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Thermal Polyamino Acids: Synthesis at Less Than 100°C

Abstract. *Thermally prepared polyamino acids, regarded as models for prebiotic protein, typically have been synthesized at 120° to 200°C. In this study, three different sets of amino acid mixtures were found to yield material of relatively high molecular weight (molecular sieving and diffusion techniques) when heated for up to 81 days at 85°, 75°, and 65°C. These temperatures, which today are generated by radiation from the sun in some terrestrial locales, probably were more common on the prebiotic earth than those above the boiling point of water. The results thus suggest that prebiotic polyamino acids may have been relatively common and widespread.*

Polyamino acids are readily prepared under simulated prebiotic conditions by heating suitable proportions of dry amino acids (*1*, *2*). Such polymers, termed proteinoids, show many properties envisionable as being important in evolutionary processes (for example, catalytic activity, morphogenesis), and they are regarded as models for prebiotic protein (*1*).

Temperatures conducive to the removal of nascent water (120° to 200°C) typically have been used in the synthesis of proteinoids, although the polymerizations are known to occur at 60°C in the presence of dehydrating agents (*1*). It has been suggested (*3*), however, that polyamino acids formed at 120° to 200°C could not have played a major role in evolutionary processes, because such temperatures probably occurred only locally on the surface of the prebiotic earth (juxtaposed with much higher, detrimental, temperatures) and would have, on long contact, destroyed any resulting polyamino acids. These objections have been rebutted [(*1*, *4*); for example igneous areas are numerous today and were more plentiful in the past; rain is a common and effective vehicle for removing proteinoid from a heat source], but with little doubt temperatures below 100°C were (and are) more prevalent (*5*).

An understanding of whether amino acid mixtures would polymerize at relatively low temperatures would thus permit inferences concerning the abundance—or scarcity—of prebiotic protein (*6*). The temperatures tested in this study (65° to 85°C) are within the range of those that are generated by solar radiation today (*7*, *8*).

Three types of amino acid mixtures were used as reactants: aspartic acid, glutamic acid, and an equimolar mixture of 16 proteinous amino acids (2 : 2 : 1 by weight), yielding 2 : 2 : 1 proteinoid; lysine as the free base and an equimolar mixture of 16 amino acids (1 : 1 by weight), yielding lysine-rich proteinoid; and the proportions of amino acids, rich in glycine, reported by Fox and Windsor (*9*) as being formed in a simulated prebiotic synthesis, yielding "Fox-Windsor" preparation. Proteinoids made from the former two sets of reactants have been well characterized (*1*); the latter set was chosen because it is representative of several sets, known to polymerize at 175°C, that may reflect the kinds and proportions of amino acids that were available prebiotically (*10*).

Each set of reactant amino acids (10 g) was slurred in triplicate in 10 ml of water, placed on a watch glass, and heated in ovens at 85°, 75°, and 65°C (± 2°C). No attempt was made to control humidity. Samples of about 2 g were removed periodically, the remaining material being reslurried in 5 to 6 ml of water (*11*). With one exception, the maximum heating time was 81 days, in contrast to the few hours typically used at higher temperatures (*1*). Unless otherwise indicated, the samples were tested without purification.

The presence of material of relatively high molecular weight in the undialyzed products was evaluated by elution patterns from a Bio-Gel P-2 column (2.0 $cm^2 \times 25$ cm, exclusion limit 1600 daltons), with the use of 0.1*M* phosphate buffer (*p*H 7.0) containing 0.2 percent sodium dodecyl sulfate and 0.02 percent sodium azide; the void volume of the column (about 21 ml) was determined with Blue Dextran (2×10^6 daltons). Relative size was also estimated by the Craig diffusion technique (*12*) (Union Carbide dialysis tubing and diffusion apparatuses that, with a 0.5-ml sample, had an area to volume ratio of about 75). Diffusion in 0.1*M* tris buffer (*p*H 8.0) was monitored at 220 nm and in some cases via ninhydrin color.

Figure 1 shows Bio-Gel elution patterns of several of the undialyzed 2 : 2 : 1 proteinoids, as well as those of the reactant amino acids and Blue Dextran. As the time of heating at 85°C increases (Fig. 1, a to d), there is a progressive increase in the amount of material eluting at Ve/Vo = 1 (elution volume/void volume), indicative (*13*) of molecules having molecular weights exceeding the exclusion limit of the gel (1600 daltons). This is accompanied by a decrease in the amount of free amino

Table 1. Diffusion data for various undialyzed 2 : 2 : 1 proteinoids. T-50 is the time required for diffusion of 50 percent of the sample; see text.

Proteinoid (time and temperature of synthesis)	Ratio, T-50 of proteinoid/ T-50 of reactants*	*N*†	Significance level‡
20 days, 85°C	1.30 ± 0.14§	4	< 0.05
48 days, 85°C	1.85 ± 0.11	4	< 0.01
81 days, 85°C	2.31 ± 0.23	4	< 0.01
81 days, 75°C	1.56 ± 0.28	3	< 0.02
81 days, 65°C	0.90 ± 0.05	3	< 0.40
81 days, 65°C (dialyzed)	2.82, 2.35	2	

*The observed T-50 value for the reactant amino acid mixture was 6.88 ± 1.03 minutes (four determinations). †Number of determinations. ‡Probability that the difference between proteinoid and reactants is due to chance (*18*). §Mean and standard deviation.

acids, as judged by ninhydrin color and the peak due to aromatic amino acids at Ve/Vo = 3. Similar trends are noted with increasing temperature of synthesis of 81 days' duration (Fig. 1, f, e, and d), although 85°C was by far the most effective temperature. The suggestion from Fig. 1f (65°C, 81-day sample) of a small amount of material eluting in Ve/Vo = 1 is substantiated by the pattern (Fig. 1g) of the *dialyzed* sample (*14*).

The 85°C, 81-day preparation contained material exceeding the exclusion limit (4500 daltons) of Bio-Gel P-6 but not that (10,000) of Bio-Gel P-10.

Elution patterns (Bio-Gel P-2) of lysine-rich proteinoids and the "Fox-Windsor" preparations showed peaks at Ve/Vo = 1 and, unlike those of the 2 : 2 : 1 proteinoids, also at Ve/Vo = 1.4 to 1.6. These latter peaks preceded the ninhydrin peak, connoting (*15*) considerable material of intermediate size. Dialyzed 81-day samples were used to verify that some polymerization occurred at 65°C.

With each type of proteinoid, minimum heating periods observed to yield material exceeding the exclusion limit of Bio-Gel P-2 were 11, 20, and 40 to 48 days, respectively, for syntheses at 85°, 75°, and 65°C. (Intermediate periods were not tested.)

The Craig diffusion technique (*12*) provided a measure of relative *average* size of the preparations (in contrast to relative amount exceeding a particular size, via gel filtration). Table 1 shows T-50 values (time required for diffusion of 50 percent of the sample) (*16*) for unpurified 2 : 2 : 1 proteinoids, normalized to the value given by the reactant amino acid mixture (*17*). The values increase, indicating larger average size, with increasing time of heating at 85°C and also with increasing temperature of heating for 81 days. Only in the case of the 65°C, 81-day sample is the relative T-50 value not significantly different (*18*) from that of the reactant amino acids; however, after preparative dialysis of this sample, the values (duplicate analyses) are more than twice that of the reactants. Dialyzed lysine-rich proteinoids and "Fox-Windsor" preparations (65°C, 81 days) also gave larger T-50 values than did their respective reactant amino acid mixtures.

Infrared spectra and positive biuret reactions showed that peptide bonds were formed during the syntheses [see (*1*)]. The coloration of the preparations, measured at 400 nm, increased with increasing time or temperature of synthesis.

Although the preparations have not as yet been characterized in detail, one demonstrated property is that of microsphere formation, observed with 2 : 2 : 1 proteinoid (85°C, 81 days). Microspheres from higher-temperature proteinoid have provided an experimental model for the formation and evolution of protocells (*1, 19*); that the lower-temperature proteinoid of this study is morphogenic is thus especially noteworthy. (The protocell stage of evolution, incidentally, would require liquid water, that is, temperatures less than 100°C.)

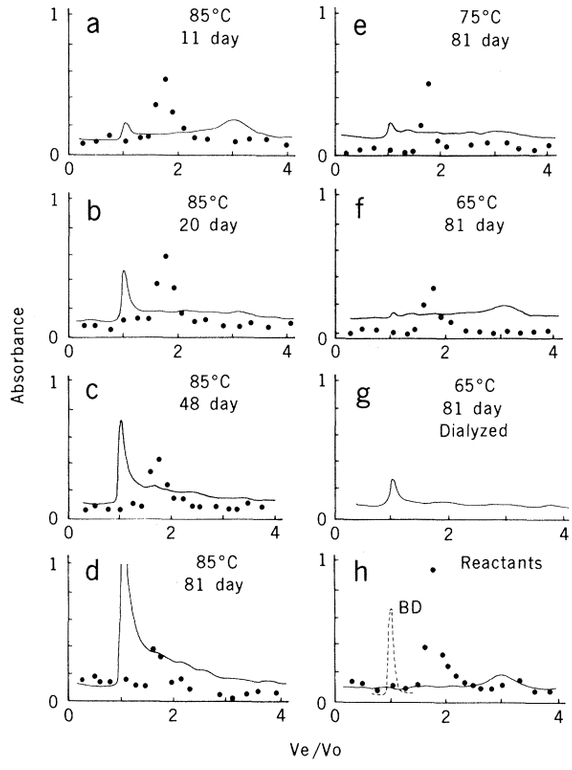

Fig. 1. Elution patterns (Bio-Gel P-2 column) of 2 : 2 : 1 proteinoids prepared at various temperatures and time periods, Ve/Vo, elution volume/void volume; *BD*, Blue Dextran. Continuous tracing, absorbance at 254 nm; (●), absorbance at 570 nm of collected fractions after reaction with ninhydrin. The patterns are of undialyzed preparations (10 mg/ml), except for panel g (dialyzed, 2.3 mg/ml). See text for details.

This study has shown that three different sets of amino acids polymerize [or oligomerize (*20*)] at temperatures well below the boiling point of water; dehydrating chemicals are not necessary. [These observations are consistent with Arrhenius theory, although extrapolations from above to below 100°C would be complicated by the heat of vaporization of nascent water at the lower temperatures (*21*).] The temperatures usually used (*1*) in the synthesis of proteinoids probably were restricted during much of the earth's prebiotic history to localities where heat from the interior of the earth reached the surface (for example, volcanoes and hot springs). The values of this study, however, may have been relatively common; temperatures as high as 85° to 90°C, generated by radiation from the sun, have been observed in contemporary deserts (*7, 8*), which occupy about one-seventh of the earth's terrestrial surface (*22*). The need for removing product from energy source may have been less at the lower temperatures, and the possibility of detrimentally high values (for example, 300° to 1000°C) being generated by solar radiation would not have been great.

Various means for forming amino acids under prebiotic conditions have been indicated by experiment (*1, 23*). Dry reactant mixtures could have resulted via dissolution of amino acids in water (rain) and subsequent evaporation, as would readily occur in arid regions; this scenario is simulated by initially slurrying the amino acids in water and then heating at environmentally realistic temperatures. By laboratory standards, the polymerizations undoubtedly occur slowly at 65° to 85°C. Time, however, was a plentiful prebiotic commodity [a feature, incidentally, that suggests that appreciable polymerization could have resulted at even lower temperatures (*24*)]. This study thus indicates that the formation of polyamino acids may have been a relatively widespread and common event on the prebiotic earth.

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14. After 24 hours of dialysis, the nondiffusible material was recovered in only 0.1 percent yield; the extent of polymerization in 81 days is thus small at 65°C. However, 2.5 percent of the 85°C sample was nondiffusible, as was 5.0 percent of a "Fox-Windsor" preparation heated at 75°C for 197 days.
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21. For the temperature range 65° to 85°C, an average activation energy of 24 ± 5 kcal/mole was calculated from the areas under the curves of Bio-Gel elution patterns and from absorbance values at 400 nm. For the range 160° to 190°C, a value of 5.5 ± 2.6 kcal/mole was obtained from published data on yield and molecular weight of thermal polyamino acids [K. Harada and S. W. Fox, *J. Am. Chem. Soc.* **80**, 2694 (19 → S. W. Fox and K. Harada, *ibid.* **82**, 3745 (1960)].
22. Kirk (*8*, p. 7). By comparison, only about 430 volcanoes have erupted in historical times [*Encyclopedia Americana* (Americana Corp., New York, 1973), vol. 28, p. 218]; hot springs and fumaroles would, of course, also afford thermal energy.
23. In almost all cases, glycine is the predominant amino acid found. A variety of sets rich in glycine, including the "Fox-Windsor" set of this study, polymerize at 175°C (*10*).
24. By using an activation energy of 24 kcal/mole (*21*), it is calculated that about 7 years at 35°C (95°F, that is, environmentally common) would yield the same extent of polymerization observed in 81 days at 65°C.
25. I thank Walter W. McAlhaney and Ricardo Moreno for capable technical assistance, John M. Herr for review of the manuscript, and Gerald T. Cowley for photographic services. Supported by grant NSG 7069 of the National Aeronautics and Space Administration.

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Isolation of a Cartilage Factor That Inhibits Tumor Neovascularization

Abstract. *A cartilage fraction isolated by guanidine extraction and purified by affinity chromatography inhibits tumor-induced vascular proliferation and consequently restricts tumor growth. This fraction contains several different proteins; the major one has a molecular weight of about 16,000. The fraction strongly inhibits protease activity.*

Tumor-induced neovascularization is inhibited by neonatal cartilage and tumor growth is thereby restricted (*1*). We now report isolation and partial purification of a cartilage fraction that inhibits the vascularization of tumors.

Segments of cartilage 1.5 by 8 cm were excised from the distal tips of scapulae of calves less than 2 weeks old and extracted in lactated Ringer solution or in 1*M* guanidine hydrochloride (0.02*M* MES, *p*H 6) for 24 hours at room temperature. Sorgente *et al.* showed that a 1*M* guanidine extract has protease inhibitory activity, and they suggest that this activity may be responsible for cartilage avascularity (*2*).

Extracts were dialyzed exhaustively against water at 4°C and centrifuged. The supernatant was lyophilized and fractionated. Sixteen fractions were obtained. The first was an aqueous extract from lactated Ringer solution. The remaining 15 were obtained from guanidine extracts. Five of these came from Sephadex G-100, three from G-200, six from ammonium sulfate precipitation, and one from the trypsin affinity column. All fractions were dialyzed exhaustively against water at 4°C, passed through 0.45-μm Millipore filters, and lyophilized. Sterile technique was followed thereafter.

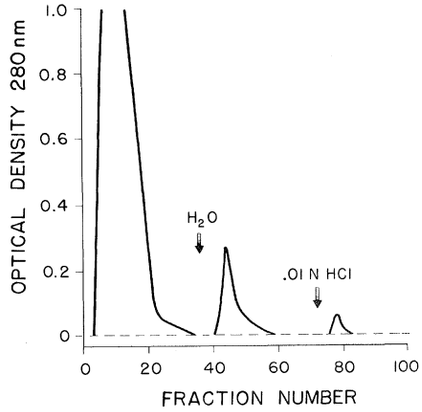

Fig. 1. Purification of protease inhibitor from cartilage by a trypsin-Sepharose affinity column. The column (1.5 by 15 cm) was equilibrated with buffer (0.05*M* tris, 0.5*M* NaCl, *p*H 8). The sample (1.45 g of guanidine extract of cartilage in 43 ml of buffer) was added at fraction 1, and buffer was added at fraction 7. The flow rate was 23 ml/hour at 4°C. Fractions of 7 ml were collected. High protease inhibitory activity was found in the HCl eluate.

Affinity chromatography with trypsin-Sepharose was used to purify the cartilage protease inhibitor. The affinity column was prepared as described by March *et al.* (*3*). We used 0.5 g of trypsin and 30 ml of packed Sepharose. Guanidine extract was passed over the affinity column, and the column was washed with buffer, distilled water, and 0.01*N* HCl (Fig. 1). The HCl eluate contained very high trypsin inhibitory activity. It migrated as one major and several minor bands on acrylamide (15 percent) slab gel electrophoresis. The major band fell between markers of myoglobin (molecular weight, 17,800) and egg white lysozyme (molecular weight, 14,400) on the gel. Approximately 500 g of cartilage produced 1 mg of this fraction.

Slow release polymers were made by mixing 5 mg of each dry fraction with 100 μl of 10 percent Elvax 40 (*4*) (an ethylene–vinyl acetate copolymer, 40 percent vinyl acetate by weight) in methylene chloride. The resulting dispersion was dried under vacuum, cut into 1-mm^3 pellets, and coated with a thin film of pure polymer. Polymer pellets containing lysozyme, alkaline phosphatase, and soybean trypsin inhibitor were prepared in identical fashion and tested in vitro to calibrate release rates and to ascertain that biological activity was not destroyed. Using agar gel diffusion assays (*5*), we found that polymers containing each of the three compounds released more than 100 ng/day of biochemically active material into aqueous media for periods longer than 100 days.

Implants of V2 carcinoma produce a neovascular response in the normally avascular rabbit cornea (*6*). Using this as a bioassay for tumor-induced vascularization, we assessed the inhibitory effect of 16 different fractions. Pellets of polymer and pieces of V2 carcinoma were placed into corneal pockets (Fig. 2A). The tumors grew as thin plaques, inducing vessels to sprout from the edge of the cornea 4 to 6 days after implantation. Vessel length and tumor diameter were measured four times each week by