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ELECTRICAL MEMBRANE PHENOMENA IN SPHERULES FROM PROTEINOID AND LECITHIN***

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Spontaneous and induced electrical phenomena resembling membrane and action potentials in natural excitable cells have been observed in artificial cells. These artificial cells were made from thermal proteinoid and lecithin in a solution of potassium acid phosphate with glycerol.

Introduction

One of the most studied attributes of the living state in modern organisms is that of excitability. Because of an extensive literature on excitability in cells, and because of accumulated knowledge of properties of proteinoid microparticles (Fox and Dose, 1977; Fox, 1980), proteinoid-containing models for excitable cells have been sought.

In somewhat related studies, excitability has been observed in internodal cells of plant tissue (Hill and Osterhout, 1934; Cole and Curtis, 1938; Findlay, 1959) and in reassembled vesicles. Droplets of protoplasm from an internodal cell of *Nitella* form semipermeable membranes on the surface under certain conditions (Yoneda and Kamiya, 1969) and display excitabilities when properly activated (Inoue et al., 1971; Takenaka et al., 1971). Other related studies are numerous (e.g. Takahashi and Miyazaki, 1971; Kennedy et al., 1977). Studies of synthetic bilayer membranes indicate that polypeptides can form ionic channels in excitable bilayers.

The early examples and the results of chemical analysis of excitable cells (Condrea and Rosenberg, 1968; Camejo et al., 1969; Ishima and Waku, 1978) suggested that excitability might be produced in an artificial cell composed of suitable polyamino acid and phospholipid.

The fact that proteinoid microparticles possess permselective membranes (Fox et al., 1969) and are capable of junction formation and communication (Hsu et al., 1971) also raised the possibility of electrical phenomena in these units. This paper describes the results of experiments in making electrically active artificial cells from proteinoids and lecithin. Various proteinoids were prepared to contain large proportions of individual amino acids in an effort to identify which amino acids most contribute to electrical activity.

Materials and methods

Proteinoid

The polyamino acids were prepared by

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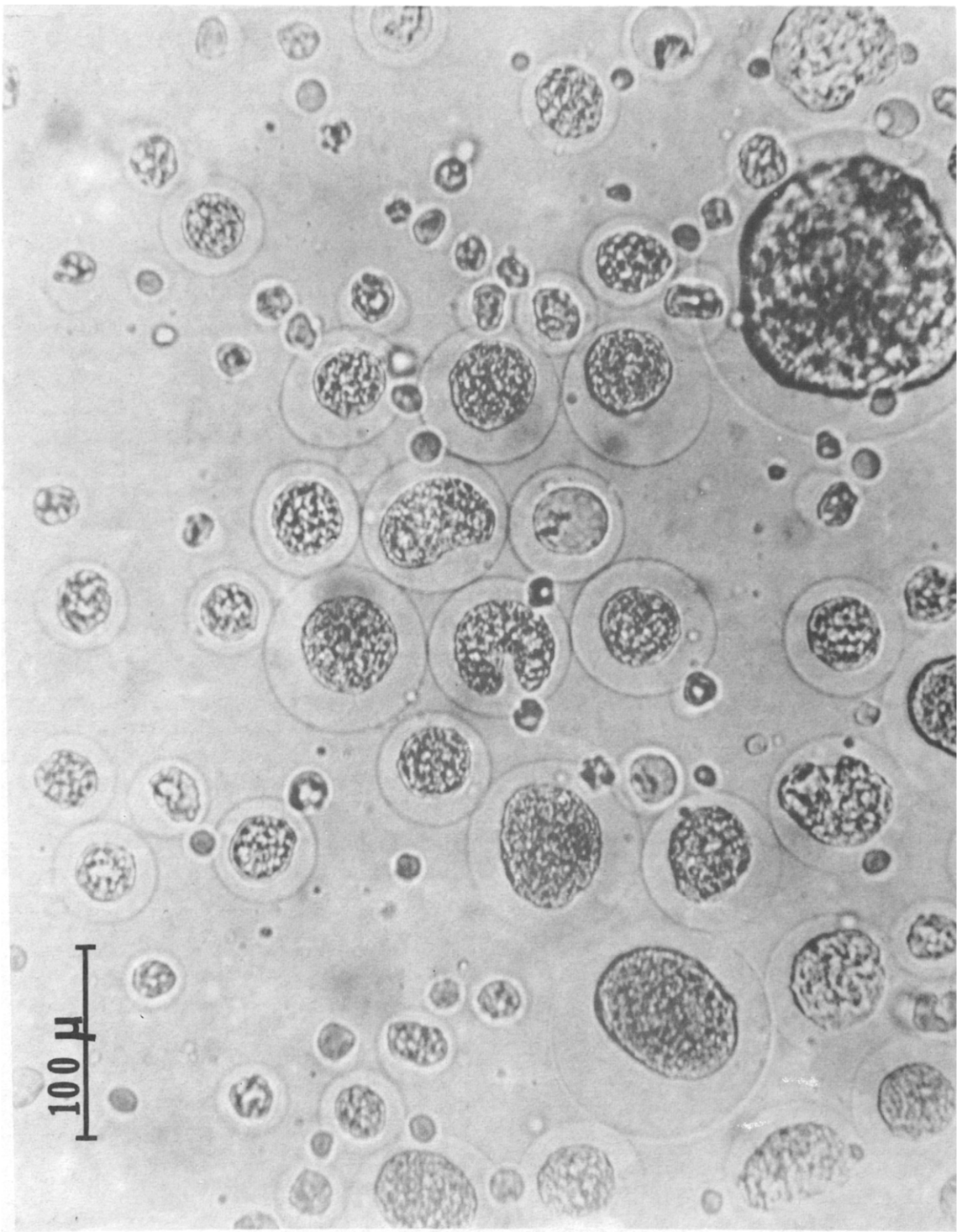

Fig. 1. Photomicrograph of the spherules with semipermeable membrane.

heating in an oil bath at 190°C for 6 h a mixture of 50 g of each individual amino acid treated with 50 g of an equimolar mixture of 18 amino acids under a nitrogen blanket (Vegotsky, 1972). The products were mechanically stirred with water for 2 h and then filtered. The dissolved fraction was dialyzed for 2 days in a cold room with two changes of water per day. The non-diffusates were lyophilized. Preliminary experiments showed that the dissolved fractions yielded the most "active" particles. The leucine-rich proteinoid that received most attention contained 14.9% leucine.

Phospholipid

Phospholipids were purified from Eastman practical grade vegetable lecithin by extraction with 50% aqueous glycerol solution containing potassium phosphate (pH 5.8).

Preparation of spherules

A suspension of 60 mg of proteinoid and 60 mg of lecithin in 3.0 ml of potassium phosphate solution with aqueous glycerol (50 : 50 v/v) was heated to boiling. This solution contained 80 mmol of phosphate (PO_4), and 50% of glycerol (pH 5.8). The solution was allowed to cool to room temperature, and then allowed to stand for a period of several hours up to 2 days. Standing was empirically observed to be helpful to the experiments. After incubation at room temperature, crusts precipitated from the supersaturated solution. They were carefully removed by use of a pipet. The original external solution was then replaced completely by the experimental external solution by a suction device. This was done by adding from the top and drawing from the bottom, while the level of the solution was kept constant. Approximately 30 min after the external solution was completely replaced, in the case of leucine-rich equimolar proteinoid, for example, a very thin, transparent, almost invisible spherical membrane emerged from the surface of the crusts (Fig. 1). This membrane formed from the crust in an external solution containing mannitol at concentrations between 150 mM and 300 mM. Mannitol was used to maintain the osmotic balance of the spherule. If the mannitol in the external solution was concentrated to more than 300 mM, it failed to produce semipermeable spherules. If the tonicity of the solution was too low the size of spherules increased rapidly until they burst. A concentration of 200 mM mannitol was found to prevent bursting of the spherules and yet maintain suitable sized spherules for several hours. The selected spherules used in this experiment were 60—150 μm in diameter. The compositions of the inside and outside solutions for the spherules are shown in Table 1.

Electrical phenomena

A simple, standard intracellular microelectrode technique was applied to monitor the electrical phenomena (Watanabe and Ishima, 1972). Figure 2 shows a schematic outline of the equipment. The tip diameter of the glass electrode was about 0.2 μm and the electrical resistance was approximately 20 MΩ, with 3 M KCl inside. The reference electrode was a silver-silver chloride-KCl agar placed in the external solution. These are employed for accurate measurements of the membrane

TABLE 1

Ionic concentrations within spherules.

Inside			Outside		
K^+	80	(mM)	Na^+	0.2	(mM)
H^+	130		K^+	0.05	
			Mg^{2+}	0.1	
			Ca^{2+}	0.5	(1.0)
PO_4^{3-}	70		Cl^-	0.25	
			SO_4^{2-}	0.1	
			NO_3^-	1.0	(2.0)
Glycerol	50%		Mannitol	200.0	(mM)
pH	5.8		pH	7.0	(Na-phosphate buffer)

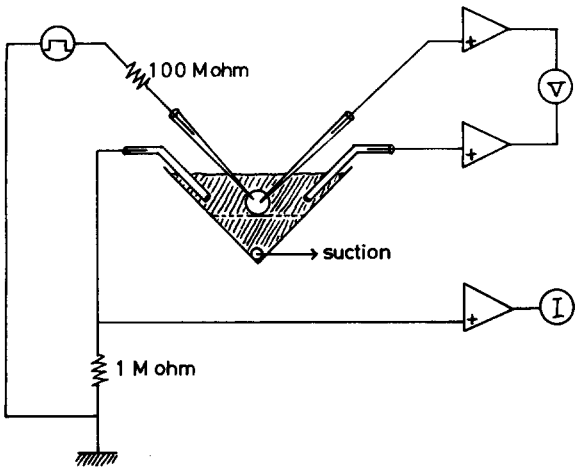

Fig. 2. Schematic diagram of the experimental setup (see text).

potential with elimination of any possible boundary potentials. For DC-differential recording of the potentials, a W-P Instrument Model 750 amplifier was used and, for current monitor, a W-P Instrument Model 701 was used. These high-input impedance amplifiers (20 000 MΩ work well for a range of electrical measurements. The output of these preamplifiers was led to a cathode ray oscilloscope (with Type 3A3 plug-in units, Tektronix) or a digital voltmeter (Elpo, Type V-529), simultaneously. In order to obtain a constant injection current for stimulations, a current clamp circuit was applied with a Tektronix Type O amplifier. Stimulator (W-P Instrument Series 800) was isolated from the ground with Model 850A Isolator (W-P Instrument). The injection current was led to a current injection glass microelectrode through a 100 MΩ resistor, which stabilized the electrode considerably. Any current through the spherule was monitored across a 1 MΩ resistor placed between the external solution and the ground. Thus, any possible current leak was eliminated, by precisely balancing the equipment throughout the experiment. A Grass camera was used to take photographs of oscilloscope traces.

Results and discussion

Of more than 100 different preparations of proteinoids, four types proved suitable for experiment. These were leucine-rich equimolar proteinoid (AMX-9, dfI), threonine-rich equimolar proteinoid (AMX-105, dfI), proline-rich equimolar proteinoid (AMX-105, dfI), and leucine-rich equimolar proteinoid (T-83, dfI). These water-soluble proteinoids formed semipermeable membranes with lecithin. Among these, the leucine-rich equimolar proteinoid alone produced pronounced electrical activities. While a proline-rich proteinoid (49.8% proline) and a threonine-rich one (19.4% threonine) also formed membranes with the same lecithin, they showed lesser electrical activities.

The individual amino acids included in proteinoids in large proportion represented quite fully the roster of amino acids common to proteins. Although vesicles containing each amino acid were not tested for electrical activity in an equalized comparison, three of the amino acids proved to offer encouragement for further investigation. These three: leucine, proline, and threonine are mainly amino acids with apolar sidechains. Such hydrophobicity may contribute directly or to aiding in the complexing with lecithin. The one amino acid of the three that does not contain a purely hydrocarbon sidechain is threonine. It will be of interest to determine if the threonine hydroxyl group coordinates ions in the manner that Kennedy et al. (1977) have suggested for serine hydroxyl.

The proteinoid crusts evidently act as ionophores because, without the proteinoid, the lipid membrane itself has an extremely high membrane resistance (Mueller and Rudin, 1969; Yoshida et al., 1972, cf. Grote and Fox, 1979). Incubation is necessary for at least several hours and sometimes for 2 days. More than 2 days of incubation improved the yields of the spherules suitable for the experiment. The period can be prolonged if there is no bacterial infection.

In various experiments, a glass microelectrode was inserted in a spherule after it aged or about 40 min after the external solution was replaced; the membrane potential was measured.

Various patterns of electrical activity along

with the steady state membrane potential were observed without any stimulating current. These fell essentially into three categories: (1) steady state membrane potential; (2) spontaneous activities (flip-flop phenomenon or potential burst); and (3) miniature potential activities at flopped phase.

The value of steady state membrane potential varied. For most spherules the value ranged between −20 and −70 mV. Figure 3 shows the cumulative data on the membrane potential of 958 spherules. The mean value was −44 mV with a standard deviation of ±16 mV. The potential was sometimes depolarized in various sizes, abruptly, and it continued to keep a relatively constant value for a while, then dropped to another hyperpolarizing level, again showing a flip-flop phenomenon (Tasaki, 1968). Figure 4 shows one of these examples. Sometimes it accompanied a series of very minor potential activities comparable to miniature potentials of the receptor membrane (Fig. 5) at the flopped phase of a considerably larger steady state potential. Some spherules generated a series of spontaneous potential bursts (Fig. 6).

These spontaneous activities, however, disappeared when the external calcium concentration was increased to more than 1 mM. A high calcium concentration stabilized the membranes and depressed the electrical activities of the spherules. In the external solution containing 1 mM calcium nitrate (with other conditions kept unaltered), the spherule was impaled by a second glass microelectrode in order to pass the stimulation

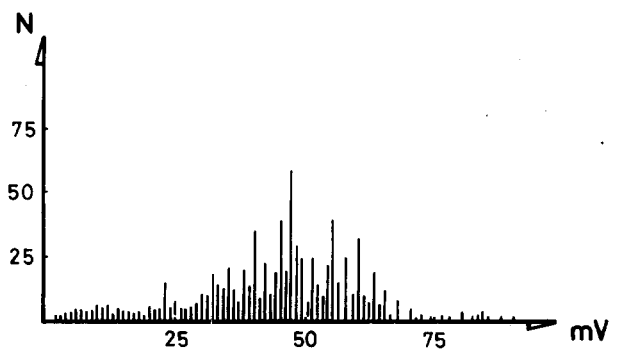

Fig. 3. The frequency distribution of the steady stage membrane potentials in 958 proteinoid-lecithin spherules.

current. The spherule was repeatedly stimulated by passing the current pulses in positive and negative directions, alternatively. Initially, the spherule produced no electrical activities showing electrotonic potential according to the intensities of the current. Approximately 15 min later, however, an abrupt potential change was observed in the course of the injection current, notwithstanding the passing current was constant during a square pulse, as is observed in the current monitor of the lower trace of the oscilloscope (Fig. 7). The magnitude of the potential jump was 13.6 mV and the resistance of the spherule in the steady state was 38 Ω/cm^2 as the diameter of this spherule was 110 μm with an 80 μm core of the proteinoid crust (Fig. 8). The range of the membrane resistance was between 8 and 150 Ω/cm^2 under this condition. Potassium ion concentration of the inside solution was estimated to be less than 80 mM according to the expansion of the diameter through the osmotic balance. When the external solution was exchanged for a solution containing 40 mM of potassium phosphate (other conditions kept unaltered), the membrane resistance of the same membrane was reduced from 37.1 Ω/cm^2 to 12.6 Ω/cm^2, and no electrical activity was observed. The value of the steady state membrane potential was −26 mV. This potential was reduced to almost zero by increasing the potassium phosphate concentration in the external solution.

Glycerol may function as a kind of detergent; it is also miscible with various kinds of lipid. After various tests, 50% of the aqueous glycerol solution with 70 mM potassium phosphate (pH 5.8) was empirically found to offer the best condition for maintenance and activity of the spherules.

Various kinds of physiological solutions for animals have been tested as external solution. In these solutions semipermeable membranes could be formed but they failed to produce any electrical activity in the spherules showing steady state membrane potential. Finally, an artificial pond water (Kishimoto and Tazawa, 1965) was found to produce electrical activities in the spherules.

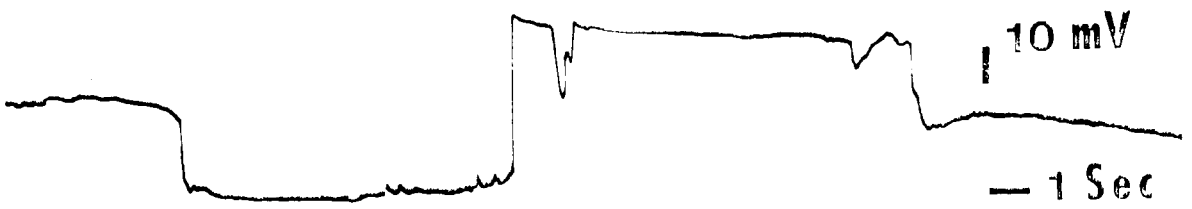

Fig. 4. Flip-flop phenomenon of the spherule. Miniature potential activities were observed on the flopped phase of the potential.

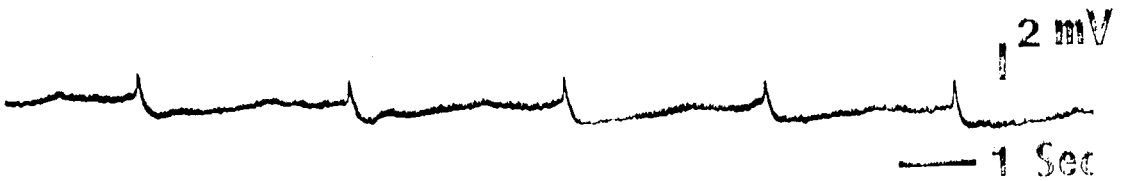

Fig. 5. Miniature potential activities. A high magnification measurement of the potential activities on the flopped phase of the potential.

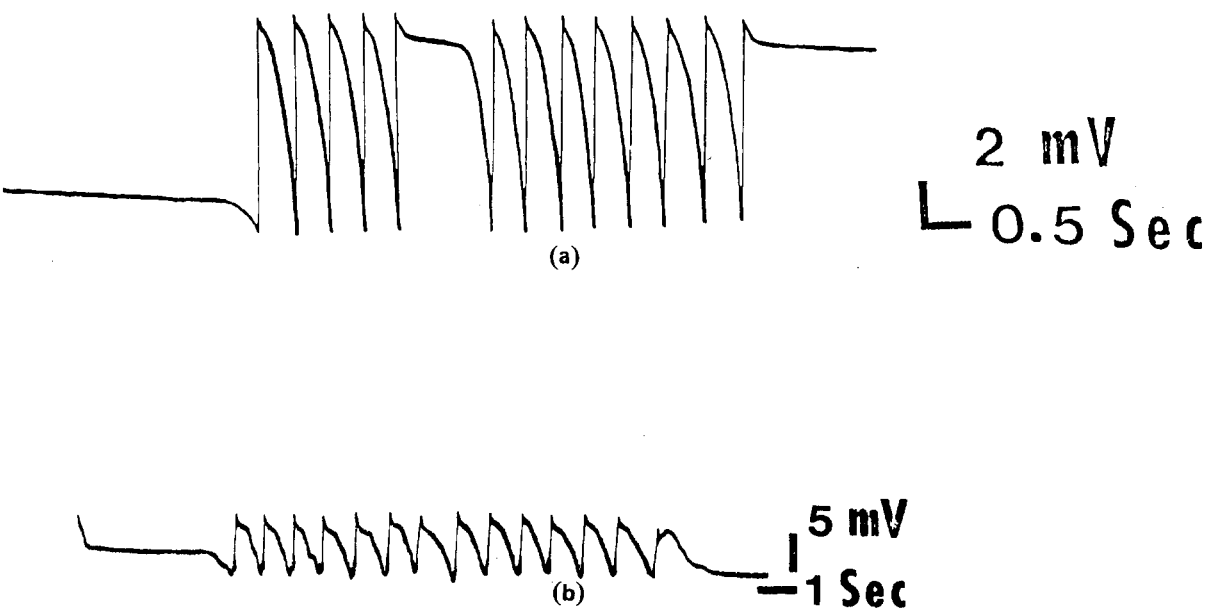

Fig. 6. Two different patterns of potential burst (a and b) observed on different spherules.

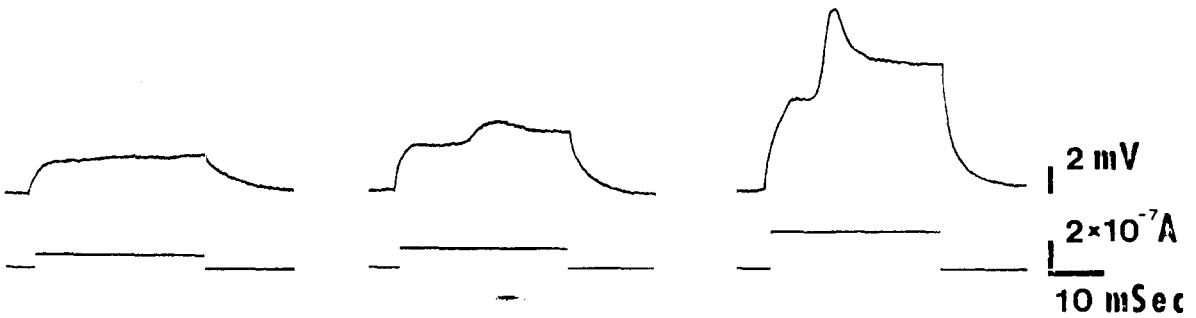

Fig. 7. Potential jumps induced by the current injection of the spherule.

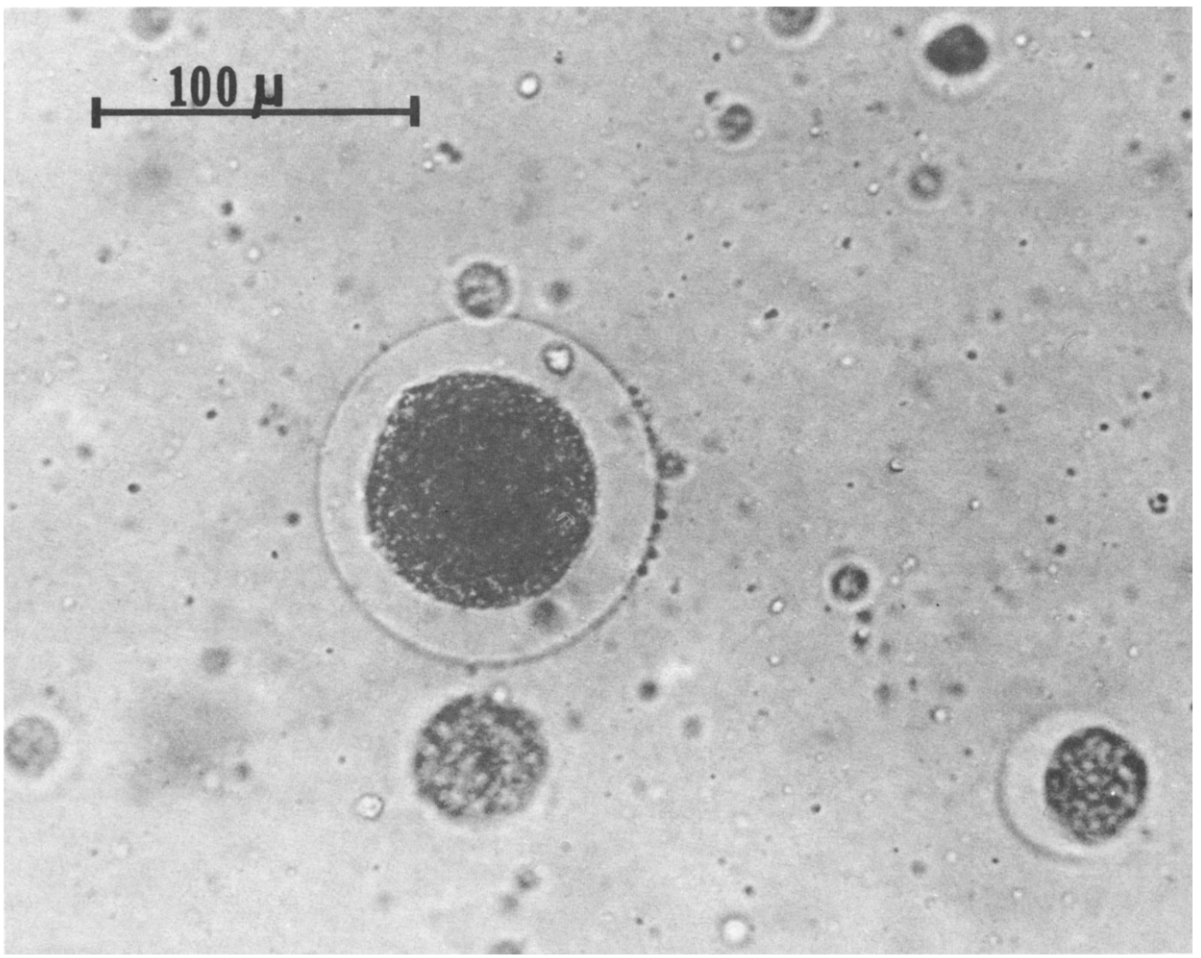

Fig. 8. A photomicrograph of typical spherule which produced the electrical activity by current injection. Diameter of the spherule was 110 μm with an 80-μm core of the proteinoid crust.

Artificial pond water is a physiological solution for *Nitella*, an excitable water plant. This is believed to be a minimum requirement for the excitation of the membrane.

Various patterns of spontaneous potential activities comparable to those of modern living excitable cells were observed notwithstanding any current leak through equipment, as proved with the current monitor. These might be related with expanding and rearranging of the membrane of the spherules in a very small area when there were considerable potential differences between the inside and outside of the membrane in the course of the development of the spherule. It was rather easy to elicit the activities by electrical stimulation in the stable state in the experimental solution containing 1 mM calcium ions if there were spontaneous activities in the standard external solution (Ca^{2+} 0.5 mM). Contribution of Ca^{2+} to compartmentation (Brooke and Fox, 1977) may explain effects on electrical activity in vesicles.

The finding of excitability in a cell-like microstructure permits investigation of coupling of biochemical and other phenomena in ways that cannot be accomplished with other models such as bilayer membranes. Although the proteinoid-lecithin vesicles are to a degree a departure from the proteinoid microspheres that have been placed conceptually on a primary evolutionary pathway (Fox and Dose, 1977), evolution itself occurred in a cell-like unit composed of both proteins and lipid, not in a simple lipid bilayer membrane (cf. Nachmansohn, 1970).

The difficulty of inserting microelectrodes in the usual size proteinoid microsphere (1—5 μm) has precluded testing the hypothesis that they are capable of electrical activity. In general, the most results have, however, been obtained in these experiments in microsystems rich in apolar amino acids (cf. Lehninger, 1975). No reason for the need of an efficient phospholipid barrier and bioelectric activity in a protocell is perceived. Indeed, Kuhn (1976) has pointed out that leakiness in a membrane would have provided, on the contrary a selective advantage to a protocell that thereby permitted unimpeded inward diffusion of metabolic intermediates from the exterior soup.

The results of the experiments with complexes of thermal proteinoid and lecithin indicate channels for passive transport of ions. The ability to generate potential pulses is not specifically a biological phenomenon since it is simulated in the non-biological systems that have been studied.

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