[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ST. JOHN'S UNIVERSITY AND INSTITUTE OF ORGANIC CHEMISTRY, UNIVERSITY OF BUDAPEST]

Chemical Studies of Polyaspartic Acids[1]

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The thermal polycondensation of aspartic acid (I) yields anhydropolyaspartic acid (IV). Various steps in an assumed mechanism, I ⟶ II ⟶ III ⟶ IV, were investigated. On heating, acetyl aspartic acid gave anhydropolyaspartic acid instead of the monomer anhydride. L-Aspartic anhydride (II) was polymerized to poly-α,β-L-aspartic acid (III) which formed IV under the same conditions as I. Partial hydrolysis of IV reformed III. The conversion of poly-α-L-aspartic acid (V) into IV was carried out by various methods. The ease of formation of the imide ring was demonstrated by the conversion of both α- and β-anilide of acetyl aspartic acid into the same succinimide derivative. Partial hydrolysis of the succinimide derivative gave mainly the β-anilide.

In preliminary communications,[3-6] we reported briefly investigations of the structure of anhydropolyaspartic acid and of α, β-polyaspartic acid. We now report additional experiments concerning the mechanism of the polycondensation of aspartic acid and rearrangement of asparagine derivatives into isoasparagine derivatives and vice versa.

Polycondensation of aspartic acid was carried out either by heating it *in vacuo* or removing the water formed by azeotropic distillation.[7] The polymeric material, referred to as anhydropolyaspartic acid (IV), was formed by the loss of two molecules of water during condensation.

Partial hydrolysis of IV in alkaline medium yielded α,β-polyaspartic acid (III). The purified free acid (III) when dried at 60–70° *in vacuo* gave analytical data in agreement with III. Like β-poly-DL-asparatic acid,[8a] γ-polyglutamic acid[8b] and α, γ-polyglutamic acid,[8c] III is also very soluble in water contrasting with α-polyaspartic acid and α-polyglutamic acid which are insoluble. It gives a strong biuret reaction which α,γ-polyglutamic acid[8c] does not.

On the basis of amino nitrogen determination (van Slyke) the molecular weights of different preparations of III were between 6000 and 12,000. Molecular weights determined by this method can be misleading because of the possibility of cyclization. However, Fox and co-workers reported that molecular weights of polyaspartic acid calculated from sedimentation constants agreed with values obtained by end-group analysis.[9]

The approximate amounts of the α- and β-linkages in III were determined by a degradation procedure similar to that used in the proof of structure of native polyglutamic acid.[10]

Anhydropolyaspartic acid (IV) was hydrolyzed to the sodium salt of III which was then converted directly to polyaspartic acid polymethyl ester (VI) using methanol–hydrochloric acid solution. The methoxyl content of the ester indicated about 90% esterification of the carboxyl groups. The polyamide (VIIa) was readily prepared by direct ammonolysis of VI after dialysis. The amidation of VI was nearly quantitative producing a polyamide in which about 90% of the carboxyl groups were in the amide form.

As expected every α-aspartyl amide residue (VIII) gave the intermediate IX by Hofmann degradation and α,β-diaminopropionic acid on subsequent acid hydrolysis. Similarly β-aspartyl amide residues (X) gave acetaldehyde *via* the intermediate XI. Acetaldehyde present in the acidic hydrolyzate of the degraded polyaspartic acid polyamide, was separated as the 2,4-dinitrophenylhydrazone, and any α,β-diaminopropionic acid isolated as the flavianate. Control experiments indicated the approximate loss of acetaldehyde (32%) and of diaminopropionic acid (40%) during the isolation procedure. The corrected amount of acetaldehyde-dinitrophenylhydrazone, obtained from degraded polyamide preparation, showed that at least 33% of the aspartic acid was incorporated with β-peptide bonds. Similarly, from the weight of α,β-diaminopropionic acid diflavianate at least 25% of the aspartyl residues had α-peptide bonds. Therefore, the ratio of α- and β-aspartyl residues in polyaspar-

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(3) J. Kovacs, I. Könyves, and Á. Pusztai, *Experientia*, **9**, 459 (1953).

(4) J. Kovacs and I. Könyves, *Naturwiss.*, **41**, 333 (1954).

(5) J. Kovacs, I. Könyves, and J. Császár, *Naturwiss.*, **41**, 575 (1954).

(6) H. Mix and J. Kovacs, *Naturwiss.*, **43**, 447 (1956).

(7) In a private communication E. Katchalski had made it known that A. Berger had successfully converted aspartic acid hydrochloride into anhydropolyaspartic acid using this procedure.

(8)(a) V. Bruckner, T. Vajda, and J. Kovacs, *Naturwiss.*, **41**, 449 (1954); *Acta Chim. Hung.*, **6**, 209 (1955); (b) V. Bruckner and J. Kovacs, *Acta Chim. Hung.*, **12**, 363–404 (1957). Summarizing review; (c) V. Bruckner, M. Szekerke, and J. Kovacs, *Naturwiss.*, **43**, 107 (1956); *Z. physiol. Chem.*, **309**, 25 (1957).

(9) A. Vegotsky, K. Harada, and S. W. Fox, *J. Am. Chem. Soc.*, **80**, 3361 (1958).

(10) J. Kovacs and V. Bruckner, *J. Chem. Soc.*, 4255 (1952); V. Bruckner, J. Kovacs, and H. Nagy, *J. Chem. Soc.*, 148 (1953).

tic acid was found to be about 1:1.3, assuming that no intramolecular transpeptidation[5] took place during the esterification, amidation, or Hofmann degradation. It was further assumed that the remaining 42% of the aspartyl residues were similarly bound, that is in the ratio of about 1:1.3.

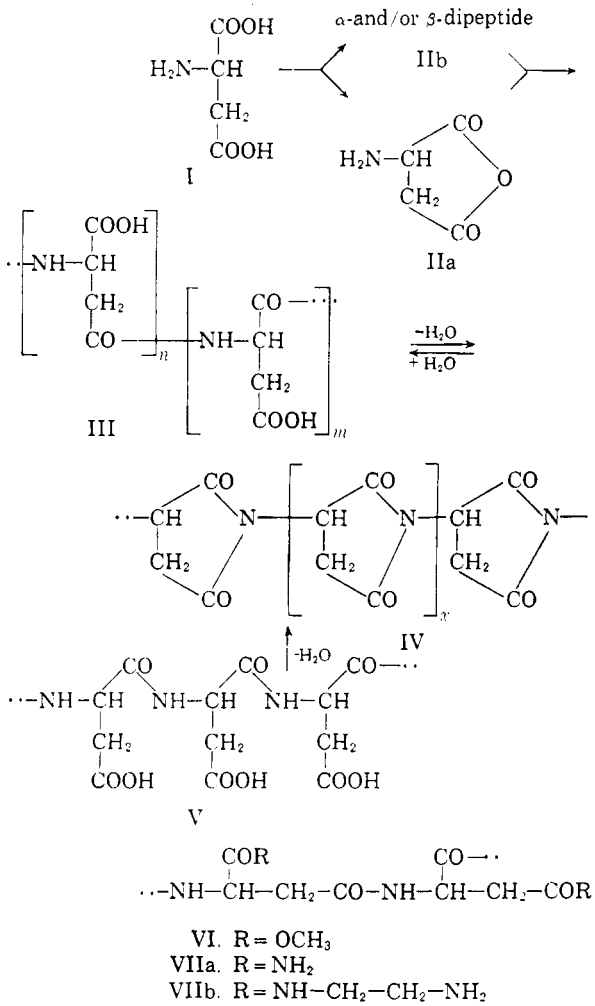
Anhydropolyaspartic acid was easily converted directly to polyamide (VIIa) and a substituted polyamide (VIIb) with liquid ammonia and ethylenediamine respectively. Hofmann degradation of the polyamide (VIIa) gave approximately the same amount of asparaginyl as of isoasparaginyl residues. A sample of anhydropolyaspartic acid, prepared by heating α,β-polyaspartic acid, when carried through the same procedure showed an excess of isoasparaginyl residues.

DISCUSSION

On the basis of the experimental work reported here the initial reaction in the polycondensation of aspartic acid may be the formation of either aspartic anhydride (IIa) or dipeptide(s) (IIb). The former would be followed by intermolecular polyacylation and the latter by polycondensation with aspartic acid or peptides. Whichever is assumed, the next product is α,β-polyaspartic acid (III).[11] However, under the reaction conditions this material is unstable and undergoes intramolecular dehydration producing IV, a polymer composed of succinimide units.[3,5,6,12]

Reaction I → II. Acetylaspartic acid was selected as a model compound to test the formation of the anhydride. However on being heated (145°, 200°) it did not form anhydride but lost water and acetic acid and was converted into a glassy polymeric material. The infrared spectrum with a double band at 5.6 and 5.86 μ, and the biuret reaction indicated that the product had a structure similar to IV. The formation of the acetylaspartic anhydride as an intermediate was suspected; however acetylaspartic anhydride did not polymerize under these reaction conditions. Raising the temperature decomposed it with the formation of acetamide. Therefore an intermolecular transacylation reaction was indicated with the formation of a polypeptide chain. This polymerization of acetylaspartic acid is unique among the acylamino acids.

On the basis of these results reaction I → IIb by direct intermolecular acylation can be expected but reaction I → IIa can not be excluded. Apparently polycondensation of aspartic acid to III through intermediate IIa or IIb is a fast reaction while III to IV is slow. On heating, aspartic acid in boiling tetralin lost the first molecule of water within seventeen hours, while the second molecule was obtained only after several days. It seems to be significant that the dehydration of α,β-polyaspartic acid (III) to anhydropolyaspartic acid (IV) under similar conditions also required several days.



VI. R = OCH_3
VIIa. R = NH_2
VIIb. R = $NH-CH_2-CH_2-NH_2$

Reaction II → III. The hydrobromide of L-aspartic acid anhydride (II) was polymerized in pyridine to α,β-L-polyaspartic acid (III). It was prepared from carbonbenzoxy-L-aspartic anhydride by decarbobenzoxylation in hydrobromic-acetic acid solution. Its structure was verified by the reaction with aniline which yielded α- and β-aspartic acid anilides.[13]

Furthermore, treatment of hydrobromide with ammonia produced L-isoasparagine together with a small amount of asparagine. One recrystallization gave chromatographically pure L-isoasparagine. With methanol, the hydrobromide gave L-aspartic acid α-methyl ester. The homogeneity of the re-

(11) In addition to these routes diketopiperazine, formed from α-dipeptide, could undergo various reactions leading to polyaspartic acid. Such possibilities are discussed in general by E. Katchalski, in *Advances in Protein Chemistry*, **13**, 333–5 (1958). The anhydride formed at the C terminal of a peptide could also participate in the polymerization.

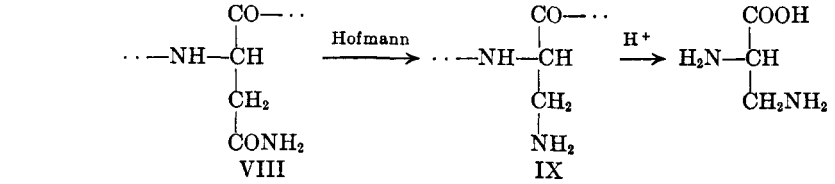
(12) J. Kovacs, I. Könyves, and Á. Pusztai, *Vegyipari Kutató Intézetek Közleményei*, **4**, 120 (1954).

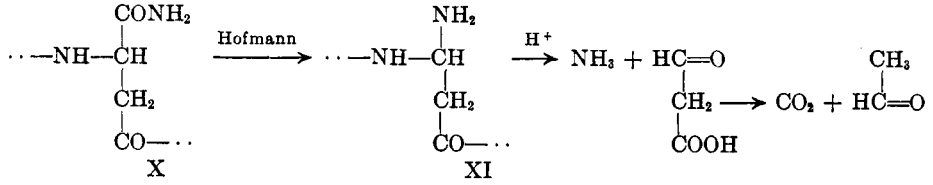
(13) L-Aspartic acid β-anilide was first reported by F. E. King and D. A. A. Kidd, *J. Chem. Soc.*, 2976 (1951). It was prepared from phthalyl-L-aspartic acid β-anilide giving m.p. 251–252°. Aspartic acid β-anilide reported in this paper gave m.p. 241–242° and $[\alpha]_D^{24}$ 33.4°; the presence of the free α-carboxyl group was confirmed by the ninhydrin reaction.

crystallized ester was established by conversion to L-isoasparagine. The structure of polymerized product was established by the degradation method employed for the proof of structure of DL-polyaspartic acid.

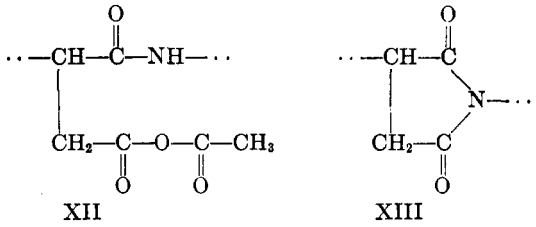
ture of which was established by degradation described previously.

In connection with step I → II it is to be noted that Harada[16] recently suggested that aspartic anhydride is not a necessary intermediate in the





Reaction III → IV and V → IV. Both poly- α, β-aspartic acid (III) and poly α-L-aspartic acid (V) were converted into anhydropolyaspartic acid (IV) under the same experimental conditions used for the pyrocondensation of aspartic acid. In addition, it was found that treatment of the latter (V) with acetic anhydride also produced IV.[14] This last reaction was accompanied by only a slight degree of racemization as determined by the [α] value of the aspartic acid obtained by the total hydrolysis of the product. The reaction probably involves the activation of the carboxyl group by a mixed anhydride formation (XII). Intramolecular elimination of acetate ion gives the polyimide (XIII).



The conversion of α,β-polyaspartic acid (III) into anhydropolyaspartic acid (IV) was followed by infrared spectroscopy[15]; the characteristic absorption bands of polyaspartic acid such as amide I at 6.01 (in potassium bromide, 6.05 is oil paste) and NH stretching near 3 μ disappeared or nearly completely disappeared; the strong amide II in polyaspartic acid appeared as a weak band in spectrum of anhydropolyaspartic acid.

The anhydropolyaspartic acids prepared from all of the above sources had the same properties; (a) they were insoluble in water and in diluted sodium bicarbonate solution, (b) they gave α,β-polyaspartic acid on partial hydrolysis, the structure of which was established by degradation described previously.

In connection with step I → II it is to be noted that Harada[16] recently suggested that aspartic anhydride is not a necessary intermediate in the free aspartic acid condensation and proposed an alternate hypothetical route involving the formation of α-aspartylaspartic acid, which had been proposed earlier by the present author.[12] Also with reference to steps IIa → III and III → IV, Harada explained polyimide formation from the anhydride through an amide intermediate by analogy, using the reaction of γ-butyrolactone with ammonia as an example. However, the formation of the polyimide (IV) through the intermediate amide (III) from the anhydride IIa or dipeptide IIb had already been reported.[5,6,12] These were apparently overlooked by Harada.

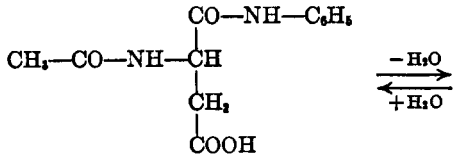
Analogous reactions which demonstrate the ease of formation of aminosuccinimide and whose probable mechanism is the same as that proposed, XII → XIII, were investigated. Both *N*-acetyl-DL-aspartic acid α-anilide (XIV), m.p. 204°, and *N*-acetyl-DL-aspartic acid β-anilide, (XV) m.p. 179°, were converted by acetic anhydride into the same imide (XVI), m.p. 162°. The higher melting anilide (XIV) was considered by Barker[17] to be the β-anilide and its dehydrated product to be the oxazolone derivative (XVII). In view of the fact that both α- and β-anilide yielded the same compound upon treatment with acetic anhydride, structure XVII must be discarded in favor of XVI as representing the dehydrated product. Moreover, the infrared spectrum of the compound XVI contained a double band at 5.60 and 5.86 μ characteristic of succinimides. Both α- and β-anilide were converted to the corresponding amides using the mixed anhydride method. The amide, m.p. 216°, obtained from the lower melting anilide (XV) when subjected to the Hofmann degradation yielded acetaldehyde while amide of the isomer, m.p. 229°, did not. Therefore the structures of the anilides must be those assigned above.

(14) Acetylation of the terminal amino groups is expected.

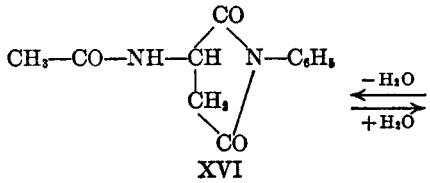
(15) We are indebted to Miss Florence D. Stefano for infrared spectra.

(16) K. Harada, *J. Org. Chem.*, **24**, 1662 (1959).

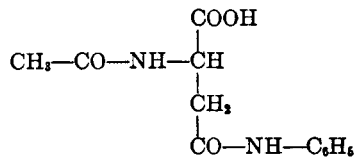
(17) C. C. Barker, *J. Chem. Soc.*, 453 (1953).



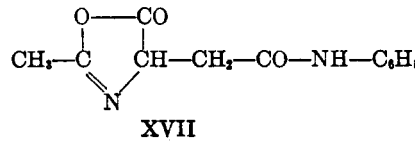
XIV (m.p. 204°)



XVI



XV (m.p. 179–182°)



XVII

Partial hydrolysis of the imide (XVI) provided a mixture of the α- and β-anilides in a ratio of about 1:8. The relative amounts of α- and β-anilide and peptides obtained from the imide derivative must be governed, at least in part, by the electrophilic character of the two carbonyl groups in the ring; attack at the α-carbonyl, corresponding to the stronger acid is favored. These experiments demonstrate that the α-amide bond in an aspartylpeptide or polypeptide can be converted to β-amide bond through the imide, and vice-versa. It appears that aspartyl peptides may give imide derivatives during manipulation elsewhere in the chain.

Ammonolysis of imide gave a mixture of asparagine and isoasparagine derivatives. The mixture contained 81% pure *N*-acetyl-isoasparagine, determined by direct isolation. It was estimated that at least 10% *N*-acetylasparagine anilide was present, from the amount of α, β-diaminopropionic acid produced in the Hofmann degradation of the mixture.

The formation of aminosuccinimide derivatives from esters of aspartic acid derivatives was reported recently by Sondheimer and Holley,[18] Battersby and Robinson.[19] Lockhart and Abraham[20] have observed a similar cyclization during the acid hydrolysis of Bacitracin A. The formation of glutarimide derivatives from acylated glutamyl peptides[21] represents an analogous reaction.

(18) E. Sondheimer and R. W. Holley, *J. Am. Chem. Soc.*, 76, 2467 (1954).

(19) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).

(20) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, 62, 645 (1956).

EXPERIMENTAL

*Preparation of anhydro-poly-*DL*-aspartic acid* (IV). (a) *By direct heating.* Thirty grams of finely powdered DL-aspartic acid was heated at 200° *in vacuo* (0.1 mm.) for 120 hr. The resulting tawny product was triturated with three 100-ml. portions of saturated sodium bicarbonate solution. The granular product was filtered, or separated by centrifugation, washed with water, 1% hydrochloric acid solution and with water until the filtrate was salt free. The yield of yellowish powder obtained after drying over phosphorus pentoxide at 100° and 15 mm. was 18 g. The sample for analysis was further dried at 180° for 200 hr.

Anal. Calcd. for $(C_4H_3O_2N)_\infty$: C, 49.5; H, 3.1; N, 14.4. Found: C, 48.6; H, 4.2; N, 14.6.

(b) *By heating in tetralin.* A suspension of 10 g. of dry finely powdered DL-aspartic acid in tetralin was refluxed for 100 hr. The water formed was removed by azeotropic distillation. After the crude product was filtered and washed with ether the polymer was further purified as described in (a). Drying was accomplished under vacuum by heating the material at 100° over phosphorus pentoxide for 12 hr.[22] The yield of light yellow anhydropolyaspartic acid was 7 g.

Anal. Calcd. for $(C_4H_3O_2N)_\infty$: C, 49.5; H, 3.1; N, 14.4. Found: C, 48.6; H, 3.7; N, 14.3.

Poly-α,β*-*DL*-aspartic acid* (III). After a solution of 9.7 g. (0.1 mole) of anhydropolyaspartic acid (IV) in 1500 ml. of 0.1*N* sodium hydroxide was allowed to stand at room temperature for 1 hr. the excess base was neutralized with 0.1*N* hydrochloric acid solution. The resulting solution was adjusted to *p*H 5.5 with 20% acetic acid and after heating to 40–50° a saturated solution of cupric acetate was added with stirring until precipitation occurred. Under these conditions the copper salt was granular and was easily collected and washed with water. The *copper salt of polyaspartic acid*, dried over phosphorus pentoxide, was obtained in 96% yield.

Anal. Calcd. for $(C_4H_4O_3N)_2Cu_\infty$: Cu, 21.8. Found: Cu, 21.5.

The copper salt from above (14.9 g.) was suspended in 50 ml. of water and hydrogen sulfide was added until a colorless solution was obtained. Following filtration the resulting solution was concentrated to 10 ml. under vacuum and dialyzed against ten 200-ml. portions of water over a period of 90 hr. At this point the material within the membrane did not give movable low molecular weight components upon paper chromatography. The solution within the dialyzing sac was concentrated to 5 ml., lyophilized and further dried under vacuum at 60°, providing 0.8 g. of polyaspartic acid (III) as a fluffy powder. This material was very soluble in water, slightly soluble in methanol and ethanol, soluble in warm dimethylformamide and gave a strong lilac biuret reaction. The R_f values for polyaspartic acid with a butanol–water–acetic acid solvent system was zero using ninhydrin as an indicator.

Anal. Calcd. for $(C_4H_5O_3N)_\infty$: C, 41.7; H, 4.4; N, 12.2. Found: C, 41.6; H, 4.8; N, 12.3.

Van Slyke amino-nitrogen determination of III using four different preparations gave molecular weights of 6100 (53 residues), 8200 (71 residues), 10,800 (93 residues) and 12,100 (105 residues).

Amino-nitrogen analysis (van Slyke) of aliquots taken at various times from a solution of anhydropolyaspartic acid (IV) in 0.1*N* sodium hydroxide (3 mg. of IV/ml. of base) gave molecular weights as 42,700, 18,680, and 12,740 at times of 0.0, 20.8, and 140 min. respectively. In another

(21) J. Kovacs, K. Medzihradszky, and V. Bruckner, *Naturwiss.*, 41, 45 (1954). *Acta Chim. Hung.*, 6, 183 (1955); V. Bruckner and J. Kovacs, *A. Magyar Tud. Akad. Kémiai Tud. Oszt. Közl.*, 3, 105 (1952).

(22) Anhydropolyaspartic acid prepared by Fox[9] is material reported to have approximately one molecule of water per residue after vacuum drying at 80°.

determination, after IV was allowed to stand at room temperature for 2 days in 0.05*N* sodium hydroxide (4 mg. of IV/ml. of base), the molecular weight was 11,670.

Preparation of anhydropoly-DL-aspartic acid (IV) *from poly-α,β-DL-aspartic acid.* Polyaspartic acid (III), (317 mg., molecular weight 12,100), was heated at 200° over phosphorus pentoxide for 5 days. The loss of water was quantitative assuming the loss of a molecule per residue of aspartic acid. As expected the material remaining was found to be insoluble in water and dilute sodium bicarbonate solution and was soluble in sodium hydroxide and warm saturated sodium bicarbonate solution.

Anal. Calcd. for $(C_4H_3O_2N)_\infty$: C, 49.5; H, 3.1; N, 14.4. Found: C, 48.8; H, 3.4; N, 14.1.

Poly-α,β-DL-aspartic acid methyl ester (VI). A solution of 0.97 g. of anhydropolyaspartic acid in 20 ml. of *N* sodium hydroxide was allowed to stand at room temperature for 1 hr. The excess sodium hydroxide was neutralized with 10 ml. of *N* hydrochloric acid and the solution was evaporated to dryness *in vacuo*. The sodium salt of the polyaspartic acid was treated with methanol-hydrochloric acid solution, prepared from 250 ml. of anhydrous methanol and 1.5 ml. of acetyl chloride. After shaking for 30 min. the clear solution was allowed to stand at room temperature for 70 hr. The solution was then filtered and evaporated to dryness *in vacuo*. The residue was triturated with 20 ml. of anhydrous methanol and the solvent was removed under reduced pressure. This manipulation was repeated five times. The hydrochloric acid free residue was treated with 30 ml. of methanol and after removal of the sodium chloride by filtration the solution was evaporated to dryness. The residue polyaspartic acid methyl ester was dissolved in 5 ml. of hot water, lyophilized, and dried at 56° for 2 hr. over phosphorus pentoxide. The yield of powdery solid, containing 3.1% ash and 19.4% methoxyl, was 950 mg. Further purification was achieved by dialyzing a solution of the polyester in 15 ml. of water against six 500-ml. portions of distilled water. The dialyzed solution provided 500 mg. of VI after lyophilization and desiccation over phosphorus pentoxide for 2 hr. at 56°.

Anal. Calcd. for $(C_5H_7O_3N)_\infty$: C, 46.5; H, 5.42; N, 10.85; CH_3O, 24.0. Found: C, 44.6; H, 5.6; N, 11.05; CH_3O, 21.7; ash, 0.2.

Poly-α,β-DL-aspartic acid amide (VIIa). The above polyaspartic acid methyl ester (CH_3O = 21.7) was treated with 60 ml. of liquid ammonia for 50 hr. in a sealed tube at room temperature. The polyaspartic acid amide obtained after evaporation of the ammonia was dissolved in 12.5 ml. of water and lyophilized. The yellow powdery polyamide gave a positive biuret test and paper chromatography indicated the absence of low molecular weight components (solvent, butanol: acetic acid: water 4:1:1; development, ninhydrin).

Anal. Calcd. for $(C_4H_6O_2N_2)_\infty$: C, 42.1; H, 5.27; N, 24.5. Found: C, 39.7; H, 5.8; N, 23.7; CH_3O, 0.33%; ash, 0.1%.

Ammonolysis of anhydropolyaspartic acid. A solution of 1.5 g. of anhydropolyaspartic acid in 40 ml. of liquid ammonia was allowed to stand at room temperature in a sealed tube for 70 hr. After 10–12 hr. the heavier yellow, viscous anhydropolyaspartic acid–ammonia phase was separated. The ammonia was evaporated and the resulting glass was allowed to stand over sulfuric acid in a vacuum desiccator. The material was then dissolved in 5 ml. of water and lyophilized. The yield of powdery yellow polyamide, containing 23.12% nitrogen, was 1 g. Five hundred milligrams of the polyamide was further purified by dialyzing against ten 100-ml. portions of water during a period of 90 hr. The dialyzed solution provided 250 mg. of amide (VIIa) after lyophilization. This polyamide gave a positive biuret reaction and a zero R_f value using butanol: acetic acid: water solvent system.

Anal. Calcd. for $(C_4H_6O_2N_2)_\infty$: C, 42.1; H, 5.27; N, 24.5. Found: C, 40.12; H, 6.06; N, 22.7; ash 0.2%.

Hofmann degradation of poly-α,β-DL-aspartic acid amides. (A) A sample of 114 mg. of polyaspartic acid polyamide (prepared from anhydropolyaspartic acid by ammonolysis) was dissolved in 2.5 ml. of 0.4*N* sodium hypochlorite solution prepared from 10% sodium hydroxide solution. After standing at room temperature for 2 hr. the reaction mixture was treated with 4 ml. of *N* hydrochloric acid. The distillate from the neutral solution was bubbled into 30 ml. of 2*N* hydrochloric acid solution saturated with 2,4-dinitrophenylhydrazine. Twenty-one milligrams of acetaldehyde-2,4-dinitrophenylhydrazone was obtained. An additional amount of acetaldehyde-2,4-dinitrophenylhydrazone was obtained by hydrolysis of the residual solution with 10 ml. of 1.5*N* hydrochloric acid followed by further distillation for 15 min. The total yield of 2,4-dinitrophenylhydrazone was 52 mg., m.p. 140–145°, which represents 23.2% of the aspartyl residues in polyaspartic acid. Control experiments indicated a 32% loss of acetaldehyde during the isolation procedure which raised the amount of β-aspartyl residues to 34.1%. The m.p. of the acetaldehyde-2,4-dinitrophenylhydrazone was 146–147° after crystallization from alcohol and this material gave no depression with an authentic sample.

The residue after distillation was further hydrolyzed by boiling with 10 ml. of concd. hydrochloric acid for 4 hr. This solution was evaporated *in vacuo* and the residue was treated with 2 ml. of concd. hydrochloric acid. After removal of the sodium chloride by filtration the solution was evaporated to dryness and the hydrochloric acid treatment was repeated twice. The residue was repeatedly dissolved in 2 ml. of water and evaporated to dryness. The hydrochloric acid-free, brown residue was dissolved in 2 ml. of water and added to a saturated aqueous solution of 500 mg. of flavianic acid. After 1 day the α,β-diaminopropionic acid diflavianate was collected by filtration and washed twice with cold water. After drying *in vacuo* at 100°, over phosphorus pentoxide, the yield was 165 mg. This represents 22.5% of the α-aspartyl residues, and 37.7%, after corrections for losses. The crude diflavianate, m.p. 222–223°, melted at 224° after crystallization from water.

Additional samples of polyaspartic acid polyamide prepared from anhydropolyaspartic acid, were submitted to Hofmann degradation and the corrected values for β-aspartyl residues were 40.8% and 30.6% and for α-aspartyl residues the values were 15.6% and 28.4%.

(B) Anhydropolyaspartic acid obtained from α,β-polyaspartic acid was also converted to polyamide and Hofmann degradation indicated this material contained 45% β- and 18% α-peptide bonds.

(C) Similarly Hofmann degradation of the polyamide obtained from α,β-polyaspartic acid polymethyl ester gave corrected values of 33% for the 6-peptide bonds and 26.4% for the α-peptide bonds.

α,β-Diaminopropionic acid diflavianate. A solution of 124 mg. of α,β-diaminopropionic acid hydrobromide[23] in 3 ml. of water was added to a saturated solution containing 1 g. of flavianic acid. After 40 hr. the diflavianate was filtered and washed with two 2-ml. portions of cold water. After drying *in vacuo* at 100° over phosphorus pentoxide the average yield was 266 mg., 60%, m.p. 223°. This material gave no depression on admixture with the diflavianate obtained from Hofmann degradation of the polyamide. An analytical sample of diflavianate was prepared by crystallization from water, m.p. 223°.

Anal. Calcd. for $C_{23}H_{20}O_{18}N_6S_2$: C, 37.7; H, 2.75; N, 11.47. Found: C, 37.41; H, 2.92; N, 11.34.

In order to determine the loss of acetaldehyde during the isolation procedure 10 ml. of a 0.503%. acetaldehyde water solution was treated with 10 ml. of 0.1*N* hydrochloric acid and after standing 15 min. the acetaldehyde was distilled into acidic dinitrophenylhydrazine reagent. The average yield of dinitrophenylhydrazone was 173 mg. (68%), m.p. 147°. The yield was unchanged when 1.5*N* hydrochloric acid was used.

Acetaldehyde was not obtained when anhydropolyaspartic acid or polyaspartic acid polymethyl ester was treated with

sodium hypochlorite solution under the conditions used for the Hofmann degradation of polyaspartic acid polyamide.

Pyrolysis of DL-*acetylaspartic acid.* DL-Acetylaspartic acid (6.176 g., m.p. 142–144°) was heated *in vacuo* at 200°. Acetic acid and water which were formed during the pyrolysis were collected in a Dry Ice trap. After 6 hr. the loss of weight was 2.365 g. (85.92%) and 2.503 g. (90.93%) after 11 hr.; the calculated loss for 1 molecule of acetic acid, and water is 2.752 g. The trap content contained 1.878 g. (88.21%) acetic acid. Infrared spectrum of the glassy polymeric material was identical with that of anhydropolyaspartic acid obtained from aspartic acid.

Anal. Calcd. for $(C_4H_3O_2N)_\infty$: N, 14.4. Found: 13.32.

Another sample of DL-acetylaspartic acid (175 mg.) was heated the same way at 148° for 4 hr. The loss of weight was 55 mg. (70.5%) and 44.5 mg. (65.4%) acetic acid was obtained from the Dry Ice trap.

DL-Acetylaspartic anhydride was recovered unchanged after heating at 148° for 4 hr.; however acetamide as a decomposition product was isolated when it was heated at 200°.

*Poly-α,β-*DL-*aspartic-(β-aminoethyl)amide* (VIIb). Anhydropolyaspartic acid (100 mg.), prepared from α,β-poly-DL-aspartic acid (molecular weight 12,100), was added in small portions to 5 ml. of anhydrous ethylenediamine. The yellow solution stood at room temperature for 20 hr. and was then refluxed for 1.5 hr. After the ethylendiamine had been distilled *in vacuo* the residue was dissolved in water and dialyzed against ten 200-ml. portions of water for 3 hr. The solution within the dialyzing sack was lyophilized; the residue was titurated with an alcohol-ether mixture, then with ether and dried under vacuum at 78° yielding 60 mg. of yellowish hygroscopic powder. The biological activity of this basic polypeptide derivative is under examination.

Anal. Calcd. for $(C_6H_{11}O_2N_3)_n$: N, 26.7. Found: N, 22.0.

The equivalent weight obtained by potentiometric titration using 0.01*N* hydrochloric acid was 167.5; calculated mean residue weight is 157.

L-*Aspartic anhydride hydrobromide.* Carbobenzoxy-L-aspartic anhydride (1.25 g., 0.005 mole) was treated with 7 g. of 15% hydrogen bromide in acetic acid at room temperature for 1 hr. Crystalline L-aspartic anhydride hydrobromide deposited. The precipitation was brought to completion by the addition of absolute ether at 0°. The precipitate was filtered with the exclusion of moisture then washed with a mixture of absolute ether and acetic anhydride, finally with absoluble ether and dried over phosphorus pentoxide. It was soluble in acetone and dimethylformamide; yield 0.85 g., 86.7%, m.p. 166–169° dec., $[\alpha]_D^{22}$ −21.26° (*c* 2.3, in dimethylformamide).

Anal. Calcd. for $C_4H_5O_3$ N·HBr: C, 24.49; H, 3.09; N, 7.14. Found: C, 24.36; H, 3.44; N, 7.11.

L-*Aspartic acid α- and β-anilide.* L-Aspartic anhydride hydrobromide was heated with an excess of aniline at 100° until a clear solution was obtained. The reaction mixture was treated with dilute sodium hydroxide solution, the aniline was extracted with ether and the slightly basic solution was acidified with dilute hydrochloric acid, to a *p*H 1.5, which precipitated the mixture of α-, and β-anilides. The yield was nearly quantitative. This mixture of anilides was dissolved in small amount of hot water and then five volumes of methanol was added to the filtered solution. After standing overnight at 2° nearly pure β-anilide separated. Recrystallization from water yielded needles of the β-anilide, m.p. 235–236° dec., $[\alpha]_D^{24}$ +33.4° (*c* 1.0, 0.1*N* hydrochloric acid). Several recrystallizations raised the m.p. to 241–242°; the mixed m.p. with a large amount of α-anilide was 220–230°.

Anal. Calcd. for $C_{10}H_{12}O_3N_2$: C, 57.68; H, 5.81; N, 13.46. Found: C, 57.42; H, 5.84; N, 13.50.

The mother liquor from β-anilide was evaporated to one fifth of its volume and the α-anilide separated as needles. After recrystallization from water the m.p. was 212° dec. Mixtures of this product with large amount of β-anilide melted over a wide range at *ca.* 200°, $[\alpha]_D^{24}$ +62.5° (*c* 0.1, 0.1*N* hydrochloric acid).

Anal. Calcd. for $C_{10}H_{12}O_3N_2$: C, 57.68; H, 5.81; N, 13.46. Found: C, 57.34; H, 5.86; N, 13.20.

The α-anilide gave a brown color and the β-anilide a violet color when paper chromatograms were developed with ninhydrin.

L-*Isoasparagine.* A solution of L-aspartic anhydride hydrobromide in dimethylformamide was added to a large excess of liquid ammonia. The ammonia was allowed to evaporate and the remaining solvent was removed by evaporation at low pressure. The residue was treated with water and then evaporated *in vacuo;* the addition of water, and evaporation were repeated. Paper chromatogram of this crude material (phenol:water) indicated the presence of isoasparagine contaminated with a very small amount of asparagine. After recrystallization from alcohol-water chromatographically pure isoasparagine was obtained. A sample which was dried over phosphorus pentoxide *in vacuo* at 78° melted at 185–188° and had a specific rotation identical with that of isoasparagine prepared by Bergmann and Zervas.[24]

α-*Methyl-*L-*aspartate.* L-Aspartic anhydride hydrobromide was moistened with small amount of acetic anhydride and then dissolved in large excess of absolute methanol. The solvent was removed *in vacuo,* and the residue dissolved in water. An excess of silver oxide was added to the water solution to remove the bromide. The reaction mixture was filtered, the filtrate was treated with hydrogen sulfide, filtered again and evaporated to dryness. The recrystallization of the solid residue was from an alcohol–water solution, yielded L-aspartic acid α-methyl ester, m.p. 167°; $[\alpha]_D^{28}$ +37.60 (*c.* 0.5, water).

Anal. Calcd. for $C_5H_9O_4N$: C, 40.81; H, 6.17; N, 9.52. Found: C, 40.56; H, 6.21; N, 9.53.

A sample of the ester was dissolved in liquid ammonia and kept at room temperature in a sealed tube. Chromatographically pure isoasparagine was obtained which gave no depression on admixture with an authentic sample.

*Poly-α,β-*L-*aspartic acid.* L-Aspartic anhydride hydrobromide (980 mg.) was added to 2 ml. of dry pyridine (dried over barium oxide). Polymer began to form in addition to crystalline pyridine hydrobromide. After heating for an hour at 70° the pyridine was removed *in vacuo.* The residue was dissolved in 10 ml. of water and evaporated to dryness under reduced pressure. This procedure was repeated five times. The residue was dissolved in 5 ml. of water and dialyzed against five 50-ml. portions of 0.005*N* hydrochloric acid over a period of 10 hr. The dialysis was continued against twenty 1000-ml. portions of distilled water over a period of 144 hr. The solution within the dialyzing sack was lyophilized then dried at 58° *in vacuo* over phosphorus pentoxide; a total of 338 mg. (58.8%) α,β-poly-L-aspartic acid was obtained.

Anal. Calcd. for $C_4H_5O_3N$: C, 41.7; H, 4.4; N, 12.2. Found: C, 41.5; H, 4.5; N, 12.0.

Van Slyke amino-nitrogen determination gave a molecular weight of 7200 (61 residues).

Poly-α,β-L-aspartic acid (250 mg.) was hydrolyzed with 2.5 ml. of 6*N* hydrochloric acid in a sealed tube at 110° for 24 hr. The reaction mixture was evaporated to dryness, and the residue was neutralized with dilute sodium hydroxide to precipitate aspartic acid: $[\alpha]_D^{22}$ +25.4° (*c* 1, 2.5*N* hydrochloric acid).

*Methyl poly-α,β-*L-*aspartate.* This polyester was prepared according to the procedure described above. The dialyzed and lyophilized product was dried at 58° *in vacuo* over phosphorus pentoxide providing a polyester in which 93% of the carboxyl groups were esterified.

Anal. Calcd. for $(C_5H_7O_3N)_\infty$: C, 46.5; H, 5.42; N, 10.85; CH_3O, 24.0. Found: C, 45.0; H, 5.8; N, 11.0; CH_3O, 22.4; ash, 0.3.

*Poly-α,β-*L-*aspartic acid amide.* The polyester was con-

(23) E. Frankland, *J. Chem. Soc.*, **97**, 1318 (1910).

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

verted into the polyamide using liquid ammonia at room temperature. Analysis of the dialyzed and dried product indicated that 92% of the carboxyl groups were converted into amide groups.

Anal. Calcd. for $(C_4H_6O_3N_2)_\infty$: C, 42.1; H, 5.3; N, 24.5. Found: C, 40.7; H, 5.9; N, 23.5; ash, 0.2.

Hofmann degradation of poly-α,β-L-aspartic acid amide. Two samples of polyamide prepared above was submitted to Hofmann degradation, and the corrected value for β-aspartyl residues was 40.2% and for α-aspartyl residues the value was 25.7%.

Preparation of anhydropolyaspartic acid from poly-α-L-aspartic acid. A. By direct heating. Poly-α-L-aspartic acid (90 mg.) was converted into anhydropolyaspartic acid according to the procedure described previously.

Anal. Calcd. for $(C_4H_5O_3N)_\infty$: N, 14.4. Found: N, 14.3; ash, 0.1. Anhydropolyaspartic acid was also prepared by heating poly-α-L-aspartic acid in tetralin and removing the water formed by azeotropic distillation.

B. *By heating with acetic anhydride.* To a solution of 50 mg. of poly-α-L-aspartic acid in 1 ml. of dimethylformamide was added 2 ml. of acetic anhydride. The mixture was heated at 100° for 3 hr. After half an hour heating anhydropolyaspartic acid slowly separated. Ten milliliters of ether was added to the cold reaction mixture and the precipitate was centrifuged, washed twice with 10 ml. of ether, and dried. Yield of tawny anhydropolyaspartic acid was 38 mg.

Anhydropolyaspartic acid obtained by procedure A or B is insoluble in sodium bicarbonate solution and gives a strong biuret reaction.

Anhydropolyaspartic acid (38 mg.) prepared by procedure B, was hydrolyzed with 10 ml. of 6*N* hydrochloric acid for 10 hr. The solution was evaporated *in vacuo* and the residue dissolved in 10 ml. of *N* hydrochloric acid; $[\alpha]_D^{20}$ +21.9° (*c*, 4.56, 5.64*N* hydrochloric acid).

Partial hydrolysis of anhydropolyaspartic acid obtained from poly-α-L-aspartic acid. Anhydropolyaspartic acid (75 mg.) prepared by direct heating of poly-α-L-aspartic acid was hydrolyzed according to the procedure described previously; yield was 38 mg. after drying at 65° for 6 hr.

Anal. Calcd. for $(C_4H_5NO_3)_\infty$: N, 12.2. Found: N, 11.8; ash, 1.1.

Hofmann degradation of poly-α,β-aspartic acid obtained from poly-α-L-aspartic acid. Poly-α,β-aspartic acid methyl ester was prepared from polyaspartic acid obtained above, by the procedure described previously. Purification was achieved by dialyzing the solution of the ester. After lyophilization and drying *in vacuo* at 65°, 86 mg. of product was obtained containing 21.2% methoxyl group (Calcd., OCH_3, 24%).

By a procedure similar to that described previously, poly-α,β-aspartic acid methyl ester was converted into poly-α,β-aspartic acid amide. This polyamide after purification by dialysis contained 21.4% nitrogen and 0.2% methoxyl group which indicated that about 74% of the carboxyl groups are amidated.

Hofmann degradation of this poly-α,β-aspartic acid amide gave corrected values of 44.8% for the β-peptide bonds and 40.8% for the α-peptide bonds.

N-Acetyl-DL-asparagine α-anilide. *N*-acetyl-DL-aspartic α-anilide (XIV) (5 g., 0.02 mole) was dissolved in a mixture of 250 ml. of dry dioxane, 50 ml. of dry chloroform, 77 ml. of dry dimethylformamide, 75 ml. of dry tetrahydrofuran and 2.8 ml. of triethylamine. The solution was cooled to 0° and 3 ml. (0.02 mole) of ethyl chloroformate was added dropwise with vigorous stirring. After 20 min., 5 ml. of concd. ammonium hydroxide was added. Stirring was continued for 7 hr. at room temperature and 200 ml. of water was added to reaction mixture. The *p*H was then adjusted to 7 by addition of hydrochloric acid. The solution was evaporated to dryness, the residue was suspended in a small amount of water, filtered, and the crystalline material was successively washed with sodium bicarbonate solution, dilute hydrochloric acid, and water. The residue, 3.5 g. (70%) melted at 223–225°. Recrystallization from alcohol then from water raised m.p. to 226–229° dec. This substance did not yield acetaldehyde when treated with sodium hypochlorite and then hydrolyzed with 5*N* hydrochloric acid.

Anal. Calcd. for $C_{12}H_{15}O_3N_3$: C, 57.81; H, 6.06; N, 16.86. Found: C, 57.63; H, 6.30; N, 16.82.

N-Acetyl-DL-isoasparagine β-anilide. The preparation of *N*-acetyl-DL-isoasparagine β-anilide was similar to that of *N*-acetyl-DL-asparagine α-anilide. Yield was 44.4%, m.p. 208–210°. The compound was recrystallized from alcohol, m.p. 214–216°, mixed melting point with a sample of the compound obtained from DL-acetamidosuccinoyl anilide with ammonia gave no depression.

N-Acetyl-DL-isoasparagine β-anilide (53 mg.) was treated with 3.3 ml. of 0.48% sodium hypochlorite solution at 0° for 5 min. and allowed to stand at room temperature for 10 min. and finally raised to 80° for 15 min. The clear solution was acidified with 5 ml. of 5*N* hydrochloric acid and distilled into a saturated solution of 2,4-dinitrophenylhydrazine in 20 ml. 2*N* hydrochloric acid. Acetaldehyde 2,4-dinitrophenylhydrazone (10.6 mg.) was precipitated, melted at 147° alone, and admixed with authentic material.

N-Phenyl-DL-acetamidosuccinimide (XVI). *A. From N-Acetyl-DL-aspartic β-anilide.* *N*-Acetyl-DL-aspartic β-anilide (XV), m.p. 177–178°, (200 mg., 0.0008 mole) and 2 ml. of acetic anhydride were heated at 95° for 1 hr. The solvent was removed *in vacuo* and the crystallization of the resulting sirup was induced by the addition of benzene. The crystals were filtered and washed with benzene. The crude imide weighed 139 mg., (70.5%), m.p. 160–162°. Recrystallization from absolute ethyl alcohol raised the m.p. to 162–164°. The infrared spectrum contained a double band at 5.60 and 5.86 μ.

Anal. Calcd. for $C_{12}H_{12}O_3N_2$: C, 62.06; H, 5.21. Found: C, 61.76; H, 5.13.

B. *From N-Acetyl-DL-aspartic α-anilide.* *N*-Acetyl-DL-aspartic α-anilide (XIV) (1.00 g., 0.004 mole, m.p. 203–204°) was converted into the imide as described above. This material was identical (mixed melting point and infrared) with the material obtained from *N*-acetyl-DL-aspartic β-anilide; yield 0.79 g., 85%.

Partial hydrolysis of N-phenyl-DL-acetamidosuccinimide. *N*-Phenyl-DL-acetamidosuccinimide (XVI) (5 g., 0.02 mole) was dissolved with stirring at 0° in 5 ml. 0.5*N* sodium hydroxide (0.025 mole). After 30 min. the clear solution was put into an icebox for an hour. The filtered solution was acidified with 3.4 ml. of 5.1*N* hydrochloric acid. The precipitate that appeared was filtered and washed with a small volume of water giving 4.7 g. (88%) mixture of *N*-acetyl-DL-aspartic anilides, m.p. 178–180°. Addition of 1 ml. of 5.1*N* hydrochloric acid to the filtrate gave a second crop of white needles, 0.45 g. (9%), m.p. 179–182°; total yield 5.15 g. (97%). This mixture of the α- and β-anilides was then treated with 30 ml. of hot alcohol and filtered. *N*-Acetyl-DL-aspartic β-anilide (750 mg., m.p. 177–178°) separated as white needles from the cooled filtrate. The undissolved material was then extracted with 80 ml. of hot dry acetone. From the filtrate a second crop of 980 mg. of β-anilide was obtained, m.p. 176–178°. The acetone-insoluble material, m.p. 191–192°, was treated again with 20 ml. of hot alcohol and filtered. The alcohol insoluble *N*-acetyl-DL-aspartic α-anilide (300 mg. 5.6%) melted at 202–204°. One crystallization of this material from water raised the melting point to 204–205°. This substance had no depression on mixed melting point with the sample prepared from *N*-acetyl-DL-aspartic anhydride and aniline; the infrared spectra showed identical bands at 5.9, 6.0, 6.1, 6.3, 6.6 μ. All the mother liquors were combined and evaporated to dryness. The residue was treated with 10 ml. of 5% sodium bicarbonate solution, filtered, and the filtrate was acidified with hydrochloric acid. The precipitate (610 mg.) which melted at 178–179° was mixed with the other crops and the combined mixture melted at 176–178°; total yield 2.34 g., 44%. This *N*-acetyl-DL-aspartic β-anilide had no depression on

mixed melting point with a sample prepared from *N*-acetyl-DL-aspartic anhydride and aniline. Infrared spectra showed identical bands at 5.8, 6.0, 6.25, 6.45, 6.7 μ.

Action of ammonia on N-phenylacetamidosuccinimide. *N*-Phenylacetamidosuccinimide (XVI) (0.75 g., 0.00324 mole) was treated with 4 ml. of concd. aqueous ammonia for 12 hr. The resulting solid was filtered and washed with water. Recrystallization from 80 ml. of alcohol gave 0.61 g. (81.2%) of *N*-acetyl-DL-aspartic α-amide β-anilide, m.p. 214–216°. This product gave no depression on admixture with a sample of the compound obtained from *N*-acetyl-DL-aspartic β-anilide by the mixed anhydride method. Hofmann degradation of 50 mg. of this substance with 3.5 ml. of 0.48% sodium hypochlorite solution gave 10 mg. of acetaldehyde-dinitrophenylhydrazone m.p. 147° alone and admixed with authentic material. A paper chromatographic analysis of the hydrolyzed mother liquor failed to indicate the presence of α,β-diaminopropionic acid. In second experiment 2.039 g. (0.0088 mole) of the imide (XVI) was treated with 30 ml. of concd., aqueous ammonia at room temperature for 16 hr. and finally evaporated to dryness. Sodium hypochlorite solution (23 ml., 30.83 mg. sodium hypochlorite per ml.) was added to the residue at 0° with stirring. The stirring was continued at room temperature for 40 min. and then at 80° for 15 min. The clear solution was acidified with 15 ml. of 6*N* hydrochloric acid and distilled into 2,4-dinitrophenylhydrazine hydrochloride solution until acetaldehyde 2,4-dinitrophenylhydrazone (87.6 mg.) was formed. The remaining solution was refluxed for 5 hr. and then evaporated to dryness. The residue was treated with 5 ml. of concd. hydrochloric acid. The undissolved sodium chloride was filtered off and the filtrate was evaporated and dried over sodium hydroxide. Semiquantitative paper chromatography (butanol, acetic acid, water; 4:1:5) indicated the presence of about 150 mg. of α,β-diaminopropionic acid (10%) in addition to aspartic acid.

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[CONTRIBUTION FROM THE NEUROSURGICAL SERVICE OF THE MASSACHUSETTS GENERAL HOSPITAL AND THE DEPARTMENT OF SURGERY OF HARVARD MEDICAL SCHOOL]

Synthesis of p-[Di(2-C14-chloroethyl)amino]-L-phenylalanine. A Study of Bis(β-hydroxyethylation) of Arylamines[1]

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A study is carried out of various methods for the bis(β-hydroxyethylation) of aryl amines with a view toward synthesizing C^{14}-labelled nitrogen mustards of high specific activity. The label would be in the biologically-active portion of the molecule. The synthesis of p-[di(2-C^{14}-chloroethyl)amino]-L-phenylalanine was carried out.

The assumption that the effectiveness of cytotoxic agents in the treatment of cancer is greatest if they preferentially concentrate in neoplastic tissue would seem to be a rational hypothesis. On this basis, the treatment of brain tumors by these compounds offers a distinct advantage over such therapy for neoplasms in other regions of the body. Tumors of the brain have a considerably altered permeability to many substances relative to adjacent normal areas[2] and consequently, it seems feasible to devise cancerocidal substances which, by cerebral perfusion, would concentrate selectively in the tumor. Work in this laboratory with aromatic boron compounds[3,4] has yielded information as to types of structures and substituents which aid or restrict the passage of organic compounds into the brain. Lipid solubility has been observed to be an important criterion in determining this rate and ease of penetration of the brain by such substances.[3,4]

Previous work with P^{32}-labelled triethylene thiophosphoramide (thio-TEPA) showed[5] that alkylating agent had a high lipid solubility and penetrated normal brain more readily and accumulated in higher concentration than in the corresponding neoplastic tissue. Based on our initial hypothesis, this would be considered an undesirable compound. On the other hand, p-[di(2-chloroethyl)amino]-L-phenylalanine had a low lipid solubility[6] as determined by the standard partitioning procedure.[3] From this consideration and the known high biological activity of melphalan,[7] the preparation of this compound with a C^{14}-label was undertaken to study its localization in brain and brain tumor as a function of its lipid solubility. The radioactive DL-compound with the label in the phenylalanine position of the molecule had been prepared[8] from carboxy-labelled benzoic acid. A synthesis incorporating C^{14} in high yield in the mustard group, however, would offer two important advantages: (1) a general method for preparing most nitrogen and sulfur mustards with a C^{14} label and (2) the label would be in the alkyl-

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