Copolymerization of the Leuchs Anhydrides of the Eighteen Amino Acids Common to Protein*

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ABSTRACT

The eighteen amino acids common to protein have been simultaneously condensed through the N-carboxy anhydrides. Nine of these monomers had first to be specially protected by groups which could subsequently be removed in a single operation. The polymer obtained resembles in quantitative composition an average natural protein. The nutritional and other significances of this type of polymer are discussed.

Anhydrocopolymers of α-amino acids prepared from the Leuchs anhydrides serve as models of protein (1,2). Thermal copolyanhydro-α-amino acids also serve as models of protein (3-5). One unique aspect of the thermal copolymers is that they may be produced so as easily to include simultaneously some proportion of each of the eighteen to twenty amino acids common to protein. These latter polymers are comparable to protein in that they possess nutritive properties for three species tested (3,6,7), some types of catalytic ability (e.g. 8,9), and other properties such as positive response to classical color tests for protein (10). Some of the characteristics of such polymers, such as catalytic type of activity, are absent in the amino acids of which the synthetic polymers are composed. Such a difference is of course analogous to that which exists between proteins and their contained α-amino acids.

In protein nutrition, the need for simultaneous availability of a variety of amino acids has long been known, from studies with mixtures of free amino acids (11). The same need has been recognized for amino acids bound in either a natural or synthetic polymer (3,11,12). One special value of maintaining caloric balance by feeding amino acids as polymers, rather than in the free state, has been pointed out by W. C. Rose (13). The fact also that free amino acids may not be tolerated as readily as are proteins has also long been known; forced feeding of protein hydrolyzates has, for example, been required in experiments with laboratory animals (14).

Inasmuch as thermal proteinoids can partially meet the need for simultaneous nutrition with amino acids linked by peptide bonds, the preparation of proteinoids through the Leuchs procedure invited attention for this investigational purpose also. The Leuchs polymers offered the promise of superior nutritional quality since the amino acids would be expected to have undergone less racemization than those condensed by heating. The latter polymers can serve also as useful materials for comparison with the thermal type. In addition, Leuchs polymers can be expected to yield compositions resembling those of natural proteins more closely than do the thermal products.

In order to pattern the composition of a Leuchs polymer after that of an average protein, a set of protein composition values obtained from a survey of purified proteins was used (15). After the amino acid profile of the hydrolyzate of the Leuchs proteinoid was obtained, it was compared with that of

a crystalline protein recorded in the literature. The profile first found in this search, and traced in this paper, was that of the α-amylase of Bacillus stearothermophilus (16).

Application of standard Leuchs procedures could not be foreseen to yield the desired polymers. For example, in the syntheses reported in this paper, debenzylation of S-benzylcysteine residues proceeded with a result differing from the behavior of homopolymers of Leuchs anhydrides of S-benzylcysteine (17). Also, the NCA of the difficultly obtainable derivatives of arginine had not been reported. The behavior of eighteen NCAs on polymerization and dederivatization could, also, not be expected to proceed smoothly. Special and separate handling of the derivatives of arginine NCA and histidine NCA had, in fact, to be developed.

MATERIALS AND METHODS

The polyanhydro-α-amino acids prepared were a proteinoid from equimolar ratios of NCAs (ERLP), one from the same monomers without histidine (ERLP-H), one containing amino acids in approximately an average ratio for protein (15; NRLP), and a polymer imitating the ratios of a thermal 2:2:3-proteinoid (18).

In the first syntheses carried out, nine free amino acids, the benzyl derivatives of three etherifiable amino acids, and O-acetyl-DL-threonine were simultaneously converted to Leuchs anhydrides by phosgene in dioxane, and the five freshly prepared and freshly recrystallized NCAs of the basic and acidic amino acids were added prior to the polymerization. In later syntheses, the process was simplified by combining nine free amino acids and seven protected amino acids in the same mixture and converting these sixteen to NCAs simultaneously, and then adding freshly recrystallized NCAs of derivatives of L-arginine and of L-histidine. This later addition of the two basic NCAs was necessary since the NCAs of protected arginine and of protected histidine cannot be prepared by the phosgene procedure used for the other amino acids. The details presented below are for preparation of one of the polymers, the NRLP.

Dry phosgene was slowly introduced with stirring into a finely powdered mixture of 0.495 g. of glycine, 0.483 g. of L-alanine, 0.420 g. of L-proline, 0.428 g. of L-valine, 0.149 g. of L-methionine, 0.480 g. of L-isoleucine, 0.480 g. of L-leucine, 0.402 g. of L-phenylalanine, 0.148 g. of L-tryptophan, 0.850 g. of O-benzyl-L-serine (19), 0.550 g. of O-acetyl-DL-threonine (20), 0.165 g. of S-benzyl-L-cysteine (21), and 0.526 g. of O-benzyl-L-tyrosine (22) in 250 ml. of dry dioxane in a water bath at 50° until a clear solution resulted. A stream of dry nitrogen was then passed through the reaction mixture for one hour at room temperature to remove unreacted phosgene. The solvent was removed by reduced pressure at 40°. The residual oil was treated with petroleum ether and dried in a vacuum desiccator. The oil was dissolved in 200 ml. of dry acetone and added to 4.0 g. of dry silver oxide. The mixture was stirred until chloride-free. The suspension was filtered, and the acetone in the filtrate removed under reduced pressure. The residual oil was dissolved in 200 ml. of dry dioxane and to this was added, freshly prepared, 0.945 g. of N^{ε}-carbobenzoxy-L-lysine NCA (23), 1.570 g. of $N^{\omega},N^{\omega'}$-dicarbobenzoxy-L-arginine NCA (24) made from 2.66 g. of $N^{\alpha},N^{\omega},N^{\omega'}$-tricarbobenzoxy-L-arginine with thionyl chloride, 0.375 g. of N^{im}-benzyl-L-histidine NCA (25) in 20 ml. of

dioxane, 1.320 g. of L-aspartic acid NCA β-benzyl ester (26), and 1.670 g. of L-glutamic acid NCA γ-benzyl ester (26). To this solution was added 0.15 ml. of triethylamine as initiator and the mixture was stirred at room temperature for one week. The solution was heated in a boiling water bath for 2 hr. By the use of reduced pressure, dioxane was reduced in volume to one-third, and the remaining liquid was poured into 1 l. of water with stirring. The polymer was collected by filtration, washed with water and dried. Yield, 8.0 g. $\overline{M.W.}$ = 3,630, $[\alpha]_D^{25}$ = -27.6° (c 2.55, dichloroacetic acid). This protected polymer is soluble in formic acid, dimethylformamide, and in dichloroacetic acid, and is slightly soluble in glacial acetic acid. It is insoluble in water, ether, and in dioxane.

For removal of the protecting groups, 4.0 g. of the polymer and 0.8 g. of metallic sodium were alternately added in portions to 100 ml. of liquid ammonia with stirring. The addition of sodium was rapid enough to maintain a blue color in the liquid. Fifteen minutes after the addition was complete as judged by persistence of blue color, the excess sodium was discharged with ammonium chloride; the ammonia was allowed to evaporate spontaneously at room temperature. The residue was dissolved in 30 ml. of water with stirring, the small quantity of undissolved material was removed by filtration, and the filtrate was acidified to pH 3 with 3 N hydrochloric acid. The precipitated fraction was collected by centrifugation, washed with water, and lyophilized. The soluble fraction was dialyzed with water for one day and lyophilized. The insoluble fraction was 0.4 g. and the soluble fraction 1.0 g. Positive reactions were observed with biuret, Sakaguchi, Millon, Pauli, and Ehrlich's aldehyde color tests. Amino acid compositions of hydrolyzates (6 N HCl) of the water-soluble fraction are depicted in Fig. 1.

The other proteinoids were prepared by using equimolar ratios of NCAs, the same with the omission of histidine-NCA derivative, and with ratios of 2 aspartic acid:2 glutamic acid: 3 of total basic-neutral amino acids to provide a polymer imitating compositionally an analogous thermal 2:2:3-proteinoid.

All of the proteinoids had water-soluble fractions whereas the protected derivatives were water-insoluble. The latter were soluble in formic acid, dimethylformamide, and dichloroacetic acid but insoluble in water, ether, and dioxane, and were positive to the color tests listed above.

Determination of molecular weights of protected polymers was accomplished by titration in dimethylformamide solution with M/50 sodium methioxide with thymol blue as indicator.

Molecular weights of unprotected polymers were determined on the Spinco Model E analytical ultracentrifuge and by C-terminal analysis (27) unless otherwise indicated.

Amino acid compositions of hydrolyzates of each fraction prepared with redistilled 6 N CHl were determined by the method of Spackman, Stein, and Moore (28) on a Model K-5000 Phoenix automatic amino acid analyzer. Tryptophan contents were determined spectrophotometrically on the unhydrolyzed polymers (29).

C-Terminal amino acid analyses were performed with the Bradbury modification of the Akabori hydrazinolysis method (27) by use of correction factors supplied by Dr. K. Harada.

To test the supposition that optically active Leuchs anhydrides retain this activity in Leuchs proteinoids as they are known to do in other polymers, the following test was performed. Histidine-free ERLP (50 mg.) was hydrolyzed with 6 ml. of 5.5 N redistilled hydrochloric acid at 110° C for 24 hr., in a sealed evacuated tube. After decolorizing with a weighed amount of Norit A, 200 mg., the sample was reduced to 2.00 ml. of 5.5 N HCl and read in a polarimeter equipped with photometer. The value obtained was corrected for the incomplete recovery of amino acids from the polymer. For comparison, L-amino acids (except for DL-threonine) in the same proportions as in the polymer were carried through the identical procedure.

The rate of hydrolysis of p-nitrophenyl acetate in the absence and in the presence of the histidine-containing proteinoids was measured with a Bausch and Lomb Spectronic 20 colorimeter at 400 mμ, with samples of each proteinoid containing equivalent amounts of histidine in polymeric form, per 5.0 ml. of pH 6.8 phosphate buffer (0.067 M) solution having 4% dioxane and 10^{-3} M p-nitrophenyl acetate, temp. 30° C. The standard free histidine concentration was 10 mg. per 1. of pH 6.8 phosphate buffer solution. Proteinoid concentration, except for histidine-free ERLP, was based on the amount of histidine in polymeric form. This was calculated from the results of amino acid analysis of the hydrolyzate. The concentration of histidine-free ERLP was the same as for other proteinoids.

RESULTS

In Fig. 1, the amino acid profile of the automatic analysis of NRLP and of the α-amylase of Bacillus stearothermophilus are compared. In order to permit close graphical comparison, the chromatograms through valine are placed one above the other and the ranges from methionine through arginine are similarly matched in the two lower tracings. The similarities of the two analyses are not merely qualitative, as the interdigitated chromatograms show. One qualitative difference is visible; some cysteic acid is produced in the synthesis or hydrolysis of Leuchs proteinoid. Also, although the α-amylase is in general quite typical of protein in its amino acid composition, it lacks tryptophan. The proteinoid contains, however, 5.0% of tryptophan.

Comparisons of analyses of the other three proteinoids indicate quite clearly that the proportion of any amino acid in the polymer can be regulated by the proportion in the reaction mixture.

In Table I are given the mean molecular weights and the specific rotations. The hydrolyzate of histidine-free ERLP showed $[\alpha]_D^{25} = +6.03°$ (c = 1.73) as against +6.20° for the mixture of L amino acids in the same proportions.

In Table II are presented amino acid compositions of ERLP, water-soluble and water-insoluble fractions. Comparison of the analyses of the protected polymer and of the freed polymer confirm the observations that removal of hydrogenolyzable groups was complete. This stage was determined during synthesis by persistence of blue color upon successive additions of sodium to the liquid ammonia solution. The absence of substituted groups is corroborated by the absence of unexplained peaks in Fig. 1. Prior to S-debenzylation the protected proteinoid gave on aqueous acid hydrolysis S-benzylcysteine but no cystine nor cysteic acid whatever. These amino acids were found in the hydrolyzate of the free proteinoid, as the analyses show. The sulfhydryl group of the Leuchs proteinoid were also determined quantitatively by amperometry. Likewise, histidine was absent from the hydrolyzate of the protected polymer, but im-benzylhistidine was recovered. Similarly, tyrosine and methionine were largely decomposed in the hydrolysis of the protected polymer, but are found in substantial proportion in the unprotected proteinoid.

Table III gives comparable data for the 2:2:3-Leuchs proteinoid.

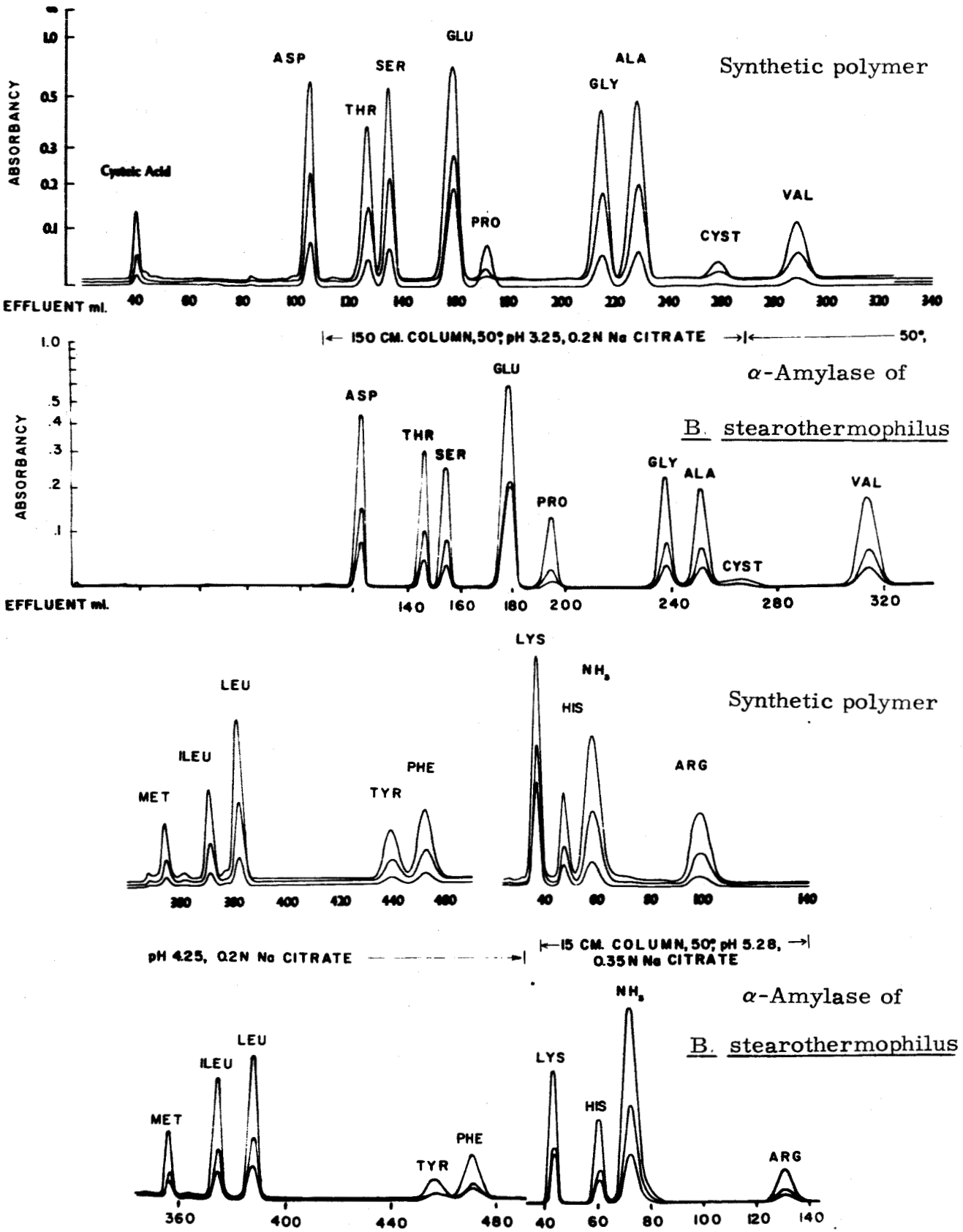

Fig. 1. Interdigitated chromatograms of hydrolyzates of synthetic and natural polymers from automatic amino acid analyzer. First and third lines of chromatograms are from natural ratio Leuchs proteinoid, second and fourth lines are from α-amylase of Bacillus stearothermophilus.

TABLE I

Constants of Leuchs Proteinoids

Proteinoid	$\overline{M.W.}$	$[\alpha]_D^{25}$
Natural Ratio	5140[a]	-47.7° (c 2.38, water)
Equimolar Ratio	3750	-15.3° (c 0.89, water)
Histidine-free Equimolar Ratio	2500	-18.8° (c 0.96, water)
2:2:3-Ratio	7030[b]	-45.4° (c 1.36, water)

TABLE II

Amino Acid Composition[a] of Equimolar Ratio Leuchs Proteinoid[b]

Amino Acid	Equimolar Ratio Leuchs Proteinoid (ERLP)			
	Protected (P) ERLP	Water-soluble[c]	Water-insoluble	C-Terminal Analysis of Water-soluble fraction
Lysine	4.49	5.86	8.68	8.89
Histidine	0.00	8.84	2.56	15.85
Arginine	6.71	2.19	4.54	tr.
Cysteic acid	0.00	.92	0.26	3.76
Aspartic acid	7.66	5.53	4.35	11.40
Threonine	5.41	5.34	3.43	6.17
Serine	3.80	6.40	3.58	11.32
Glutamic acid	8.02	8.84	6.32	5.49
Proline	7.10	7.31	3.63	5.45
Glycine	5.46	6.64	3.29	5.81
Alanine	7.01	8.07	6.39	6.16
Half-cystine	7.29[d]	5.00	2.16	4.30
Valine	6.55	3.60	8.17	2.39
Methionine	2.91	4.12	6.77	2.22
Isoleucine	5.77	2.53	7.98	2.01
Leucine	8.05	5.12	11.43	2.79
Tyrosine	1.56	4.07	7.49	2.95
Phenylalanine	7.21	4.64	9.08	3.04
Tryptophan	- [e]	4.98	- [e]	tr.

[a] g. amino acid residue/100 g. polymer

[b] $\overline{M.W.}$ = 3,000, by C-terminal analysis

[c] Recovery in analysis, 83%

[d] S-benzylcysteine

[e] Not det'd.

TABLE III

Amino Acid Composition[a] of

2:2:3-Leuchs Proteinoid[b]

Amino Acid	Proteinoid	(2:2:3) Thermal Proteinoid	2:2:3-Leuchs Proteinoid water-soluble fraction[c]	C-Terminal Analysis of water-soluble fraction
Lysine	6.91	5.85	9.77	8.72
Histidine	0.00	2.19	2.85	6.27
Arginine	2.49	2.53	1.81	tr.
Cysteic acid	0.00	0.00	2.68	7.07
Aspartic acid	57.39	54.50	41.23	41.97
Threonine	0.15	0.00	1.05	tr.
Serine	0.25	0.00	0.17	5.14
Glutamic acid	13.60	12.59	15.35	9.14
Proline	6.11	0.66	5.94	6.33
Glycine	1.84	3.32	2.72	6.82
Alanine	4.06	4.70	5.48	8.54
Half-cystine	2.32[d]	3.78	0.14	tr.
Valine	1.13	1.32	0.90	tr.
Methionine	0.09	2.07	1.58	tr.
Isoleucine	0.96	1.36	1.11	tr.
Leucine	1.34	1.25	1.71	tr.
Tyrosine	tr.	2.07	1.86	tr.
Phenylalanine	1.37	1.80	2.23	tr.
Tryptophan	[e]	[e]	1.43	tr.

[a] g. amino acid/100 g. polymer

[b] M = 7,000 by C-terminal analysis

[c] Recovery in analysis, 70.8%

[d] S-benzyl cysteine

[e] Not det'd

In Table IV are recorded the influence of various polymers containing histidine, and of a Leuchs proteinoid lacking histidine, upon the relative rate of hydrolysis of p-nitrophenyl acetate.

TABLE IV

Influence of Various Forms of Histidine on Rate of Hydrolysis of p-Nitrophenyl Acetate

Compound	Conc'n of compound/l.	Conc'n of histidine/l.	Rate relative to that of histidine
L-Histidine	10 mg.	10	1.0
NRLP	441	10	2.9
(2:2:3) LP	415	10	1.7
ERLP	173	10	2.7
Histidine-free ERLP	200	-	0.5
Histidine-free ERLP plus histidine	200 + 10	10	2.1

DISCUSSION

Figure 1 demonstrates that the composition of a protein can be closely imitated by suitably combining the NCAs of the eighteen amino acids common to protein. Nine of the amino acids are first appropriately protected by derivatives; the substituting groups need to be simultaneously removable by hydrogenolysis. No attempt was made to pattern the natural ratio Leuchs proteinoid precisely on the composition of the α-amylase of Bacillus stearothermophilus. What was done, instead, was to attempt to produce a composition which was typical of all proteins, since all proteins fall into a moderately narrow range compositionally (15). The α-amylase, then, except for lacking tryptophan, was a typical protein in composition and proved to be comparable to the synthetic polymer. Figure 1 brings out these comparisons graphically, especially in the lysine-histidine-arginine-series of peaks, in the tyrosine-phenylalanine series, and in the methionine-isoleucine-leucine series. A small inversion in quantitative relationship appears between glycine and alanine, but not in the relationship to valine. In the aspartic acid-threonine-serine-glutamic acid-proline relationships the relative amounts are again comparable, except for a slight inversion between threonine and serine in the comparison of synthetic polymer with the natural polymer.

When the data of the Leuchs natural ratio proteinoid are compared with those of the equimolar ratio proteinoid (Table II) and of the 2:2:3 variety (Table III), proportions in the polymer are found to vary with proportions in the reaction mixture. Thus, in principle, any composition may be imitated in synthesis.

Accordingly, for nutritional investigation of amino acids bound by peptide bonds, and for other investigations, the Leuchs polymers containing as many as eighteen types of amino acid offer unique value as easily varied models of protein. The utility to nutritional investigation has been shown by the fact that the polymers are utilized by Tetrahymena pyroformis R (7). The ease of preparing Leuchs proteinoids with single amino acids omitted affords an opportunity to determine more precisely the role of individual amino acids in protein nutrition (12).

The fact that Leuchs proteinoids show some diversity in constitution is brought out by comparison of the water-soluble and water-insoluble fractions of the equimolar ratio LP of Table II. A principal point made by this comparison is that both fractions of the proteinoid contain some proportion of all of the amino acids reacted. This type of result was found with the fractions of each of the polymers.

Correlations between types of amino acid and water solubility or insolubility can be inferred by comparing individual values between columns 1 and 2 in Table II. The water-insoluble proteinoid is markedly higher in the contents of lysine, arginine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine. All of these amino acids are all of those which have the largest straight chain hydrocarbon moieties in the residue.

Comparison of the amino acid contents of the proteinoid (Table II) with the molar proportions of the amino acids in the reaction mixture shows some differences between composition of reaction mixture and of reaction product. In the reaction mixture, each amino acid is present as such or as derivative to the extent of 5.5 molar %. Three are present in the reaction product to a figure 50% greater than in the reaction mixture: lysine, leucine, and phenylalanine. One, cystine, is present to a markedly less degree. Slightly more than half of the cystine introduced into the reaction mixture is found in the product. The loss of cystine is easily explained by the known instability of cystine NCA (29).

The C-terminal analyses of the 2:2:3-proteinoid of Table III reveal ten amino acids in this position in traces only. Accordingly, less heterogeneity by this criterion is indicated for this polymer than is observed in the ERLP (Table II). A tentative explanation for this effect is based on the relatively large proportion of aspartic acid which may induce some ordering. In the thermal 2:2:3-proteinoid, the proportion of aspartic acid is also approximately 50%, and evidence for limited heterogeneity in that polymer was obtained (18).

The results of Table IV show that each of the histidine containing Leuchs proteinoids is more active than the equivalent weight of free histidine in catalyzing the hydrolysis of p-nitrophenyl acetate. The most active was found to be the NRLP, which was approximately 3 times as active as the amount of histidine it contained, when tested in the free state. Of most relevance is the fact that while the histidine-free proteinoid is but half as active as histidine, the same material tested simultaneously with the standard amount of histidine shows activity twice as great as that of the histidine alone. From this result, one may infer a) that the polymer enhances the intrinsic activity of histidine in this reaction and b) that appropriate incorporation of the histidine, as by peptide bonds, enhances such activity further.

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