[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

On the Preparation and Properties of Some Amino Acid Amides[1]

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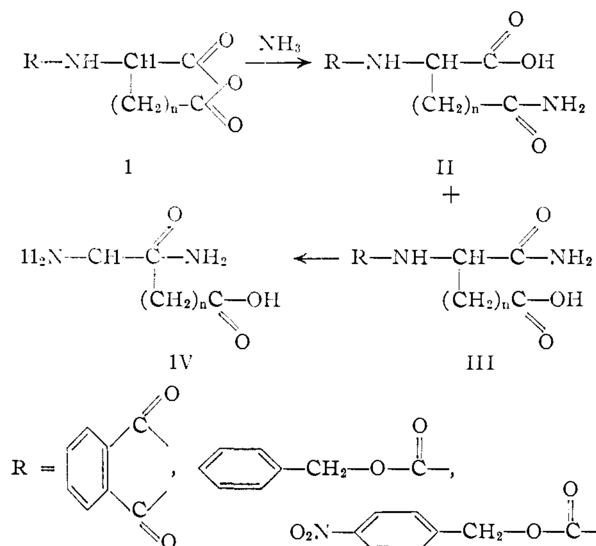
The preparation and properties of the amides of a number of the commonly occurring amino acids have been studied. Included are syntheses of DL-isoasparagine and L-isoglutamine from the *p*-nitrobenzyloxycarbonyl derivatives of the corresponding amino acids and the preparation of L-isoglutamine from diethyl L-glutamate hydrochloride. The apparent dissociation constants of the α-amino groups of the amino acid amides as well as the paper chromatographic behavior of the amides is reported.

In connection with studies conducted in this Laboratory on the ammonolysis of peptides and proteins,[3] it was necessary to prepare the amides of most of the commonly occurring amino acids for use as reference compounds in the identification of the ammonolysis products of peptides by the technique of paper chromatography. Although most of the amino acid amides have been prepared previously,[4] it seemed desirable, in view of the increasing importance of the amino acid amides in naturally occurring products,[5] to record the information we have obtained on various methods of preparation and on the properties of these compounds.

Eleven of these amides (see Table III) were prepared in the present study as the DL-derivatives through ammonolysis of the corresponding methyl or ethyl esters of the amino acids by procedures similar to those of Fischer and Köenigs.[4a] These amides were isolated in the present instance as their acetate salts, which, for the main part, have not been previously described in the literature. The preparation of isoglutamine and isoasparagine presented special problems because of the presence of two carboxyl groups in the parent amino acids.

Many of the methods which have been described for the preparation of the monoamides, as well as α- and β-peptides, of aspartic acid have involved the use of a suitably blocked aspartic anhydride derivative (I, $n = 1$).[6] In order to use this type of intermediate, there must be a preferential opening of the anhydride ring in favor of the desired product. There is considerable evidence to indicate that the direction of the ring opening depends on both the nature of the blocking group and the reaction conditions.[7] In the synthesis of isoasparagine described here, the *p*-nitrobenzyloxycarbonyl (PNBC) group[8] was used as the means of blocking the amino group of the aspartic acid.



It was of considerable interest to determine to what extent the anhydride ring was opened to give the isoasparagine derivative, under various conditions, when PNBC was used as a blocking agent. A method of analyzing the reaction mixture obtained upon ammonolysis of the anhydride was devised. This analysis involved both countercurrent distribution and paper chromatography and took advantage of the fact that the PNBC group has a strong light absorption at 268 mμ. Thus, the derivatives containing this group were conveniently detected in countercurrent fractions or on paper chromatograms by their absorption of ultraviolet light. The results obtained after ammonolysis of PNBC-DL-aspartic anhydride (I, $n = 1$, R = PNBC) under three different reaction conditions are shown in Table I.

TABLE I

PRODUCTS OBTAINED FROM THE REACTION OF PNBC-ASPARTIC ANHYDRIDE AND AMMONIA

Conditions	PNBC-Aspartic acid (a), %	PNBC-Iso-asp-NH_2 (b), %	PNBC-asp-NH_2 (c), %	Ratio (b)/(c)
Ammonium hydroxide	4	61	35	1.7
Anhydrous ammonia in dioxane	20	49	31	1.6
Ethanol–ammonium hydroxide	4	67	29	2.3

These results indicate that the anhydride opened in favor of the α-isomer under all the conditions studied and that the distribution of the α- and β-isomers was similar under both anhydrous and aqueous conditions. The large amount of PNBC-as-

(1) This work was supported in part by grants from Eli Lilly and Company and the National Institute for Arthritis and Metabolic Diseases.

(2) Part of this work was performed during the tenure of a du Pont Postgraduate Fellowship.

(3) R. W. Chambers and F. H. Carpenter, THIS JOURNAL, **77**, 1527 (1955).

(4) (a) E. Fischer and E. Köenigs, *Ber.*, **37**, 4585 (1904); (b) P. S. Yang and M. M. Rising, THIS JOURNAL, **53**, 3183 (1931).

(5) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954); V. du Vigneaud, H. C. Lawler and E. A. Popenoe, *ibid.*, **75**, 4880 (1953).

(6) J. S. Fruton, *Advances Protein Chem.*, **5**, 1 (1949).

(7) (a) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932); F. E. King and D. A. A. Kidd, *J. Chem. Soc.*, 2976 (1951); (b) S. W. Tanenbaum, THIS JOURNAL, **75**, 1754 (1952).

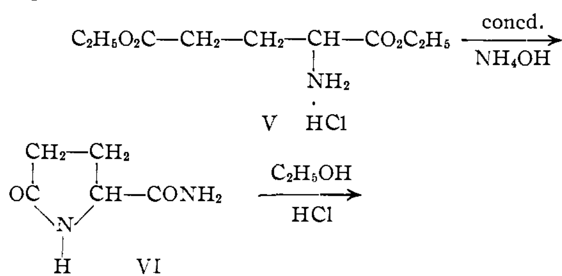
(8) F. H. Carpenter and D. T. Gish, *ibid.*, **74**, 3818 (1952). The abbreviation PNBC will be used in this communication for the *p*-nitrobenzyloxycarbonyl group.

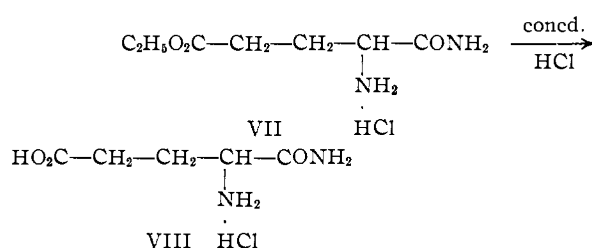
partic acid found in the the anhydrous reaction mixture was apparently due to the insolubility of the anhydride (I, n = 1, R = PNBC) in the dioxane–ammonia mixture resulting in its failure to react during the interval allowed for the ammonolysis reaction. The unreacted anhydride then hydrolyzed to give PNBC-aspartic acid when the reaction mixture was subjected to countercurrent distribution. Of the conditions studied, ethanol–ammonium hydroxide appeared to be the most favorable for the production of PNBC-isoasparagine. A similar result was reported by Tanenbaum for the opening of phthaloyl-L-aspartic anhydride.[7b]

Since the mixture of PNBC-isoasparagine, PNBC-asparagine and PNBC-aspartic acid was difficult to purify, isoasparagine was prepared without isolation of intermediates. The DL-isoasparagine was readily separated from the DL-asparagine and DL-aspartic acid in the reaction mixture by recrystallization from water and alcohol. The over-all yield of DL-isoasparagine by this method was 35% based on PNBC-DL-aspartic acid.[9]

Various derivatives of glutamic anhydride, involving different blocking groups on the amino group, as well as different reaction procedures and conditions have been used for the synthesis of isoglutamine as well as glutamine.[7,10] The use of this type of intermediate presents the same problems as those described above for the preparation of isoasparagine in that preferential opening of the anhydride (I, n = 2) to give the α-isomer must occur in order for the synthesis to be practical for the preparation of isoglutamine. In the work described here PNBC was used to block the amino group of glutamic acid. Although no detailed quantitative studies were made, it was found that the best yield of PNBC-L-isoglutamine was obtained when the PNBC-L-glutamic anhydride (I, n = 2, R = PNBC) was opened with anhydrous ammonia in dioxane. After the PNBC group had been cleaved by hydrogenolysis, the reaction mixture was freed of small amounts of L-glutamic acid by the use of a weak base ion exchange resin.[11] The L-isoglutamine could then be readily separated from L-glutamine by recrystallization from water and ethanol. By this procedure, L-isoglutamine was synthesized in an over-all yield of 31% from PNBC-L-glutamic acid.

L-Isoglutamine was also prepared by the following series of reactions





This synthesis was investigated because of the report of Angier, *et al.*,[12] on the preparation of γ-carbethoxy-α-aminobutyramide hydrochloride (VII) from diethyl L-glutamate hydrochloride (V). Removal of the γ-ester group of VII would result in a convenient and unequivocal synthesis of L-isoglutamine. It was found that the γ-ester group of VII could be hydrolyzed in good yield with concentrated hydrochloric acid without concomitant hydrolysis of the α-amide group. The success of this reaction appeared to rest on the extreme lability of the γ-ester and on the insolubility of L-isoglutamine hydrochloride (VIII) in concentrated hydrochloric acid which caused VIII to precipitate from solution as it was formed. However, despite the expenditure of considerable effort, the yields reported by Angier, *et al.*, for the preparation of VII could not be repeated, much less improved, and as a result the over-all yield of L-isoglutamine from V amounted to only 10%.[13]

Electrometric titrations were carried out on 14 amino acid amides in order to determine the effect of the amide group on the basic strength of the amino group. The apparent pK_a values (pK'_a) of these amides are shown in Table II. By comparing these values with those for the free amino acids,[14] it readily can be seen that the conversion of the carboxyl group to an amide group is attended by a drop in the amino pK'_a of about 1.5 pH units. Thus, the amino group in a free amino acid is roughly thirty times as strong a base as the amino group in the corresponding amide. Furthermore, comparison of the amino pK'_a values of the monoamides of aspartic and glutamic acids with the free amino acids indicates that the effect of the amide group on the basicity of the amino group falls off rapidly as the distance between the amino and the amide group increases. This effect is similar to that noted with the amino acid esters.[15] It is also interesting that the amide group effect is almost as large as the ester group effect. For example, the pK'_a value for the esters of the aliphatic α-amino acids is about 7.7,[15] while that for the corresponding amides is about 8.0 (see Table II). The titration data obtained with histidinamide monoacetate is of particular interest. The pK'_a values for the acetate, imidazole and amino groups were found to be 4.52, 5.78 and 7.64, respectively. The titration

(9) A synthesis of DL-isoasparagine which involves the use of the mixed anhydride of chloroformic acid and N-benzyl-DL-aspartic acid has recently been reported by Y. Liwschitz and A. Zilkha, THIS JOURNAL, **76**, 3698 (1954).

(10) F. E. King, B. S. Jackson and D. A. A. Kidd, *J. Chem. Soc.*, 243 (1951).

(11) H. K. Miller and L. Waelsch, *Arch. Biochem. Biophys.*, **35**, 176 (1952).

(12) R. B. Angier, C. W. Waller, B. L. Hutchings, J. H. Booth, J. H. Mowat, J. Semb and Y. SubbaRow, THIS JOURNAL, **72**, 74 (1950).

(13) Since this work was completed, J. M. Swan and V. du Vigneaud, *ibid.*, **76**, 3110 (1954), have published an unambiguous synthesis of L-isoglutamine which proceeds in much better yield than either of the methods reported here.

(14) D. M. Greenberg, "Amino Acids and Proteins," Charles C. Thomas Publ., Springfield, Illinois, 1951, p. 430.

(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 99.

curve of this amide indicates that histidinamide acetate may be useful as a wide range buffer (pH 4–8) in biological systems.

TABLE II

CHROMATOGRAPHY AND TITRATION DATA FOR AMINO ACID AMIDES

Compound	Solvent A[a] R_f	Solvent B[b] R_f	Ninhydrin color[c]	Dissoc. of amino group pK'_a
Asparagine	0.09	0.16	Tan	8.86
Isoglutamine	.10	.28	Purple	7.88
Isoasparagine	.11	.19	Purple	7.95
Glutamine	.12	.18	Purple	9.28
Glycinamide	.21	.24	Yellow	8.03
Aspartic diamide	.22	.22	Yellow-green	7.00
Histidinamide	.26	.20	Blue-grey	7.64
Serinamide	.27	.28	Yellow	7.30
Prolinamide	.28	.34	Yellow	8.82
Alaninamide	.30	.30	Blue-grey	8.02
Valinamide	.52	.45	Purple	8.00
Methioninamide	.58	.46	Purple	7.53
Tyrosinamide	.61	.43	Purple	7.57
Leucinamide	.64	.58	Purple	8.00
Phenylalaninamide	.65	.56	Purple	7.45
Tryptophanamide	.69	.54	Purple	7.55

[a] Pyridine–isoamyl alcohol–water.[17] [b] 1-Butanol–acetic acid–water.[16] [c] Reagent of Levy and Chung[18] which was made 1% in acetic acid.

The amides reported here were subjected to paper chromatography in two solvents (1-butanol–acetic acid–water[16] and pyridine–isoamyl alcohol–water[17]). The R_f values obtained for these materials along with the color they exhibited on development with the ninhydrin reagent of Levy and Chung[18] which was made 1% in acetic acid are reported in Table II.

Experimental[19]

Amino Acid Ester Hydrochlorides.—The Fischer esterification procedure was used for the preparation of the amino acid ester hydrochlorides shown with their melting points in Table II. In early preparations the esterification was carried out at room temperature, essentially as described by Synge,[20] but later it was found that in most cases the conditions described by Vaughn and Eichler[21] gave the desired product in a shorter time and often in better yield.

Amino Acid Amides.—Most of the amino acid ester hydrochlorides were converted to the free ester as described by Fischer.[22] In a few cases a method involving triethylamine was used. In several instances where the Fischer method was compared with the triethylamine method better yields were obtained by the latter procedure. In this procedure the ester hydrochloride was dissolved in methanol (10–15 ml. per 5 g. of ester hydrochloride) and decomposed with 1 eq. of redistilled triethylamine. Ether (*ca.* 200 ml.) was added, and the mixture was cooled for 1 hour in an ice–salt-bath. After the precipitate of triethylamine hydrochloride had been removed by filtration and washed with ether, the combined filtrate and washings were concentrated under reduced pressure to give the free ester. The free base, prepared by either method, was transferred to a flask containing 50 ml. of methanol previously saturated with ammonia at 0°. After 3 days the solvent was removed under reduced pressure. The residue (usually a solid) was repeatedly dissolved in small portions of methanol followed by evaporation to dryness under reduced pressure. This procedure was repeated with benzene. The crude product was dissolved in a small amount of methanol and 1 eq. of glacial acetic acid was added. The amide acetate was precipitated with ethyl acetate. Recrystallization was usually carried out in methanol by the addition of ethyl acetate. The melting points and analyses of the amides prepared in this manner are shown in Table III.

Aspartic Diamide.—Crude aspartic diamide, as well as its acetate salt, was extremely hygroscopic. When the hygroscopic acetate (precipitated from methanol by addition of ethyl acetate) was collected on a filter, washed copiously with ether and placed in a vacuum desiccator over phosphorus pentoxide, a solid material was obtained which became slightly sticky on exposure to air, but remained crystalline, m.p. 112–114°. The filtrate was concentrated to dryness under reduced pressure. The dry solid was redissolved in dry methanol, and absolute ether was added until the solution became turbid. The solution was cooled to −20° and decanted from a small amount of gummy solid. Addition of more ether gave a precipitate which was removed by filtration and dried as described above. This material melted at 136–137° and was not hygroscopic (see Table III).

L-Prolinamide Hydrochloride.—The sirupy L-proline ethyl ester hydrochloride, which did not crystallize readily, was converted to the free ester by the method of Fischer and then to the free amide. Two recrystallizations from benzene yielded a crystalline product, m.p. 102–104°.[23] Attempts to convert the free amide to the acetate salt failed to yield a crystalline product. The crystalline hydrochloride (see Table III) was prepared as described by Hammer and Greenstein.[24]

Quantitative Determination of the Reaction Products Obtained by Ammonolysis of p-Nitrobenzyloxycarbonyl-DL-aspartic Anhydride. (A) **Reaction Conditions.**—Samples (100 mg.) of PNBC-DL-aspartic anhydride were treated with either 3 ml. of concentrated ammonium hydroxide, with 3 ml. of ethanol–ammonium hydroxide (6.7 ml. of concentrated ammonium hydroxide diluted to 100 ml. with 95% ethanol), or with 3 ml. of purified dioxane[25] followed by 2 ml. of purified dioxane containing 0.7 mmole of ammonia per ml. These reaction mixtures were shaken mechanically for 1.5 hours and then allowed to stand at room temperature for 30 minutes. The solvent was removed under reduced pressure, and the residues were stored in a vacuum desiccator over sulfuric acid.

(B) **Countercurrent Distribution.**—Two-mg. samples of PNBC-aspartic acid, PNBC-asparagine and PNBC-isoasparagine were subjected, individually, to countercurrent distribution in ethyl acetate and phosphate buffer (4 parts 2 *M* sodium dihydrogen phosphate to 1 part of 2 *M* dipotassium hydrogen phosphate, pH *ca.* 5.3). After 24 transfers had been completed, 2 ml. of 3 *M* phosphoric acid was added and the tubes were equilibrated. The optical density at 268 mμ of the upper layers was determined in a Beckman model DU spectrophotometer. From the results the distribution constants (K) were calculated by the formula $N = nK/(K + 1)$, where N is the hypothetical tube in which the maximum optical density occurred and n is the number of transfers.[26] The K values for the PNBC derivatives of aspartic acid, asparagine and isoasparagine were found to be 0.17, 0.29 and 1.24, respectively.

The reaction mixtures described in (A) were dissolved in 10 ml. of water and 1-ml. aliquots were subjected to a 30 transfer distribution. The peak corresponding to PNBC-isoasparagine was well separated from a second peak containing PNBC-aspartic acid plus PNBC-asparagine. From these data the percentage of PNBC-isoasparagine present in the reaction mixture was calculated.

(16) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(17) P. Edman, *Arkiv. Kemi, Mineral. Geol.*, **22A**, 1 (1945).

(18) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

(19) The analyses were performed by the Microchemial Laboratory, Department of Chemistry, University of California, Berkeley. Melting points, unless otherwise noted, are capillary melting points and are uncorrected.

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

(21) J. R. Vaughn and J. A. Eichler, THIS JOURNAL, **75**, 5556 (1953).

(22) E. Fischer, *Ber.*, **34**, 433 (1901). The aqueous solution of histidine methyl ester was extracted with ethyl acetate instead of ether.

(23) V. E. Price, L. Levintow, J. P. Greenstein and R. B. Kingsley, *Arch. Biochem.*, **26**, 92 (1950), reported 99°.

(24) D. Hammer and J. P. Greenstein, *J. Biol. Chem.*, **193**, 81 (1951).

(25) According to E. Eigenberger in A. Weissberger and E. Proskauer, "Organic Solvents," Oxford University Press, New York, N. Y., 1935, p. 139.

(26) L. C. Craig and D. Craig in A. Weissberger, "Techniques of Organic Chemistry," Interscience Pub., Inc., New York, N. Y., 1950, Vol. III, p. 171.

TABLE III

AMINO ACID ESTER HYDROCHLORIDES AND AMIDE SALTS

Amino acid	Ester·HCl Type	Ester·HCl M.p., °C.	Amide salt M.p., °C.	Yield, %	Salt[a] formula	Nitrogen, % Calcd.	Nitrogen, % Found	Neut. equiv. Calcd.	Neut. equiv. Found
Glycine	Ethyl[b]	145–148[c]	122–124	69	$C_4H_{10}O_3N_2$	20.88	20.23	134	129
DL-Leucine	Ethyl	106–110[d]	140–141	65	$C_8H_{18}O_3N_2$	14.75	14.36	190	190
DL-Valine	Methyl	112–113[e]	140–143	66	$C_7H_{16}O_3N_2$	15.92	15.85	176	173
DL-Phenylalanine	Methyl	156–157[f]	139–140	29	$C_{11}H_{16}O_3N_2$	12.54	12.71	224	222
DL-Methionine	Methyl	109–111[g]	143–146	27	$C_7H_{16}O_3N_2S$	13.47	13.26	208	205
DL-Serine	Methyl	133–134[h]	117–119	57	$C_5H_{12}O_4N_2$	17.07	17.09	164	165
DL-Alanine	Ethyl[b]	81–83[i]	136–137	77	$C_5H_{12}O_3N_2$	18.92	18.73	148	147
DL-Tyrosine	Ethyl[j]	105–106[k]	159–161	64	$C_{11}H_{16}O_4N_2$	11.68	11.45	120	117
DL-Tryptophan	Methyl	221–222[l]	126–127	56	$C_{13}H_{17}O_3N_3$	15.95	15.60	263	269
DL-Histidine	Methyl	191–193	151–152[m]	50	$C_8H_{14}O_3N_4$	26.15	25.94	107	107
DL-Aspartic acid	Methyl	111–114	136–137	54[n]	$C_6H_{13}O_4N_3$	20.88	21.53	201	202
L-Proline	Methyl[o]	.....	179–181[p]	55	$C_5H_{11}ON_2Cl$	18.69	18.65	151	149

[a] All salts are acetates except L-prolinamide hydrochloride. [b] Commercial sample. [c] C. S. Marvel, *Org. Syntheses*, **14**, 46 (1934), reported 142–143°. [d] F. Röhman, *Ber.*, **30**, 1978 (1897), reported 112°. [e] A. Darapsky, J. Germscheid, C. Kreuter, E. Englemann, W. Engels and W. Trinius, *J. prakt. Chem.*, **146**, 219 (1936), reported 116.5°. [f] T. Curtius and E. Müller, *Ber.*, **37**, 1261 (1904), reported 158°. [g] C. A. Dekker, S. P. Taylor, Jr., and J. S. Fruton, *J. Biol. Chem.*, **180**, 155 (1949), reported 109–111°. [h] A. M. Mattocks and W. H. Hartung, *ibid.*, **165**, 501 (1946), reported 112–114°. [i] T. B. Johnson and A. A. Ticknor, THIS JOURNAL, **40**, 642 (1918), reported 86.5–87°. [j] L-Tyrosine ethyl ester (commercial sample) was completely racemized in converting it to tyrosinamide. [k] E. Fischer, *Ber.*, **34**, 451 (1901), reported 108–109°. [l] D. O. Holland and J. H. C. Nayler, *J. Chem. Soc.*, 285 (1953), reported 225°. [m] Monoacetate. [n] Diamide, crude product. W. Grassman and O. Mayr, *Z. physiol. Chem.*, **214**, 185 (1933), reported m.p. 144–145°. [o] Not isolated as a crystalline product. [p] $[\alpha]^{23.5}D$ −68.4° (*c* 2, ethanol); Hammer and Greenstein[24] reported m.p. 182°; $[\alpha]^{23}D$ −70.7°.

(C) **Chromatography.**—Samples (10 mg.) of the reaction mixtures were dissolved in 1.0-ml. portions of dimethylformamide, and aliquots (10, 20 and 30 λ) of each reaction solution were spotted on Schleicher and Schuell No. 507 filter paper (13 × 14 cm.). Chromatography was performed by the ascending technique in quart, screw cap jars in *t*-butanol–methyl ethyl ketone–water–diethylamine (40:40:20:4).[27] Two spots, one corresponding to PNBC-aspartic acid and a second corresponding to PNBC-asparagine plus PNBC-isoasparagine, were detected with an ultraviolet lamp.[28] The spots were cut out, placed in 10 × 100 cm. test-tubes and allowed to stand with occasional shaking in exactly 4.0 ml. of 95% ethanol-water (1:1) for 1.5 hours. The percentage of PNBC-aspartic acid in each reaction mixture was obtained by reading these solutions at 268 mμ in a Beckman model DU spectrophotometer and comparing the optical density obtained from the PNBC-aspartic acid spot with that obtained from the PNBC-asparagine-PNBC-isoasparagine spot. From this information and the countercurrent data, the percentage of PNBC-asparagine present in the reaction mixture was calculated by difference.

***p*-Nitrobenzyloxycarbonyl-DL-aspartic Anhydride.**—PNBC-Aspartic acid[29] (5.0 g., 0.017 mole) was suspended in 25 ml. of acetic anhydride and converted to PNBC-DL-aspartic anhydride by the method of Bergmann and Zervas.[30] The clear solution was cooled in an ice–salt-bath and the product was precipitated by the addition of 75 ml. of absolute ether followed by 100 ml. of petroleum ether (b.p. 90–100°). The yield was 4.32 g. (92%), m.p. 160–164°. Decolorization with charcoal (Darco) and two recrystallizations from dioxane yielded 3.25 g. (69%) of material, m.p. 163–164.5°. The product was dried over phosphorus pentoxide *in vacuo* at 57° for analysis.

Anal. Calcd. for $C_{12}H_{10}O_7N_2$: C, 48.98; H, 3.40; N, 9.52. Found: C, 49.12; H, 3.85; N, 9.68.

***p*-Nitrobenzyloxycarbonyl-DL-isoasparagine.**—PNBC-DL-Aspartic anhydride (0.968 g., 3.26 mmoles) was dissolved in 10 ml. of ethanol–ammonium hydroxide (6.7 ml. of concentrated ammonium hydroxide diluted to 100 ml. with 95% ethanol). Almost all of the solid dissolved before the solution turned cloudy and a precipitate formed. The mixture solidified after about 1 hour. The solid was dissolved by the addition of 5 ml. of water, and the solution was acidified with concentrated hydrochloric acid. The solution was cooled, and crystallization was induced by scratching the wall of the flask. The product was collected on a filter and washed with a mixture of ethanol and ether and finally with ether; yield 0.88 g. (85%), m.p. 160–161°. After this material had been recrystallized from 60 ml. of water and dried *in vacuo* over phosphorus pentoxide, the yield was only 0.39 g. (45% recovery), m.p. 162–163°.

Anal. Calcd. for $C_{12}H_{13}O_7N_3$: C, 46.30; H, 4.21; N, 13.49. Found: C, 46.23; H, 4.32; N, 13.32.

Preparation of DL-Isoasparagine without Isolation of Intermediates.[31]—PNBC-DL-Aspartic acid (2.61 g., 8.37 mmoles) was converted to DL-isoasparagine by the methods described above except that at each step the solvent was removed under reduced pressure and then the reaction mixture was subjected to the next step without any purification. The crude reaction products from ammonolysis were subjected to hydrogenolysis over palladium in ethanol–glacial acetic acid (2:1). After removal of the solvent and the slightly volatile toluidine acetate, the crude reaction mixture was dissolved in 10 ml. of water, decolorized with charcoal (Darco) and the product was precipitated with 100 ml. of ethanol. The crystals were collected on a filter, washed with cold ethanol and dried over phosphorus pentoxide *in vacuo;* yield 0.482 g. (43% based on PNBC-DL-aspartic acid). Chromatography (see below) indicated that the product was contaminated with a small amount of asparagine. A second crop, obtained by cooling the filtrate for 2 hours in the refrigerator, was almost entirely asparagine. Recrystallization of the first crop from water and ethanol yielded 0.38 g. (79% recovery) of chromatographically pure isoasparagine. The over-all yield based on PNBC-DL-aspartic acid was 35%. A sample was dried *in vacuo* over phosphorus pentoxide at 56° for analysis.

Anal. Calcd. for $C_4H_8O_3N_2$: C, 36.36; H, 6.06; N, 21.21. Found: C, 36.39; H, 6.21; N, 20.81.

***p*-Nitrobenzyloxycarbonyl-DL-asparagine.**—The method of Gish and Carpenter, procedure B,[29] was used, starting with 6.6 g. (4.86 mmoles) of DL-asparagine. After the reaction was complete, the voluminous white solid was centrifuged down and the supernatant filtered. The solid was suspended in 80 ml. of water and stirred for a few minutes. The suspension was centrifuged and filtered as before. This procedure was repeated three more times, and after the last addition of water only a small amount of yellowish side product remained. The product was isolated from the aqueous solution according to the general method of Gish and

(27) R. R. Redfield, *Biochim. Biophys. Acta*, **10**, 344 (1953).

(28) Model MR-4, George Gates and Co., Inc., Franklin Square, L. I., N. Y., equipped with a germicidal lamp and a filter giving fluorescence activation in 2537 Å. region.

(29) Prepared by the method of D. T. Gish and F. H. Carpenter, THIS JOURNAL, **75**, 950 (1953).

(30) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(31) We are indebted to Mr. Michael Gumbman for assistance in the early stages of this work.

Carpenter.[29] The yield was 11.29 g. (73%), m.p. 147–148°. Two recrystallizations from water containing a small amount of ethanol raised the m.p. to 159–160° (72% recovery). The product was dried over phosphorus pentoxide *in vacuo* at 100° for analysis.

Anal. Calcd. for $C_{12}H_{13}O_7N_3$: C, 46.35; H, 4.21; N, 13.50. Found: C, 46.30; H, 4.04; N, 13.19.

p-Nitrobenzyloxycarbonyl-L-glutamic Anhydride.—PNBC-L-Glutamic acid[29] (2 g., 6.1 mmoles) was suspended in 15 ml. of acetic anhydride and heated in a boiling water-bath for exactly 5 minutes. The solvent was removed under reduced pressure and the residue dried overnight *in vacuo* over phosphorus pentoxide and paraffin. The yield was 1.89 g. (quantitative). Recrystallization from dioxane by the addition of ether gave 1.70 g. (90% recovery), m.p. 156–158°, $[\alpha]^{24}D$ −34.2° (*c* 2.5, dioxane).

Anal. Calcd. for $C_{13}H_{12}O_7N_2$: C, 50.65; H, 3.92; N, 9.08. Found: C, 50.61; H, 4.17; N, 8.93.

p-Nitrobenzyloxycarbonyl-L-isoglutamine.—PNBC-L-Glutamic anhydride (1.5 g., 4.87 mmoles) was dissolved in 25 ml. of purified dioxane by warming the mixture on a steam-bath. The solution was cooled to room temperature and ammonia gas was passed into the solution for a few minutes. A solid formed almost instantaneously. After the reaction mixture had been allowed to stand for 1.5 hours, the solvent was removed under reduced pressure, and the residue was dried *in vacuo* over phosphorus pentoxide; yield 1.67 g. (quantitative for the ammonium salt). The salt was dissolved in 20 ml. of hot water, and the solution was filtered, acidified with concentrated hydrochloric acid and rapidly cooled to room temperature. The product was collected, washed with water and dried *in vacuo* over phosphorus pentoxide and sodium hydroxide; yield 1.03 g. (65% recovery). After the material had been recrystallized from water and then from ethanol, the yield was 0.645 g. This material melted as follows. When it was heated rapidly on the hot stage, all the solid melted at about 130–135°. When it was placed on the block at 120° and heated slowly, the solid changed crystalline form (to long needles) at 130–135° and the needles then melted at 166–170°. Another recrystallization of the product from alcohol and then from acetone by the addition of ether, yielded 0.410 g. of material which showed the same melting point behavior. The over-all yield was 25% of the theoretical amount based on the anhydride, $[\alpha]^{24}D$ +4.0° (*c* 10, dimethylformamide). The product was dried for analysis over phosphorus pentoxide.

Anal. Calcd. for $C_{13}H_{15}O_7N_3$: C, 47.99; H, 4.67; N, 12.91. Found: C, 47.66; H, 4.64; N, 12.40.

L-Isoglutamine.—PNBC-L-Isoglutamine (200 mg., 0.615 mmole) was dissolved in 10 ml. of 95% ethanol–acetic acid (1:1). Hydrogenolysis was carried out in the usual manner in the presence of 40 mg. of palladium oxide catalyst. The catalyst was removed by filtration and the solvent removed under reduced pressure. The residue was dried *in vacuo* over phosphorus pentoxide; yield 0.120 g., m.p. 171–172°. Paper chromatography of this material gave a single, purple, ninhydrin positive spot; $[\alpha]^{24}D$ +19.4° (*c* 3, water). Bergmann and Zervas[30] reported $[\alpha]^{22}D$ +21.1° (*c* 6.5, water) and Melville[32] reported $[\alpha]^{18}D$ +19.1° (water). Swan and du Vigneaud[13] reported $[\alpha]^{21}D$ +20.5° (*c* 6.1, water).

Preparation of L-Isoglutamine without Isolation of Intermediates.—PNBC-L-Glutamic acid (5.0 g., 15.4 mmoles) was converted to L-isoglutamine by the procedures described above except that at each step the solvents were removed under reduced pressure and then the reaction mixture was subjected to the next step without further purification. After hydrogenolysis and removal of most of the *p*-toluidine acetate *in vacuo*, the residue weighed 1.53 g. Paper chromatography (see below) indicated that this residue was a mixture of glutamic acid, glutamine and isoglutamine. Recrystallization from water–ethanol gave 0.81 g. (53% recovery) of crystalline material which, by chromatography, was found to be mainly isoglutamine but which still contained some glutamic acid and glutamine. This material was dissolved in 40 ml. of water and passed through a weak base ion-exchange resin[33] as described by Miller and Waelsch.[11] The effluent was evaporated to dryness under reduced pressure. The residue was recrystallized from water–ethanol and dried *in vacuo* over phosphorus pentoxide. The yield was 0.74 g. (31% over-all yield based on PNBC-glutamic acid), m.p. 175–176°, $[\alpha]^{24}D$ +20.5° (*c* 3, water).

Anal. Calcd. for $C_5H_{10}O_3N_2$: N, 19.17. Found: N, 19.01.

γ-Carbethoxy-L-α-aminobutyramide.—Diethyl L-glutamate,[34] m.p. 114–116° (hot stage), $[\alpha]^{23}D$ +21.3° (*c* 7, ethanol) was converted to γ-carbethoxy-L-α-aminobutyramide by the method of Angier, *et al.*[12]; the yield was 12% based on the diester hydrochloride, m.p. 194–195°, $[\alpha]^{23}D$ +22.8° (*c* 2, water). Angier, *et al.*,[12] reported m.p. 197–198°, $[\alpha]^{26}D$ +21.2° (*c* 2, water).

L-Isoglutamine.—γ-Carbethoxy-L-α-aminobutyramide hydrochloride (1.0 g., 4.75 mmoles) was dissolved in 10 ml. of concentrated hydrochloric acid (sp. gr. 1.188) at room temperature. Precipitation of the product began after 15 minutes. After 2 hours, the product was collected on a sintered glass funnel and washed, dropwise, with cold ethanol until the odor of hydrochloric acid was no longer detectable, then with ether and dried; yield 775 mg. (90%), m.p. 214–216°. Part of this material (500 mg.) was dissolved in 5 ml. of hot water, cooled to room temperature and adjusted to *p*H 5–6 with triethylamine. Crystallization was induced by addition of ethanol, cooling and scratching the wall of the flask. The product weighed 270 mg. (68% based on the hydrochloride), m.p. 173–174°, $[\alpha]^{24}D$ +19.9° (*c* 3, water).

Titration of Amino Acid Amides.—Accurately weighed samples of the amino acid amides (approximately 0.1 mmole) were dissolved in 5 ml. of 0.15 *M* sodium chloride. Standard hydrochloric acid (0.2 ml., 0.992 *N*) was added and the titration was carried out with 1 *N* standard alkali in a nitrogen atmosphere using an automatic electrometric titration apparatus. Neutral equivalents were calculated by subtracting the meq. of acid added from the total meq. of alkali required for the titration. From these data and the titration curves, the pK'_a values were calculated.[35]

Chromatography of Amino Acid Amides.—Standard solutions of the amino acid amide acetates (0.05 *M*) were prepared. Two lambda aliquots were chromatographed in jars on strips (6 × 22 in.) of Whatman No. 1 filter paper by the descending technique using 1-butanol–acetic acid–water (4:1:5)[16] and pyridine–isoamyl alcohol–water (7:7:6).[17] The papers were dried, sprayed with a ninhydrin–collidine reagent described by Levy and Chung[18] which was made 1% in acetic acid and heated in an oven for 5 minutes at 90° to develop the spots.

In order to separate isoglutamine from glutamine and isoasparagine from asparagine it was necessary to run the butanol–acetic acid–water chromatograms for 48 hours, allowing the solvent to run off the paper. Isoglutamine was not separated from glutamic acid, nor isoasparagine from aspartic acid in this solvent, but they were separated by pyridine–isoamyl alcohol–water in 48 hour runs.

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(32) J. Melville, *Biochem. J.*, **29**, 179 (1935).

(33) Amberlite IR-4B, Rohm and Haas Co., Philadelphia, Penn.

(34) Prepared by the method of H. M. Chiles and W. A. Noyes, THIS JOURNAL, **44**, 1798 (1922).

(35) A description of the apparatus and typical titration curves are to be found in the doctoral dissertation of Robert Warner Chambers, University of California, Berkeley, 1954.