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A MODEL FOR THE ORIGIN OF STABLE PROTOCELLS IN A PRIMITIVE ALKALINE OCEAN

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When a mixture of the eighteen proteinous amino acids are suitably heated in the dry state with seawater salts, a copolyamino acid results. One fraction of this polymer is found, through isoelectric focusing, to consist of a mixture of acidic and basic proteinoids, each of sharply limited heterogeneity. When one fraction of the seawater proteinoid is dissolved in hot water, and the solution is cooled, proteinoid microspheres result. These have properties in common with simpler types, but are also stable at pH values to 9, in common with microspheres prepared by mixing acidic and basic proteinoids. These processes thus constitute a simple model for the origin of a protocell stable in a primitive alkaline ocean.

A model for the origin of reproducing protocells (Fox, et al., 1967; Hsu, et al., 1971; Fox, 1973a; Hsu, 1974) on Earth requires a prior accumulation of amino acids in locales on the surface of the primitive planet.

Experiments designed to simulate subsequent polymerization of the amino acids [or alternatively, (a) evaporation of solvent water and (b) polymerization of the dried amino acid residue at the same temperature] at 70—200° C (Harada and Fox, 1965; Young, 1965; Fox and Harada, 1960) have yielded a variety of proteinoids (copolyamino acids). Many of the proteinoids assemble easily in aqueous solution to form microspheres (Fox, 1968). The numerous protein-like properties of proteinoids and cell-like properties of the microspheres have been described elsewhere (Fox and Dose, 1972). The results are consistent with the view that the formation of proteinoids (preprotein) on the primitive Earth was frequent and rapid.

The enzymelike activities that have been catalogued for thermal proteinoids (Fox, 1974a) have been studied especially in acidic proteinoids, which are rich in aspartic acid and/or glutamic acid, and in basic proteinoids, which are rich in lysine (Rohlfing and Fox, 1969). The observed variations in arrays of activity are a function of the amino acid composition. However, the assembly and stability of the microspheres from any one kind of proteinoid is limited by pH.

Acidic proteinoid alone forms enormous numbers of microspheres at pHs below 6 but the spherules dissolve at higher pH. Microspheres from neutral proteinoid tend to be stable at pHs as high as 7, but dissolve above that value. Basic proteinoid is quite soluble in water, but addition of sodium chloride to the solution results in microspheres stable up to pH 12 (Rohlfing, 1975). A combination of acidic and basic proteinoids yields "mixed" microspheres that are stable up to pH 8.5, and also stable in acidic solution (Fox and Yuyama, 1963). The microsphere of mixed proteinoids would require independent syntheses of acidic and basic proteinoids (presumably preceded by some fractionation of amino acids into acidic and basic types), followed by subsequent transport and mixing, prior to assembly into mixed microsphere systems.

Such a sequence of special fractionations is no longer conceptually required. Thermal polycondensation of amino acids in the presence of seawater salts has been found to yield a single polymer that contains both acidic and basic components, as determined by isoelectric focusing. On treatment with water, this "sea-

water proteinoid" then forms microspheres that are stable over a broader and higher pH range than previously observed for proteinoid microspheres (Fox and Dose, 1972). As noted in preliminary reports of work with seawater proteinoid, similar processes could have occurred in primitive lagoons (Fox, 1973; Snyder and Fox, 1973).

The limited heterogeneity observed in the seawater proteinoids reflects similar findings from this and other laboratories (Dose and Rauchfuss, 1972; Saunders and Rohlfing, 1972; Fox, 1974a) on other thermal polyamino acids.

1. Experimental

1.1. Materials

Acrylamide, N,N'-methylenebisacrylamide (BIS), N,N,N',N'-tetramethylenediamine (TEMED), and ammonium persulfate were obtained in electrophoresis grade from Eastman Kodak Co. Ampholine carrier ampholytes, pH 3-10, were purchased from LKB Productor AB. All other chemicals were reagent grade and were used as purchased without further purification.

1.2. Methods

1.2.1. Artificial Seawater

One l of trace MBL Formula (Cavanaugh, 1956) artificial seawater was prepared by combining 24.72 g NaCl, 0.62 g KCl, 1.36 g $CaCl_2 \cdot 2$ HOH, 4.66 g $MgCl_2 \cdot 6$ HOH, 6.29 g $MgSO_4 \cdot 7$ HOH, 0.089 g KBr, 0.003 g NaF, 0.037 g $SrCl_2 \cdot 6$ HOH, 0.024 g H_3BO_3 and diluting to volume. Sodium bicarbonate (0.180 g) was added just before use.

1.2.2. Seawater Proteinoid

The synthesis of the seawater proteinoid was set up to model a two step process of (a) evaporation of the water in a geologically aqueous solution followed by (b) polymerization of the dried amino acid residue at the same temperature employed for evaporation. [The elevated temperature and dry state of the solid are required for thermodynamic reasons (Fox and Dose, 1972; Fox, 1974b)].

Fifteen grams of the eighteen common (Fox, et al., 1970) L-α-amino acids in equimolar proportions was slurried in 375 ml of artificial seawater (4% slurry). The slurry was evaporated to a thick paste, and heated under nitrogen flow in a 190° C oil bath for 7 hr. The final reaction mixture temperature was 180° C. [1] The crude product was an orange-brown friable solid (22 g). Eleven grams of the product was slurried in 100 ml of water and the suspension was dialyzed with stirring against seven changes of 400 ml of water each. The first dialysis occupied 6 hr while the next five each spanned 24 hr. Approximately 20 mg of NaN_3 was added at each change to prevent microbial growth. Combined diffusates and filtered retentate were lyophilized; undissolved solids were dried over P_2O_5 (20 mm Hg).

The composition of amino acids employed reflects what could have happened in a complex matrix; it does not indicate the exact nature of a primitive mixture of amino acids.

1.2.3. Neutral Proteinoid

Neutral proteinoid was prepared as described (Fox and Waehneldt, 1968, no. 55). The procedure is essentially identical to that for seawater proteinoid except that salts were omitted.

1.2.4. Microspheres

A 4% (40 mg/ml) slurry of proteinoid (crude or fractionated) in distilled water or artificial seawater was heated to boiling for 30 sec. The hot, yellow-brown supernatant was separated from undissolved tarry solids. On cooling to room temperature, the solution deposited microspheres. Morphological and colloidal stability of microspheres to titration with dilute HCl or NaOH was monitored by optical microscopy (Leitz Ortholux); a Radiometer pH meter

[1] Reaction at 80°C for 12 days yielded small amounts of proteinoid.

(Model PHM4c) was used to measure pH of microsphere suspensions.

1.2.5. Gel Isoelectric Focusing

The apparatus consisted of a Bio-Rad Model 200 electrophoresis system and a Beckman Spinco RP-2 power supply. Gels were formed in 5 × 125 mm glass tubes treated with dimethyldichlorosilane before use. The following stock solutions were made from distilled water saturated with nitrogen (Smith, 1968):

(A) Monomer stock solution: 14 g acrylamide and 0.3675 g BIS/50 ml solution

(B) Initiator stock solution: 0.07 g ammonium persulfate/50 ml solution.

Gels were prepared by rapidly mixing 5 ml (A), 5 ml water, 0.1 ml TEMED, 1 ml ampholyte solution and 10 ml (B), then filling each glass tube. Gelation occurred after 5 min; the length of the gel bed was 115 mm. Gel tubes were installed in the apparatus and the top of each bed was layered with 0.1 ml of a 40% sucrose solution. The cathode compartment (top) was filled with 2.9% diethanolamine and the anode compartment with 1% H_2SO_4 (Wellner, 1971).

Sample solutions were prepared containing 40 mg/ml of each proteinoid or fraction in a 1 M urea solution containing 2% ampholytes. Five lambdas of this solution was layered on a gel bed under the sucrose solution.

Electrofocusing was carried out at 120 V for 5—10 hr. Proteinoid component bands were located in the gels by their strong fluorescence under 254 nm irradiation.

1.2.6. Miscellaneous

Amino acid composition of unhydrolyzed and acid hydrolyzed proteinoid fractions were determined by established methods (Fox et al., 1963). Molecular weight distributions were estimated from: gel permeation chromatography on Bio-Gel P-10 (0.5% sodium dodecyl sulfate as eluent; Fish, 1971) and Sephadex LH-20 (methanol as eluent); dialysis with Spectrapor 3 membrane tubing of 3500 m.w. cut off, and ultrafiltration with Bio-Rad hollow fibre devices. Ash content of fraction 2 was determined after ignition to constant weight below 900° C. The extent of amino acid racemization was measured by ORD on 0.05—0.4% filtered aqueous solutions with a JASCO ORD/UV-5 spectrophotometer. Established procedures were used for the biuret test (Clark, 1964) and the Gram stain (Fox and Yuyama, 1963).

2. Results

The four fractions of seawater proteinoid obtained are listed in Table 1, with the properties studied. Fraction 2 was studied in greatest detail because of its outstanding tendency to yield a dense population of microspheres at a high pH.

Table 2 compares fraction 2 of seawater proteinoid and a similar fraction of neutral proteinoid. The amino acid compositions for the two proteinoids are comparable except for the higher content of tyrosine in the seawater polymer.

Although most properties observed (Table 3) are common to all thermal proteinoids (Fox and Dose, 1972), a new characteristic emerged on examination of the microsphere preparations.

The seawater proteinoid microspheres begin to form upon slight cooling of a hot solution. The most significant feature of these units (Fig. 1) is their stability at high pH. As stated earlier, mixed microspheres are stable above pH 7. This fact suggested that the proteinoid contains both acidic and basic components. Gel isoelectric focusing verified this inference. As shown in Fig. 2, acidic and basic proteinoids contain families of components with pH values entirely in the acidic and basic regions of their gels respectively. Seawater proteinoid, as thus observed, contains both acidic and basic polyamino acids, each of limited heterogeneity.

The mobility previously observed in acidic proteinoid microspheres is found to exist in greater degree in those described here. Brownian motion has been observed continuously for as much as ten days, during which fraction 2 spheres settled out only gradually on glass surfaces.

TABLE 1

Properties of fractions of seawater proteinoid.

Fraction	Composition by Source	Wt (g)	Free Amino Acids/ Solid (wt %)[c]	Proteinoid[a] Solid (wt % of solid)	Moisture[b] (wt %)	Molecular Weight Range of Major Component	pH of Suspension of Microspheres
1	Diffusates 1 + 2	7.6	20.9	42.0	16.3	<3,000	7.9
2	Diffusates 3 — 7	1.3	19.2	18.6	9.2	3,000—10,000	9.1
3	Dissolved retentate	0.1	8.4	19.2	—	>10,000	—
4	Undissolved solids	0.4	trace	14.6	12.1	10,000	7.5

[a] Weight % hydrolyzable to amino acids; fraction 1, in addition to salts, probably contains large proportions of diketopiperazines.
[b] Weight loss after 48 hr at 110° C over P_2O_5 (0.1 mm Hg).
[c] From analysis of unhydrolyzed product.

TABLE 2

Amino acid composition of seawater proteinoid and neutral proteinoid.

Amino acid	Seawater	Neutral
Lys	5.0	8.4
His	4.1	3.3
Arg	5.0	3.4
Asp	2.4	4.6
Thr	1.6	0.3
Ser	0.2	0.2
Glu	14.8	10.3
Pro	3.6	2.7
Gly	8.3	8.3
Ala	12.4	12.2
Cys/2	2.1	5.2
Val	3.4	9.7
Met	6.5	6.8
Ileu	2.4	4.7
Leu	3.9	6.0
Tyr	15.3	5.1
Phe	6.9	4.6

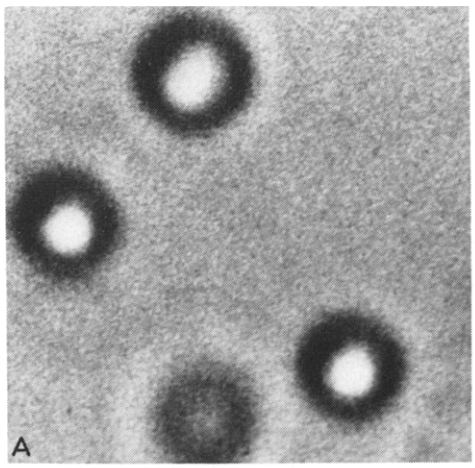
Expressed as mole % (NH_3 omitted from the calculations).

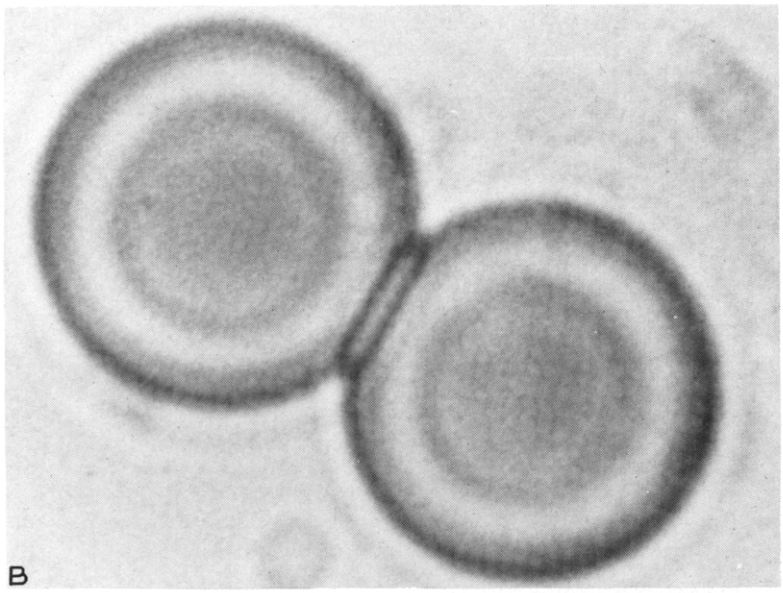
TABLE 3

Characteristics of fraction 2 (Table 1).

Proteinoid:
1) 12% ash
2) Complete racemization (ORD)
3) Positive biuret test
4) Limited heterogeneity
5) Composition similar to that of protein
6) Molecular weights of many thousand
7) Recoverability of amino acids on hydrolysis
8) Tendency to aggregate to cell-like structures

Microspheres:
1) Microscopic size range (<3μ)
2) Stability from pH 8.7—9.9 when freshly prepared
3) Stability from pH 4.7—9.5 on aging 10 days
4) Positive Gram stain
5) Uniformity of size
6) Numerousness
7) Associative patterns, including formation of junctions
8) Ability to bud





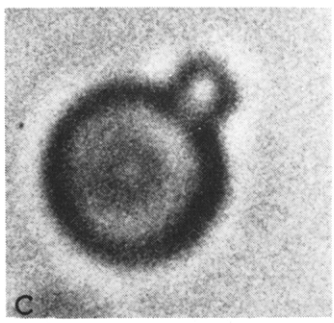


Fig. 1. Seawater proteinoid microspheres. A) Approximately 1 μm in diameter. A more concentrated slurry yields larger spherules, B and C, e.g., 120 mg/ml yields 2.5 μm spherules, many with buds, C.

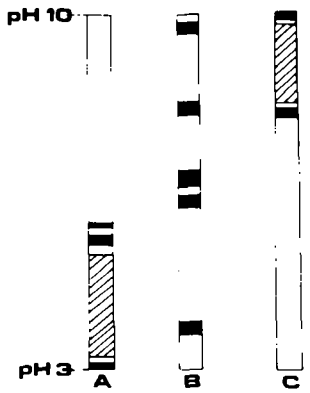


Fig. 2. Gel isoelectric focusing of proteinoids at 25°C. Seven percent crosslinked acrylamide gels, 5 mm × 115 mm; cathode (top) contained 2.9% ethanolamine, anode contained 1% H_2SO_4; 120 V/5 hr, A: acidic proteinoid, B: seawater proteinoid, C: basic proteinoid. Solid areas represent well defined bands; cross-hatched areas are diffuse.

3. Discussion

The composition of thermal proteinoids has been shown to be precisely, although not necessarily linearly, related to the proportions of amino acids in the reaction mixture (Fox et al., 1963; Fox and Waehneldt, 1968) from which they are prepared. By varying these proportions, one can produce an almost limitless variety of proteinoids, each of remarkably limited heterogeneity. With possible co-reactants ("prosthetic groups" in some cases) there is theoretically encompassed most or all organic and inorganic compounds that participated in protobiological evolution. As a minor example, inorganic phosphates or polyphosphates affect the composition and yield of proteinoids (Fox, 1968). Dose and Rauchfuss (1972) have reported the production of acidic and basic proteinoids in a single preparation by heating an amino acid mixture with sodium polyphosphate.

The principal effect of seawater salt on the composition of proteinoid is in the tyrosine content. The saline matrix apparently increases the relative incorporability, and perhaps stability, of the tyrosine. The known ability of tyrosine to form magnesium complexes (Greenstein and Winitz, 1961) and the high content of tyrosine in this polymer may be a significant contribution to the ash content of fraction 2. The fact that the proteinoid forms in the presence of seawater salts extends the knowledge of the ruggedness of this polycondensation, as earlier described for other geological materials such as basalt (Fox, 1965).

The stability of seawater proteinoid microspheres at high pH is explained by the fact that it contains both acidic and basic components analogous to mixed acidic plus basic microsphere systems. In such systems, interactions of a basic macromolecule with an acidic one would give a complex, less soluble than either component. Thus the simple, two-step conceptual sequence: amino acids → preprotein → protocell is modified by these experiments. The presence of seawater salts provides a route to base-stable microspheres of mixed proteinoids and obviates the need for independent syntheses of acidic and basic proteinoids on the primitive Earth.

Aside from a possible very early period of an acidic ocean on the early Earth (Rubey, 1964), the primitive ocean is generally believed to have been at about pH 7.3-8.1. The seawater proteinoid is thus of interest as one model for the origin of protocells in such an ocean. Moreover, the origin of cellular polymerizations yielding proteins and nucleic acids can be thought of as favored by high pH as they are in contemporary cells (Kirsch et al., 1960; Aposhian and Kornberg, 1962). Budding (Fig. 1B) has been shown to function as a necessary step in a primordial reproductive cycle involving separation of the buds (cf. Lehninger, 1970) by mechanical shock or thermal treatment (Fox et al., 1967). Those units that happened to have prolonged mobility would accordingly have had enhanced opportunity for mechanical separation or for travel into warmer waters, which could cause separation followed by accretive growth. Seawater proteinoid microspheres would thus have been relatively highly adapted to reproductive events. For instance, a model protoribosome capable of making peptides from phenylalanine

and ATP (Fox et al., 1974) has as a necessary component a basic proteinoid.

Vaporization of water from a primitive lagoon and exposure of the resultant evaporite deposit of inorganic salts and amino acids to temperature of 70 to 200° C provide a simple process that could have occurred on perivolcanic and other regions of the Earth's surface (Fox, 1964). The model and concept are strengthened by the finding that seawater proteinoid yields cell-like structures that are stable in an alkaline ocean. Rohlfing's basic microspheres (1975) provide an alternative explanation with a microunit of other properties. The fact that spherules of seawater proteinoid are relatively stable also in warm water is consistent with the geological model of Turcotte et al. (1974). Turcotte et al. have linked the first cells also to microfossils, and to a global thermal event resulting from a Moon-Earth interaction. Although such an event was conceptually not necessary to produce the first proteinoid microspheres, a grand burst of protocells could have resulted at that time (3 billion years ago).

In other simulations of settings such as the one examined here, seawater salts have been shown to increase the yields of purine nucleosides from reactions between purine bases and ribose at temperatures near the boiling point of water, but in the absence of water (Fuller et al., 1972). Questions of temperature, presence or absence of water, sequence of events, etc. in the general theory as raised by others, have been critically discussed in two recent reviews (Fox, 1973b, 1974c).

Seawater proteinoid, in the context of a thermal model of origins, provides experimental support for the concept that life could have arisen in the sea (Fox et al., 1959), as earlier promulgated by Oparin (1924).

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