tri-peptide is hydrolyzed. It may be that the products of hydrolysis have an inhibitory effect on the further conversion or possibly, but improbably, there is an equilibrium between the di-DNP-val-his and the unrecognized product.

Conclusions

Our earlier experiments[2] on the release of the N-terminal products of DNP-globin showed the almost immediate release of DNP-val-leu from the α chains. Then, simultaneously, there followed the hydrolysis of the val-leu bond and of an unknown bond from the β chains: the hydrolysis of both bonds yielded DNP-valine. Inasmuch as the hydrolysis of the val-leu bond was a straightforward, uncomplicated reaction for which a first-order reaction rate constant could be obtained, it was possible to calculate a reaction rate constant for the hydrolysis of the unknown bond. On the basis of this calculated constant, the β chains release DNP-valine more slowly than does the DNP-val-leu from the α chains and, indeed, 20% of each β chain should still be unhydrolyzed after 22 hr. in refluxing 6 *N* hydrochloric acid. If 20% of each of the two β chains is indeed unhydrolyzed after 22 hr., it provides a reasonable explanation of our earlier finding that DNP-globin contained 3.6 N-terminal valyl residues[3]: 20% of two chains equals 0.4 N-terminal residue and thus the actual number of N-terminal residues would be four.

Because this kinetic interpretation of the results is difficult to correlate with the known rates of hydrolysis of peptide bonds, serious consideration had to be given to the possibility that the rate constant was an apparent one only and that actually the destruction of DNP-valine in the course of the hydrolysis of the β chains was greater than the destruction of DNP-valine in the course of the hydrolysis of the α chains. These possibilities have been discussed in detail in previous publications.[2,3]

The present kinetic study of the hydrolysis of the DNP-peptides from the β termini has failed to provide an unequivocal answer to these questions. Because of the side reactions that occur during the hydrolysis of di-DNP-val-his and di-DNP-val-his-leu, the calculation of a reaction rate constant is meaningless. Comparison, therefore, cannot be made with the constant for the β chains that was calculated from the hydrolysis of DNP-globin.[2] If the constant is correct, 20% of di-DNP-val-his should be unhydrolyzed after 22 hr. in refluxing 6 *N* hydrochloric acid. Actually, at most, only a few per cent. may be detected. One might, therefore, conclude that the constant for the hydrolysis of the β chains is very similar to that of DNP-val-leu of which about 5% is unhydrolyzed after 22 hr. This conclusion is unwarranted because of the complicated course of the hydrolysis of di-DNP-val-his and because of our ignorance as to whether or not unrecognized products may still be present after 22 hr. On the other hand, if both the di-DNP-val-his and the unrecognized product are essentially hydrolyzed in 22 hr., the recovery of only 85 to 90% of the DNP-valine must mean that the destruction of DNP-valine is different in the course of the hydrolysis of the two chains.

One can conclude only that our original difficulty in arriving at an integral number of N-terminal residues in DNP-globin is associated with the different hydrolytic behavior of the two types of chains, but it cannot be decided with certainty whether the difference lies in the rates of hydrolysis or in the degree of destruction.

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PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT AND OCEANOGRAPHIC INSTITUTE OF THE FLORIDA STATE UNIVERSITY]

The Characterization of Polyaspartic Acid and Some Related Compounds[1,2]

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The "polyaspartic acid" obtained by heating aspartic acid above the boiling point of water is shown to be a mixture consisting of polyimide molecules with tightly held water in a ratio of almost one molecule of water per imide linkage. This water is removable by rigorous drying under reduced pressure at 200°. The polyimide polyhydrate can be converted to a true peptide-type polyaspartic acid by brief warming with dilute sodium hydroxide. The relationships between these various species have been clarified by study of the infrared analyses and in other ways. A typical average molecular weight for polyaspartic acid is 11,000, as determined by end-group assay. Data are presented to show that slightly hydrolyzed diketopiperazines may yield in chemical analyses results which resemble closely those from linear peptides. Rapid methods for distinction between diketopiperazines and polyamino acids were developed and are evaluated. These studies showed that the polymer is definitely not a diketopiperazine.

Syntheses of polypeptides and other protein-like materials in the past have included stepwise procedures,[3] fabrication of materials with recurrent amide linkages as exemplified by nylon[4] and the conversion of amino acids to "polyamino acids" by various methods.[1,5–7] For the most part, poly-

(1) Contribution No. 95 of the Oceanographic Institute of the Florida State University, Tallahassee. Presented at the Miami meeting of the American Chemical Society, April 7, 1957. Aided by Grant RG-4666 of the National Institutes of Health, Public Health Service and the General Foods Corporation.

(2) Except for the title, the term polyaspartic acid is not used without qualification in this paper until the evidence for the prevalent structures is reviewed.

(3) E. Abderhalden and A. Fodor, *Chem. Ber.*, **49**, 561 (1916).

(4) W. H. Carothers, U. S. Patent 2,071,250 (1937).

(5) E. Katchalski, *Advances in Protein Chem.*, **6**, 123 (1951).

(6) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 737 (1953).

(7) C. H. Bamford, A. Elliott and W. E. Hanby, "Synthetic Polypeptides," Academic Press, New York, N. Y., 1956.

amino acids have been prepared through the oxazolidinediones[5,7] (N-carboxyamino acid anhydrides, Leuchs' anhydrides).

Although several workers have prepared polypeptides by dry heating of amino acids,[8-11] the thermal approach has appeared to be unpromising because of reactions such as deamination, decarboxylation and cyclodehydration.[5,7,12,13] This view is typified by the comment of Curphey[12] that "In the simpler and more complex α-amino acids, the propinquity of the amino and carboxy groups favours dimerization to diketopiperazine or its appropriate alkyl derivatives and accordingly the preparation of linear polypeptides using amino-acids is one of difficulty."

Experimental findings in these laboratories have resulted in a re-evaluation of the possibility of producing linear peptides by pyrocondensation of amino acids. Enzymic experiments with amino acid derivatives have suggested that the amino acids themselves might lead to an ordering of residues in any chain formed thermally[14,15] in a way that would correspond to the orders observed in nature. The results of experiments that ensued demonstrated that a number of amino acids which fail to give linear peptides when heated alone do so in concert. The dicarboxylic amino acids, glutamic acid and aspartic acid, appear to be critical in these copolymerizations. Of these two, aspartic acid yields a homopolymer, for which adequate characterization thus becomes necessary.[5]

The possibility that polymers of aspartic acid are diketopiperazines rather than linear peptides has been examined critically with the finding that they are largely or entirely linear. The likelihood of dimerization to diketopiperazines was particularly apparent for other amino acids when one combination gave rise almost entirely to diketopiperazines.[16]

A search for adequate methods for distinguishing between diketopiperazines and polypeptides demonstrated that many of the usual methods for characterizing polypeptides are ambiguous. The possibility of the DKP (diketopiperazine) from phenylalanine for example, being mistaken for polyphenylalanine is a strong one, as shown in Table I. Table I emphasizes the need for particular care in interpreting data of these types and for obtaining conclusive proof of structure for polypeptides, particularly when the corresponding diketopiperazines are not soluble in water. Reconsideration of some of the "polypeptides" recorded in the older literature may well be in order, because of possible analytical confusion of polypeptides and slightly hydrolyzed diketopiperazines.[17]

Several amino acids have been reported to lead to diketopiperazines on individual heating.[7,16,18] It is of signal interest that concerted heating of two or more amino acids leads some combinations of amino acids to polymerize to linear peptides.[16] Studies of this sort have also indirectly led to a thermal theory of biochemical origins.[15,16,19,20] In this latter realm of interpretation it is of particular interest to find and study thermal or other presumably primordial conditions which will yield variegated polyamino acids.

TABLE I

COMPARISON OF PROPERTIES OF THE DIKETOPIPERAZINE FROM PHENYLALANINE AND THE EXPECTED PROPERTIES OF POLYPHENYLALANINE[a]

Property	Phenylalanine DKP	Polyphenylalanine[a]
Solubility in water	Very slight	Very slight[b]
Dialysability in water	Non-diffusible[c]	Non-diffusible
Reactivity in ninhydrin test	None	None
Product of total hydrolysis	Quantitative yield of phenylalanine	Quantitative yield of phenylalanine
C, H, N analysis	C, 73.4; H, 6.2; N, 9.5[d]	C, 73.4; H, 6.2; N, 9.5[d]
Amino nitrogen (Van Slyke)[e] assay	0.09%[d,f]	0.09%[d]

[a] For calculations, a molecular weight of 15,000 was used. [b] C. H. Bamford, A. Elliott and W. E. Hanby, "Synthetic Polypeptides," Academic Press, New York, N. Y., 1956, p. 313. [c] Due to insolubility of phenylalanine DKP in water. [d] Calculated values. [e] G. Kainz and F. Schöller, *Biochem. Z.*, **327**, 292 (1955). [f] Assuming 0.02% of the diketopiperazine is hydrolyzed to the dipeptide in the 5 minute Van Slyke reaction period. Diketopiperazines are known to be somewhat unstable in acid and base.[17]

Formation of aspartic acid polymers by heating aspartic acid in the dry state was first reported by Schiff[9] who suggested that the reaction products were tetra- and octa-aspartic acid anhydrides. Kovacs and co-workers[10] proposed that heating aspartic acid results in formation of a reactive internal anhydride which condenses to give a polyimide which is readily converted in turn to an open polyamino acid in alkaline solution. Kovacs' concepts have been extended in this study.

Products of pyrocondensation of aspartic acid, alone or with other amino acids, give positive biuret tests. The positive biuret tests were not considered sufficient evidence for polypeptides as the predominant thermal product from heating amino acids, since diketopiperazines may be present and aspartic acid peptides respond uniquely when treated with alkaline copper solution[21] (Table II). It was therefore thought necessary to synthesize the appropriate controls and to use a number of criteria for distinguishing between linear and cyclic condensation products. Rapid methods for positive distinction between polypeptides and diketopiperazines were sought.

The production of 3,6-diketopiperazine-2,5-diacetic acid (aspartic acid DKP) was accordingly

(8) E. Schaal, *Ann.*, **157**, 24 (1871).
(9) H. Schiff, *Chem. Ber.*, **30**, 2449 (1897); *Ann.*, **307**, 231 (1899).
(10) J. Kovacs, I. Könyves and A. Pusztai, *Experientia*, **9**, 459 (1953); J. Kovacs and I. Könyves, *Naturwiss.*, **41**, 333 (1954).
(11) A. B. Meggy, *J. Chem. Soc.*, 1444 (1954).
(12) E. G. Curphey, *Chemistry and Industry*, 783 (1956).
(13) J. Noguchi and T. Hayakawa, THIS JOURNAL, **76**, 2846 (1954).
(14) S. W. Fox, M. Winitz and C. W. Pettinga, THIS JOURNAL, **75**, 5539 (1953).
(15) S. W. Fox, *Amer. Scientist*, **44**, 347 (1956).
(16) S. W. Fox, A. Vegotsky, K. Harada and P. D. Hoagland, *Ann. N. Y. Acad. Sci.*, **69**, 328 (1957).
(17) I. S. Yaichnikov, *J. Russ. Phys. Chem. Soc.*, **58**, 879 (1926); *C. A.*, **21**, 1124 (1927).
(18) E. Erlenmeyer and A. Lipp, *Ann.*, **219**, 208 (1883).
(19) S. W. Fox, J. E. Johnson and A. Vegotsky, *Science*, **124**, 923 (1956).
(20) S. W. Fox, *J. Chem. Educ.*, **34**, 472 (1957).
(21) M. I. Plekhan, *Zhur. Obshchei. Khim.*, **22**, 1633 (1952); *C. A.*, **47**, 2226 (1953).

TABLE II

BIURET REACTIONS AND ABSORPTION MAXIMA OF COPPER COMPLEXES OF ASPARTIC ACID POLYMER AND RELATED COMPOUNDS

Compound	Visual color	Copper complex spectral absorption max., mμ
Aspartic acid	Trace blue[a]	.....
Glycylglycine	Blue	625–650
Glycine DKP[b]	Blue	620–640
Aspartic acid DKP dimethyl ester	Blue	630
Aspartic acid DKP	Blue	630
3 Glycylglycine: 1 aspartic acid polymer	Blue-violet	570–590
L-Asparagine	Blue-violet	560–580
Valylglycylphenylalanine	Violet	555–565
1 Glycylglycine: 3 aspartic acid polymer	Lilac	545–565
Aspartic acid polymer	Lilac	540–550
Blood albumin	Lilac	535
Aspartic acid DKP diamide	Lilac	530

[a] Trace blue due to copper sulfate reagent. [b] The diketopiperazine from glycine.

TABLE III

PAPER CHROMATOGRAPHY OF POLYASPARTIC ACID AND OF ASPARTIC ACID DKP

Material	Solvent system	R_f
Polyaspartic acid I[a]	A[b]	..
Aspartic acid DKP	A	0.24
Polyaspartic acid I[a]	B[c]	..
Aspartic acid DKP	B	0.15

[a] Polysuccinimide derivative; see text. [b] Butanol: acetic acid:water/4:1:1. [c] Lutidine:water/65:35.

Paper chromatographic methods for checking contamination of polypeptides by diketopiperazine also were employed[25] (Table III) as well as the infrared spectra (Table IV).

Determination of the molecular weight of the polymer posed the difficulties that are observed with compounds that are not readily soluble and are subject to hydrolysis. Methods which would minimize these effects were sought. The average molecular weight was determined by sedimentation velocity and by end-group assay. The sodium salt of polyaspartic acid was prepared in order to test the concept that a polycyclic imide which is first formed is converted to a true linear peptide by alkaline treatments.[10]

Finally, a comparison of aspartic acid polymers of other workers with the preparation from this Laboratory was tabulated (Table V).

Experimental[26]

Preparation of 2,5-Dibenzyl-3,6-diketopiperazine.—This compound was synthesized by refluxing phenylalanine in ethylene glycol.[22] *Anal.* Calcd. for $C_{18}H_{18}N_2O_2$: N, 9.5. Found: N, 9.5.

Preparation of 3,6-Diketopiperazine-2,5-diacetamide.—The diamide was synthesized by the reaction of diethyl fumarate with ethanolic ammonia.[23,27]

The diamide m. 303–305° (dec., block). *Anal.* Calcd. for $C_8H_{12}N_4O_4$(DL): N, 25.0. Found: N, 24.9.

Preparation of Dimethyl 3,6-Diketopiperazine-2,5-diacetate.—The procedure of Fischer and Koenigs[24] was used with several slight modifications.[28] Fischer and Koenigs started with L-aspartic acid whereas the DL compound was used here, as well as the L form; the L diester m. 247–248°, in close agreement with the literature.[24]; the DL diester m. 209–210°. *Anal.* Calcd. for $C_{10}H_{14}N_2O_6$(DL): N, 10.9. Found: N, 11.1.

Preparation of 3,6-Diketopiperazine-2,5-diacetic Acid.[24]—The L diacid in the literature and the DL synthesized here both m. >300°. The over-all yield from aspartic acid was about 7%. The DL was analyzed. *Anal.* Calcd. for $C_8H_{10}O_6N_2$: N, 12.2. Found: N, 11.9.

TABLE IV

SUMMARY OF INFRARED ABSORPTION SPECTRA OF POLYASPARTIC ACID AND RELATED COMPOUNDS[a]

Compound	Approximate wave number, cm.$^{-1}$ of characteristic peaks: 3300[b]	3200[c]	3080[d]	1780[e]	1715[c]	1650[d]	1600[f]	1550[d]	1280[d]
Aspartic acid DKP	..[g]	S[h]	W[i]	..	..	S	S	..	M[j]
Polyaspartic acid I	..	..	..	W	S	..	..	M	M
Sodium polyaspartate	M	..	..	..	..	S	S	S	..
Phenylalanine DKP	..	W	..	..	..	S	..	..	..
Leucine–phenylalanine[k] polymer	..	W	..	..	..	S	..	..	..

[a] Nujol mulls. [b] Secondary amide (*trans*-bonded NH). [c] Secondary amide (*cis*-bonded NH). [d] Characteristic absorption bands for amides. [e] Characteristic absorption bands for cyclic imides. [f] Carboxylate ion absorption. [g] No absorption. [h] Strong absorption. [i] Weak or slight absorption. [j] Moderate absorption. [k] The spectra of phenylalanine DKP and the presumed polymer of leucine and phenylalanine were essentially superposable over the entire infrared range.

attempted through heating asparagine in ethylene glycol[22] and by conversion of aspartic acid DKP diamide to the diacid. Crystalline aspartic acid DKP diamide was not obtained in glycol by the method of Sannie.[22] Aspartic acid DKP diamide was synthesized by heating diethyl fumarate with ethanolic ammonia at 100° under pressure.[23] Treatment of the diamide with sodium hydroxide, barium hydroxide or nitrous acid each failed, however, to yield the desired product. The procedure of Fischer and Koenigs[24] which was used to convert dimethyl L-aspartate to aspartic acid DKP through the DKP diacid dimethyl ester was applied to the L and DL compounds. The DKP diacid was thus obtained and used for control tests.

(22) C. Sannie, *Bull. soc. chim.*, [5] **9**, 487 (1942).

(23) M. S. Dunn and S. W. Fox, *J. Biol. Chem.*, **101**, 493 (1933).

(24) E. Fischer and E. Koenigs, *Chem. Ber.*, **40**, 2048 (1907).

(25) F. Reindel and W. Hoppe, *ibid.*, **87**, 1103 (1954).

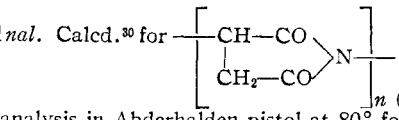
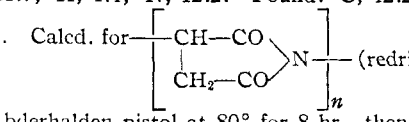
(26) All melting points are uncorrected. Dumas nitrogen and other analyses were performed under the direction of Dr. T. Shiba of Osaka University, Japan, except for sodium polyaspartate which was analyzed by both Clark Microanalytical Laboratory and Dr. Shiba. Infrared spectra were determined by Miss Martha Brackett and Miss M. T. Esquivel of Florida State University and Mr. T. Inui, of Osaka University. The visible spectra of copper complexes were obtained by Dr. Gordon Tollin on a Beckman DK recording spectrophotometer.

(27) An attempt to prepare the diamide from equal volumes of an ethanolic solution of diethyl fumarate and of concd. ammonium hydroxide failed to deposit the diamide. Paper chromatography of the reaction liquor revealed that a number of ninhydrin-reactive compounds had formed. All of these could be hydrolyzed to aspartic acid.

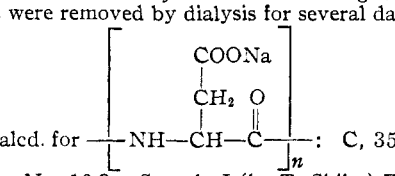
(28) The Hillman procedure (*Z. Naturforsch.*, **1**, 682 (1946)) was used to neutralize the dimethyl ester amine hydrochloride. The over-all yield was improved by decreasing the heating period for condensing the dimethyl ester from 3 days to 6 hours.

Preparation of Valylglycylphenylalanine.—The synthesis of this tripeptide has been reported.[29]

Preparation of Amino Acid Polymers.—The products of Table III were prepared by heating 0.5 or 1.5 g. of the amino acid or equimolar mixtures of thoroughly ground amino acid pairs in open tubes at 200° for 2 or 3 hours. The reaction products were washed with acetone to remove tars. The material insoluble in acetone was then dialyzed until concentrated aliquots from within the sac failed to give ninhydrin tests. This non-diffusible fraction was then concentrated to dryness in a vacuum desiccator. The aspartic acid polymer was analyzed.

Anal. Calcd.[30] for  (redried for analysis in Abderhalden pistol at 80° for several hours) C, 41.7; H, 4.4; N, 12.2. Found: C, 42.2; H, 3.8; N, 12.6. Calcd. for  (redried for analysis in Abderhalden pistol at 80° for 8 hr., then at 200° for 8 more hours)[31] C, 49.5; H, 3.1; N, 14.4. Found: C, 47.6; H, 3.0; N, 14.0.

Preparation of Sodium Polyaspartate.—The sodium salt of polyaspartic acid was prepared by dissolving the polymer in 2 *N* NaOH and heating the solution at 100° for 15 min. Excess alkali and any low molecular weight hydrolysis products were removed by dialysis for several days.

Calcd. for  : C, 35.0; H, 2.9; N, 10.2; Na, 16.8. Sample I (by T. Shiba) Found: C, 33.1; H, 3.4; N, 9.9. Sample II (by Clark Microanalytical Laboratory) Found: C, 33.3; H, 3.7; N, 10.2; Na, 15.5. In each case, carbon analysis was corrected for sodium carbonate formation.

Biuret Tests.—For the biuret tests, 40 mg. of each sample was used. A standard procedure was followed.[32]

Infrared Spectra.[26]—All infrared spectra were run as Nujol mulls. The characteristic peaks of aspartic acid DKP, aspartic acid polymer, sodium polyaspartate, phenylalanine DKP and a leucine–phenylalanine polymer are reported (Table IV).

Paper Chromatography.—The paper chromatograms were all of the ascending type. The chromatograms were developed for 23–24 hours in each solvent system. The method of Reindel and Hoppe was used for identification of the diketopiperazine.[25]

Molecular Weight by Sedimentation Velocity.[33]—Two determinations of S_{20} were made, both at *p*H of 8.5 and $\mu/2$ of 0.1, but at different concentrations. The apparent diffusion coefficient was measured roughly in the second determination.

Molecular Weight by the DNFB Method.—The DNP derivative of aspartic acid polymer was prepared and extracted.[34] As a precaution against contamination of the sample by small amounts of breakdown products, the DNP derivative was dialyzed for several days. The product was hydrolyzed by refluxing in 6 *N* HCl for 12 hours.[35] The molecular weight was estimated by the method of Katchalski, Grossfeld and Frankel.[36] DNP-Aspartic acid was assayed on a Beckman DU Spectrophotometer.

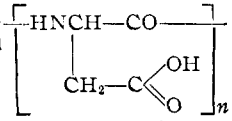
Results and Discussions

In five days of dialysis, aspartic acid polymer diffused 10% by weight and 20% by weight whereas aspartic acid DKP diffused 100% (98% in 1 day). Aspartic acid DKP is thus one of the more soluble diketopiperazines and diffuses readily. Table III confirms this behavior and furthermore demonstrates that aspartic acid DKP is not held by the polymer nor the dialysis membrane. The failure of aspartic acid polymer to give discrete spots with the chlorinating reagent[25] may be explained as due to the fact that the substance is a polycyclic imide virtually devoid of –CONH– radicals.

The biuret tests of polymers obtained, by heating at 200° for 3 hr., indicate that aspartic acid yields a linear polymer but that leucine or phenylalanine do not do so.[37] When amino acids were heated in pairs, the two which failed to give a biuret-positive product, leucine and phenylalanine, failed also to copolymerize. When aspartic acid was heated with leucine, phenylalanine or valine, the products were biuret-positive; in each of these, one of the reactants was an amino acid, aspartic acid, which individually displayed a tendency to form biuret-positive products. It cannot be stated from these data, however, that both amino acids were in peptide chains. For these tests, the authors are indebted to the work of Dr. Mavis Middlebrook at Iowa State College.

The results recorded in Table II demonstrate the failure of the biuret test for peptides with less than two amide bonds. Asparagine uniquely absorbs most strongly about 580 mμ. This fact, already noted,[21] additionally required testing aspartic acid DKP. This latter compound fails to give a biuret test, forming instead a blue complex as has been recorded for dipeptides.[38] Similar blue complexes were the result of the biuret test on glycylglycine, glycine DKP and aspartic acid DKP dimethyl ester. Aspartic acid DKP diamide, possessing four amide linkages, is strongly biuret-positive. The biuret behavior of aspartic acid polymer is comparable to that of valylglycylphenylalanine and of blood albumin. The two mixtures of glycylglycine and polymer, 3:1 and 1:3, were tested to determine whether there is a basis for estimating proportions of linear peptides in mixtures of compounds of the type being studied. The observed shifting of the absorption spectra after addition of glycylglycine suggests that the biuret test affords some promise in this connection.

(29) S. W. Fox, T. L. Hurst and K. F. Itschner, THIS JOURNAL, **73**, 3573 (1951).

(30) The same analysis would be given by  but this possibility was ruled out by infrared studies.

(31) The possibility that rigorous drying converts a peptide to a polyamide is ruled out by the infrared studies which demonstrate that neither the partially dried nor rigorously dried material contains peptide bonds.

(32) P. B. Hawk, B. L. Oser and W. H. Summerson, "Practical Physiological Chemistry," The Blakiston Co., New York, N. Y., 1953, p. 153.

(33) The authors are grateful to Dr. D. P. Waugh and R. W. Hartley, Jr., of Massachusetts Institute of Technology, for their ultracentrifugal studies on the polymer and their helpful interpretation.

(34) E. F. Mellon, A. H. Korn and S. R. Hoover, THIS JOURNAL **75**, 1675 (1953).

(35) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

(36) E. Katchalski, I. Grossfeld and M. Frankel, THIS JOURNAL, **70**, 2094 (1948).

(37) The alternative explanation of formation of a copolymer of leucine and phenylalanine, which was too insoluble to react with alkaline copper sulfate solution, was precluded by other studies. See also ref. 16.

(38) P. A. Kober and A. B. Haw, THIS JOURNAL **38**, 457 (1916).

TABLE V

COMPARISON OF POLYASPARTIC ACID POLYMERS PREPARED IN DIFFERENT WAYS

Polypeptide	Conditions for polymerization	Mean mol. wt.	Analyses, % C	H	N	Biuret test	Ninhydrin test
DL-Aspartic acid	Heating at 200°[a]	11000[b]	42.2 (47.6)[c]	3.8 (3.0)[c]	12.6 (14.0)[c]	+	−
DL-Aspartic acid	Heating at 180–200°[d]	8200[e]	....[f]	...	....	+	+
DL-Aspartic acid	Heating at 190–200°[g]	1000[h]	....	...	....	+	..
DL-Aspartic acid	From N-carboxy anhydride of aspartic acid β-benzyl ester[i]	4000, 7000, 8200, 9300, 18,600 and 20,000 for different preparations[e]	40.8	4.3	11.9	+	−
L-Aspartic acid	(Heating benzyl aspartate[k])	9200[e]	41.0	4.3	12.0	..	..

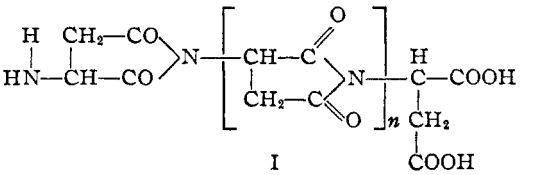
[a] As described in this paper. [b] From assay of DNP- aspartic acid in DNP-polymer. [c] After rigorous drying. [d] J. Kovacs, I. Könyves and A. Pusztai, *Experientia*, 9, 459 (1953). From analysis of amino nitrogen of an isolated fraction. [f] No information given. [g] H. Schiff, *Chem. Ber.*, 30, 2449 (1897). From elementary analysis of salt. [i] Initial starting material was L but product was racemic. [j] M. Frankel and A. Berger, *J. Org. Chem.*, 16, 1513 (1951). [k] A. Berger and E. Katchalski, THIS JOURNAL, 73, 4084 (1951).

The mean molecular weight of aspartic acid polymer was determined by two different methods.

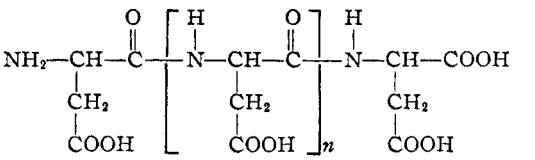
The sedimentation coefficients by one method[33] were found to be highly dependent upon concentration, making calculation of S_{20} at infinite dilution difficult. The sedimentation constant was estimated to lie between 1.6 and 2.0 svedbergs. A fairly Gaussian distribution of molecular weight was indicated by the broad and somewhat symmetrical boundaries obtained. The diffusion coefficient estimated in the second determination was too high to be real because of the heterogeneity in molecular size. The minimum molecular weight was calculated on the basis of a spherical model by Hartley[33] and suggests a mean value of 4600–6600. For a randomly coiled polymer, the molecular weight might be two to three times the minimum value, *i.e.*, 9,000–20,000.

The molecular weight value obtained by assay of terminal DNP-aspartic acid in the DNP-derivative of the polymer is in accord with a diffuse polymer. Two successive determinations by this method[36] gave 11,000 for the molecular weight of this polymer.

The infrared spectra of phenylalanine DKP and the "leucine–phenylalanine polymer" are so nearly identical that there is little doubt that most of this preparation consisted of phenylalanine DKP. The infrared spectrum of each compound is in accord with the interpretation of a cyclic lactam, and they lack the distinguishing peaks of linear peptides.[7,39,40] The absorption spectrum of "polyaspartic acid" is unique. It is obviously quite different from that of aspartic acid DKP. It is also markedly different from typical polypeptide spectra. "Polyaspartic acid' lacks bonds at 3300 and 3080 cm.$^{-1}$ and has only moderate absorption at 1550 cm.$^{-1}$, all of which are found in synthetic polypeptides.[39] This material has, however, strong absorption at 1715 cm.$^{-1}$ and mild absorption at 1780 cm.$^{-1}$, features which are found in cyclic imides.[39] From this line of evidence, a polycyclic structure is proposed for this polymer (I) identical to that postulated by Kovacs, Könyves and Pusztai.[10] This structure might be expected to hydrolyze easily to II and to recyclize upon acidification.[10,41] Structure I is in complete accord with the infrared absorption spectrum of an imide type of polyaspartic acid (Table IV) and saponification from



I



II

I to II would explain the positive biuret tests.[42] The C,H,N analysis (Experimental) after rigorous drying was in fair agreement with the values calculated for I. With less exhaustive drying, analysis of the polymer was in agreement with either structure II, or structure I with roughly stoichiometrically bound water. The latter interpretation was supported by the infrared studies already discussed and a finding of sloping at 3500-3300 cm.$^{-1}$, presumably due to –OH stretching absorption.

The tenacity displayed by the polyimide structure for water molecules is of interest in suggesting searches for similar structures in protein molecules which are known to bind water tightly.[43]

Further confirmation of the structure of "polyaspartic acid" came from a study of the sodium salt. The alkali-treated polymer no longer absorbed at 1780 and 1715 cm.$^{-1}$ (Table IV). It did, however, absorb at 3300, 1650, 1600 and 1550 cm.$^{-1}$, in accord with structure II.

A comparison of aspartic acid polymer made in different laboratories by different methods shows

(39) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," John Wiley and Sons, Inc., New York, N. Y., 1954, pp. 175–179.

(40) The authors are indebted to Mr. T. Inui of Osaka University for a detailed independent interpretation of the spectra in this paper.

(41) D. L. Swallow and E. P. Abraham, *Biochem. J.*, 65, 39P (1957).

(42) The possibility that a polyimide could give a positive biuret test without prior hydrolysis should also be considered. For example, see M. M. Rising, F. M. Parker and D. R. Gaston, THIS JOURNAL, 56, 1178 (1934). Although II is depicted as an α-peptide, β-linkages are undoubtedly also present.[10]

(43) O. L. Sponsler, J. D. Bath and J. W. Ellis, *J. Phys. Chem.*, 44, 996 (1940); H. R. Bull, THIS JOURNAL, 66, 1499 (1944).

both similarities and differences (Table V). The mean molecular weights of the different polymers are about 10,000 except for the polymer described by Schiff in 1897.[9] In the cases reported, the polymer gave positive biuret tests as would be anticipated from the molecular weights.[38] Samples of polyaspartic acid prepared by Frankel and Berger and in this Laboratory gave negative ninhydrin tests, but the one described by Kovacs and coworkers gave a positive test. The analytical data for the polymer described in this paper already have been discussed. Such analyses are subject to possible misinterpretations due to the tenacity of water of hydration and also to the possibility of more than one polymeric form, *i.e.*, I and II.

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[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Countercurrent Distribution Studies with Ribonuclease and Lysozyme

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A search has been made for systems which will permit fractionation by countercurrent distribution of ribonuclease, lysozyme and chymotrypsinogen. Systems for the first two are reported in which there is no sign of denaturation. These proteins behave as nearly ideal solutes as far as countercurrent distribution is concerned.

Introduction

In the earliest attempts in this Laboratory to fractionate a protein by countercurrent distribution (C.C.D.) two types of system were found which would partition insulin.[1] One achieved partition in a useful range of partition coefficient by the use of salt as an important component of the system. The other contained a complexing agent, such as dichloro- or trichloroacetic acid which modified the hydrophilic nature of the molecule and thus increased partition in the alcohol phase. In neither type of system was there any evidence of denaturation or loss of biological activity even though the operation was carried out at 25° and required several days.

Since insulin is known to be a very stable protein even in an environment often quite different from that in which it occurs, the stability found was not considered representative of proteins in general. Indeed, it was soon found that most other proteins would not show anything like this stability. Nonetheless, up to the present time, a number of other proteins have been successfully distributed. These include adrenocorticotropins,[2] growth hormone,[2] lactogenic hormone,[2] lysozyme,[3] casein[4] and serum albumin.[5] Evidence was found in the serum albumin study that the complexing agent itself had a certain stabilizing effect at lower *p*H values where proteins tend to be more alcohol soluble.

This did not seem to hold true with most of the other proteins thus far studied. In many cases an initial partition ratio within a usable range was found but on carrying out the distribution, clearly defined bands were not obtained because of a progressive shift of the partition ratio (K). An inconsistency of the determined K and the position of the band on the pattern offered further evidence of slow transformation.

Aside from the question of denaturation, there is the question of adherence to ideal solute behavior. With serum albumin[5] an extremely narrow distribution band was found which was explained on the basis of solute binding. A similar behavior had been noted in the earlier insulin work[1] and somewhat later in the A.C.T.H. work.[2] If solute binding could produce a band too narrow, it could also produce one which would be too broad, an observation which usually denotes inhomogeneity in countercurrent distribution work.

It therefore would be very helpful to have an independent fractionation method available for cross checking the fractions, provided such a method had high resolving power and was suited to the small amount of substance available in the fraction. Ion-exchange chromatography provides such a method in the case of ribonuclease,[6] chymotrypsinogen[7] and lysozyme[8] and has been widely accepted as the most reliable method for studying the purity of these substances. For this reason these proteins have been chosen for one of the initial phases of our partition studies with proteins.

Experimental

The sample of ribonuclease used was obtained from Armour and Company, Lot 381-059. The lysozyme was also Armour material, Lot 381-187. The chymotrypsinogen was obtained from Worthington Biochemical Company.

The distribution equipment used included the previously described automatic 200-tube machine with 10-ml. capacity[9] and a new 1000-tube machine of similar construction but with 2-ml. capacity per phase in each cell.

Reagent grade ammonium sulfate, redistilled water and absolute ethanol were used to prepare the system which contained $(NH_4)_2SO_4$, H_2O and C_2H_5OH in the weight ratio, respectively, of 16.5:57.6:25.9. In practice it was found convenient to prepare this system by mixing three volumes of an ammonium sulfate stock solution (40 g. in 100 ml. of water), one volume of water and two volumes of ethanol. Slightly more lower phase was formed than the upper phase. A phase diagram of this three-component system at 25° was constructed and from the diagram it could be determined that the system used is near the critical point and the compositions of the upper and lower phases are, respectively,

(1) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952).

(2) C. H. Li, "Advances in Protein Chemistry," Vol. XI, Academic Press, New York, N. Y., 1956, p. 101.

(3) E. Craenhals and J. Leonis, *Bull. soc. chim. Belges*, **64**, 58 (1955).

(4) P. von Tavel and R. Signer, "Advances in Protein Chemistry," Vol. XI, Academic Press, New York, N. Y., 1956, p. 237.

(5) W. Hausmann and L. C. Craig, THIS JOURNAL, **80**, 2703 (1958).

(6) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).

(7) C. H. W. Hirs, *ibid.*, **205**, 93 (1953).

(8) H. H. Tallan and W. H. Stein, *ibid.*, **200**, 507 (1953).

(9) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).