^{13}C n.m.r. study of the structure of poly(aspartic acid)

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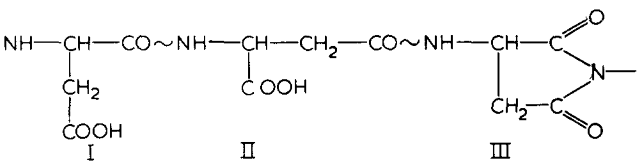
From hydrolysis of the product of thermal polycondensation of aspartic acid, poly(succinimide), under various conditions, a series of samples of thermal poly(aspartic acid) was obtained. These polymers differed by molecular mass and, from n.m.r. spectral evidence, by the contents of α and β peptide bonds, depending mostly on the pH of hydrolsysis. The contents of α and β bonds found were further confirmed by analysis of the n.m.r. spectra of the complex of poly(aspartic acid) with Co(II). Also, spectra of poly(aspartic acid) obtained by debenzylation of poly(β-benzyl-L-aspartate) described in our previous work were analyzed in greater detail. A comparison of racemic and optically pure polymers has shown that the n.m.r. spectra are only slightly affected by stereoregularity, and that stereoregularity is not the cause of the splitting of the various carbon bonds. Analysis of the complicated structure of the spectra, mainly of the carbonyl group, leads us to the conclusion that the distribution of the α and β bonds is random in all the polymers studied.

Keywords Structure; preparation; nuclear magnetic resonance spectroscopy; peptide bonds; alpha bond; beta bond; succinimides; poly(α, β-D,L-Asp); poly(α-L-Asp)

INTRODUCTION

Aspartic acid, as a trifunctional monomer, can be incorporated into polymers by α (I) or β (II) peptide bonds, or in the form of a cyclic imide (III):



Poly(aspartic acid) (poly(Asp)) is usually prepared by debenzylation of poly(β-benzyl aspartate)[1] or by thermal polycondensation[2]. The most suitable method for the identification of I and II and determination of their populations is n.m.r. spectroscopy[3,4]. By this method it was found that most samples prepared by both methods actually contain both I and II[4,5]. Succinimides (III) are best determined by potentiometric titration[5].

The first method of preparation is currently used for obtaining poly(Asp) for various physico–chemical studies in polypeptide research. The finding that the samples studied so far probably always contain β bonds led to a re-examination of the conformational behaviour of poly(Asp)[6]. It was found that deionization of poly(Asp) in dilute solution induces a conformational transition, and that the conformation generated by deionization probably is an α-helix.

Following the Fox evolution theory[7], thermal poly(α-amino acids), the so called proteinoids, are considered as prebiotic ancestors of present proteins. A strong argument against this theory was the assumption that polymers formed by thermal polycondensation contain almost exclusively 'unnatural' ω peptide bonds[8]. Aspartic acid is of key importance in the preparation of proteinoids, because it promotes polymerization[2] and makes the preparation of the copolymers of all natural amino acids possible[9]. Andini *et al.*[10] state that thermal poly(Asp) contains only II and they therefore assume that further studies of proteinoids are not worthwhile because it is very improbable that conditions could be found leading to a polymer containing both types of bonds. By analysis of n.m.r. spectra of poly(Asp), this statement from Andini *et al.* was found to be incorrect[4].

Thermal poly(Asp) and its derivatives are also the subject of research for possible medical applications (see refs 4 and 11–13).

In this paper, our previous studies of the structure of poly(Asp)[4,5,14] are extended. We have prepared a series of thermal polymers which we have analysed in a similar way as the samples of poly(Asp) as prepared by debenzylation of poly(β-benzyl-L-aspartate) in a previous study[5]. This broader study made it possible to investigate to what extent the results of the determination of the populations of I and II are affected by stereoregularity, and also to investigate the distribution of structures I and II. Spectra of the complexes of poly(Asp) with Co(II) are also included; the presence of the structures I and II in the polymers can be confirmed by their analysis.

MATERIALS AND METHODS

Poly (D,L-succinimide) (poly(Suc)) was prepared by the thermal polycondensation of D,L-aspartic acid[12] and fractionated by precipitation with water from dimethylformamide solution[13].

Samples of thermal poly(D,L-aspartic acid) (designated as T-poly(Asp) with the corresponding number from *Table 1*) were prepared by hydrolysis of 1 g (10 mmol) of finely ground poly(Suc) at room temperature by the following procedures:

(A) Poly(Suc) was dissolved in 1 mol l^{-1} NaOH (100 ml) for 2 min and stirred for a further 30 min[2,10,15].

(Aa) To a suspension of poly(Suc) in 10 ml H_2O with a pinch of thymolphtalein, an equivalent amount of 2 mol l^{-1} NaOH was added dropwise, each drop after discolouration (pH <9.4). After the last addition, the solution was stirred for a further 2 h (pH ~ 10)[4].

(B) A suspension of poly(Suc) in 10 ml buffer (4 mol l^{-1} KH_2PO_3 or 2 mol l^{-1} Na_2CO_3 and 4 mol l^{-1} NaCl, pH adjusted by conc. HCl) was dialysed against 1 litre of the

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same buffer for 10 days.

(C) To a solution of poly(Suc) in 10 ml dimethylformamide, 10 mmol triethylamine and 1 ml H_2O were added. The solution was stirred for 2 h.

(D) The suspension of poly(Suc) in 10 ml H_2O was kept at pH 9.0 by means of a pH-stat adding 0.1 mol l^{-1} NaOH until the reaction was completed (about two days).

The conditions of preparation of various samples are summarized in *Table 1*. After the reaction the solutions were acidified with acetic acid and dialysed against 0.1 mol l^{-1} HCl and then against streaming water, thickened at 40°C under reduced pressure to a $\sim 20\%$ solution and precipitated by addition dioxane (50 ml). The precipitate was washed with dioxane, methanol and ether and dried at room temperature and pressure, above H_2SO_4. For the determination of the mole fraction of imides the samples were also isolated by lyophilization from a 20% solution. T-poly(Asp)IX was desalted by gel filtration through a column packed with Sephadex G-25. T-poly(Asp)X is the 5th (last-but-one) fraction obtained by fractionation of the 2% water solution of T-poly(Asp)IX by dioxane.

The preparation of samples of poly(aspartic acid) by debenzylation of poly(β-benzyl-L-aspartate) and their charateristics were described previously[5]. They are designated as B-poly(Asp) and corresponding number (see *Table 1* in ref 5). Their molecular mass is about 3×10^4 (refs 5, 6) and they contain L-aspartic acid only (with the exception of B-poly(Asp)II, which is racemic).

Elemental analysis, amino-acid analysis, potentiometric titration, determination of mole fraction of imides, mass and i.r. spectroscopy and viscometry were performed as described previously[4–6]. The weight average of the molecular masses was determined by the sedimentation equilibrium method according to Chervenka[16] using an analytical centrifuge, Spinco model E.

The ^{13}C n.m.r. spectra were measured at 15 MHz using the FX-60 JEOL spectrometer, and at 50.3 MHz using a Varian XL-200 spectrometer. The details of the measurements have been described[4]. The samples of poly(Asp) were dissolved in H_2O (D_2O for the Varian spectrometer) and 4 mol l^{-1} NaOH yielding a solution of pH ~ 9; the final pH of the solution was adjusted by 4 mol l^{-1} HCl. The final concentration was $20 \pm 1\%$ (w/v) for the measurements on the JEOL spectrometer and $10 \pm 1\%$ (w/v) for the measurements on the Vairan spectrometer. Complexes with Co(II) were prepared by addition of $CoCl_2.6H_2O$ (Fluka, p.a.) to the solution. The concentration of Co(II) was expressed as the mole ratio, f, of Co(II) to the aspartic acid residue.

RESULTS AND DISCUSSION

Preparation of thermal polymers

The product of the thermal polycondensation is poly(succinimide) ((poly(Suc)) III. Up until now thermal poly(Asp) has always been prepared by its hydrolysis in excess NaOH in an aqueous suspension[2,4,10,13,15]. We have performed this reaction under somewhat different conditions and have identified the reaction products. The effects of pH, ionic strength and molecular mass of the original poly(Suc) were followed; the reaction was also performed in solution (see *Table 1*).

The results of elemental analysis, potentiometric titration and amino-acid analysis are shown in *Table 2* and they exhibit good agreement. All polymers contain about 10% water which cannot be removed without polymer damage[5]. Elemental analysis was in good agreement with the theory after correction for water contents. In amino-acid analysis, no other ninhydrin-positive substance was detected except aspartic acid. I.r. spectra exhibited absorption bands of amide II, amide I and carboxyl at 1530, 1660 and 1730 cm^{-1}, and did not show imide absorption at 1720 cm^{-1}. Mass spectra, after acid hydrolysis, agreed with the spectrum of aspartic acid.

The presence of succinimide units (III) in thermal poly(Asp) may be due either to incomplete hydrolysis of III, or to their formation during isolation. The content of III in the polymers was determined prior to isolation, i.e. after hydrolysis and desalination, and after

Table 1 Preparation of T-poly(Asp)samples. 1 g of poly(Suc) was hydrolysed under the following conditions

Sample	Hydrolysis procedure[a]	pH of hydrolysis	Starting poly (Suc)[b] fraction number	$M_w \times 10^{-4}$	Medium	Yield (%)
I	A[b]	9.4–14	5	3	H_2O	63
Ia	A[a]	13–14	5	3	1 mol l^{-1} NaOH	65
II	B	8	5	3	4 mol l^{-1} potassium phosphate buffer	81
III	B	7,3	5	3	"	79
IV	B	9	5	3	"	78
V	B	9	3	4.4	"	88
VI	B	11.2	5	3	2 mol l^{-1} Na_2CO_3 buffer and	74
					4 mol l^{-1} NaCl	60
VII	C	11[c]	5	3	Dimethylformamide, triethylamine and H_2O	80
VIII	D	9	5	3	H_2O	
IX	B	8	8	1.6	4 mol l^{-1} potassium phosphate buffer	96
X[d]	B[d]	9	–	–	"	–
XI	B[e]	7,3	5	3	"	76
A	A[f]	13–14	–	0.8	1 mol l^{-1} NaOH	–

[a] Procedures (in detail in text) at room temperature: Aa – dissolved in NaOH, B – dialysis against a buffer, C – in solution in dimethylformamide with 10 mmol of triethylamine, D – pH maintained by means of pH-stat

[b] Preparation and fractionation of poly(Suc) and preparation of T-poly(Asp)I have been described[4]. $\bar{M}_w$ was determined by light-scattering method[13]

[c] Apparent pH

[d] 5th fraction of T-poly(Asp)IX

[e] At 65°C

[f] T-poly(Asp)A was kindly provided by Dr. Andini, preparation has been described by Andini *et al.*[10]

Table 2 Characterization of T-poly(Asp)samples

	Molar fraction of aspartic acid[a]			Molar fraction of succinimide[b] x 10^2				
Sample	N	PT	AA	after hydrolysis	after lyophilization	after precipitation with dioxane	$[\eta]^c$ (ml/g)	$\bar{M}_w \times 10^{-4}$
I	0.92	0.89	0.90	0.24	9.6	0.52	29	2.9^e
Ia	0.88	0.88	0.88	0.30	11.6	0.27	12	1.5^f
II	0.92	0.91	0.89	0.10	11.4	0.17	44	3.5^e
III	0.90	0.90	0.92	0.31	14.9	0.23	43	3.7^f
IV	0.88	0.89	0.88	0.17	12.0	0.71	44	3.8^e
V	0.93	0.92	0.91	0.09	8.0	0.05	70	5.5^f
VI	0.84	0.86	0.89	0.19	8.5	0.28	29	2.8^f
VII	0.88	0.85	0.86	0.59	8.0	0.32	28	2.7^f
VIII	0.90	0.91	0.90	0.25	13.3	0.41	43	3.7^f
IX	0.88	0.88	0.86	0.15	15.1	0.12	16	1.8^f
X	0.85	0.88	0.85	0.28	10.7	0.23	5	0.8^f
XI	0.91	0.89	0.88	0.17	10.2	0.43	43	3.7^f
A	–	0.91	–	–	–	0.44	12	1.5^f

[a] In the theoretical amount calculated from the weight of the sample; determined by: N = elemental analysis, PT = potentiometric titration, AA = amino acid analysis

[b] Related to the aspartic acid content determined by potentiometric titration

[c] Intrinsic viscosity in 0.2 M NaCl, pH = 7.3

[d] Weight average molecular weight

[e] Determined by equilibrium sedimentation measurement

[f] Estimated according to sedimentation measurements and the intrinsic viscosity

Table 3 Population and distribution of α and β linkages in various samples of poly(aspartic acid)

			Measured CONH				Calculated[b] CONH		
Sample		CH_2 β	$\alpha\alpha$	$\alpha\beta$	$\beta\beta$	β^a	$\alpha\alpha$	$\alpha\beta$	$\beta\beta$
Thermal polymers	Poly(D,L-Asp) I	0.75	0.07	0.37	0.56	0.75	0.06	0.38	0.56
	Poly(D,L-Asp) II	0.60	0.16	0.47	0.36	0.60	0.16	0.48	0.36
	Poly(D,L-Asp) III	0.55	0.18	0.50	0.31	0.56	0.19	0.49	0.31
	Poly(D,L-Asp) IV	0.62	0.15	0.47	0.37	0.61	0.15	0.47	0.37
	Poly(D,L-Asp) V	0.62	0.16	0.49	0.34	0.59	0.17	0.48	0.35
	Poly(D,L-Asp) VI	0.73	0.07	0.41	0.52	0.73	0.07	0.39	0.53
	Poly(D,L-Asp) VII	0.75	0.07	0.38	0.55	0.74	0.07	0.38	0.55
	Poly(D,L-Asp) VIII	0.70	0.08	0.44	0.47	0.69	0.09	0.43	0.48
	Poly(D,L-Asp) IX	0.60	0.11	0.46	0.42	0.65	0.12	0.46	0.42
	Poly(D,L-Asp) XI	0.57	0.17	0.49	0.34	0.59	0.17	0.48	0.35
Debenzylated polymers	Poly(L-Asp) I	0.55	0.14	0.59	0.27	0.56	0.19	0.49	0.31
	Poly(D,L-Asp) II	0.75	0.05	0.31	0.65	0.76	0.04	0.36	0.58
	Poly(L-Asp) III	0.20	0.79	0.19	0.02	0.12	0.77	0.21	0.01
	Poly(L-Asp) V	0.20	0.61	0.34	0.05	0.22	0.61	0.34	0.05
	Poly(L-Asp) VI	0.10	0.72	0.23	0.04	0.16	0.71	0.27	0.03
	Poly(L-Asp) VII	0.10	0.64	0.30	0.06	0.21	0.62	0.33	0.04

[a] Calculated as $\beta = \beta\beta + 1/2\alpha\beta$

[b] Calculated for Bernoulli trial statistics with experimental values β (column 5)

isolation. The results are shown in *Table 2*. From the results it is clear that hydrolysis was complete in all cases, and that the imides are re-formed during lyophilization. A polymer without imides can be prepared by precipitation with dioxane from a ~20% solution. Polymers prepared in this way were used for further analyses.

At a higher pH, hydrolysis of poly(Suc) leads to a decrease in the degree of polymerization, at pH 7.3–9 such a decrease does not take place (compare *Tables 1* and *2*).

The kinetics of the poly(Suc) hydrolysis were followed by pH-static NaOH titration at room temperature. At pH > 10.5 the reaction was practically instantaneous, at pH 8–10 it took about two days, at pH = 7.3 it took about ten days. The obtained kinetic curves did not follow any conventional kinetic equation, even at the beginning of the reaction; this is probably due to the very complicated reaction conditions (in suspension with gradual dissolution; during the reaction the polymer acquires charge with a different effective pH in its vicinity, and this changes during the reaction).

The mole fractions of I and II can be affected by conditions of hydrolysis (compare *Tables 1* and *3*). The determining factor is the pH of the medium: the lower the pH, the higher the proportion of I in the product. The ratio of I and II is almost unaffected by ionic strength and temperature. Also for the solution reaction, the effect of pH is decisive. The molecular mass of the original poly(Suc) has no effect on the ratio of I and II.

^{13}C *n.m.r. spectra of T-poly(Asp) and B-poly(Asp)*

For the structural studies of poly(Asp), samples of T-poly(Asp) I–XI and B-poly(Asp)II (*Table 1*) containing racemic aspartic acid, and samples of B-poly(Asp) I, III–IX (*Tables 1* and *2* of ref 5) containing only L-aspartic acid were used. The samples also differed by their contents of α and β peptide bonds and by molecular mass. The ^{13}C n.m.r. spectra of all these samples were measured to elucidate the reason for the complicated structure of bands[4,5].

Contents of α and β peptide bonds. As shown in our previous paper[4], in ^{13}C n.m.r. spectra of CH_2 and CH groups of poly(Asp), two bands corresponding to α and β peptide bonds can be resolved. The basic structural information, the mole fraction of β peptide bonds, can be determined from the ratio of integrated intensities of these bands in the spectra of CH_2 groups. These data for the whole series of measured samples are shown in *Table 3*. The relative error of this determination is about 10%, if the contents of both peptide bonds are comparable, and 20% in other cases. In *Table 3*, samples of B-poly(Asp) VIII and IX are not included, because their contents of β peptide bonds are less than 0.1 and cannot be determined by this method with sufficient precision. In samples of lower molecular mass, lines corresponding to end groups could be observed in the n.m.r. spectra.

The presence of α and β bonds in the peptide chain was detected[4] by means of n.m.r. spectra of aqueous solutions of poly(Asp) at various pHs. Another possibility for proving the presence of α and β bonds in poly(Asp) is through complex formation with metal ions. It is known that interaction of metal ions with poly(α-amino acids) can be followed by means of n.m.r. spectra[17,18]. ^{13}C n.m.r. spectra of an aqueous solution of T-poly(Asp) IV in the presence of various amounts of Co(II) at pH = 7.0 are shown in *Figure 1*. In this figure, in the spectrum form T-poly(Asp) without Co(II) the band of the CH_2 group is resolved into two lines, whereas the bands of CONH and COOH exhibit a more complicated structure. By addition of Co(II) to the aqueous solution of T-poly(Asp)IV, the CH band is also resolved into two lines, and a gradual broadening and shift of some lines also takes place, while other lines are only slightly affected (*Figure 1b, c*). The complex is formed through the COOH groups[18]. In the case of α bond, the COOH group is next to the CH_2 group, in case of a β bond it is next to a CH group. Therefore in the presence of Co(II), the CH (β) line in the n.m.r. spectrum is broadened and shifted more than the CH (α) line, and the CH_2 (α) line more than the CH_2 (β) line. The mole fraction of β bonds determined from the integrated line intensities of CH and CH_2 bands is 0.58, in agreement with the data in *Table 3*. The ^{13}C n.m.r. spectra of the complexes of poly(Asp) with Co(II) therefore confirm our original interpretation and indicate a further possibility for determining the contents of α and β peptide bonds.

Stereoregularity

In other poly(α-amino acids), bond splitting as a consequence of chain stereoregularity was observed only exceptionally and in very strong fields[19]. In order to find out if our original interpretation was not affected by stereoregularity, we have compared n.m.r. spectra of samples of T-poly(Asp) and B-poly(Asp) differing by chain stereoregularity, contents of α and β peptide bonds and molecular mass. The spectra of samples with different stereoregularity, similar contents of β peptide bonds and comparable molecular mass had a very similar shape. Only small differences in the spectra of CH_2 and CONH groups were found, which could be ascribed to the effects of polymer chain stereoregularity. For samples of B-poly(Asp) with a pure L-configuration, the chemical shift of the CH_2 band corresponding to an α peptide bond is equal to 40.1 ± 0.1 ppm, whereas for all samples of T-poly(Asp) and for sample B-poly(Asp)II (racemic samples) the respective shift was equal to 40.55 ± 0.1 ppm. The chemical shifts in the spectrum of the CH_2 group in a β structure are not affected by chain stereoregularity and are equal to 39.25 ± 0.1 ppm in all spectra. The complicated spectrum of the CONH group exhibits a peak at 174.65 ppm in B-poly(Asp); in samples of T-poly(Asp) an additional peak appears at 174.9 ppm. These changes of the structure of the CONH band connected with differences in chain stereoregularity are not sufficient to affect the determination of the populations of α and β peptide bonds from these bands.

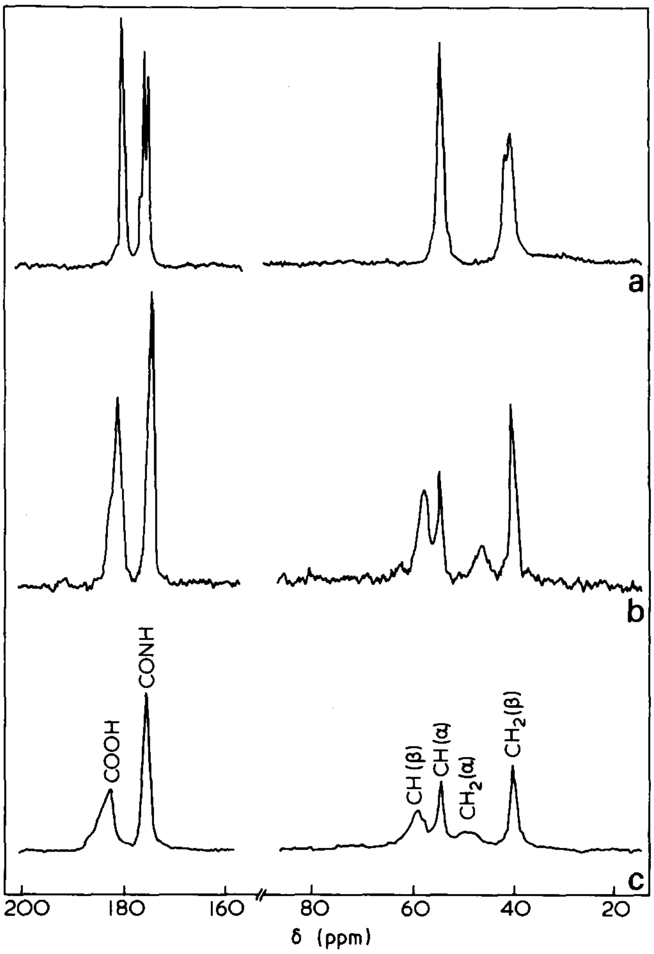

Figure 1 Effect of Co(II) on the 15 MHz ^{13}C n.m.r. spectrum of T-poly(Asp) IV at pH = 7.3 and room temperature. (a) f = [Co(II)]/[aspartic acid residue] = 0; 5750 scans; (b) $f = 1.5 \times 10^{-2}$, 9800 scans; (c) $f = 2.3 \times 10^{-2}$, 7500 scans

Microstructure

Bands in ^{13}C n.m.r. spectra of poly(Asp) samples exhibit a complicated structure, if the sample contains a mixture of α and β peptide bonds. The most complicated are the spectra of the CONH groups (*Figure 2*). The number of resolved lines depends on the molecular mass of the sample (e.g. the structures of the spectra of T-poly(Asp)IX and T-poly(Asp)X were identical, but the spectrum of T-poly(Asp)X was better resolved). Irrespective of molecular mass, the carbonyl carbon

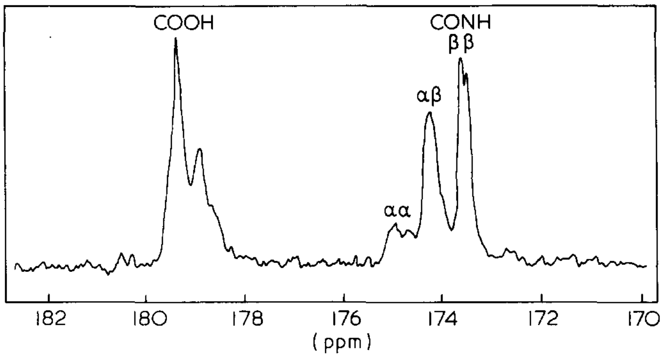

Figure 2 ^{13}C n.m.r. spectrum of T-poly(Asp) IX at 50.3 MHz and pH = 9; mole fraction of β bonds ~0.6

spectra of all poly(Asp) samples exhibit, in principle, three bands with chemical shifts 174.65, 173.55 and 174.18 ppm which are sensitive to peptide bond populations. In poly(Asp) samples with a small population of β bonds, the band at 174.65 ppm is the strongest and the bands at 173.55 and 174.47 ppm are the weakest; the band at 174.18 ppm is most pronounced in samples with the mole fraction of β bonds ~ 0.5. Based on these observations, the bands at 174.65, 174.18 and (173.55 + 173.47) ppm were assigned to the two-unit sequences $\alpha\alpha$, $\alpha\beta + \beta\alpha$, $\beta\beta$. Based on this interpretation, the populations of two-unit sequences were determined as shown in *Table 3*. The population of β bonds calculated from the populations of two-unit sequences agrees well with data obtained directly from the integrated intensities of CH_2 carbon bands (*Table 3*) thus confirming the proposed interpretation. In *Table 3* the distribution of the integrated intensities observed in the CONH spectra of various poly(Asp) samples is compared with the distribution calculated on the basis of Bernoullian statistics. A very good agreement between the calculated and observed values is obtained, indicating a random distribution of α and β peptide bonds in all poly(Asp) samples measured.

With most samples, in the spectrum of the COOH group carbon, two lines can be resolved. However, the shape of the ^{13}C n.m.r. spectra of the COOH group exhibits little dependence on sample composition, the line at 178.2 ppm is stronger than the line at 178.8 ppm in all samples of poly(Asp) measured. These two lines are therefore not suitable for the determination of the population of the two types of peptide bonds.

CONCLUSIONS

The results of our structural analysis of thermal poly(Asp) support the evolution theory of Fox. Thermal poly(Asp) contains both α and β bonds and their ratio can even be modified by the pH of the medium in which hydrolysis takes place. It is not improbable that under primitive prebiotic conditions, hydrolysis of poly(Suc) might have taken place at a pH near to neutrality, where poly(Asp) with a mole ratio almost 1:1 of I and II is formed. With a random distribution of I and II, the polymer contains some long sequences of one type which could lead to the formation of ordered structures and superstructures.

Poly(Asp) prepared for studies of conformational structure by debenzylation of poly(β-benzyl-L-aspartate) probably always contained some amount of β bonds. Their distribution in the polymer is random. As will be shown in a future paper, the presence of β bonds in poly(Asp) affects its conformational behaviour. Most controversial literature data on the conformation of poly(Asp) can be explained by the presence of β bonds and their random distribution in the chain.

Poly(Suc) can be hydrolysed without substantial reduction of the degree of polymerization, and the resulting polymer does not contain succinimide units. This finding is important, primarily for practical applications of thermal poly(Asp) and of its derivatives.

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