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INTERACTIONS BETWEEN DIVERSE PROTEINOIDS AND MICROSPHERES IN SIMULATION OF PRIMORDIAL EVOLUTION

LAURA L. HSU * and SIDNEY W. FOX

Institute for Molecular and Cellular Evolution, University of Miami, Coral Gables, FL 33134, USA

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Experiments demonstrating an incorporation of different enzymelike activities into a single preparation of proteinoid microspheres provide a conceptual basis for the primitive lengthening of protometabolic pathways. An enhancement of one enzymelike activity by another proteinoid in the same microsphere has been found. This effect, plus the pathway-lengthening propensity of combinations of microspheres, indicates selective advantages contributing to adaptive protoselection.

Data reported in this paper also bring into purview the concept of internally controlled variation. Inferences are derived for the origin of protosexuality in protocells.

When allowance is made for a closer relationship to the environment than that needed in contemporary selection, the fundamental mechanistic requirements of protoevolution are regarded as met by the proteinoid microsphere.

Many kinds of polyamino acid have been prepared by heating the monomeric amino acids (Fox and Dose, 1972). Under simple conditions of dehydration, various mixtures of amino acids polymerize in specific patterns to yield macromolecules of limited heterogeneity (Dose and Rauchfuss, 1972). The necessary information is inherent in the particular mixtures of reactant amino acids (Fox, 1975-a,b); (information is defined elsewhere, and the interpretation that such polymers are informational is explained on the basis of the evidence and the definition; Fox, 1974). The specific nature of each of the polymers is manifest, for example, in the fact that various thermal polyamino acids have differing catalytic activities (Rohlfing and Fox, 1969; Dose, 1971); some of these polymers have been shown to be true catalysts (Rohlfing and Fox, 1969). Proteinoids, however, are not proteins; the conceptual relationship of the two is essentially that of proteinoids (enzymoids) → protocells → proteins (enzymes; Fox, 1974).

Cell-like microstructures (proteinoid microspheres) are formed in vast numbers by contact of proteinoid with water, especially by cooling of hot aqueous solutions. The physical and morphodynamic properties have been catalogued (Fox and Dose, 1972). In view of the laboratory conditions used for abiogenic (Florkin, 1975) production of microspheres, the appearance of these bodies provides a geologically plausible simulation (cf. Knight, 1974; Florkin, 1975; Lehninger, 1975) of the origin of protocells.

Some of the qualities that have been identified in proteinoid microspheres suggest properties necessary to organic evolving systems. For example, proliferation, through "budding", was reported in 1967 (Fox et al.). Bud-like appendages that appeared on the microspheres separated therefrom, and were stained and allowed subsequently to incubate in fresh supersaturated solution of proteinoid. The stained "buds" behaved as physical nuclei around which polymer accreted to yield full-

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sized acidic proteinoid microspheres (within a range of diameter of 3—7 μm). Acidic proteinoid microspheres were shown to form junctions with one another frequently and spontaneously (Hsu et al., 1971). Ultrasmall packets of proteinoid, endoparticles, separate within the walls of microspheres either spontaneously or by appropriate manipulation in the laboratory. The endoparticles were shown to pass between microspheres through junctions of the latter. The junctions are accordingly communicative channels, and provide a mechanism for physical variation (Hsu et al., 1971; Hsu, 1972).

Data in this paper extend earlier data (Hsu, 1972), which show that endoparticles meet within the communicative intermicrospheric system and sometimes form couplets. Single or coupled endoparticles released from acidic microspheres have been found to function as single or multiple accretion nuclei, which accumulate fresh acidic proteinoid to form a second "generation" of microspheres.

These mechanisms for simulating intragenerational and intergenerational communication have been studied with a single kind of microsphere made from the acidic 2 : 2 : 1-proteinoid (2 : 2 : 1 denotes the proportions by weight of amino acids in a reaction mixture of glutamic acid : aspartic acid; equimolar mixture of 18 amino acids). Accordingly, the question of whether the interactions can occur between different kinds of proteinoid polymer and microspheres arises. Another question is whether functions associated with different kinds of proteinoid polymer can be combined within single microspheres in this manner.

This paper describes some ways in which various proteinoids and microspheres interact. One example of increased functional capability as a result of interactions between two different proteinoids in one particulate form is reported.

1. Materials and methods

1.1. Preparation of proteinoids

Proteinoids used in this work were mainly of the 2 : 2 : 1 type and variations thereon. The 2 : 2 : 1 proteinoid is an acidic proteinoid. Variations of this proteinoid are appropriately qualified (Table 1). Details of the polycondensation are given elsewhere (Fox and Dose, 1972). Table 2 contains the analyses of hydrolyzates of each of these proteinoids.

Not all substrates acted upon by proteinoids have been shown to be altered by

TABLE 1

Reaction mixtures for preparation of proteinoids.

Proteinoid	Parts by weight of 2 : 2 : 1 mixture	Parts by weight of other components
2 : 2 : 1 (acidic)	100	0
Glycine-rich-2 : 2 : 1 (Gly-2 : 2 : 1)	100	25 glycine
Proline-rich-2 : 2 : 1 (Pro-2 : 2 : 1)	100	25 proline
Phenylalanine-rich-2 : 2 : 1 (Phe-2 : 2 : 1)	100	25 phenylalanine
Histidine-rich *	80	20 histidine HCl
Hemoproteinoid **	99.5	0.5 hemin
Neutral proteinoid No. RRI-20	0	equimolar mixture of 18 amino acids ***

* Histidine-rich proteinoid contained 6.5% histidine.

** Hemoproteinoid was prepared as proteinoid 73a, described by Dose and Zaki, in which heme content was 20% (Dose and Zaki, 1971).

*** Amino acids are: Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Lys, Leu, Met, Phe, Pro, Ser, Thr, Try, Tyr, Val, each in the L form (except Gly).

TABLE 2

Amino acid compositions of proteinoids (mole %; calculated without NH_3).

Amino acid	Proteinoids						
	2 : 2 : 1	Gly-2 : 2 : 1	Pro-2 : 2 : 1	Phe-2 : 2 : 1	His-rich	Hemo-proteinoid	Neutral
Lys	1.1	0.5	1.5	0.8	1.5	<0.1	5.9
His	0.2	0.2	0.5	0.3	6.5	0.5	2.5
Arg	0.5	0.4	0.3	0.1	0.6	—	2.3
Asp	76.	46.	74.	59.	61.	65.	4.4
Glu	13.	6.4	10.	14.1	15.2	32.	6.2
Pro	0.3	0.5	7.0	0.3	—	—	0.5
Gly	0.9	35.	0.8	0.6	1.5	<0.1	7.4
Ala	2.0	1.2	1.1	1.1	3.3	0.1	8.8
$\frac{1}{2}$ Cys	0.4	6.2	0.1	0.1	1.0	—	12.1
Val	0.6	0.4	0.5	0.5	1.5	—	6.6
Met	0.8	0.6	0.7	0.6	1.5	—	6.1
Ile	0.4	0.2	0.4	0.3	0.7	—	2.8
Leu	0.8	0.4	0.8	0.8	1.3	—	8.4
Tyr	0.9	0.6	0.9	—	1.8	1.0	9.1
Phe	1.0	0.7	1.0	21.	1.7	1.0	11.9
Others	0.4	0.6	0.6	0.3	0.6		4.6

truly catalytic action (Rohlfing and Fox, 1969). The activities studied in the experiments reported here, as stated, have specifically been demonstrated to be catalytic by Oshima (1968) for phosphataselike, and Dose and Zaki (1971) for peroxidaselike proteinoids. These are the activities specifically studied in the experiments reported here.

1.2. Preparation of proteinoid microspheres

Microspheres composed of a single proteinoid were prepared by brief boiling of a mixture of 25 mg proteinoid per ml of water. The clear hot proteinoid solution was decanted and allowed to cool slowly to room temperature.

Microspheres composed of two proteinoids were prepared by incubating small-sized proteinoid particles (about 1 μm) of one kind of proteinoid in particle-free, warm solution of another kind of proteinoid, and allowing to cool slowly. The accretion medium was prepared by boiling 25 mg of an appropriate proteinoid per ml water. The hot, clear proteinoid solution was decanted and allowed to cool to 45°C. It was filtered through a millipore filter while maintained at 45°C. Seed proteinoid microparticles were gently pipetted into the accretion medium without shaking, and the mixture was allowed to cool slowly to room temperature.

Three kinds of seed proteinoid microparticles were used in preparing two-proteinoid microspheres. In cyclic proliferation experiments, endoparticles extruded from one generation of microspheres were used as seeds to form subsequent generations of microspheres. In mutual accretion tests, seeds were prepared by the same method as in preparing single proteinoid microspheres. The cooling process, however, was abbreviated to yield smaller microparticles.

The seeds used in accretion tests and in cyclic proliferation experiments were stained by either crystal violet or safranine solutions. Seeds used in preparation of two-proteinoid microspheres employed in studying alteration of catalytic rates were not stained. Outer layers of hemoproteinoid microspheres were dissolved away in distilled water to yield smaller particles of about 1 μm diameter which were

then seeded into histidine-rich proteinoid medium.

Heating conditions used in preparation of microspheres from histidine-rich proteinoid and hemoproteinoid were adjusted to allow for maximal rate-enhancing activity. Active boiling tends to enhance the peroxidaselike activity of hemoproteinoid in solution (Dose and Zaki, 1971). Hemoproteinoid used in experiments in this report was brough to a boil ten times by quickly passing tubes containing aqueous solutions of hemoproteinoid through the flame. On the other hand, heating in aqueous solution is known to reduce the phosphataselike activity of histidine-rich proteinoid (Oshima, 1968) as it does for esteraselike catalytic activity (Rohlfing and Fox, 1967). The solutions yielding histidine-rich proteinoid microspheres reported in this paper were, accordingly, heated at 70°C only, for 30 min.

1.3. Assays of activity

Phosphataselike activity (Oshima, 1968) of washed proteinoid microspheres was assayed at room temperature in pH 4.0 acetate buffer (0.015 M) containing 10 mg NPP (*p*-nitrophenyl phosphate disodium salt) per ml of buffer. A few drops of toluene were added to inhibit microbial growth. Stability of washed microspheres in the test medium was ascertained beforehand by determinations of dry weight; no significant loss of weight from the solid phase was observed after 24 hr incubation at room temperature. Microspheres were used in equivalents of 1 mg dry weight per 2.5 ml of buffer. Before each spectrophotometric reading, the reaction mixture was centrifuged to settle the microspheres. Particle-free portions of the reaction mixture were removed for assay and then returned to the original mixture to continue incubation. Spectro-

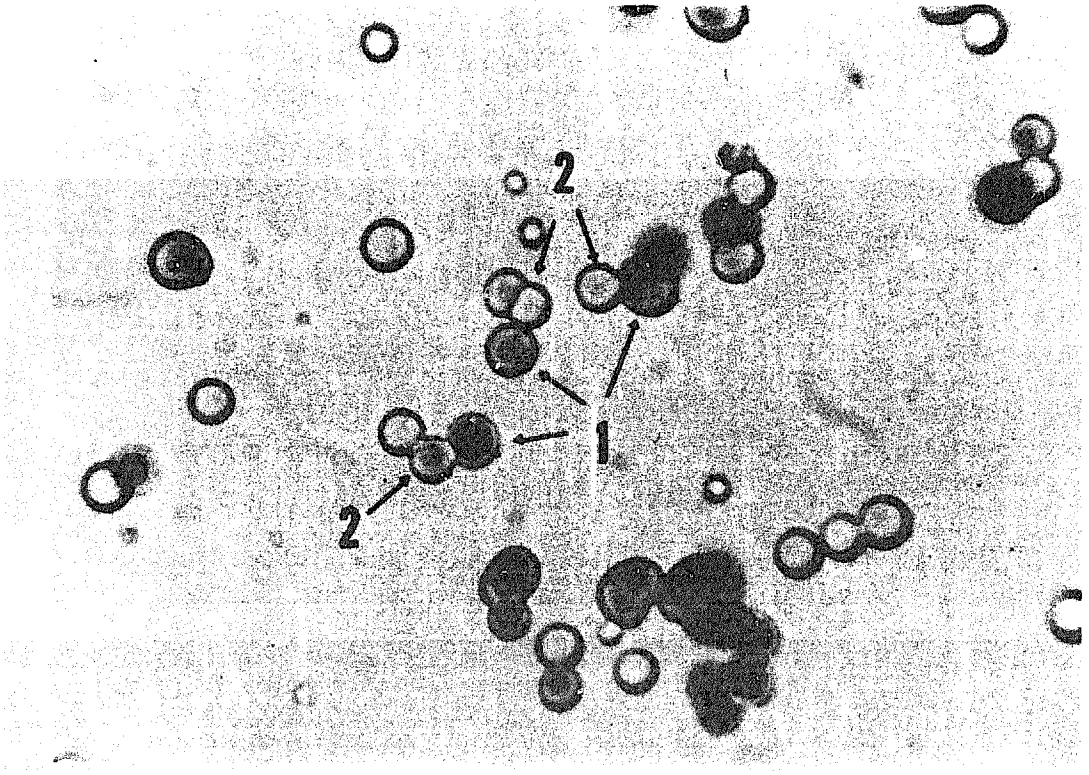


Fig. 1. Communicative junctions are formed between different varieties of acidic proteinoid microsphere. (1) The blue (dark grey) units are proline-rich proteinoid microspheres and the plain acidic proteinoid units (2) are stained pink (lighter grey). These microspheres are about 4 μm in diameter.

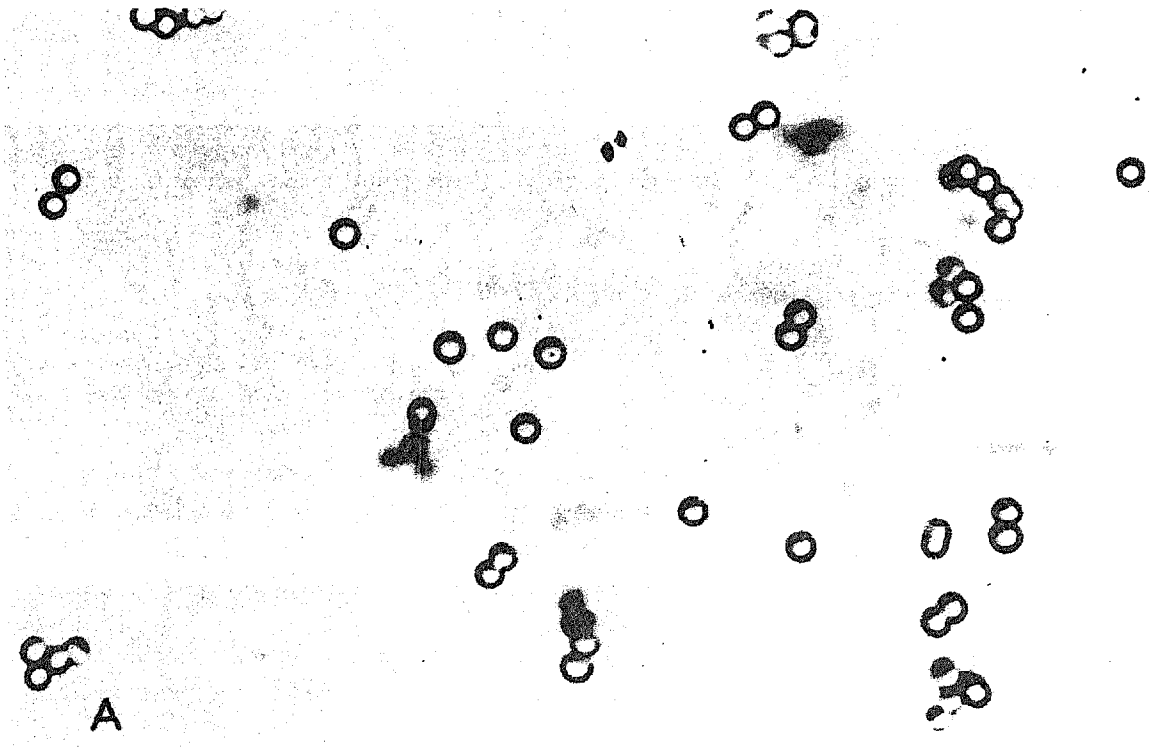


Fig. 2A. A two-hour-old sample of acidic proteinoid microspheres showing single units and couplets. These microspheres are about 4 μm in diameter.

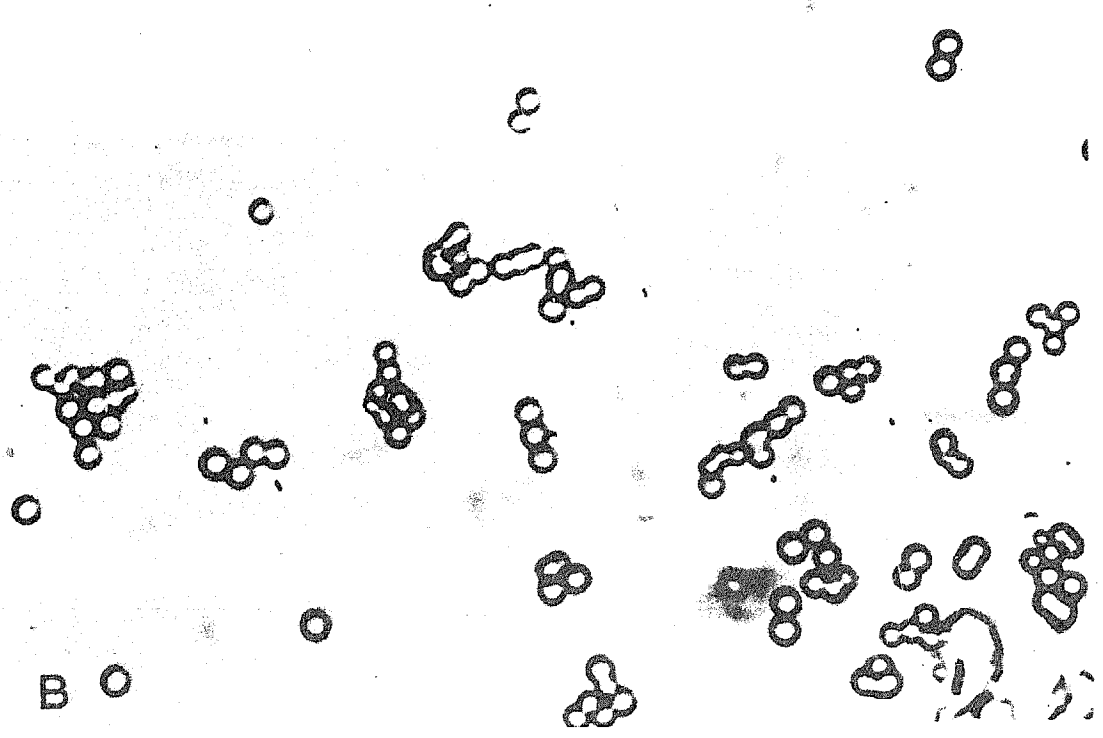


Fig. 2B. The same field as 2A after 10 sec of gentle tapping on the cover slip. The single and doublet microspheres collided with one another to form clusters.

photometric readings were taken at 400 nm at 1 hr intervals on clear liquids.

Peroxidaselike activity (Dose and Zaki, 1971) was assayed at room temperature in pH 4.0 acetate buffer (0.015 M) containing 0.22% guaiacol, which was first dissolved in a few drops of acetone to render it miscible. Washed microspheres were used in equivalents of 1 mg dry weight per 2.5 ml of buffer. Reaction was started by adding 0.02 ml of 3% fresh aqueous solution of hydrogen peroxide per 2.5 ml of test mixture. Spectrophotometric readings of the particle-free portion of reaction mixtures were taken at 436 nm at 20-minute intervals.

2. Experiments and results

2.1. Formation of communicative junctions between microspheres made of different proteinoids

In an experiment designed to determine whether communicative junctions can be established between microspheres made of two different kinds of acidic proteinoid, proline-rich 2 : 2 : 1-proteinoid was boiled in distilled water tinted with methylene blue. In a second test tube, 2 : 2 : 1-proteinoid was boiled in safranine-tinted (pink) water. The samples were allowed to cool slowly to room temperature in a hot water bath. Each of the two varieties of acidic proteinoid formed dense populations of microspheres stained pink or blue. These two kinds of microsphere (aged up to 2 hr after reaching room temperature) were mixed and gently shaken. Microscopic examination showed that junctions were formed between like and unlike microspheres (Fig. 1).

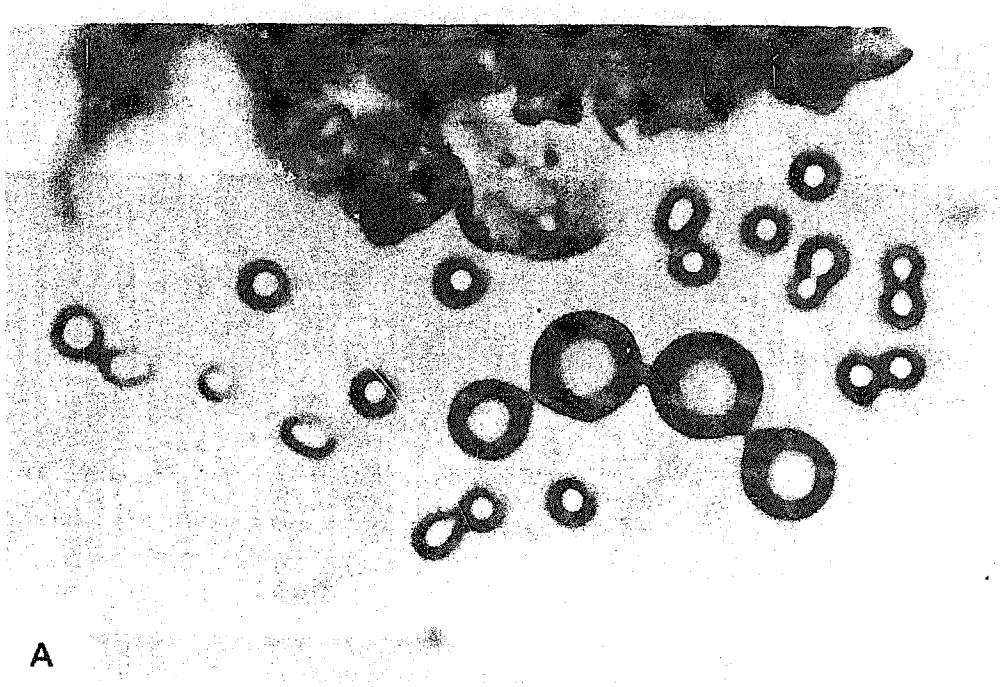
2.2. Effect of aging on junction formation between proteinoid microspheres

Coupled structures have been found to form only between and among newly precipitated ("juvenile") microspheres. The walls of acidic proteinoid microspheres up to a few hours old are relatively plastic; when these units come into contact with one another, junctions are formed (Fig. 2A,B). The walls and junctional structures of microspheres become increasingly stable with time; i.e., "aged" microspheres are no longer able to form new junctions. The effect of aging can, however, be reversed. Stabilized microspheres reassume juvenile qualities by dilution with distilled water or to slight increases in pH or temperature. Each of these conditions involves limited dissolution of the outer layers of the matured (stable) microspheres. Juvenile versatility is also regained by deposition of new proteinoid from the medium. Rejuvenated microspheres can make new junctions (Fig. 2A,B) or break old ones as in Fig. 3A,B. The rejuvenated state is not a permanent property. Rejuvenated units appear to age in the same way as newly precipitated microspheres.

2.3. Simulated cyclic protoreproduction by accretion around endoparticles

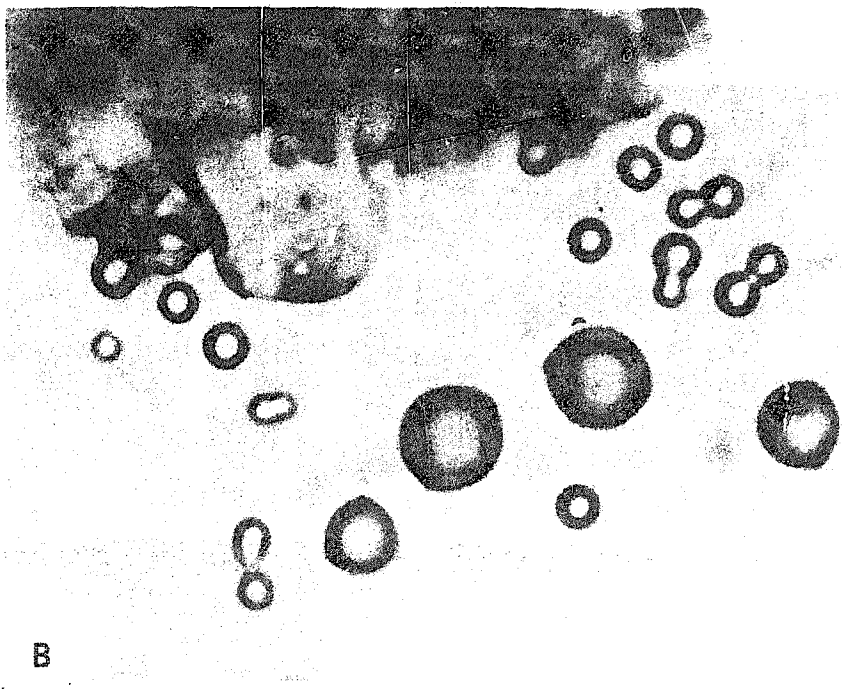
The spontaneous formation of endoparticles in acidic proteinoid microspheres may be induced; the process can be hastened by laboratory manipulation. The walls of acidic proteinoid microspheres were first stained and fixed with Gram's crystal violet (one part stain; 9 parts water) and Gram's iodine solution. These fixed microspheres were then partially dissolved by water (Fig. 4). Endoparticles were formed inside the fixed microspheric walls and released into the medium through holes in the walls (Hsu et al., 1971). Endoparticles were isolated, stained (1/10 dilution Gram's crystal violet) and seeded into unstained, particle-free warm solutions of proteinoid and allowed to cool slowly to room temperature. The results show normal-sized microspheres (3—7 μm diameter) that contain stained accretion centers (Fig. 5). Upon accretion of new proteinoid from the medium, the stain of the endoparticles gradually infiltrated the newly accreted proteinoid layers.

This study shows that endoparticles ex-



A

Fig. 3A. Two week old sample of acidic proteinoid microspheres is shown after 3 min in medium diluted with distilled water. The group of 4 microspheres are 3—5 μm in diameter.



B

Fig. 3B. The same specimen as 3A at 5 min after dilution of medium showing the microspheres have separated.

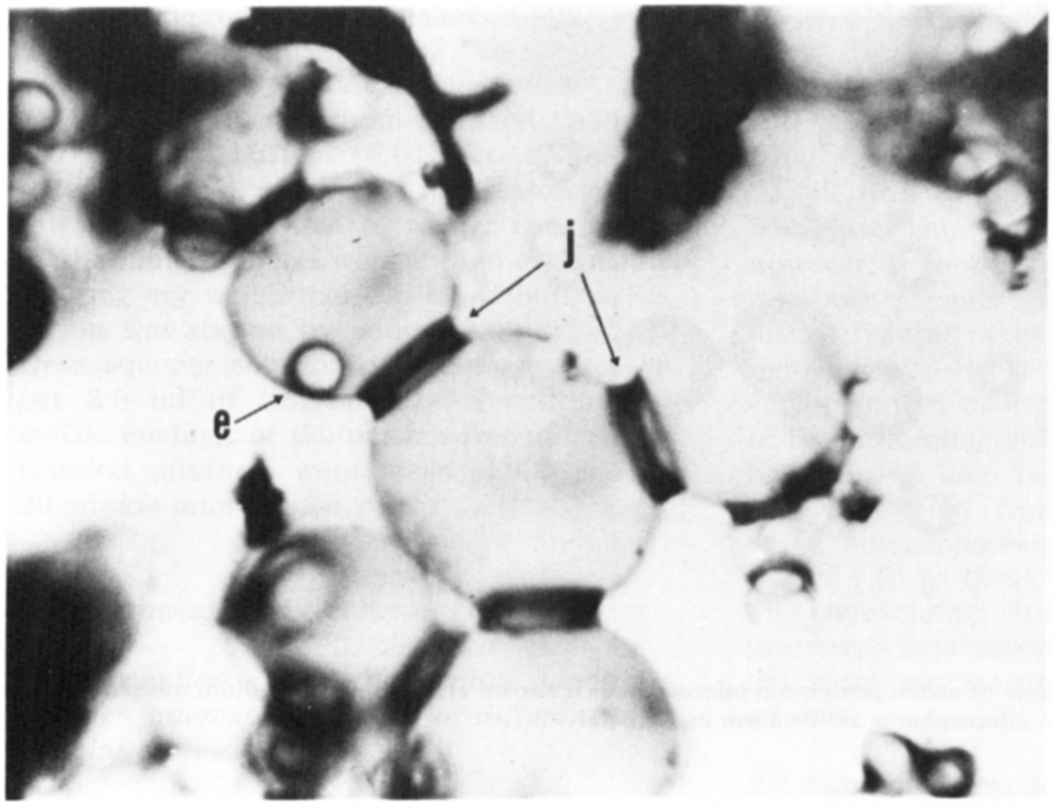


Fig. 4. Connecting structures between acidic proteinoid microspheres are shown (j). The cluster was aged three months, then stained and fixed with 1/10 aqueous solution of Gram's crystal ciolet and Gram's iodine solution. An endoparticle (e) remained inside the microsphere joined at 10 o'clock. Microspheres are 4—6 μm in diameter.

TABLE 3

Accretive compatibility between various proteinoids.

Accretion seeds	Accretion medium			
	2 : 2 : 1	Gly-2 : 2 : 1	Pro-2 : 2 : 1	Phe-2 : 2 : 1
2 : 2 : 1	+	+	+	—
Gly-2 : 2 : 1	+	+	+	—
Pro-2 : 2 : 1	+	+	+	—
Phe-2 : 2 : 1	+	—	+	±
Neutral proteinoid	—			
Ground glass (green)	0			

+ = positive mutual accretion (microspheres formed around stained nuclei).
0 = no evidence for mutual accretion (microspheres present but without colored nuclei).
— = no microspheres of any kind present.
± = Hot solutions of Phe-2 : 2 : 1, filtered and slowly cooled, formed small microparticles (about 1 μm diameter). Fresh Phe-2 : 2 : 1 accreted poorly around Phe-2 : 2 : 1 seeds. Fresh Phe-2 : 2 : 1 did not accrete around seeds of other kinds of acidic proteinoid tested.

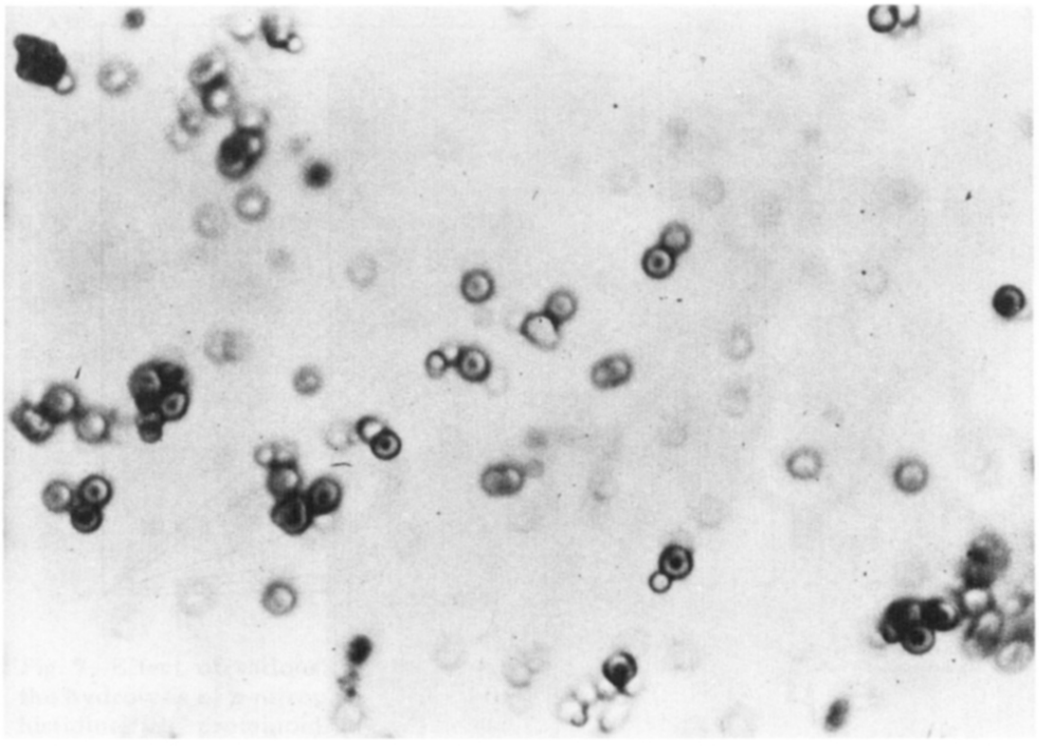


Fig. 5. Proteinoid in solution accumulated around stained endoparticles to form normal-sized microspheres. Microspheres with 0—3 endoparticles as accretion nuclei may be seen. The average diameter of these microspheres is 4 μm.

truded from the inside of microspheres behave as nuclei for the accretive growth of a subsequent generation of microspheres. This kind of "protoreproduction" was carried to the third generation. The behavior observed indicated that subsequent cycles can be continued indefinitely.

2.4. Formation of microspheres around multiple endoparticles from microspheres made of different proteinoids

Proline-rich 2 : 2 : 1-proteinoid endoparticles were stained light purple with crystal violet. A preparation of 2 : 2 : 1-proteinoid endoparticles was stained pink with safranine. These two lots of stained endoparticles were mixed and then seeded into a freshly boiled unstained solution of 2 : 2 : 1-proteinoid, which was then cooled to 45°C and filtered at that temperature. The seeded, warm proteinoid suspension was allowed to cool slowly to room temperature. Proteinoid solution accreted around the stained endoparticles. Microspheres containing one or both kinds of endoparticle were observed (Fig. 6).

2.5. Compatibility between different proteinoids to form microspheres by accretion

Growth by accretion of like proteinoid ("self-accretion") has been reported in earlier publications (Fox et al., 1967; Hsu, 1972). In order to test for possible selective accretion between unlike proteinoids in the formation of microspheres, cross-accretion experiments were performed. The results are recorded in Table 3. Each test used stained seeds of one kind of proteinoid and incubated them in unstained media of another kind of proteinoid. The proteinoids used are listed in Table 1.

2.6. Combination of enzymelike properties in two-proteinoid microspheres

This set of experiments was done to determine whether two rate-enhancing functions

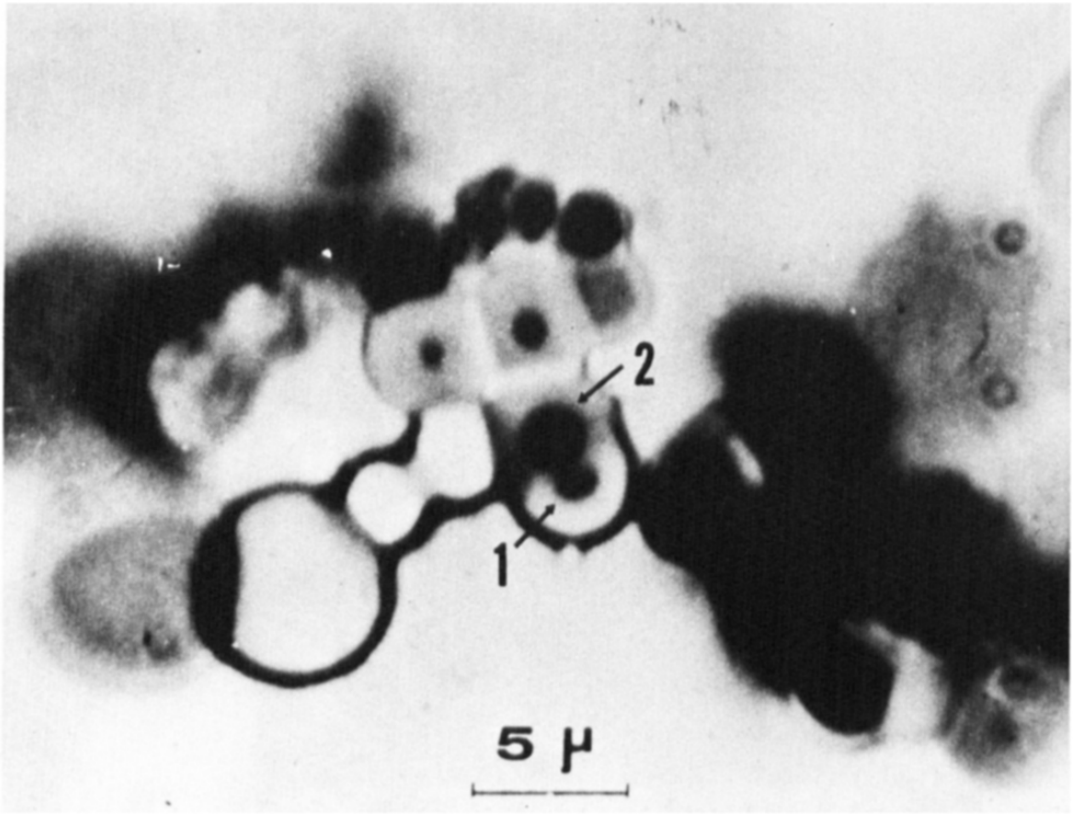


Fig. 6. Recomposed microspheres containing two kinds of endoparticle: (1) red (lighter grey) indicates plain acidic proteinoid endoparticle; (2) blue (darker grey) indicates proline-rich proteinoid endoparticles. Unstained acidic proteinoid around either one or both kinds of stained endoparticle. Microspheres range 3—7 μm in diameter.

intrinsic to each of two different proteinoids could be combined by accretion to form a new variety of microsphere having both functions. Two kinds of acidic proteinoid (see Table 1) were used in making microspheres; 2 : 2 : 1-proteinoid containing hemin (hemoproteinoid) and 2 : 2 : 1-proteinoid containing elevated levels of histidine (histidine-rich proteinoid). Each of the proteinoids formed microspheres within the range of 2—7 μm in diameter. Two-proteinoid microspheres were prepared by seeding hemoproteinoid endoparticles into histidine-rich 2 : 2 : 1-proteinoid media. Combined proteinoid microspheres resembled the single-proteinoid microspheres in size and appearance.

Each of the two kinds of single-proteinoid microsphere and the two-proteinoid microsphere (used in equivalents of 1 mg dry weight per 2.5 ml of test medium) was tested for phosphataselike and peroxidaselike activities. The results are shown in Figs. 7 and 8. The relative phosphataselike activity per unit weight of these three kinds of proteinoid microsphere was as follows:

histidine-rich proteinoid microsphere > two-proteinoid microspheres > hemoproteinoid microspheres.

The results for peroxidaselike activities showed an order of:

two-proteinoid microspheres > hemoproteinoid microspheres > histidine-rich proteinoid microspheres.

3. Discussion

The formation of communicative junctions between different proteinoid microspheres provides one type of physical basis for channel-

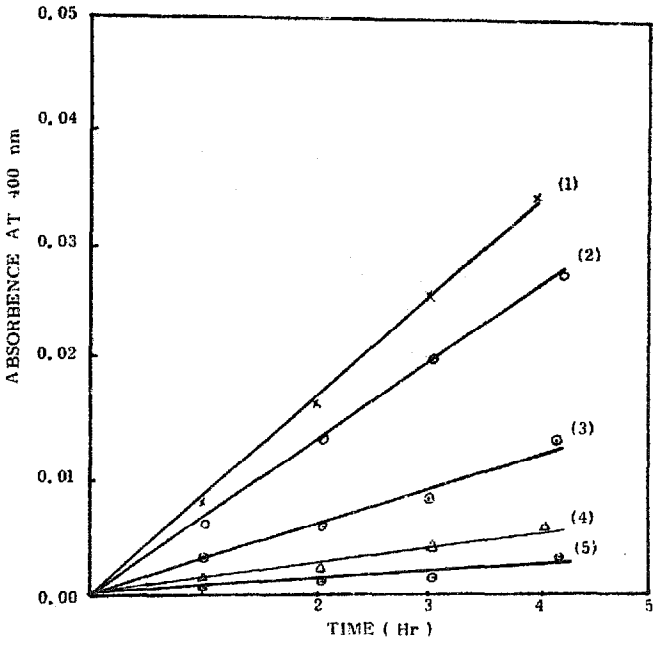


Fig. 7. Effect of various proteinoid microspheres on the hydrolysis of *p*-nitrophenyl phosphate (NPP); (1): histidine-rich proteinoid microspheres; (2): hybrid microspheres composed of hemoproteinoid and histidine-rich proteinoid; (3): hemoproteinoid microspheres; (4): free histidine equivalent to the content of histidine in (1); (5): no proteinoid present: spontaneous rate. Readings were taken at 400 nm at 1-hour intervals.

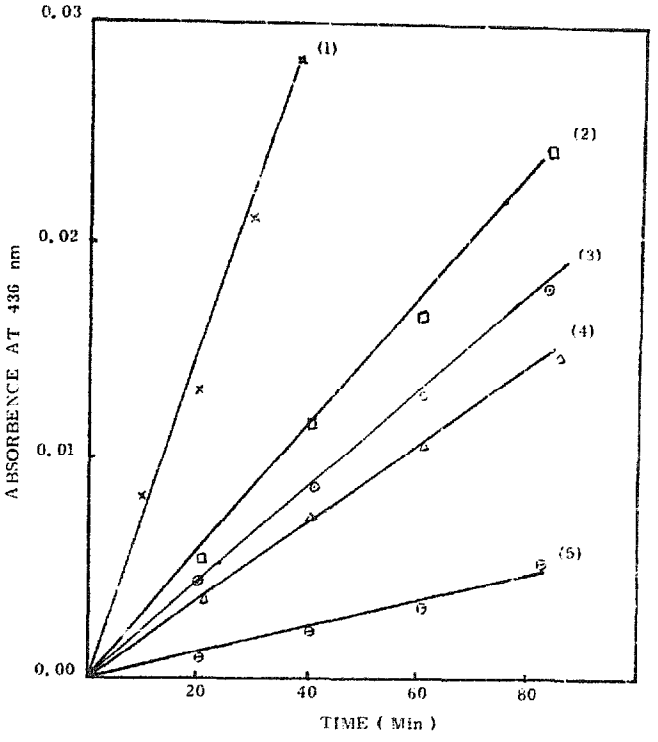


Fig. 8. Effect of various proteinoid microspheres on peroxidation of guaiacol in the presence of H_2O_2. (1): hybrid microspheres composed of hemoproteinoid and histidine-rich proteinoid; (2): hemoproteinoid microspheres; (3): free hemin equivalent to the hemin content of (2); (4): histidine-rich proteinoid microspheres; (5): no proteinoid present: spontaneous rate. Readings were taken at 436 nm and recorded at 10 min intervals.

led and, therefore, nonrandom variation. The potential for variation through communicative systems (Hsu, 1972) is further enhanced by the fact that junctions can be established not only once but for an unlimited number of times between microspheres through the rejuvenation and maturation processes.

A kind of selectivity is evident in the mutual accretion pattern between various proteinoids. This indicates that certain physicochemical bases for compatibility may be operating in the accretion process. In the case of phenylalanine-rich acidic proteinoid, a high proportion of phenylalanine in the proteinoid significantly altered the accretive ability of 2 : 2 : 1-proteinoid. In this case, bias in accretion is related to a single amino acid in the polymer. Phenylalanine-rich acidic proteinoid is a poorly accreting proteinoid in solution but particles served as accretion nuclei for 3 out of 4 varieties of acidic proteinoid tested (Table 3). This proteinoid is, thus, better suited to be seeds than it is to be capsular material under the tested conditions. With the exception of the phenylalanine-rich variety of 2 : 2 : 1-proteinoid, the other three varieties showed mutually compatible accretion.

Neutral proteinoid seeds in 2 : 2 : 1-proteinoid accretion medium showed the absence of microspheres. 2 : 2 : 1-Proteinoid in the presence of ground glass, however, did form microspheres typical of this proteinoid. The neutral proteinoid seeds appear to have interfered with the microsphere-forming ability normally associated with 2 : 2 : 1-proteinoid alone.

Microspheres composed of only one pro-

teinoid showed one kind of catalytic activity, whereas the two-proteinoid microspheres exhibited two kinds of catalytic activity. Physical combination of different proteinoid polymers in hybrid microspheres is thus shown to have functional significance. In the case of phosphataselike activity, the relative potency was predictable on the basis of the relative proportions of histidine-rich proteinoid present per unit weight of the different kinds of microsphere. What was not predicted was the distinctly higher peroxidaselike activity of the hybrid proteinoid microspheres compared to an equal amount of hemoproteinoid microspheres. Evidently, optimal peroxidaselike action is related to synergistic interactions between the two component proteinoids in the hybrid microspheres.

Proteinoid microspheres exhibit properties comparable to the most basic qualities of contemporary organic evolving systems, i.e., the ability to reproduce *, the ability to undergo controlled variation, and the susceptibility to natural selection (Merrell, 1962; Solbrig, 1966; Stebbins, 1966). As first stated by Oparin (Young, 1974), a true understanding of evolution requires a grasp of relevant processes from their cosmic beginnings (cf. also Fox, 1975b). Early stages in evolution are simulated by the microspheres.

When internal fragments (endoparticles) from one generation of microspheres participate in the formation of subsequent generations of new microspheres, a simple kind of intergenerational communication, or "protoinheritance", may be inferred. Cycles of protoinheritance and accretive growth, i.e. "protoreproduction", have the essential physical qualities of reproduction.

The data in this paper further demonstrate that functions associated with different proteinoid polymers are combined in new units of hybrid microspheres. Functional variations in proteinoid microspheric populations can, in turn, be susceptible to natural selection, protoselection (Kenyon, 1974).

Proteinoid microspheric systems are thus shown to be internally integrated and self-sufficient evolvable systems (protoevolution). For example, it is now possible to consider how extensions in protometabolic pathways may have evolved in structured and protoreproducing microspheres. Increasingly more complex enzyme-like systems could have developed through innumerable cycles of protoreproduction and protoselection. Other steps in the evolution of proteinoid microspheres could include the development of energy-trapping mechanisms and the acquisition of a nucleic acid type of genetic apparatus (Fox, 1974).

* Logically, precontemporary systems must have had some of the properties of the contemporary cell, but not all of them, nor in as full measure. Accordingly, some of the same terms, e.g., reproduction, are used in this paper for models of the primordial and for the contemporary cell; in some cases the term "proto" is employed as a prefix.

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