotting 2-μl aliquots of the solutions. The overreacted products were identified by comparison of the R_f's with those of reference compounds prepared by the NCA method by sequential syntheses.

Reaction of the NCA of L-Proline with ^{14}C-L-Phenylalanine at pH 13.5.—To stirred solution of 826 mg of ^{14}C-L-phenylalanine (5 mmoles, 50 μcuries) in 40 ml of water in a Waring Blendor at 1° adjusted to and maintained at pH 13.5 with a concentrated solution of aqueous potassium hydroxide was added (over a period of 20 sec) 776 mg (5.5 mmoles) of the NCA of proline; the mixture was stirred for 2 min. After removing an aliquot for the electrophoretic analysis described below, the reaction mixture was brought to pH 7, causing the single-spot L-propyl-L-phenylalanine monohydrate to crystallize. The yield was 890 mg (63.5%). Recrystallization of this dipeptide from water gave long needles (749 mg) which were dried at 100° for 2 hr *in vacuo* to yield the anhydrous product, $[\alpha]^{25}_{589}$ −40.3° (*c* 1, 6 *N* HCl) (lit.[34] −41.7°).

Anal. Calcd for $C_{14}H_{19}N_2O_3$: C, 64.10; H, 6.92; N, 10.68. Found: C, 64.45; H, 7.23; N, 10.92.

The Spinco amino acid analysis after acid hydrolysis gave $Pro_{0.99}Phe_{1.00}$.

Reaction of the NCA of L-phenylalanine with ^{14}C-L-Proline at pH 13.5.—A solution of 5 mmoles (50 μcuries) of ^{14}C-L-proline was allowed to react with 5.5 mmoles of the NCA of phenylalanine by the procedure described above. The slightly cloudy reaction mixture was filtered and an aliquot was withdrawn for the electrophoretic analysis described below. The pH of the bulk of the solution was brought to 2 and the solution was extracted three times with 25-ml portions of ethyl acetate. The combined extracts were dried over magnesium sulfate and the solvent was removed *in vacuo*. The residue (978 mg, 64% yield) was single-spot material by electrophoresis at pH 7. Crystallization from ethylacetate–*n*-hexane gave crystals of 1-(α-carboxyphenethylcarbamoyl)proline, dec pt 158°.

Anal. Calcd for $C_{15}H_{20}N_2O_5$: C, 58.81; H, 5.92; N, 9.15; equiv wt, 153. Found: C, 58.63; H, 5.77; N, 9.09; equiv wt, 156.

A Spinco amino acid analysis after acid hydrolysis gave $Pro_{1.00}Phe_{0.94}$.

Analysis of Reactions of the NCA of Proline with ^{14}C-Phenylalanine and of the NCA of Phenylalanine with ^{14}C-Proline at pH 13.5. A.—Electrophoresis using 20-μl samples of the above reaction mixtures on S + S 598 paper in pH 7 phosphate buffer, at a voltage gradient of 11.3 v/cm for 4 hr gave the following radioactive peaks when measured with a recording count rate meter–strip scanner. For the first-named reaction, only one peak (prolylphenylalanine and phenylalanine) was observed at 0.9 cm toward cathode relative to point of application. For the latter reaction, one peak was found at 20.4 cm (hydantoic acid) toward the anode relative to the point of application, a second peak at 0.5 cm (phenylalanylproline), and a shoulder at 1.5 cm (unreacted proline). The radioactivity in the hydantoic acid peak represented 68% of the total on the strip.

B.—Thin layer chromatography on silica gel using a butanol–acetic acid–water system (10:1:3) gave the following radioactive zones: for the first-named reaction, phenylalanine (27%) R_f 0.456, prolylphenylalanine (73%) R_f 0.276; for the latter reaction, hydantoic acid (72%) R_f 0.615, phenylalanylproline (23%) R_f 0.354, proline (5%) R_f 0.115.

Reaction of a Large Excess of the NCA of Proline with ^{14}C-Phenylalanine at pH 12.5.—To a stirred nitrogen blanketed solution of 165.2 mg of ^{14}C-L-phenylalanine (1 mmole, 100 μcuries) in 25 ml of freshly boiled, distilled water at 1° adjusted to and maintained at pH 12.5 with a 10 *N* solution of sodium hydroxide were added six portions of the NCA of proline in a Waring Blendor; five portions consisted of 1 mmole of NCA each and the last portion consisted of 5 mmoles. The addition required about 8 min. Subsequent to the addition of each portion of NCA (when no more base addition was required), 50-μl aliquots were withdrawn for analysis. Analysis of 5-μl samples by tlc on silica gel using the butanol–acetic acid–water system (10:1:3) gave two radioactive peaks with R_f values of 0.344 and 0.45 representing prolylphenylalanine and phenylalanine, respectively. The analytical results are summarized in Table V.

TABLE V

RESIDUAL PHENYLALANINE AND PHENYLALANINAMIDE AFTER ADDITION OF EXCESS NCA OF PROLINE AT PH 12.5

	Residual nucleophile, %, NCA added, mmoles						
	1	2	3	4	5	10	15
Phenylalanine	52.5	8.8	2.0	0.6	0.3	0.1	
Phenylalaninamide			44.7		22.9	4.4	2.3

Reactions of the NCA of Proline with ^{14}C-Phenylalaninamide.[21,42] **A. Synthesis of the Amide.**—To a solution of 25 ml of dry, peroxide-free dioxane (saturated at *ca.* 5° with anhydrous ammonia) was added a solution of 600 mg of the NCA of ^{14}C-L-phenylalanine (3.14 mmoles, 47 ± 2 μcuries/mmole) in 4 ml of dioxane. After removal of solvent and excess ammonia, the residue was taken up in methylene chloride and the solution was filtered through Celite. The solvent was removed *in vacuo* and the residue was crystallized from a chloroform–*n*-hexane mixture to give crystalline L-phenylalaninamide, mp 92–92.5° (376 mg, 73% yield), with an activity of 49 μcuries/mmole.

Anal. Calcd for $C_9H_{12}ON_2$: C, 65.83; H, 7.37; N, 17.06. Found: C, 65.87; H, 7.49; N, 16.94.

The product gave one radioactive, ninhydrin-positive spot on electrophoresis on S & S 598 paper, in pH 4.2 acetate buffer, at a voltage gradient of 11.3 v/cm for 3.25 hr. It moved 22.7 cm toward the cathode. Material prepared essentially as described above, mp 91–94°, $[\alpha]^{25}_{589}$ +20.1° (*c* 1.04, water), showed $\lambda_{max}^{CHCl_3}$ 2.83, 2.97, 3.35, 5.97, and 6.46 μ. The hydrochloride salt of L-phenylalaninamide, $[\alpha]^{25}_{D}$ +20.7 (*c* 1, water), has been described.[42]

B. Reaction of the NCA of Proline with ^{14}C-Phenylalaninamide at pH 10.2.—A stirred, freshly boiled solution of 0.45 *M* boric

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acid (25 ml), blanketed with nitrogen at 1°, containing 164.2 mg of the labeled amide (1 mmole) was adjusted to pH 10.2 with a 10 *N* solution of sodium hydroxide. Over a period of about 10 sec, 155.2 mg of the NCA of proline (1.1 mmole) was added. The pH of the solution was maintained at 10.2 by the continued addition of aqueous alkali. Tlc of the reaction mixture on silica gel using a butanol–acetic acid–water system (10:1:3) gave radioactive spots at R_f 0.25 (prolylphenylalaninamide, 83%) and at R_f 0.41 (phenylalaninamide, 17%).

C. Reaction of an Excess of the NCA of Proline with ^{14}C-Phenylalaninamide at pH 12.5.—To a stirred, nitrogen-blanketed solution of 164.2 mg of the above ^{14}C-amide (1 mmole) in 25 ml of freshly boiled, distilled water at 1°, adjusted to and maintained at pH 12.5 with a 10 *N* solution of sodium hydroxide, were added consecutively over an 8-min period five portions of the NCA of proline consisting of 1, 2, 2, 5, and 5 moles, respectively. The reaction mixture was sampled subsequent to the addition of each portion of the NCA after no more base addition was required to keep the pH at 12.5. The aliquots were analyzed for residual ^{14}C-phenylalaninamide by tlc as described above. The results are summarized in Table V.

Reaction of Excess of the NCA of Alanine with ^{14}C-Phenylalanine.—To a solution of 165.1 mg of ^{14}C-phenylalanine (1 mmole, 100 μcuries) in 25 ml of freshly boiled water were added consecutively over about 8 min five portions of the NCA of alanine consisting of 1, 2, 2, 2, and 3 mmoles, respectively, and finally one portion of 5 mmoles of the NCA of proline. The reaction was carried out at pH 12.5 and sampled essentially as described directly above for the reactions of the NCA of proline. Radiochemical analysis of thin layer plates gave the following percentages of residual ^{14}C-phenylalanine at successive stages of the reaction: 63% (1 mmole of NCA), 8.4% (3 mmoles of NCA), 4.6% (5 mmoles of NCA), 4.2% (10 mmoles of NCA of alanine followed by 5 mmoles of proline).

Registry No.—L-Valyl-L-serine, 13588-94-8; L-leucyl-L-valine, 13588-95-9; L-alanyl-L-leucine, 3303-34-2; L-phenylalanine-L-leucine, 3303-55-7; glycyl-L-phenylalanine, 3321-03-7; L-tyrosyl-L-serine, 13588-99-3; L-alanyl-L-serine, 3303-41-1; L-tryptophenyl-L-leucine, 13123-35-8; L-prolyl-L-phenylalanine, 13589-02-1; L-isoleucyl-ε-*t*-Boc-L-lysine, 13612-78-7; L-phenylalanyl-L-arginine, 13589-03-2; L-methionyl-L-tyrosine, 13589-04-3; L-alanyl-L-phenylalanine, 3061-90-3; L-isoleucyl-L-tryptophan, 13589-06-5; L-valyl-L-histidine, 13589-07-6; N-(α-carboxyphenethylcarbamoyl)phenylalanine, 13589-08-7; α,4-dibenzyl-2,5-dioxo-1-imidazolineacetic acid, 13589-09-8; 1-(α-carboxyphenethylcarbamoyl)-proline, 13589-10-1; L-phenylalanylamide, 5241-58-7.

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Oxygen-Sensitive Reactions of Proteins and Peptides. III. Chromogenicity and Cystine-Related Structures

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Further insight into the structural requirements supporting the oxygen-sensitive color reactions of solutions of proteins or peptides containing fully combined cystine moieties in ammonia or certain other amines has been gained with studies of simpler model compounds. A generalized structure for the latter is $(S(CH_2)_nCHXCOY)_2$. To maintain the chromogenic properties, it was found that n must equal 1, though the CH_2 group could be altered to $C(CH_3)_2$, but reactivity was greatly diminished. Also, the two S atoms could not be separated by a methylene group. The group X was expanded from NHCHO to NHCOR and $NHSO_2Ar$ and was found chromogenically reactive if suitable groups were at Y. Also, X could be NHCOOR and with this substituent 1,1,3,3-tetramethylguanidine displayed not only color but also thermochromism which it failed to do when X was NHCOR. It was found that X could even be H or NH_2, but with these groups the chromogenic effect was greatly restrained. Structural variations at Y included OR, NH_2, NHR, and NR_2. The speed and intensity of color development diminished progressively in the above order; esters were the most reactive and the disubstituted amide displayed scarcely any reactivity. Finally, it was shown that Y could be H or CH_3, showing that the minimum essential structural skeleton to support the oxygen-sensitive, thermochromic reactions is $(SCH_2\underset{|}{C}HCO)_2$. Syntheses of compounds necessary for these structural studies are reported.

In paper I of this series,[1] a chromogenic thermochromic reaction of liquid anoxic ammonia was reported with proteins and peptides containing fully combined cystine groups, *i.e.*, cystine having both carboxyl and both amino groups combined in amide or peptide bonds. In paper II, it was shown[2] that similar oxygen-sensitive, thermochromic solutions resulted when such proteins or peptides were treated anoxically with primary amines containing the group CH_2NH_2. An oxygen-sensitive chromogenic reaction was observed also if 1,1,3,3-tetramethylguanidine was used as the reactive base, but these blue solutions failed to show thermochromic properties in the experiments reported. The blue solutions resulting from the reaction of keratins with anoxic ammonia have been recently investigated by the electron spin resonance technique, but no signals were observed, indicating the absence of free radicals.

In the present paper, model compounds have been studied to gain further insight regarding the minimal structural requirements in the chromogenic compounds. The fragment $SSCH_2CH(NHCOR)CONHR'$ of fully combined cystine reveals these groups: the 2-amido group NHCOR, the 1-amido group CONHR', the carbon chain, the methylene group contiguous to the disulfide group, and the disulfide group itself. All

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