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ALTERATIONS IN NUCLEAR MEMBRANE STRUCTURE DURING THE CELL CYCLE IN PHYSARUM POLYCEPHALUM A SLIME MOLD

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I hereby recommend that this dissertation prepared under my direction by George H. Kieffer

entitled Alterations in Nuclear Membrane Structure During the Cell Cycle in Physarum polycephalum a Slime Mold

be accepted as fulfilling the dissertation requirement of the degree of Ph.D.

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SIGNED: George H. Kieffer

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ABSTRACT

Electrophoretic mobilities of suspensions of isolated nuclei from the slime mold, Physarum polycephalum were measured at various times during the cell cycle in a modified Choucroun electrophoretic cell. It was found that electrophoretic mobility underwent significant variations during the cell cycle with the most marked differences being noted in the first hour following nuclear division. Two less severe changes were observed approximately eight and eleven hours after division. The fluctuations in mobility were attributed to alterations in the nuclear membranes mediated by nuclear and/or cytoplasmic activity in response to differential cellular demands. Electron microscopic observations of the membranes, particularly during the first hour after division, indicated a large amount of membrane surface activity.

Fragments of old nuclear envelopes were observed to be broken down in vacuoles located in the cytoplasm and new membranes were presumed to be assembled or synthesized at the nuclear surface. Nuclear evaginations or blebs were noted to arise concurrent with new membrane formation. Membranous whorls found attached to the outer nuclear membrane, free in the cytoplasm or in association with mitochondria were also observed. The role of these whorls in cellular

activity was not determined although mitochondrial formation is suggested. The nature of the bleb contents was also undetermined, however, it is postulated that they are involved in genetic transfer culminating in vesicle or organelle formation.

Presumably, the changes in electrophoretic mobility were manifestations of the observed increase in surface activity particularly new membrane formation.

INTRODUCTION

Membranes play a central role in the organization of living cells. Not only do they constitute phase boundaries which separate the cell from its environment and subdivide the cell interior into many compartments, but biological membranes also serve as loci for many important biochemical reaction systems. As a phase boundary, they are necessarily the site of much of the physical and chemical activity associated with transport of substances from one phase to another. The demonstrated role of membranes in oxidative phosphorylation (Lehninger, Wadkins, Cooper, Devlin and Gamble, 1958) and photosynthesis (Arnon 1956; Sager 1958) point up the biochemical function of cellular membrane systems. The sets of chemical events which constitute these pathways presumably require the spatial structure of intact membranes for efficient function. It is clear that these and other functional activites must "ultimately derive from the properties of the molecular components of the membrane and from the detailed geometry of membrane structure," (Thompson, 1964).

The nuclear envelope, regarded by some as a cytoplasmic derivative, (e. g. Moses, 1964) is thought to facilitate and regulate the nucleocytoplasmic interrelationship of complex cells. It can be regarded as a specialized surface separating the two cellular phases,

the nucleus and the cytoplasm. Whatever enters or leaves the nucleus must pass a barrier of two membranes and whatever material is included between them. To facilitate the many cellular activities, these membranes apparently assume a functional role.

Nuclear surface activity has often been observed correlated with a cellular event. Gay and co-workers (1956) present evidence implicating the nuclear membranes in the transfer of genetic material from the nucleus to the cytoplasm. In active nuclei of Drosophilia salivary gland, outpocketing or blebs in the envelope, formed in conjunction with specific chromosome bands, extend and pinch off into the cytoplasm. It is to be noted that nuclear blebbing is a fairly common occurrence having been observed by several investigators (Moses, 1956; Merriam, 1961; Wischnitzer, 1963; Kessel, 1963). However, in these instances, seldom is the production of blebs accompanied by a deposition of nuclear material in the cytoplasm. It has been suggested that the elaboration of blebs constitutes a means of conveying information contained in the membranes to the cytoplasm (e. g. Porter and Machado, 1960).

Structural variations in nuclear membranes correlated with age were reported by Merriam (1962). It was demonstrated that envelopes from immature frog oocytes had more pores per unit than mature envelopes. Variations in thickness of pore diaphragms were also noted, young stages being much more variable than older ones.

A unique membrane activity in which the membranes of the nuclear envelope were implicated in the fertilization process was described by Bell (1963) in fern oocytes. Active spermatozoid capture by nuclear evaginations was observed. Evaginations from the nucleus cease when the egg is fertilized. This dual function of membrane activity affords both a physiological and an adaptive advantage, since an effective block to polyspermy is obtained after fertilization.

Numerous ultrastructural studies (Daniels and Roth, 1964; Robbins and Gonates, 1964; Wilson and Kyle, 1966) have described the rupturing of the nuclear envelope at prometaphase and its reconstruction after karyokinesis. Wilson and Kyle described extensive infoldings of the nuclear membranes prior to envelope rupture in human brain tumor cells. Daniels and Roth report that fragments of the nuclear envelope may be conserved intact during mitosis and reused to form the new envelope at telophase. Robbins and Gonates emphasized the endoplasmic reticulum as the precursor for the nuclear envelope.

The nuclear envelope has been suggested as assuming a functional role in organelle formation (Hoffman and Grigg, 1958; Brandt and Pappas, 1959; Bell and Muhlethaler, 1964; Pannese, 1966). The close physical relationship between mitochondria and the nuclear envelope might imply either an interchange of material between nucleus and mitochondria or actual formation of mitochondria at the nuclear

surface. Micrographs of this association have been discussed in terms of the origin of mitochondria from the nuclear envelope (e.g. Bell and Muhlethaler, 1964).

Porter and Machado (1960) suggest that some membranous systems of the cytoplasm may take their origin from the nuclear envelope. A new system of endoplasmic reticulum elements was seen to form, presumably from fragments of the old nuclear envelope, during mitosis in Allium. From these elements a new nuclear envelope, a phragmoplast and a new cell surface were formed. Palade (1959) hypothesized a dynamic equilibrium for the membrane systems of the cell with the nuclear envelope, the endoplasmic reticulum, the Golgi system, the vesicles and organelles and the plasma membrane all being interchangeable at a point in time.

From these and other observations it might be concluded that the membranes of the nuclear surface are capable of alteration in response to cellular demands. However, the apparent subtlety of these changes makes their study difficult. A simple and direct approach for investigation of surface properties of membranes is provided by the microscopic method of electrophoresis. In this method, migration of suspended particles in an electric field is measured. It has been extensively used in the study of yeast, bacteria, erythrocytes and viruses (reviewed by Brinton and Lauffer, 1959). Presumably, minute differences in surface membrane structure are manifested by

alterations in surface charge, detectable as a change in electrophoretic mobility (μ). A large amount of experimental evidence has been gathered which shows that the mobility of a microscopically visible particle is independent of size, gross shape or orientation, over a wide range (Abramson, 1929; Brinton and Lauffer, 1959). In the case of "packaged" particles as represented by cells or nuclei, the nature of the contents does not alter the surface charge on the membranes (Kishimoto and Lieberman, 1965).

The first to describe the electrophoretic mobility of isolated cells were Ambrose, James and Lowick (1956). These authors found that cancer cells from hamster kidney and rat liver showed a higher mobility when compared to similar non-cancerous cells. It was at once suggested that the increased electrophoretic mobility was due to the pathological condition of the cancer cells and an electrophoretic method for diagnosis of cancerous cells was propounded. Subsequent investigation by Eisenberg, Ben-Or and Doljanski (1962), however, associated the increase in mobility with growth. Comparing regenerating liver cells obtained from adult, partially hepatectomized rat and normal adult rats, a rapid rise in the μ was observed in the hepatectomized rats beginning almost immediately after partial hepactectomy. Mobility reached a peak and returned to normal values upon termination of the restorative process. The μ of cells from liver of newborn animals as compared with adult cells also showed about 40% higher μ value. It was shown further that mobility decreased with increasing age.

Since much cytoplasmic activity is mediated through the nucleus, (Mazia, 1956) it was presumed by Kishimoto and Lieberman (1964; 1965) that an electrophoretic study of isolated nuclei might provide insight into the role of nuclear membranes in mediating nucleocytoplasmic relationships. Partial hepatectomy of rat liver was found to lead to a change in the membranes of liver nuclei detectable as an increase in electrophoretic mobility. Similar results were noted in kidney cell nuclei.

From these experiments, it is apparent that cancer cells and regenerating tissue both demonstrate increased mitotic activity and presumably it is the phenomenom which is being measured electrophoretically. Mayhew (1966), using parasynchronized human osteogenic sarcoma whole cells, found them to have a higher electrophoretic mobility during the mitotic peak phase than at other times in the mitotic cycle. Weiss (1966) attributed changes in mobility to alterations in the metabolic rate.

These and other electrophoretic studies of suspended whole cells or free nuclei leave some unanswered questions. In many of the experiments reported, cells obtained from tissue culture methods are used. Investigation has revealed that tissue cultured cells might undergo basic physiological as well as morphological changes which do not occur in cells in vivo, (Paul, 1965). Secondly, since in some studies sarcoma cells are used, the effects of their pathological state

on membrane structure has not been determined. A third difficulty arises from the fact that in a given cell population, different stages of development are represented. Different manifestations of cellular physiology and morphology are possible at varying times in the cell cycle. Efforts at synchrony through alteration of some aspects of the environment again may effect a change in the cell and/or its membranes.

Physarum polycephalum, an acellular slime mold, as used in this study, minimizes many difficulties of more specialized or pathological cells. Numerous diverse studies have been performed with Physarum because of its several rather unique properties. It is not cellular in the vegetative phase but forms a multinucleate plasmodium. Division of nuclei throughout a plasmodium occurs somewhat synchronously. Thus, for some purposes the plasmodium may be regarded as a single giant "cell." The "naked" protoplasm is capable of moving freely about over the culture surface; the cytoplasm within the plasmodium network maintains a rather constant rythmic streaming during this migration. Small bits of cytoplasm may be removed from the migrating plasmodium for study without apparent harm or interference with function to the organism. Finally, axenic culture on defined media has been successfully accomplished by Daniels, Babcock, Sievert and Rusch (1963).

Howard (1931) has described in detail the life history of Physarum polycephalum. It usually exists as a multinucleate plasmodium but when the environment becomes unfavorable particularly through a scarcity of nutrients and upon proper light stimulus, stalked sporangia are produced each containing several nuclei. The mature capsules liberate multinucleate spores which under favorable conditions form flagellates that multiply by binary fission and develop into myxamebae. The free-living myxamebae eventually coalesce to form a new plasmodium. Under adverse conditions, particularly dehydration, the organism can also form a hard sclerotium and remain dormant until conditions become favorable once more.

Since the vegetative Physarum is acellular, it does not undergo cytokinesis. Growth occurs through addition to the protoplasmic mass. An increase in the number of nuclei occurs concurrently within the protoplasmic mass. Nuclei throughout a single plasmodium divide every 10 - 20 hours with no apparent diurnal periodicity. However, the degree of synchrony decreases with increasing age of the culture, (Nygaard, Guttes and Rusch, 1960).

Cytological observation shows that each division follows a typical mitotic pattern, but occurs within the nucleus (Howard, 1932). The nuclear membrane remains intact throughout the process until the daughter nuclei separate.

The typical interphase nucleus is identified by its single, centrally located rather small nucleolus, and peripherally distributed chromatin. (Plate I). About one hour prior to mitosis the nucleolus increases in size and the chromatin moves away from the periphery of the nucleus. With the beginning of prophase, the nucleolus occupies a peripheral position and becomes crescent-shaped. Disintegration of the nucleolus follows, accompanied by chromosomal condensation. Elongation of the chromosomes then takes place filling the entire nuclear space. After further condensation the chromosomes migrate toward the equator of the nucleus and spindle fibers become apparent. Although the individual spindle fibers seem to have a common attachment, no centriole or centriole-like structure is observed. Furrowing of the nuclear envelope occurs at late telophase to complete karyokinesis. Immediately after division, the nucleolar material reappears in the form of tiny particles and within 1.5 - 2 hours these prenucleoli fuse until all nuclei possess a single nucleolus. A time of approximately 15 minutes is required to complete the mitotic events from prophase to telophase. Guttes, Guttes and Rusch (1961) report a chromosome number of about 20; but the lack of sufficient resolution and the small size of the nuclei, (ave. 3μ - 4μ) precluded an accurate count.

In this acellular slime mold then is found the favorable requisites for a study of the nuclear envelope as it relates to activity

during the cell cycle. It was postulated that because of the dynamic nature of membranes, perceptible differences in the nuclear membranes should parallel cellular activity.

A two fold approach was utilized in this study. The method of microscopic electrophoretic analysis was used to ascertain qualitative differences in the membranes during the cell cycle. This was followed by electron microscopic observations of these membranes.

It was found that electrophoretic mobility underwent significant variations during the cell cycle. The most marked differences were noted in the first hour following nuclear division. Two less severe changes were observed approximately eight hours and eleven hours after division. The fluctuations in mobility were attributed to alteration in the membrane mediated by nuclear and cytoplasmic activity in response to differential cellular demands. Electron microscopic observations of the membranes, particularly during the first hour after division, indicated a large amount of membrane surface activity.

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mitochondria was observed. The role of these whorls in cellular activity was not determined although mitochondrial formation is suggested. The nature of bleb contents was also undetermined, however, it is postulated that they are involved in genetic transfer culminating in vesicle or organelle formation.

Presumably, the changes in electrophoretic mobility were manifestations of the observed increase in surface activity particularly new membrane formation.

MATERIALS AND METHODS

An axenic culture of Physarum polycephalum was obtained from the McArdle Cancer Laboratory, University of Wisconsin at Madison, through the generosity of Dr. H. P. Rusch. This culture was maintained and successfully propagated on a 2% non-nutrient agar medium in 15 centimeter Petri plates. Sterile oatmeal sprinkled over the agar surface served as the food source as suggested by Howard (1931). The pH of the medium ranged between 7.2 and 5.0, the limits ascertained by Merrill (1959) for maximum plasmodial migration. Sterile technique were employed to forestall mold or bacterial contamination. Every attempt was made to keep procedures uniform.

Small bits of plasmodium were removed from a migrating organism with a sharp scalpel blade to determine the stage of development of the nuclei. No apparent harm or interference with function was observed. Immediately after excision, the fragments were fixed in 70% ethyl alcohol for two minutes, followed by staining in Congo Red also for two minutes. Squash preparations were made of the fixed and stained tissue and observed under the oil immersion lens of the compound microscope.

Time zero was arbitrarily established as the time of nucleolar disappearance since this event corresponds to nuclear division and is easily identified. All subsequent time measurements were based on nucleolar disappearance.

Only three to four day old cultures were used, since it was found that after this length of time, the plasmodium had grown to a satisfactory size so as to give adequate yields of fresh material. Secondly, after four days, the degree of synchrony changes, cultures becoming less synchronous with age (Guttes, Guttes, and Rusch, 1961).

The electrophoretic cell was constructed from two pieces of plexiglass modified from the design of Choucroun (Guillermond and Choucroun, 1936).

The overall dimensions of the cell were 70mm x 45mm x 4mm. Two U-shaped channels, were cut from 2mm thick plexiglass (Fig. 1). These were firmly affixed to a second layer of 2mm thick plexiglass with epoxy glue. A fitted piece of cover glass was glued between the inner arms of the two U's. Two thicknesses of cover glass were glued to the inner edges of the two U's. An observation chamber 6mm x 8mm x 0.07mm was then formed to hold the suspension to be studied. It was necessary to line the walls of the viewing chamber with the same material since calculations for electrophoretic mobility are based on this assumption (reviewed by Brinton and Lauffer, 1959).

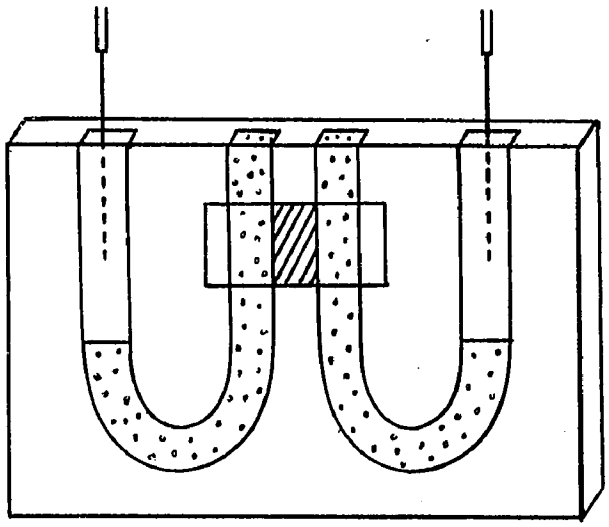


Fig. 1.--The Electrophoretic Cell. An actual size drawing of the electrophoretic cell used to measure mobility of suspended nuclei of slime mold. The stipled areas indicate the agar phase, the clear areas, $CuSO_4$. The viewing chamber is represented by cross hatching. (See text for a complete description.)

The cell is assembled by adhering a piece of plexiglass large enough to cover the bottom 1/3 and the outer arms of the two U's, to the face of the cell with molten paraffin. The remainder of the inner arms is covered by cover glass. Both U's with the exception of the upper 2/3's of the outer arms are filled with molten agar to form the inner phase. A 2% non-nutrient agar mixture is prepared using a 0.005M Na_2 So_4 at pH 7.3 aqueous solution as the solvent. This is allowed to harden. A 0.5% solution of $CuSO_4$, the external phase, is placed in the outer arms of the U's and copper wires are inserted as electrodes. The ends are sealed with molten paraffin making sure that no bubbles are trapped in the electrolyte. The suspension to be examined is introduced into the chamber by means of a wire loop placed in contact with the cover glass. Surface tension and adhesion of the suspension with the glass walls distribute the sample throughout the chamber. Again, it is vitally important that bubbles be eliminated from the chamber to minimize the problem of convection. When these conditions are met, the cover glass is sealed with several drops of molten paraffin. During observation, the electrophoretic cell rests on the microscope stage.

Linear distance was determined by means of a fitted ocular micrometer. The external electrical field was supplied by a Spinco Duostat DC power supply, electrical current being held constant at 0.5 ma with minor fluctuations during the course of an experiment.

The phenomenom of electroosmotic streaming is a problem which cannot be eliminated or counteracted and must therefore be taken into account. This occurrence results from the movement of an electrolyte in response to an electrical current. Since the system involved with here is closed, the net flow of liquid in the cell must be zero, the flow next to the walls being counterbalanced by a flow in the opposite direction in the center of the cell. Therefore, the velocity of the electrolytic solution varies with the depth of the cell. For plane parallel cells whose two walls are composed of the same material and whose width is large compared to its thickness, stationery levels, independent of the cell depth and electroosmotic velocity, have been calculated to fall at the 0.21 and 0.79 level of the whole cell depth (reviewed by Brinton and Lauffer, 1959). Consequently, in each experiment, total depth of the cell was first determined using the microscope fine adjustment micrometer and the stationery level calculated before time measurements were made.

A switch capable of reversing the electrical field was included in the circuit. Two determinations, one in either direction, were made for each nucleus measured and an average taken. A stopwatch was used for determining the time required to travel 50 microns.

Nuclei from a single plasmodium were used for each experiment. They were harvested at some specified time after nucleolar disappearance. Extraction was performed in a 0.25M sucrose

solution with 0.005M Na_2SO_4 added at 0-4°C and pH adjusted to 7.3. One milliliter of solution per one hundred milligrams of fresh material was determined to give adequate yields of suspended nuclei. To break the plasmodium and free the nuclei, 10 strokes of a 5ml Misco glass tissue homogenizer was used. Microscopic observation indicated that no apparent damage was done to the nuclei using this process.

This suspension was next centrifuged at 250g on a Servall refrigerated centrifuge for fifteen minutes at 0°C. The resulting supernatant containing nuclei and small bits of cell debris was used for determination of electrophoretic mobility. To check on the "cleanliness" of these membranes, a pellet was prepared from this suspension and viewed with the electron microscope. Although the nuclei were ruptured in pellet formation, the nuclear membranes do not show an excessive amount of cytoplasmic contaminants (Fig. 1; Plate II).

Between 10 and 20 nuclei per sample suspension were measured and the electrophoretic mobility for each nucleus was calculated.

All mobilities were computed from the equation,

$$\mu = 1A/tIR_s \text{ (Black and Smith, 1962)}$$

where μ = electrophoretic mobility
A = cross sectional area of the cell in cm^2
1 = distance of path in cm
t = time in seconds to negotiate 1
I = amperage
R_s = resistivity of the suspending solution

The units of electrophoretic mobility are expressed as μ/sec/vot/cm.

In all of these experiments the following were held constant: $l = 0.005$ cm, $I = 0.0005$ amps and $R_s = 200$ ohm-cm. The values to be determined in each experiment were A and t.

The results from these experiments were subjected to a data analysis in which the following was calculated for each experiment: 1) the range, 2) the arithmetical mean, 3) the standard deviation and 4) the standard error of the mean at 95% confidence level. This information was then compared on a master graph.

Several methods of preparation were employed for electron microscopic observation. Since only small fragments were necessary, a single whole plasmodium provided all of the tissue for one experimental series. However, it was found that as much tissue was removed from a single organism, nuclear division was hampered.

Immersion for one hour in glutaraldhyde buffered with either phosphate or collidine buffer both at pH 7.4 and $4^{o}C$ served as the primary fixative. The plasmodium was then removed and left overnight in buffer under refrigeration. Postfixation was performed in 2% OsO_4 at $4^{o}C$ buffered as before. Dehydration through a graded series of alcohols was followed by embedding in Maraglas 732 (Erlandson, 1964). The tissues were left in Maraglas overnight in the cold to insure penetration. Polymerization was affected in the oven at $60^{o}C$.

An alternative method of preservation using unbuffered $KMnO_4$ was also used. This fixative has the property of preserving only

membranes with none of the other cellular or nuclear constituents such as the nucleic acids, being preserved (Pease, 1964). These tissues were also passed through graded alcohols and embedded in Maraglas as described above.

Two samples of tissue were collected simultaneously from the same experimental organism. One of these was preserved using the glutaraldehyde - OsO_4 method, the other in $KMnO_4$. Thus, membranes could be studied without the interference of non-membrane components in the permanganate-fixed material and compared to parallel sections fixed by the alternative method in which cellular or nuclear events might be recognized.

Thin sections were cut on a Porter-Blum ultramicrotome MT-2, and mounted on 300 mesh, uncoated copper grids. The glutaraldehyde - OsO_4 fixed sections were double stained in uranyl acetate for 30 minutes followed by a 15 minute exposure to Reynold's lead citrate (Reynolds, 1963). Uranyl acetate is used to increase contrast of the nucleic acids while lead is primarily a membrane "stain." The $KMnO_4$ sections were not stained. All observations were made on a Phillips EM100B and a Phillips EM200 electron microscope.

RESULTS

The results of the electrophoretic study are presented in Table I and Fig. 2. It is immediately apparent that the electrophoretic mobility of suspensions of isolated nuclei does change at various times in the cell cycle. At T_0 the average mean mobility of two samples was 0.55 μ/sec/volt/cm. This corresponds to the onset of mitosis, the nucleolus having disappeared. Division will be accomplished within the next 10-15 minutes. The next readings were taken beginning at $T_{0.5}$ after mitosis was complete. These values, from 0.5 to 1.5 hours, show the most dramatic changes which occur during the cell cycle of Physarum. A steady decrease in electrophoretic mobility takes place attaining its minimum at about 0.75 hours after T_0. The mean mobility is placed between 0.1 and 0.2 μ/sec/volt/cm. These values are only for nuclei that were actually moving and were measured in the field of the microscope. Many nuclei ceased to move completely and could not be measured. This zero mobility is not accounted for in the compilation of the data. The dotted line is included to indicate that many nuclei were not moving at all. Another problem with measurement was encountered during this time interval. Many nuclei began a slow migration with the induction of an electrical field but for some unexplained reason, stopped moving. When the field was

TABLE 1.--Data analysis of electrophoretic mobilities of isolated nuclei in Physarum polycephalum. T (in hours) is based on the time after nucleolar disappearance. Standard error is calculated at the 95% confidence level.

No.	T	n	mean	range	Std. Dev.	Std. Error
1	0	20	0.58	0.42-0.68	0.081	0.018
2	0	10	0.52	0.41-0.66	0.075	0.023
3	0.5	20	0.29	0.20-0.51	0.067	0.013
4	0.75	16	0.18	0.16-0.20	0.019	0.008
5	0.75	10	0.12	0.08-0.17	0.024	0.010
6	1.0	10	0.24	0.19-0.26	0.024	0.007
7	1.0	20	0.25	0.16-0.32	0.041	0.009
8	1.25	20	0.29	0.21-0.39	0.054	0.012
9	1.5	16	0.27	0.21-0.43	0.066	0.017
10	1.5	20	0.35	0.24-0.62	0.095	0.021
11	1.5	15	0.38	0.26-0.46	0.059	0.015
12	2.0	9	0.46	0.36-0.61	0.100	0.033
13	2.0	10	0.48	0.32-0.73	0.480	0.058
14	2.0	15	0.49	0.33-0.64	0.084	0.022
15	2.5	15	0.52	0.33-0.69	0.114	0.029
16	2.5	20	0.63	0.41-0.86	0.119	0.026
17	3	20	0.55	0.35-0.86	0.120	0.027
18	3	12	0.61	0.41-0.80	0.139	0.039
19	3	20	0.53	0.39-0.81	0.123	0.028
20	4	19	0.58	0.37-0.74	0.105	0.024
21	4	16	0.69	0.49-1.00	0.123	0.031
22	5	10	0.71	0.55-0.88	0.111	0.035
23	6	20	0.66	0.47-0.98	0.128	0.029
24	7	20	0.65	0.52-0.80	0.111	0.025
25	8	16	0.50	0.37-0.59	0.064	0.016
26	8	10	0.37	0.30-0.42	0.044	0.017
27	9	20	0.66	0.39-0.83	0.095	0.021
28	10	20	0.67	0.53-1.00	0.109	0.022
29	11	20	0.56	0.39-0.70	0.110	0.024
30	12	14	0.42	0.31-0.57	0.062	0.017
31	13	15	0.40	0.30-0.56	0.077	0.020

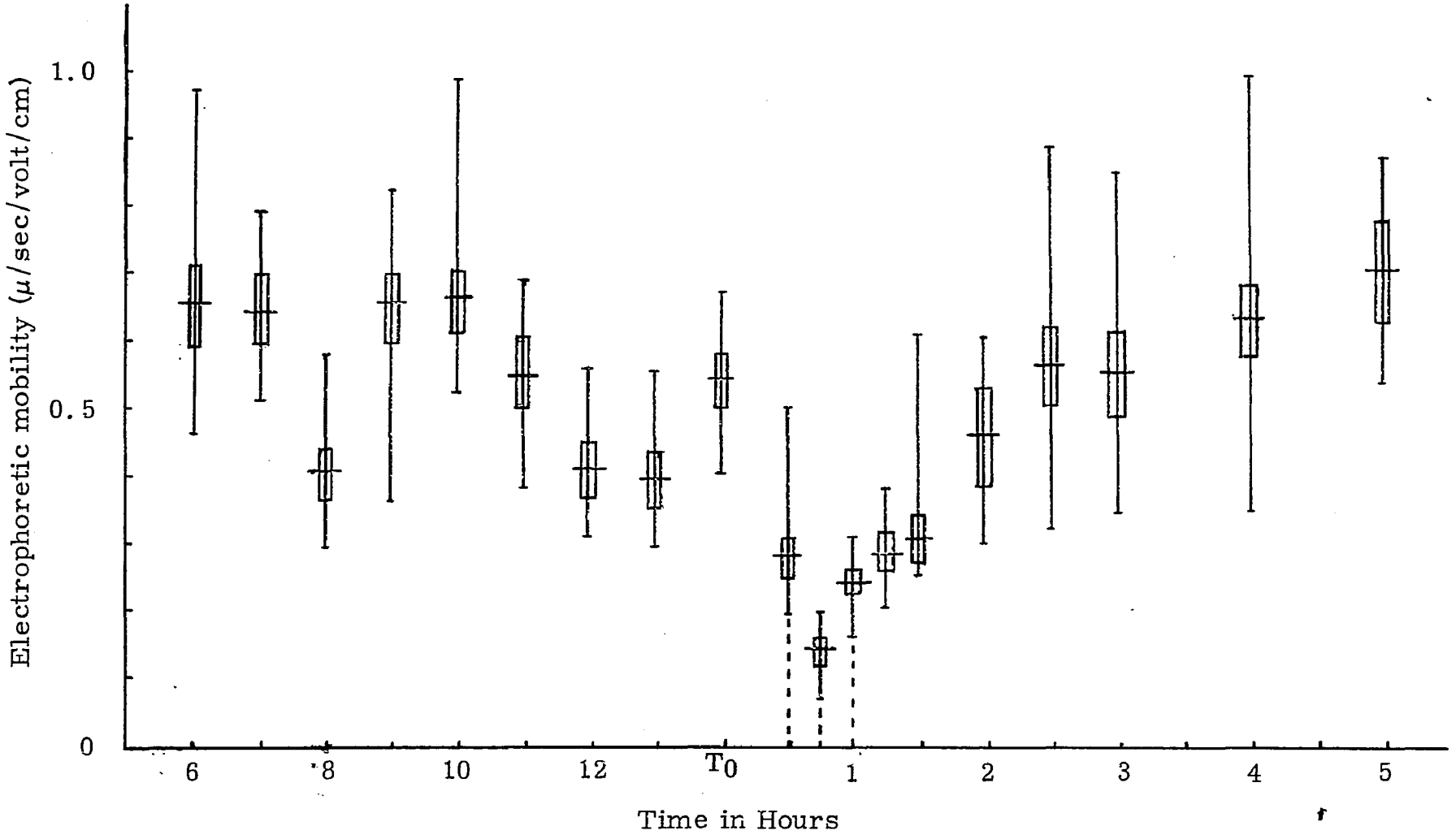


Fig. 2.--Dice-Larras comparison of electrophoretic mobilities of isolated nuclei in slime mold. T is calculated from the time after nucleolar disappearance. The T scale is skewed to facilitate analysis. Minimum electrophoretic mobility occurs in the interval between 0.5 - 2 hours after nucleolar disappearance. The dotted lines indicate no movement of suspended nuclei.

reversed, a very small movement was noted but was of such a minute nature that it could not be measured. Possibly electroosmotic steaming was responsible for the very small movement. Statistically, a comparison of the intervals during this period readily indicated that mobility rates rates are significantly different. It is this stage of the cycle that was most intensively studied with the electron microscope.

At T_2 hours, the mobility again corresponds to the pre-mitotic level. Small fluctuations occur over the next few hours, probably arising from experimental error. According to Brinton and Lauffer (1956), the top and bottom planes of the electrophoretic chamber should not fluctuate more than a few degrees from side to side and from end to end. Variations in the geometry of the chamber can lead to varying results. Some degree of significance can be attributed to the fact that there is a large amount of overlap in ranges of these experimental values; in fact, the extremes of these values do show a considerable degree of correlation. Also, measurements of individual nuclei were made at random, not all nuclei in a given suspension were measured. Individual differences in the membranes of nuclei could account for minor fluctuations in electrophoretic mobility.

At T_8 hours the mobility again decreases but soon recovers and approaches the previous level. At T_{12} and T_{13}, a decrease in μ again occurs. It is doubtful however, that these fluctuations are significant. The overlap of the extremes suggest that the mobilities

are not a result of membrane differences but of experimental procedure as described previously. However, the possibility of a subtle change in the membranes corresponding to a nuclear or cytoplasmic event should not be ruled out until these events have been subjected to more exhaustive investigation.

Reciprocals computed from the average of mean electrophoretic mobilities are presented in Table II and Fig. 3. Examination of these calculations reveal that a standard value of μ persists during the major part of the cell cycle. Again, a dramatic change in μ is in evidence during the first hour after division. Three more less severe fluctuations occur at T_8, T_{12}, and T_{13}.

The most striking result of this study is the decreasing mobility occurring during the first hour after division. Many nuclei stopped completely; however, only those moving were measured. If, as suggested, size, shape and contents have no effect on electrophoretic mobility, with only net change on the surface contributing to the phenomenom, then it is possible that a morphological or structural change, correlated with a physiological event, could be observed in the nuclear envelope or nucleus to explain this overt behavior. These changes could be manifested morphologically as alterations in membrane thickness, staining characteristics, differences in the intramembrane space, variation in size, number and/or shape of the nuclear gaps or some other membrane-associated event. Electron

TABLE 2.--Reciprocals of mean electrophoretic mobilities in slime mold. T (in hours) is based on the time after nucleolar disappearance.

T	$1/\mu$
0	0.018
0.5	.035
0.75	.067
1.0	.040
1.25	.035
1.5	.032
2.0	.021
2.5	.018
3	.018
4	.016
5	.014
6	.015
7	.015
8	.025
9	.015
10	.015
11	.018
12	.025
13	.025

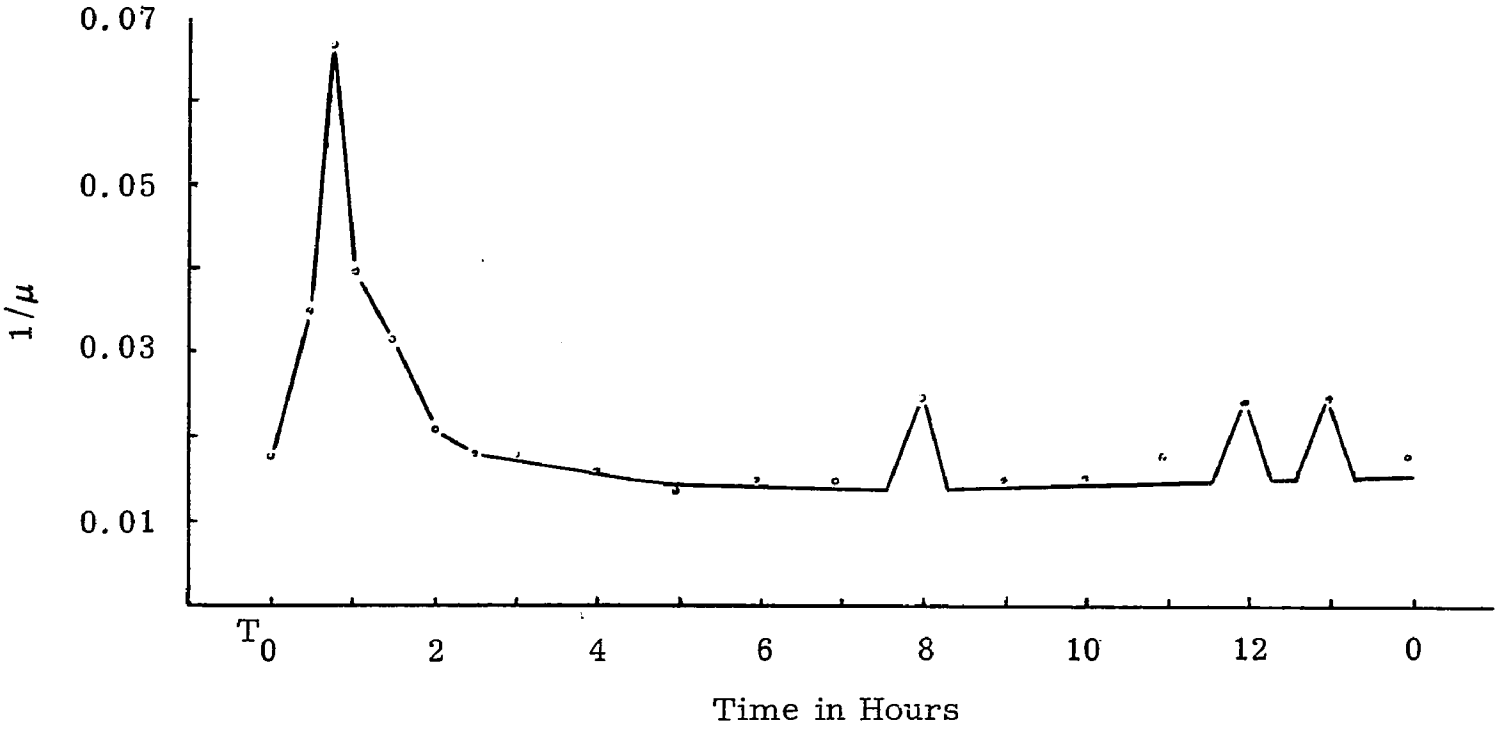


Fig. 3. --Reciprocals of the mean electrophoretic mobilities in slime mold. A standard mobility persists during the major part of the cell cycle with a marked difference occurring during the first two hours after division.

microscopy of the nuclei during the time of minimum mobility could indicate structural alterations related to this activity.

The electron micrographs of the cell cycle can allow one to form the hypothesis that visible alterations in the nuclear membrane structure are correlated with electrophoretic mobility variations observed during the cell cycle. The typical interphase nucleus is identified by its single, centrally located nucleolus (Fig. 2, Plate II). The nuclear envelope is composed of the characteristic double membrane system described by many researchers (reviewed by Moses, 1964), both membranes of the envelope appearing to have the same thickness. The total thickness of the envelope is approximately 200A, the average thickness of a single membrane being 75A. This compares favorably with the values for such membranes reported by Andre and Rouiller (1957). The trilaminar units of Robertson's unit membrane are exhibited by these individual membranes (Robertson, 1960). The dark staining external layers of the membrane measure approximately 25A, while the lighter inner layer is also 25A.

Membrane discontinuities, places where the inner and outer membranes are joined to form gaps or pores, are randomly distributed in the envelope. The average diameter of these gaps is approximately 400A. They are most clearly observed in preparations fixed with $KMnO_4$. The pore in cross section appears to have a fine diaphragm. The significance of this structure is undetermined, but the

observations of Callan and Tomlin suggested that it is continuous with the inner membrane while Watson proposes it as an optical effect of sectioning (reviewed by Moses, 1964). Merriam (1962) noted the presence of dense material susceptible to trypsin digestion which was in these passages. The appearance of such material was not observed in this study. Annuli of the type described by Swift (1956), Watson (1959), and Wischnitzer (1958) were not seen in any of the material examined.

It is assumed that nuclear-cytoplasmic transfer occurs during the interphase period. This transfer of genetic information may be facilitated through contact between the chromatin and the inner membrane (Gay, 1956; Moses,1964). However, it is noted that a small, clear space of about 100 A exists between the membrane and the chromatin. This would suggest that a diffusion-type mechanism must exist if the genetic material is to be successfully passed out of the nucleus; however, the nature of this material and the mechanism of passage in slime mold are undetermined.

As the nucleus approaches division, the late G_2 period, the nucleolus becomes very much enlarged (Fig. 3, Plate II). This sudden increase in size would require an active assimilation of pre-formed materials such as nucleic acids or proteins or an active growth process. Nucleolar enlargement prior to division is not a unique event, having been observed in the fungus, Basidiobolus

ranaum (Robinow, 1963). The increase in size is perhaps a requisite event for division which is imminent. Several investigators suggest that the nucleolar material becomes part of the mitotic apparatus (LaFontaine, 1963; Robinow, 1963). Slime mold would offer an ideal circumstance for investigation of the fate of the nucleolar material since the nuclear envelope remains intact and the problem of cytoplasmic dilution would be eliminated.

In the prophase nucleus, the nucleolus migrates to a peripheral position and becomes smaller (Fig. 1, Plate III). It is not clear whether this is a result of condensation or loss of nucleolar material in the nucleoplasm. Large pores in the immediate vicinity of the peripherally placed nucelolus suggest that some sort of polarity exists. The number of pores in this region increases in comparison to those regions removed from the nucleolus. It would appear possible that a transfer is occurring at this time via the pores.

The previously dispersed chromatin concentrates into compact masses away from the nuclear membrane as the nucleus elongates with the peripheral nucleolus locating one of the poles. The full, rounded appearance of the nucleus disappears as the envelope becomes convuluted, these infoldings becoming very severe as mitosis proceeds. Richards (1960) hypothesizes that a significant part of the nuclear material does not become incorporated into the daughter nuclei but that the mass of the prophase nucleus decreases before division. The

non-chromosomal components of the nucleus are labile, diffusing out of the nucleus. The "clear" zone around the nucleus is said to represent such diffusion.

In late prophase, the nucleolus completely disappears from the nucleus and chromosomal condensation is complete (Figs. 2a and b, Plate III). Spindle fibers become apparent at this time although their appearance was suggested in the early prophase nucleus. Nuclear pores are evident with no change in their size or shape indicated and they appear to be randomly located around the nucleus.

At metaphase, the chromosomes complete migration to the center of the nucleus (Fig. 1, Plate IV). Spindle fibers are very pronounced and appear to have a common point of attachment in the nuclear periphery although no centriole is obvious. Here, as in the light micrographs, no aster formation is indicated. In cross section, the nuclear envelope appears more convuluted than in longitudinal section (Fig. 2, Plate IV). Pores are again observed, appearing irregularly in the envelope. It is perhaps fortuitous that pore diaphragms are more easily recognized at this time. However, this circumstance may be related to a physiological occurrence of unknown origin.

The anaphase nucleus is denoted by the movement of chromosomes away from the nuclear equator (Fig. 3, Plate IV). The relation of spindle fibers in orientating this poleward migration is readily

apparent, although no thickening of the fibers is seen as the chromosomes approach the poles. Few anaphase nuclei were observed, presumably because of the difficulty in obtaining nuclei during the short time involved. Mittermayer, Braun, Chayka and Rusch (1966) report a metaphase-anaphase period of only five minutes in slime mold.

At telophase, the chromosomes lose their morphological identity and the whole mass becomes amorphous and condensed (Fig. 4, Plate IV; Fig. 1, Plate V). The original nuclear envelope is much too large to contain the chromatin, and presents a very much contorted appearance (Fig. 2, Plate V). No breaks appear in the membrane. The excess membranes fold back on themselves, becoming very convuluted and difficult to trace. Furrowing of the membrane begins at a point approximately half way between the chromatin masses with no apparent clues as to exact location being indicated by the micrographs. Spindle fibers can no longer be indentified. Even at this stage pores still appear in the membrane.

At the completion of karyokinesis, the chromatin mass comes to lie immediately adjacent to the nuclear envelope (Fig. 3 and 4, Plate V), no other materials appear to be present in the nucleus. The tremendous amount of excess membrane pinches off and forms vacuoles (Fig.3 and 4, Plate V); various stages of breakdown are observed in these vacuoles as digestion of the old membranes is affected.

At no time, however, does the envelope around the chromatin appear to break.

Whorls of membranous material having the appearance and dimensions of a single nuclear membrane occur attached to the outer nuclear membrane. (Fig. 1, Plate XI). Their appearance in association with the nuclear envelope is first noted during the hour after mitosis and presumably is involved in much of the observed surface activity. Whorls of similar appearance are also seen in the cytoplasm and affixed to mitochondria (Figs. 2, 3 and 4, Plate XI). However, the later observations did not always occur concurrent with decreased mobility, but were recognized at other, presumably non-specific, times during the cell cycle. The direction of movement of the membranous components of these whorls, whether into or out from the nuclear envelope, was not determined. This event may be an indication of new membrane incorporation or the construction of a cytoplasmic organelle. In either case, nuclear membranes appear to be actively engaged and further research is necessary to determine the role of these whorls in cellular function.

Presumably, the new nuclear envelope is a combination of newly synthesized membranes, possibly of cytoplasmic origin and a small portion of the old envelope that still surrounds the division nucleus.

After mitosis, the division nuclei undergo a very rapid enlargement (Fig. 1, Plate VI). This, according to Richards (1960), is probably not due to new synthesis since the process occurs much too rapidly, asserting that the necessary constituents diffuse in rapidly from the cytoplasm. It would be of interest to investigate this "inward flow" of material as to its nature and mechanism, since membrane changes would be necessary to accomodate such an active process. The amorphous appearance of the chromatin disappears as it is dispersed throughout the enlarging nucleus. Nuclear pores are present.

Later in early interphase, the nucleolus reappears as small, disjointed osmiophilic masses (Figs. 2a and b, Plate VI). The diffuse chromatin becomes densely distributed adjacent to the inner membrane of the envelope. Such "plastering" of the inner surface suggests that a very rapid transcription of nuclear information is occurring at this time. According to some investigators (Merriam, 1961; Moses, 1964), disruption of the nuclear envelope at division facilitates distribution of some nuclear information. Since in slime mold the nuclear membrane persists during division, another mechanism for rapid distribution must be affected. This could be accomplished through a "demand response" (Gay, 1956) process in which large amounts of genetic information are passed quickly from the

nucleus to the cytoplasm; such a response may be mediated by the membrane.

It is significant that this period corresponds exactly to the previously reported decrease in electrophoretic mobility of suspended nuclei. Apparently, some aspect of membrane structure which occurs only at this time accounts for these variations. The number, size and morphology of the pores does not change during this time inferring that they are not implicated in the process. No noticeable change in intramembrane spaces or membrane thickness was noted.

Eruptions in the outer membrane of the envelope exemplify the next event in nuclear morphology (Plates VII - X). These "blebs" correlate with that time when electrophoretic mobility is at its lowest value. Although they are observed in the glutaraldehyde-OsO_4 fixed material, they are most obviously seen in the permanganate fixed sections (Plate VII). In the osmiun prepared sections, these blebs appear to contain a darkly staining, osmiophilic substance (Plates VIII and IX). The blebs enlarge to varying sizes and then separate from the outer membrane. Breaks in the nuclear membrane are not observed suggesting that a "pinching" mechanism accounts for the disjunction (Fig. 1, Plate X). Cytoplasmic vesicles of apparent "bleb" origin are observed in the vicinity of these nuclei. It should be emphasized that not all of the nuclei at this stage demonstrate blebbing at any one time. However, if the production is a very rapid one, the

possibility of it being preserved would not be great. The important aspect of bleb formation is the requisite alteration of the nuclear envelope that makes this event possible and presumably mediates this activity. It is suggested that this difference in membranes was detected by the decrease in electrophoretic mobility and not the formation of the bleb.

Following bleb formation, the chromatin becomes dispersed more uniformly throughout the nucleus. Some chromatin is still found adjacent to the inner membrane but the amount is reduced. Presumably, transcription also occurs during the long interphase period. However, no more blebbing was observed, the normal routes for genetic transfer apparently being sufficient to supply the demands of the cytoplasm.

Approximately two hours after division, the new nucleolus has become completely snythesized and fused to form the characteristic single structure of the interphase nucleus. No further detectable alterations in membrane structure appear until the next division cycle approaches when a repeat performance ensues.

DISCUSSION

The nuclear surface appears to be a highly active region in Physarum. Presumably, this activity is related to physiologic events important to cell function. At this interface, materials from both the nucleus and the cytoplasm may be gathered and through interaction, mediated by alterations in membrane structure, bring to realization cellular potential.

A remarkable correlation between nuclear surface activity and change in the electrophoretic mobility of these nuclei has been demonstrated. If the charge on a particle suspended in an electrial field is independent of the size, shape, orientation or contents of the particle, surface features being solely responsible, the measurements of electrophoretic mobility described in these experiments would indicate that slime mold nuclei have the lowest charge density at approximately 0.5 to 1 hour after mitosis has occurred. This finding is in agreement with that of Mayhew (1966) who noted a decrease in mobility of whole cancer cells after division. He further observed that mobility rose to its highest level in the late G_2, during mitosis and early G_1 periods. After incubation with the glycosidic enzyme, neuraminidase, the increase in mobility was not demonstrated. In fact, he found that he could reduce the mobility to the same value irrespective of the stage in the cell cycle after enzyme treatment. This enzyme is known to

split off terminal N-acetylneuraminic acid residues bearing ionized carboxyl groups (Cook, Heard and Seaman, 1961). The high mobility was thought to be caused by a higher concentration of ionized neuraminic acid carboxyl groups exposed. These may be uncovered by a stretching of the cell surface during the peak of mitotic division.

A change in mobility was shown by Kishimoto and Lieberman (1964), to be effected by a loss of some ethanol-soluble material that decreases the net nuclear charge on the nuclear membrane. This finding would indicate that a discrete molecular substance is involved in the mobility phenomenom.

Eisenberg et al (1962), assumes that the release of intercellular bonds during cell division exposes ionizable groups which increase the charge density of the cells.

Weiss (1966) was able to lower mobility by altering the temperature above and below an optimum level. Similar results were achieved after the cells were incubated in DNP. These findings suggested that interference with the metabolic rate brought about a reduction in electrophoretic mobility. He submits three possible mechanisms whereby a cell can alter its mobility in relation to its metabolic rate. The most plausible of these mechanisms, as he suggests, requires conformational changes at the cell surface which result in ionized carboxyl groups being reversibly uncovered.

Thus, the major opinion favors the view that the change in electrophoretic mobility is associated with or contingent upon the uncovering of ionizable groups with a corresponding addition or loss of charge. No new synthesis of molecular membrane components is required. However, in none of the systems cited, did the nuclear envelope remain intact during division. The very rapid enlargement of the post division nuclei in slime mold plus the observed concurrent surface activity would require a new source of nuclear membrane. A large amount of membrane must be assembled or synthesized quickly to supply this requirement. Stretching of existing membranes could not account for this increase since the micrographs indicate that the formed membranes appear to be loosely arranged in the envelope. The occurrence of membrane vacuoles immediately following division would also eliminate old membranes as a source.

Presumably, new synthesis occurs rapidly since full nuclear size is acquired within one hour after division. Membranous outpocketings and whorls, found in association with the nuclear envelope at this time, would also require a rapid synthesis. It is hypothesized that these new membranes are as yet molecularly "undifferentiated", identified by their different electrophoretic mobilities. Upon differentiation, mobility returns to a standard value. Experimental evidence does indicate that there are structural variations in different membranes, (Sjostrand, 1964). If, indeed, the membrane systems of the

cell are in dynamic equilibrium with the nuclear membrane, the endoplasmic reticulum, the Golgi system, the vesicles and organelles and the plasma membrane, all being interchangeable at a point in time as suggested by Palade (1959), then modification at the molecular level within a specified membrane would be necessary.

Membrane alterations could be under direct genetic control (cited by Gay, 1956). Swift hypothesizes a synthetic cycle in which active chromosomal loci may serve as sites for formation of protein filaments which radiate to the nuclear membrane in bundles (Swift, 1956). Where these bundles intersect the membrane, new membrane components are formed. The filaments may extend into the cytoplasm and participate in the synthesis of new membrane systems parallel to the nuclear envelope. A second mechanism for membrane modification is presented by Manganiello and Phillips (1965), in reticular systems. Newly formed protein from ribosomes attached to cytoplasmic membranes is transferred directly to adjacent sites on the membrane where some of these proteins become incorporated as structural components of that membrane. Such systems could account for variation in molecular structure as dictated by physiological demands of the system involved. In slime mold, membrane heterogeneity could be manifested as a change in electrophoretic mobility.

Manganiello and Phillips (1965) have demonstrated a method for membrane analysis in which the isolated membranes are disrupted through the action of the detergent, deoxycholate. The products of the membrane are then analyzed electrophoretically. Employing this method, slime mold membranes might be analyzed for their molecular components. A comparative study relating cell activity with nuclear membrane components might further illucidate the dynamic properties of these intracellular membranes.

A more direct approach is suggested by Cunningham, Stiles and Crane (1965). Various cell membranes have specific surface structures after phosphotungtic acid treatment, recognizable in the electron microscope. It is suggested that this method may be useful in the identification of isolated membranes and particularly to interpret molecular arrangements in cell membranes.

An alternative explanation for changes in electrophoretic mobility must be considered in view of the large amount of surface activity observed during the first hour after division. The previously cited studies suggest that changes in mobility during mitosis result from distortions in the membrane surface exposing previously buried ionizable groups. In slime mold, such activites as membrane evaginations and new membrane formation could account for these changes through a deforming of the nuclear surface. However, it is conjectured that this hypothesis is not too valid. So far as has been

determined, no mention of a decrease in mobility approaching zero has been published. The fluctuations previously reported (e.g. Mayhew, 1966; Eisenberg,et al, 1964), were in the order of ±0.3 - 0.4 of some standard value. If deformations alone accounted for the decreased mobility in slime mold nuclei, surface events presumbly would be cataclysmic. Furthermore, the occurrence of membrane protrusions was not observed in all the nuclei studied in any one section. Although no attempt was made to quantitize their occurrence, the number of nuclei actively engaged in surface activity was less than half. However, all nuclei were electrokinetically similar. This would imply that the formation of a bleb is a very rapid process and that membrane events preceding evagination determine the course of specialization. Also, blebbing was observed to occur only at a specific time in the cell cycle. No other structues were noticed in proximity to the evaginations. Thus, no other initiator appears to stimulate the activity. Even if the membrane does respond to an unknown gene product, presumably it must be receptive requiring specialization since the evaginations appear only once during the cell cycle. For these reasons, the hypothesis of an "undifferentiated" or newly synthesized membrane seems to correlate with the dramatic change in electrophoretic mobility.

The micrographs of the nuclear outpocketings indicate that they contain a substance of presumed nuclear origin. Examination of the

bleb interiors of glutaradehyde-osmuim fixed materials discloses a fine granular material. It is not as opague as the nucleic acid of the chromatin nor as coarse as ribosomes or nucleolar particles both of which contain RNA. The density of the vesicle is similar to that of the cytoplasm which might indicate the presence of a protein. Closer observation reveals a laminar structure resembling the cristae of mitochandria in some (Figs. 1 and 3, Plate IX).

The nucleus as a site of mitochondrial formation, has been reported in several communications. Early investigators, describing the de novo synthesis of mitochondria, remarked on their proximity to nuclei (cited by Novikoff, 1961). Hoffman and Grigg (1958), in actively dividing cells in onion root tip, rat thymus gland and mouse lymph nodes, claimed the mitochondria were formed within the nucleus from invaginations of the nuclear envelope and subsequently were extruded into the cytoplasm. Brandt and Pappas (1959) noting that the limiting membranes of mitochondria and postdivision nuclei were continuous suggested a nuclear origin of mitochondria through progressive foldings of the envelope. Evidence implicating both membranes of the nuclear envelope in mitochondrial formation was presented by Bell and Mühlethaler (1964). Complex evaginations from the nucleus were interpreted as being the first stage of mitochondrial genesis with development of the cristae occuring after the vesicle detaches. Recently, Pannese (1966), described the appearance of

membranous whorls associated either with the nuclear envelope or with some mitochondria. He hypothesizes that these whorls may be related to the formation of new mitochondria.

Similar whorls of membrane have been observed in slime mold, particularly during the period following division (Fig. 1, Plate XI). These membranous structures have a morphology analogous to a single nuclear membrane and often appear attached to the nuclear surface. They have also been observed free in the cytoplasm as well as in association with mitochondria (Figs. 2 and 4, Plate XI). If, indeed, these are mitochondrial precursors, large amounts of membrane must be synthesized immediately following division. The correlation between decrease in electrophoretic mobility and elaboration of these new membranes is postulated as another indication of membrane modification required for a specific activity, in this case, organelle formation.

Analysis of the blebs in Figs. 1 and 2, Plate VIII does not seem to indicate a whorl origin. They are still attached to the nuclear surface and organization of the contents appears to be proceeding. If the laminar structures will eventually result in mitochondria, then a second mechanism, again implicating differential membrane activity, is indicated.

However, it has not been established that these structures will result in new mitochondria. The structural relationship between

nuclear outpocketings and the cytoplasm has been interpreted as indicative of nucleocytoplasmic interchange (Gay, 1956). The event assumes commutability in which nuclear membranes participate either actively or passively. The mode of transcription between the nucleus and the cytoplasm has been the subject of much research (Rebhun, 1956; Merriam, 1961; Kessel, 1963; Gay, 1956). It is generally agreed that messenger must pass from the nucleus to the cytoplasm but little evidence of a cytological nature has been demonstrated to indicate such transport. Several mechanisms for effecting transfer are possible. The most direct pathway would involve a straight diffusion in which molecular messenger passes out of the nucleus via nuclear pores. Pore diameters are well within the range to allow for such transfer (Feldherr, 1965; Wiener, Spiro, and Lowenstein, 1965). The continuities between the outer membranes of the nuclear envelope and those of the endoplasmic reticulum could also facilite distribution. Feldherr (1964) demonstrated that pore material could selectively accumulate and bind certain substances to be transported across the nuclear envelope. Such a mechanism could accomodate passage of messenger aggregates. Hoshino (1961), noted deep invaginations of the inner nuclear membrane into the nucleoplasm of Ascites cells. These were interpreted as being extensions of the perinuclear space in which synthesis occured, the product then being passed through the cytoplasmic membrane system.

Outpocketings of the nuclear envelope in which one or both of the membranes contributed to formation of a cytoplasmic vesicle have frequently been observed. Helen Gay (1956), using Drosophila salivary glands, noted an intimate structural and configurational association between chromosomal materials and nuclear membrane outpocketings. These so-called "blebs" were formed by localized evagination of the dual layered envelope and were interpreted as a possible mechanism for transport of materials of chromosomal origin to the cytoplasm. The contents of these packages are unknown since the small size of the vesicles precludes cytological analysis. Other investigations suggest that they may contain DNA, as evidenced by the presence of Feulgen-positive particles in the cytoplasm. The possibility that they may contain some other material is not eliminated.

The nuclear envelope remains intact in Physarum during division. At no time is there free communication between the nuclear contents and the cytoplasm. This could impose a barrier to the transcriber function in slime mold since a mechanism for rapid distribution of messenger has been postulated to occur at the time of membrane fragmentation in the cells exhibiting nuclear breakdown during division. An alternative system to accomodate rapid dispersion in slime mold could conceivably involve the packaging of large amounts of genetic information and distributing the membrane-bound messengers to various points in the cytoplasm. This process or "demand

response" in effect would replace complete membrane rupture with localized, controlled eruptions. At no time does the nuclear envelope manifest breaks or discontinuities even when a bleb detaches from the nuclear surface. Bleb formation is followed by the appearance of a stalked vesicle still affixed to the outer nuclear membrane. The vesicle appears to be "pinched" off at the base of the stalk, again implicating a dynamic but controlled membrane process. The nuclear membrane must be able to replace material lost at a sufficient rate so as to maintain a harmonious interaction of rapid membrane synthesis and membrane loss. However, no information is available which suggests the control mechanism for these processes. During the remainder of the cell cycle, blebs were no longer observed which might be interpreted as meaning that more subtle mechanisms for transfer would prevail.

This study has shown that in slime mold only the outer membrane participates in bleb formation. This would prohibit the direct inclusion of DNA in the formed vesicles as postulated by Gay, since the inner membrane imposes an extra barrier. It was also remarked that the chromatin adjacent to the inner membrane in slime mold did not directly contact the membrane but was separated from it by a small space. These observations indicated that no continuity exists between nuclear DNA and the formed vesicles precluding a DNA component.

If the chromatin adjacent to the inner nuclear envelope is actively transcribing, then the contents of the vesicles could be of a genetic nature. Apparently the material would have to traverse the inner nuclear membrane to reach the cisterna of the nuclear envelope, and remain there since this space is for a short time continuous with the forming vesicle. If true, this would imply differential permeability properties between the inner and outer nuclear membranes. The observation of only the outer membrane participating in evagination also indicates that the two membranes differ structurally if the capacity for bleb formation resides in the membrane. This would require a distinction in differentiation. This hypothesis is susceptible to investigation via the technique of microelectrophoresis.

Since electrophoretic properties reside only on the surface of the nucleus, (Brinton and Lauffer, 1959), a comparison of the two membranes composing the nuclear envelope is possible. It is known that citric acid causes complete removal of the outer membrane from rat liver nuclei (Kishimoto and Lieberman, 1964). Physical properties reflected by chemical change in the nuclear membrane could presumably be evidenced as differences in electrophoretic mobility.

If product transfer depends on diffusion through the inner membrane barrier, the particle must of necessity be small. This could account for the fine grained appearance of the bleb contents. The synthesis of an intermediate in the cisterna as postulated by

Hoshino should not be ignored until further investigation is more revealing. Labeling experiments including autoradiographic and electron microscopic techniques could be an approach to the problem. Pulse labeling immediately following mitosis with tritiated uridine as an indicator of RNA, or a labeled amino acid to identify a protein could be resorted to.

It would also be of interest to investigate the dependence of bleb formation on RNA and/or DNA synthesis. Again, electrophoretic measurements could be employed since it is postulated that chemical modification of the membrane is a prerequisite for blebbing. Synthesis of either of these could be blocked through any number of chemical agents. Mobility measurements plus microscopic observation would provide the techniques for verifications of a suspected relationship.

Up to this point, the emphasis has been on the transcriber function of the nucleus. A second nuclear function, a repositor one in which genetic material is conserved and propagated (Moses, 1964), is a necessary aspect of cell activity. It is well known that DNA synthesis in many organisms occurs periodically and occupies only part of the reproductive cycle. Actively growing Physarum synthesize DNA mainly during the first three hours after mitosis, there being no G_1 or presynthetic period (Rusch and Sachsenmaier, 1963). The reported nuclear blebs occur approximately one third of the way through the

synthesis period. All of the activities so far described occur at the peak of the S period. One might inquire as to the possibility of a nucleus performing both the transcriber and the repositor functions simultaneously.

DNA replication occurs asynchronously among the chromosome set, as shown by Plaut and Nash (1964), in labeling experiments of polytene chromosomes of Drosophila. Because discontinuous labeling was observed, different numbers of DNA units were thought to be engaged in replication at any one time. The time necessary for replication is not very long. Plaut and Nash suggest that individual DNA units may be copied in a few minutes. Gross and Caro (1965), experimenting with E. coli, found that the entire bacterial chromosome could be duplicated in less than one hour.

Since in slime mold, a chromosome number of about twenty has been reported, (Guttes, Guttes and Rusch, 1961), during any one time in the synthetic period a large amount of chromatin is not being replicated. Presumably then it is available to perform the transcriber function simultaneous with replication of other DNA units.

The interpretation that nucleocytoplasmic interaction through the mediation of nuclear membranes is based primarily on the occurrence of blebbing in Drosophila larvae. In these observations both membranes of the nuclear envelope were seen to evaginate in vesicle formation. In slime mold, eruption occurs only in the outer

membrane. It therefore, seems appropriate to inquire as to whether or not these two activities are concerned with the same cellular event, namely, the transfer of genetic information from the nucleus to the cytoplasm.

Blebbing phenomena in which only the outer membrane participates have been observed in other cells. Afzelius (1957) described irregularly shaped bodies in sea urchin oocytes which consist of a dense mass of granules enclosed by membranes possessing a structure similar to the nuclear membranes. These bodies were found to contain RNA and were occassionally observed in close association with the nucleus from which they were thought to be derived by an outpocketing of nuclear membrane. Hsu (1963) noticed several blebs of the outer membrane in oocytes of the tunicate, Boltenia. He supposed that as the bleb was set free in the cytoplasm, its membrane disintegrated, the "naked" blebs forming strings of ribosomes. Wischnitzer (1963) also noted a blebbing of the outer nuclear membrane in oocytes of Rana, but since vesicle formation was only occasionally observed, it was felt that it occurred in response to some transient physiological demand of unknown significance. Kessel (1963) has demonstrated in young oocytes of Necturus that the number of blebs on the outer membrane can be very numerous. Long chains of vesicles were formed from fusion of detached blebs. He also observed that they could be produced over a considerable period of time in the cell cycle. Palade

(1955) observed membrane systems in the cytoplasm which were structurally similar to nuclear membranes and designated them as "idiosomes." The term annulate lamellae was first used by Swift (1956) to describe these groups of porous and parallel lamellae which may or may not be arranged in stacks. Rebhun (1956) observed similar membrane units in oocytes of Spisula and named them "periodic lamellae." Since then these structures have been observed in a variety of cells other than oocytes.

Annulate lamellae, the present designation, are composed of lamellar units, each of which consists of two parallel membranes separated by a cisterna of approximately 20-40 millimicrons in diameter. The ends of a lamella are fused giving an appearance of a flattened sac. Their membranes are composed of periodically arranged annuli whose pores posses a fine structure comparable to the annuli present in the nuclear envelope. They are generally free of ribosomes although the region of the cytoplasm adjacent to them shows a greater density than the remaining cytoplasm, suggesting a diffusion of substance from the lamellae into the surrounding cytoplasm. The significance of these structures in terms of actual cell function is still largely obscure.

The morphological similarity of annulate lamellae to the nuclear envelope coupled with the frequent occurrence of lamellae in close proximity to the nucleus, implies an origin from the nucleus or one

that is common with the nuclear membrane. In fact, Afzelius considered these lamellar structures to be remnants of the nuclear membrane remaining after nuclear breakdown in metaphase. A delamination of the nuclear membrane was postulated by Rebhun while Merriam (1959) asserted that their original formation was intranuclear, the membranes being subsequently sloughed off into the cytoplasm. Hsu observed lamellar elements on both sides of the nuclear envelope. Vesiculation of the outer nuclear membrane with ensuing fusion of the formed vesicles as advanced by Kessel seems to be most satisfactory explanation of lamellae formation currently.

Although blebbing of the outer membrane has been observed in slime mold, structures resembling annulate lamellae have never been noted. Vesiculation, however, has been seen in conjunction with nuclear blebbing. Vesicules, with membranes approximating the morphology of a single nuclear membrane have frequently been recognized in the cytoplasm. Large vesicles with dimensions greater than the individual blebs have also been observed. This would indicate that several vesicles may fuse, an event paralleling the one described by Kessel.

In sea urchin oocytes, many cytoplasmic vesicles, apparently ribosomes, are seen in the region of the annulate lamellae, expecially near their ends. Although this condition has been observed by several investigators, it has not been determined whether or not the vesicles

were actually fusing to form annulate lamellae, or the lamellae were giving rise to vesicles by budding (Swift, 1956; Merriam, 1959). However, it might be well to reemphasize that the functional significance of annulate lamellae is completely unknown. The only characteristic shared by tissues in which these structures have been described is that they are identified with rapidly growing or differentiating tissue, such as embryonic or fetal tissue, and their existence is short lived.

Annulate lamellae have been observed in both vertebrate and invertebrate cells but none as primitive as slime mold. It might be conjectured that in slime mold, because of its phyletic position, the middle stage of lamellae formation is bypassed. Whereas in more complex life forms the sequence might be, bleb - vesicle - annulate lamellae - modified vesicle - physiologic function, in slime mold the progression could read, bleb - vesicle - fusion - modified vesicle - physiologic function. Modification for function could be accomplished by the fusion process rather than annulate lamellae formation.

The supposition that these outpocketings are related to the formation of new endoplasmic reticulum must not be eliminated. The most rapid growth in slime mold occurs immediately following nuclear division. With this new construction of large amounts of cytoplasm, a growing reticular system would be required. In the Bennett model (1956) of "membrane flow," the source of some new cell membranes is the nuclear envelope. Thus, the bleb as observed in slime mold at

least, may be the manifestation of reticular formation. This hypothesis however, is not too likely in view of the similarity between bleb formation in slime mold and that described in other organisms but its possibility cannot be completely ignored.

The proposed relationship between physiological activity and morphological alteration involving formation, detachment and fusion of nuclear membrane blebs might represent an expression of a latent membrane potentiality serving to produce a directed response in the cell. Schultz (1952) has suggested that "the properties of nuclear membranes may vary according to the chromosomes most intimately associated with them during their production. This variation might afford a basis for differential cell function and cell differentiation." The present studies sustain this point of view. In the foregoing discussion it has been implied that detached membrane segments are expressions of genetic potentials, endowed to the membrane by chromosomal activity. The investigations of Kessel in which he describes the ordered migrations of formed vesicles and lamellar units to specific and different locations in the cytoplasm hints at this potential. The appearance of outpocketing only at specified and predictable times provides further credence to the concept of membrane modification.

Alterations may be effected in more than one way. A protein, functioning enzymatically or structurally within the membrane could

execute change. Gain or loss of ions could also alter membrane properties. Bell (1962) has drawn attention to the presence of polysaccharide as a component of cell membrane capable of bestowing a versatile range to membrane properties. Other organic constituents such as the previously mentioned sialic acid could also effect change. These molecular alterations in the nuclear membrane, mediated by specific chromosomal loci and operative only at designated times, could produce subtle but required alterations in basic membrane patterns.

The emerging picture in slime mold thus seems to be of membranes of heterogeneous molecular construction containing within their very structure the information necessary to realize genetic potential as functional cell organelles.

Explanation of Figures in Plate I

The cell cycle of slime mold, Physarum polycephaslum. Plasmodium was prepared using a squash technique and stained with Congo Red. x 2400.

Fig. 1. Typical interphase nucleus with a central nucleolus.

Fig. 2. About 0.5 hour before mitosis. Overall size of the nucleus and the nucleolus has increased.

Fig. 3. Early prophase nuclei. The nucleoli are peripherally located.

Fig. 4. Prophase nuclei. The nucleoli are assuming a crescent-shape.

Fig. 5. Late prophase nuclei. The extreme crescent-shape of a disintegrating nucleolus is indicated.

Fig. 6. Late prophase nuclei. The nucleoli have disappeared and chromosome condensation is complete.

Fig. 7. Prometaphase nuclei. Condensation of the nuclear contents has begun as noted by the clear peripheral area.

Fig. 8. Metaphase nuclei displaying the equatorial position of the chromosomes. Spindle fibers occur only within the envelope with a common poleward attachment for these fibers.

Fig. 9. Late anaphase and early telophase nuclei.

Fig. 10. Late telophase nuclei showing furrow formation.

Fig. 11. Late telophase nucleus. An elongation is apparent within the nuclear envelope. Fiber attachment is noted.

Fig. 12. Early postdivision nuclei. The daughter nuclei are irregularly shaped and much reduced in size.

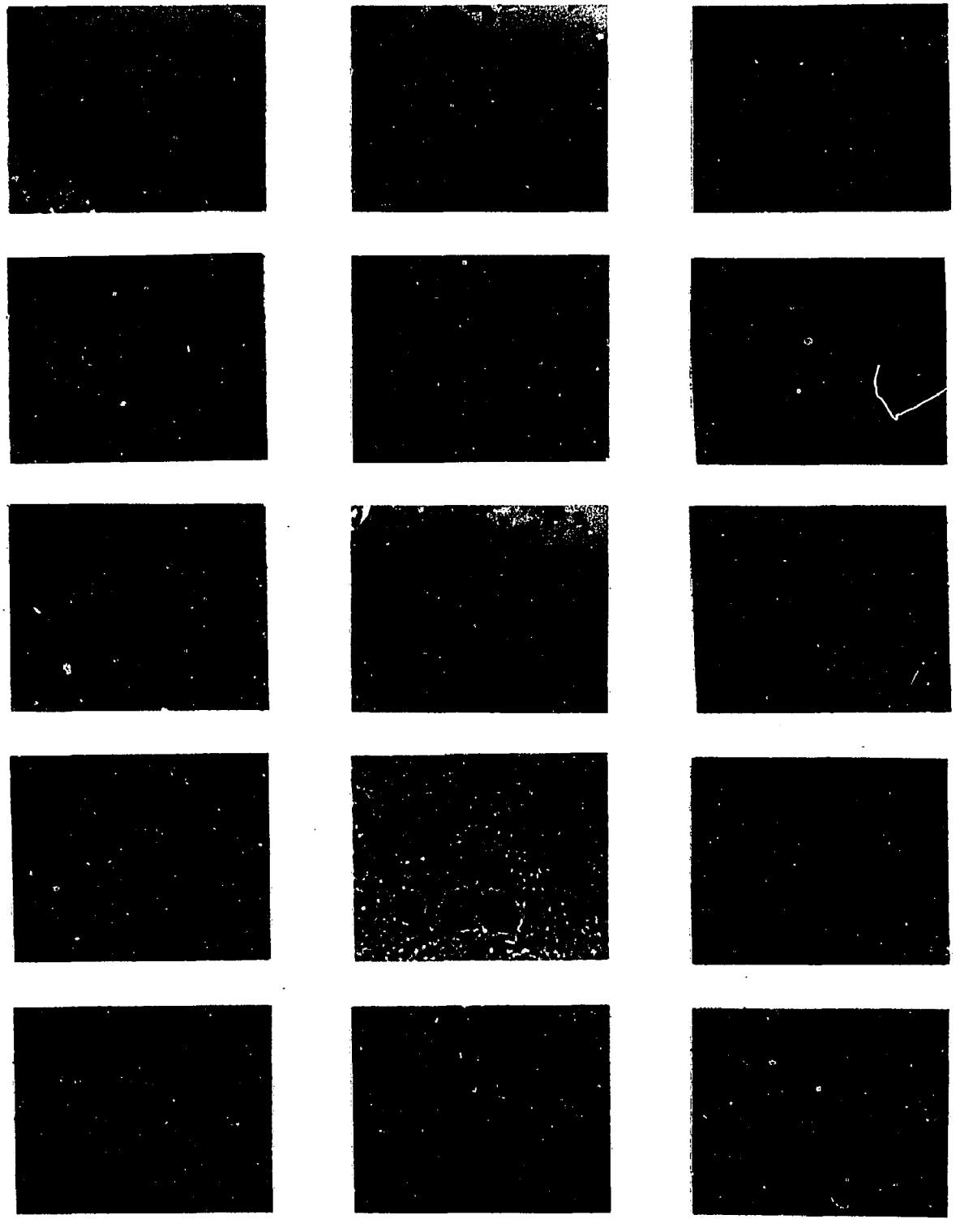
Fig. 13. Interphase nuclei about 40 minutes after division. Very rapid nuclear enlargement has occurred.

Fig. 14. Interphase nuclei about one hour after division. Nucleolar fusion is shown.

Fig. 15. Interphase nuclei about 1.5 - 2 hours after division. Fusion of nucleolar material is at various stages. A typical mononucleolar nucleus is indicated.

PLATES OF MICROGRAPHS

Plate I



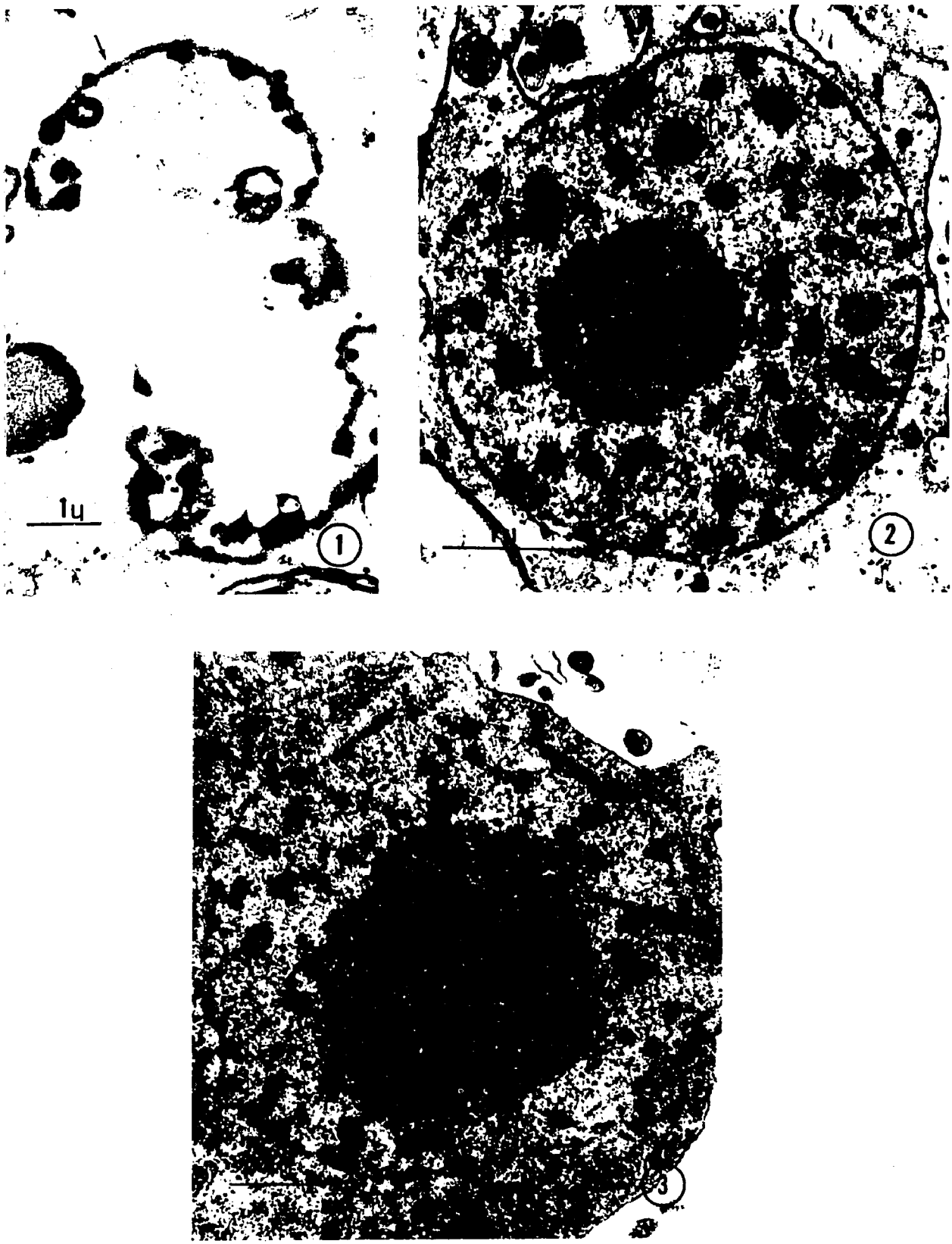
Explanation of Figures in Plate II

Fig. 1. Electron micrograph of a nuclear envelope obtained from a pellet. The outer membrane is indicated.

Fig. 2. EM micrograph of a typical interphase nucleus. A single, centrally located nucleolus and scattered chromatin are characteristic.

Fig. 3. EM micrograph of a late interphase nucleus, approximately one hour prior to division and indicated by nucleolar enlargement.

Plate II



Explanation of Figures in Plate III

Fig. 1. EM micrograph of a prophase nucleus. The peripheral position of the nucleolus and condensing chromatin are shown.

Fig. 2 a & b. EM micrographs of late prophase nuclei.
2a. The nucleolus has disappeared and chromatin condensation is well advanced.
2b. Spindle fibers are indicated.

Plate III

Explanation of Figures in Plate IV

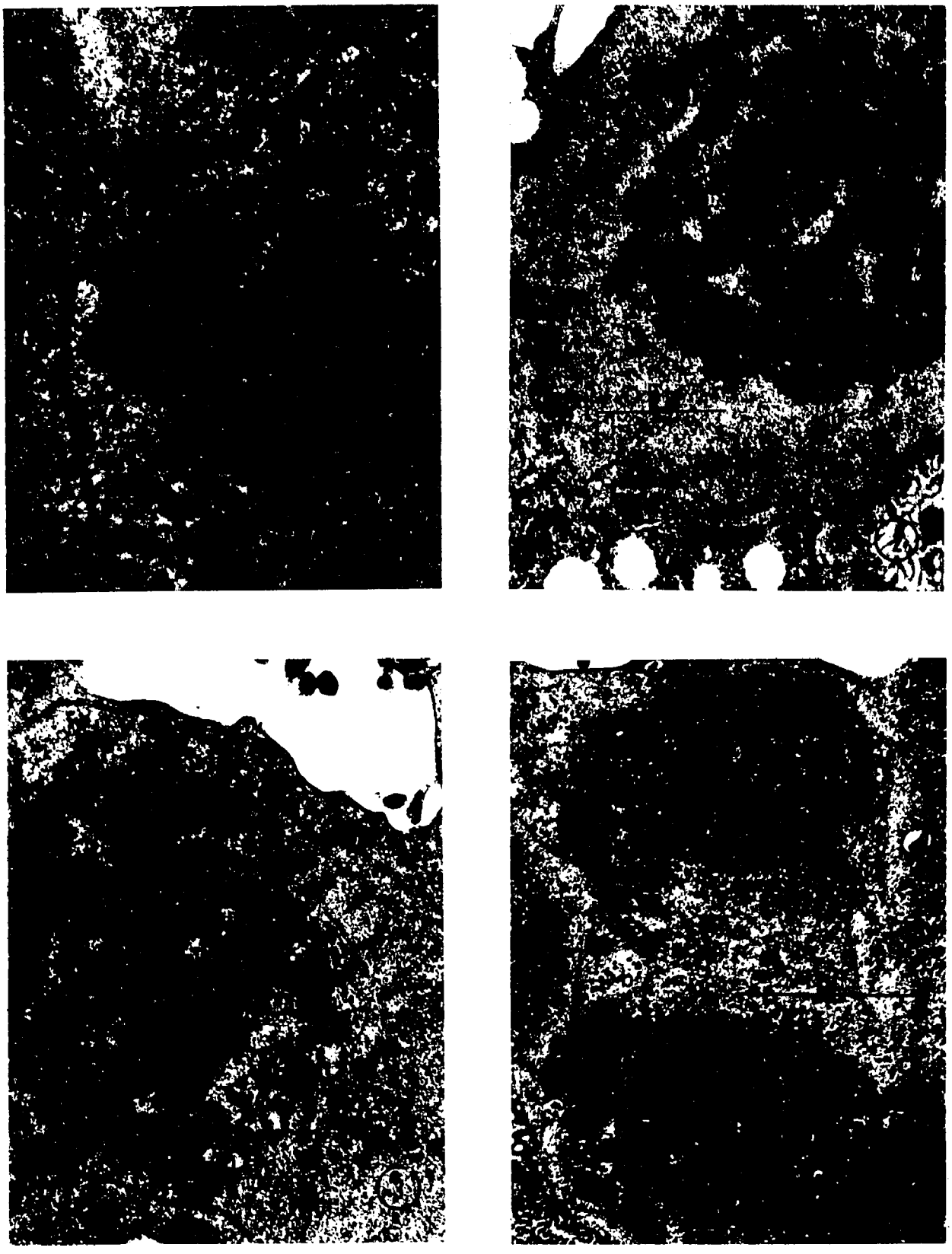
Fig. 1. EM micrograph of a metaphase nucleus in longitudinal section. Spindle fibers are indicated. No aster or centriole appears to be present.

Fig. 2. EM micrograph of a metaphase nucleus in cross section. Chromosomes are equatorial and spindles in xS are observed at a.

Fig. 3. EM micrograph of an anaphase nucleus. Spindles are shown.

Fig. 4. EM micrograph of a telophase nucleus. Furrowing is indicated at a. Excess membranes appear to fold back on themselves at b.

Plate IV



Explanation of Figures in Plate V

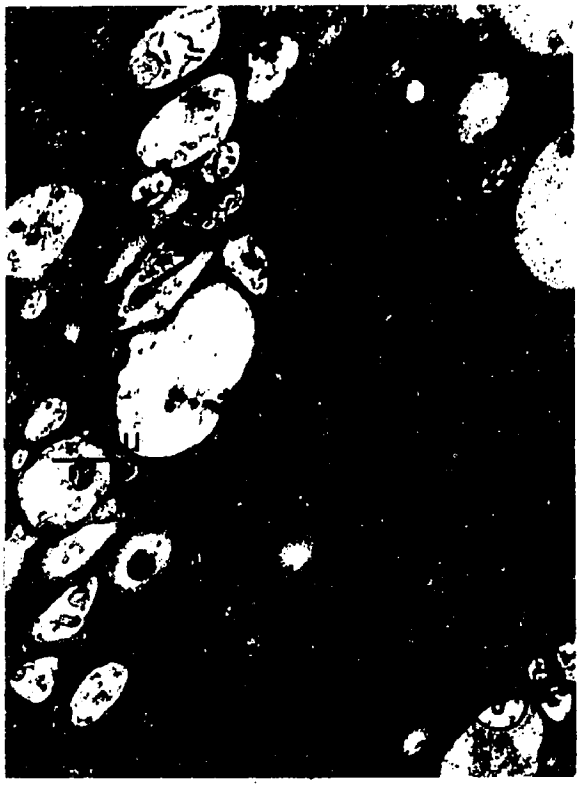
Fig. 1. EM micrograph of a division nucleus. Furrowing is almost complete. The chromatin mass has taken on an amorphous appearance.

Fig. 2. EM micrograph of late telophase nucleus. Numerous infoldings of the nuclear envelope are evident. Spindle fibers are demonstrated.

Fig. 3. EM micrograph of a post division nucleus. The chromatin and nuclear membrane are intimately related, (a). Vacuoles containing old nuclear membranes indicate various stages of digestion (b).

Fig. 4. EM micrograph of a post division nucleus. Excess membranes are indicated in the cytoplasm.

Plate V

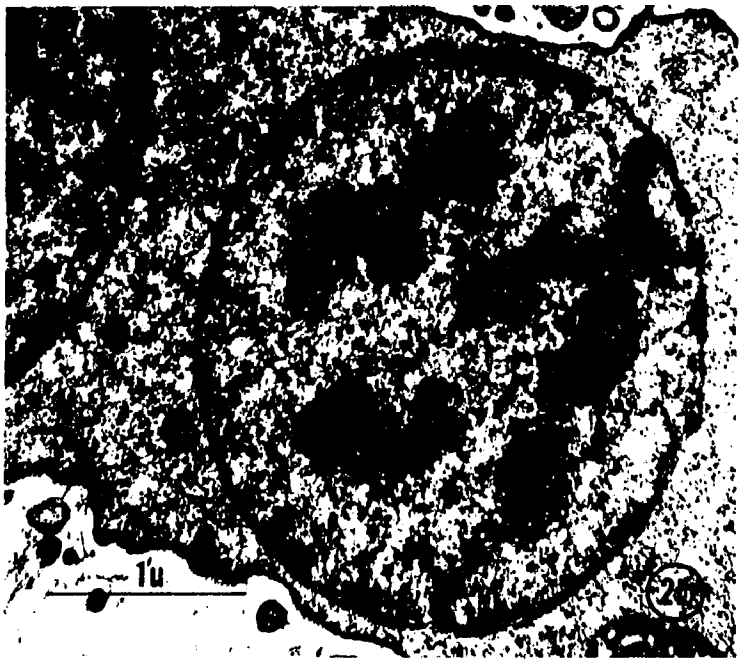


Explanation of Figures in Plate VI

Fig. 1. EM micrograph of an early interphase nucleus. The chromatin mass diffuses into an enlarging nucleus. The nucleus is still irregularly shaped.

Fig. 2a & b. EM micrographs of transcribing nuclei. The nucleolus is observed reforming and large amounts of chromatin are found adjacent to the inner nuclear membrane. This event is seen to occur approximately thirty minutes after division.

Plate VI

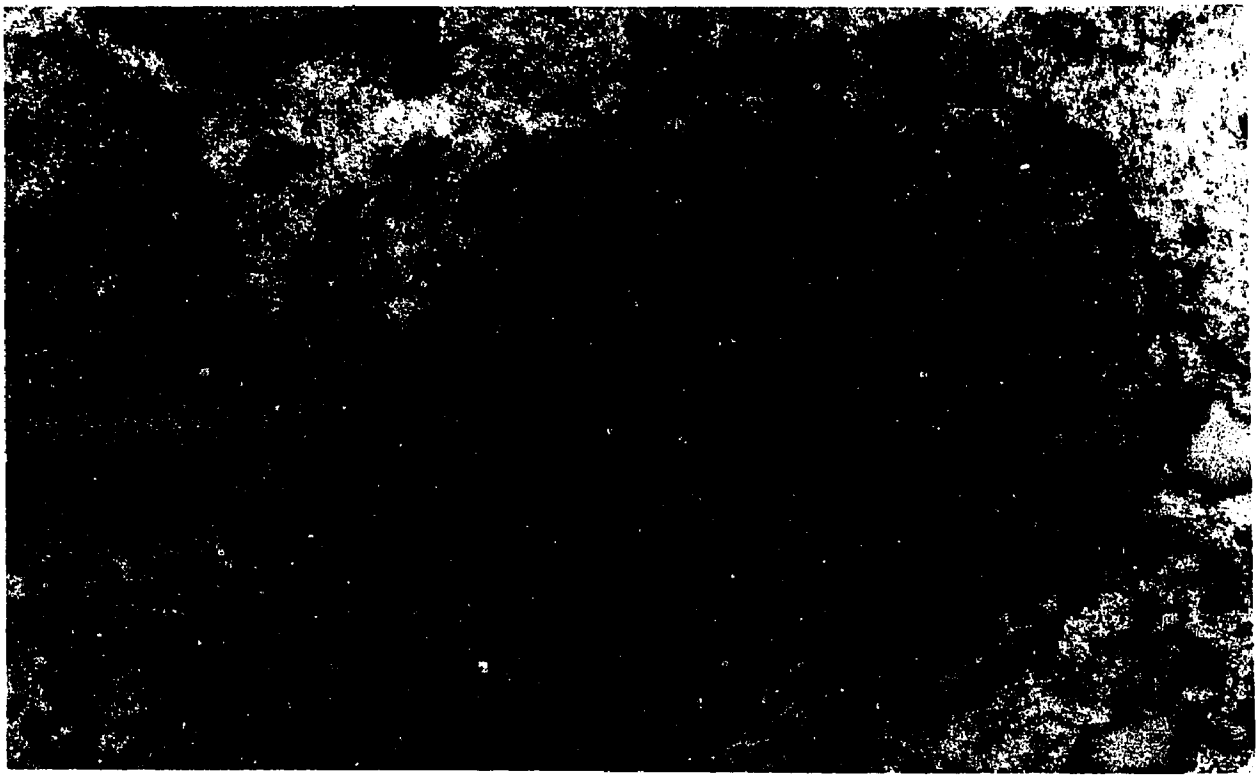


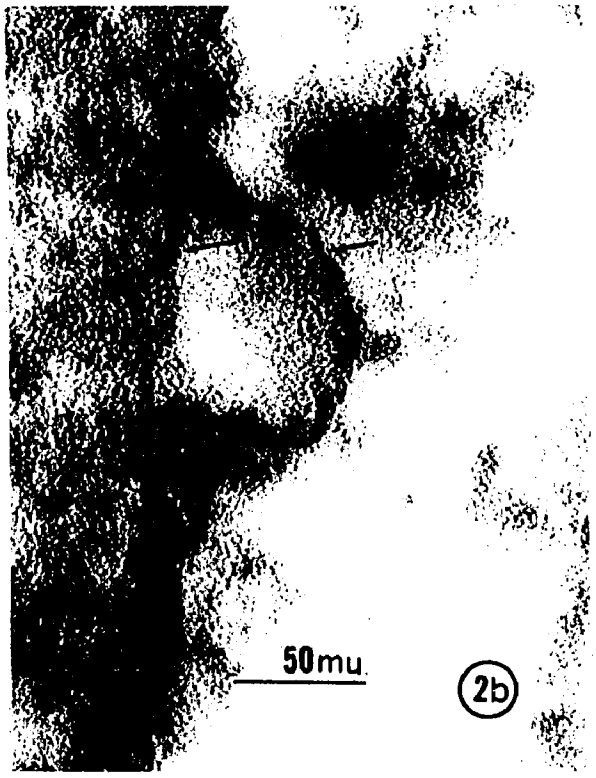
Explanation of Figures in Plate VII

Fig. 1. EM micrograph of bleb formation, $KMnO_4$ fixation. Several eruptions involving only the outer membrane of the nuclear envelope are observed. Vesicles of presumed bleb origin are indicated.

Fig. 2 a, b. Enlargement of Fig. 1. Outpocketings of only the outer membrane are emphasized. The typical unit membrane of the individual membranes is shown.

Plate VII



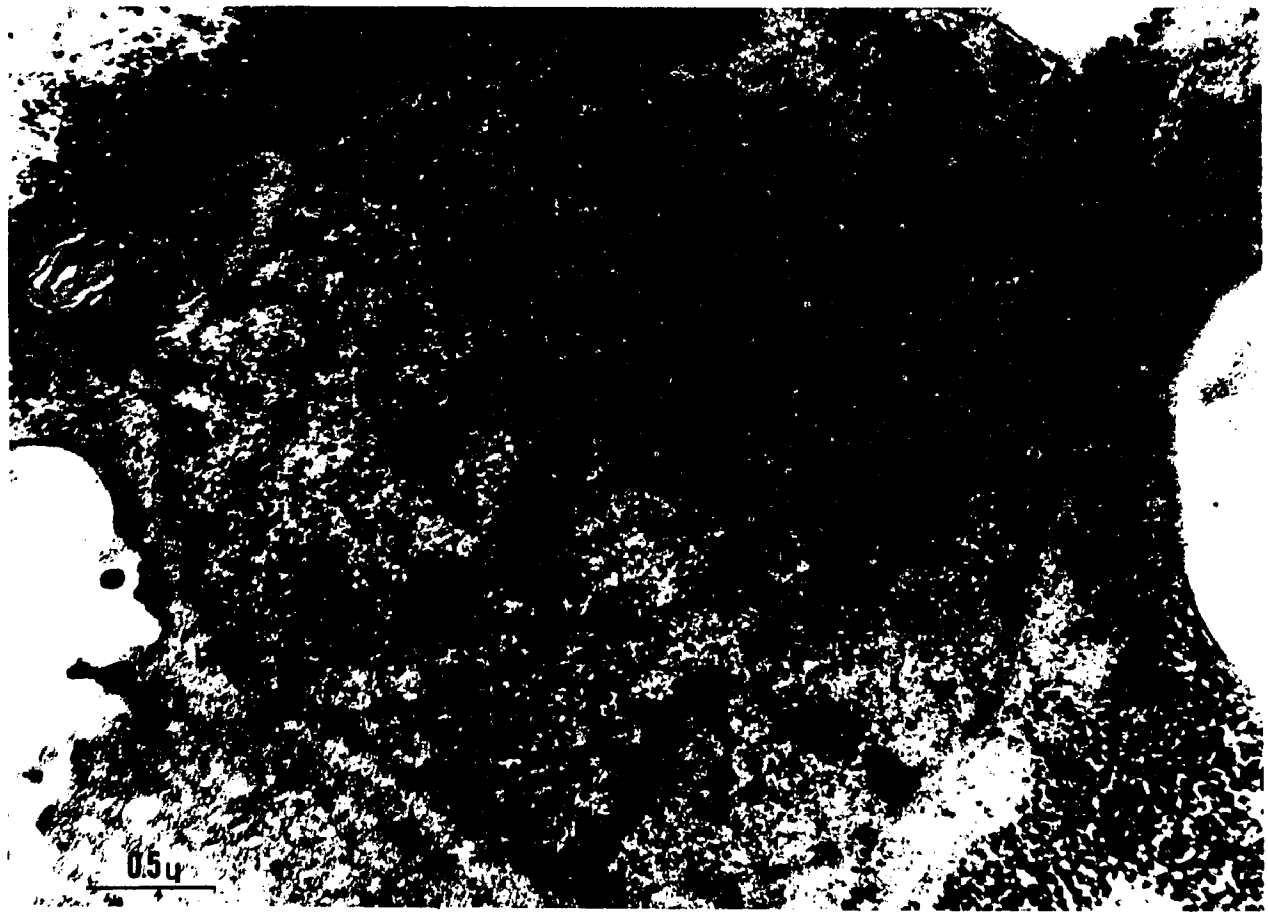


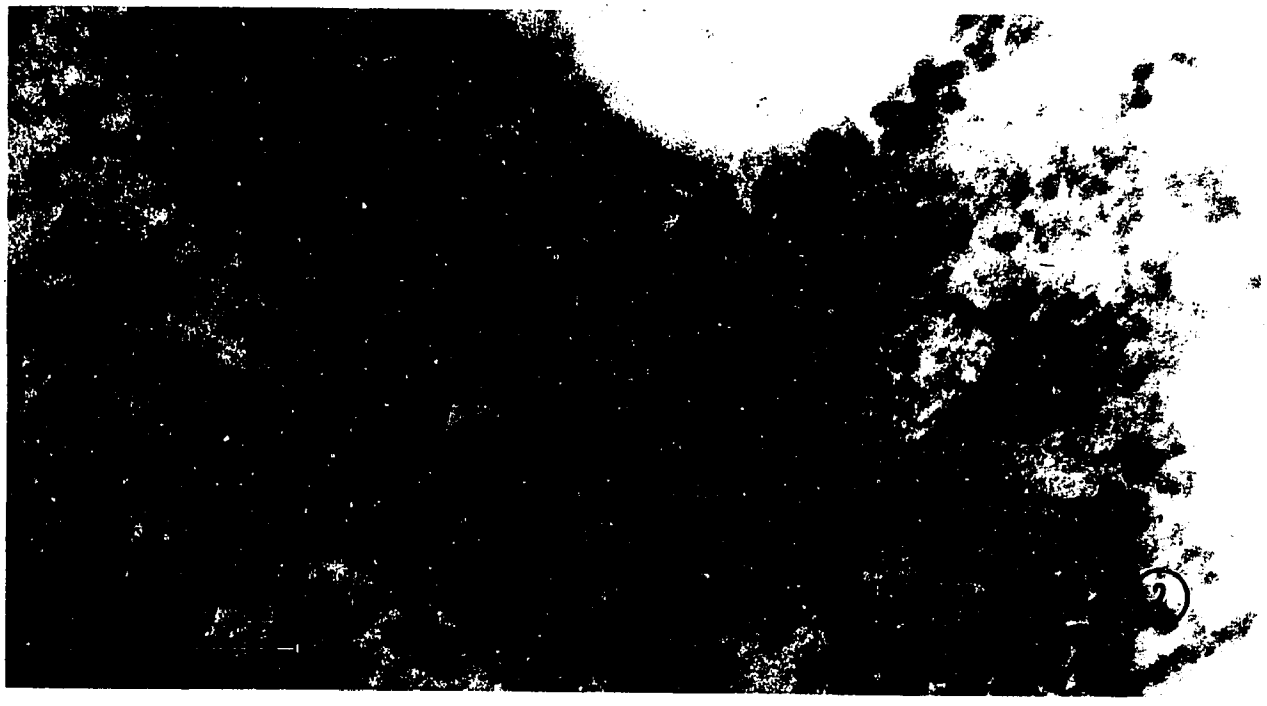
Explanation of Figures in Plate VIII

Fig. 1. EM micrograph of bleb formation. GA-osmium fixation. The bleb at A appears to be rounded, enclosed by a single membrane. A double, elongated bleb appears at B.

Fig. 2. Enlargement of Fig. 1. The constriction at A indicates the bleb is soon to be pinched off. The laminar structure of bleb contents is shown at B.

Plate VIII





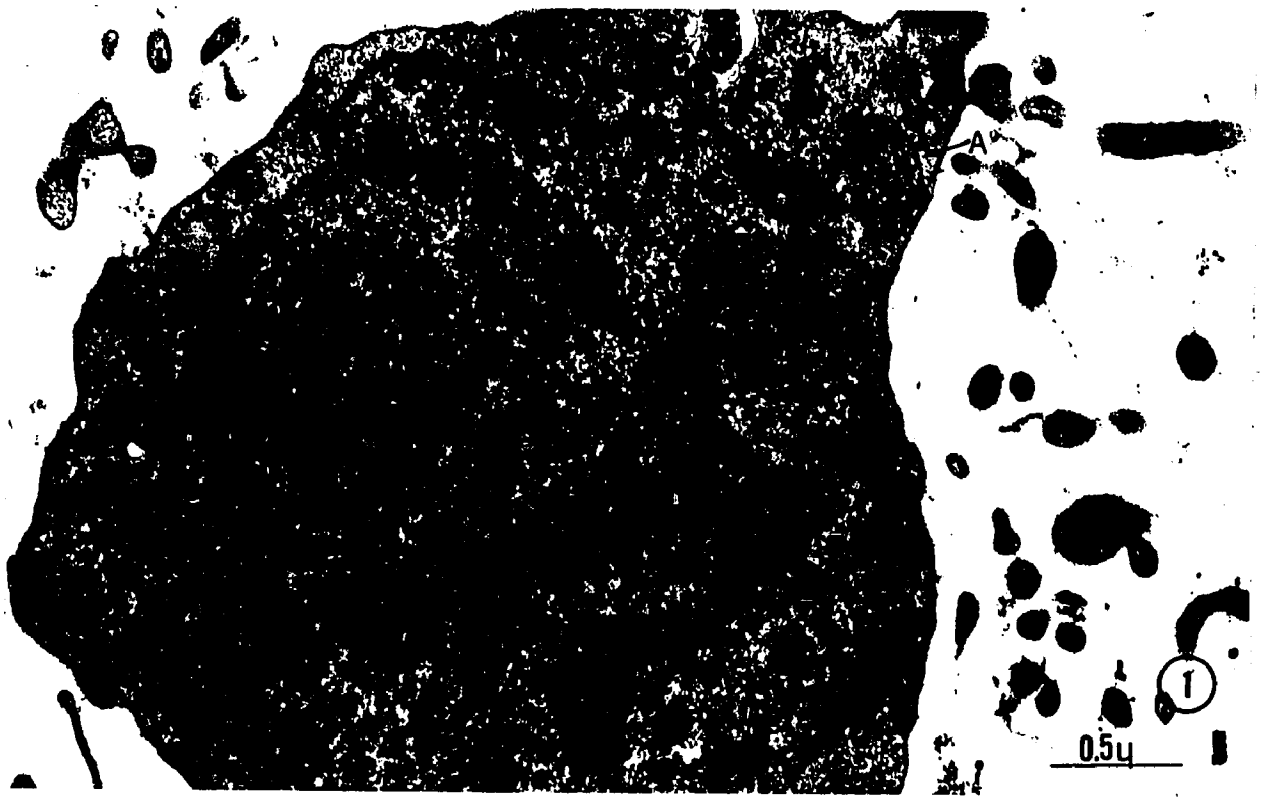
Explanation of Figures in Plate IX

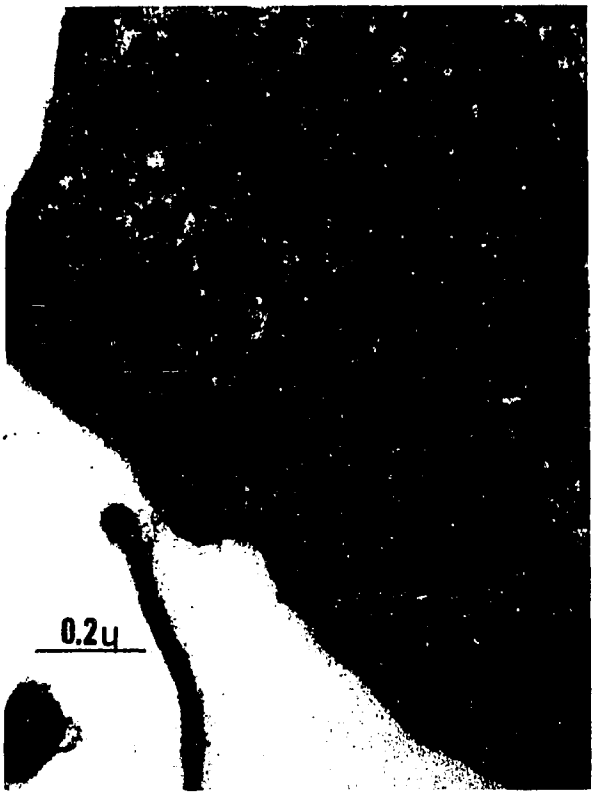
Fig. 1. EM micrograph of bleb, GA-osmium fixation. A budding of the bleb appears at A. Several vesicles of presumed nuclear origin are shown at B.

Fig. 2. An enlargement of the vesicles in Figure 1 at B. A single membrane encloses these structures. The contents appear to have the same density as the cytoplasm.

Fig. 3. An enlargement of the bleb in Fig. 1 at A. Budding or fusion of blebs is shown. The laminar structure of bleb contents is also indicated.

Plate IX





Explanation of Figures in Plate X

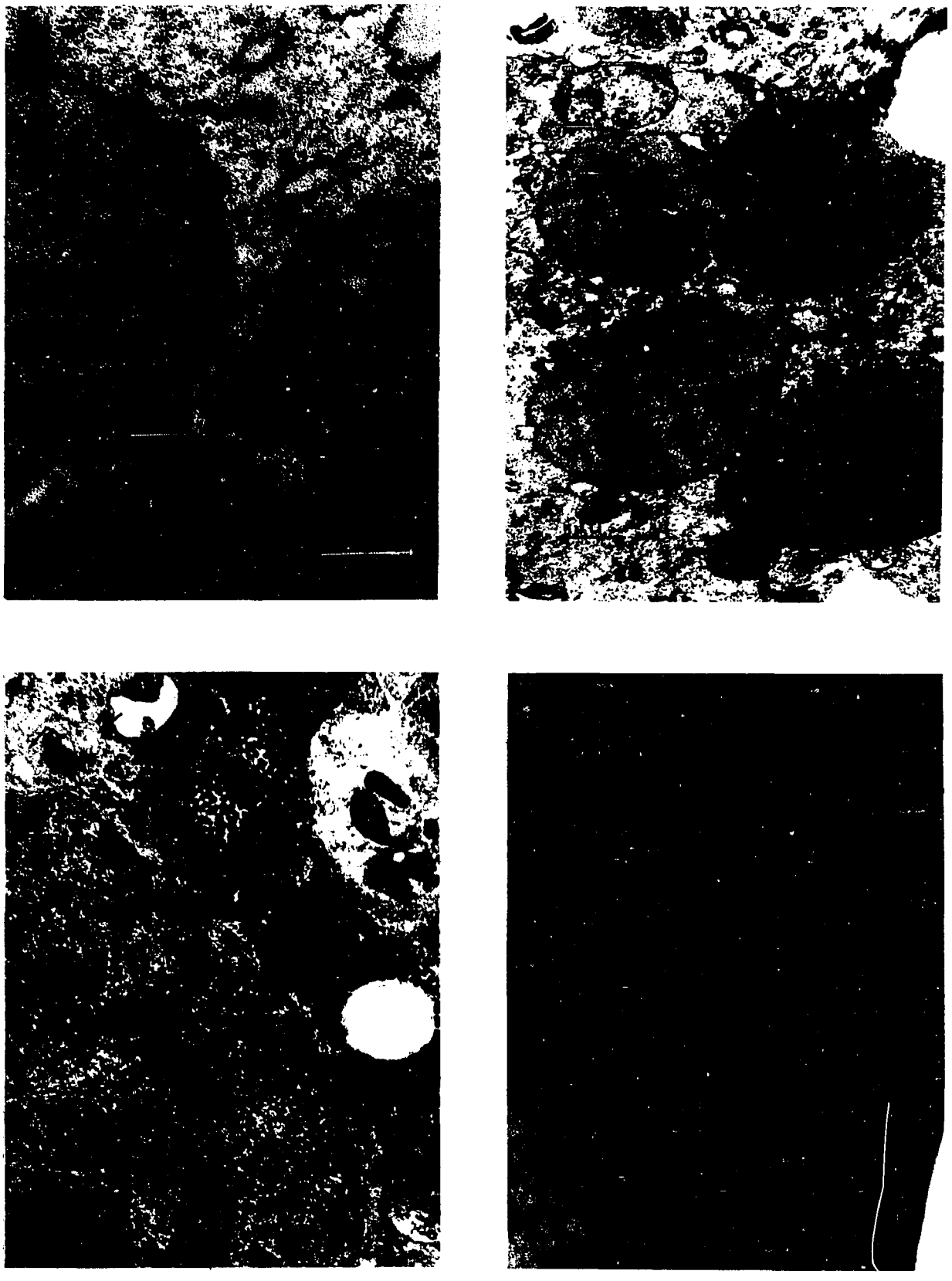
Fig. 1. EM micrograph of bleb formation. A stalked bleb appears to be pinched off at a. ($KMnO_4$ fixation.)

Fig. 2. EM micrograph of several nuclei demonstrating blebbing. Note the structured vesicles in the cytoplasm. ($KMnO_4$ fixation.)

Fig. 3. EM micrograph of surface activity in interphase nuclei. Several blebs are shown. (GA-osmium fixation.)

Fig. 4. EM micrograph of surface activity in interphase nuclei. Fusion of vesicles is indicated at a. An elongated vesicle possibly resulting from fusion is shown at b. A mitochondrion appears to be attached to the outer nuclear membrane at c.

Plate X



Explanation of Figures in Plate XI

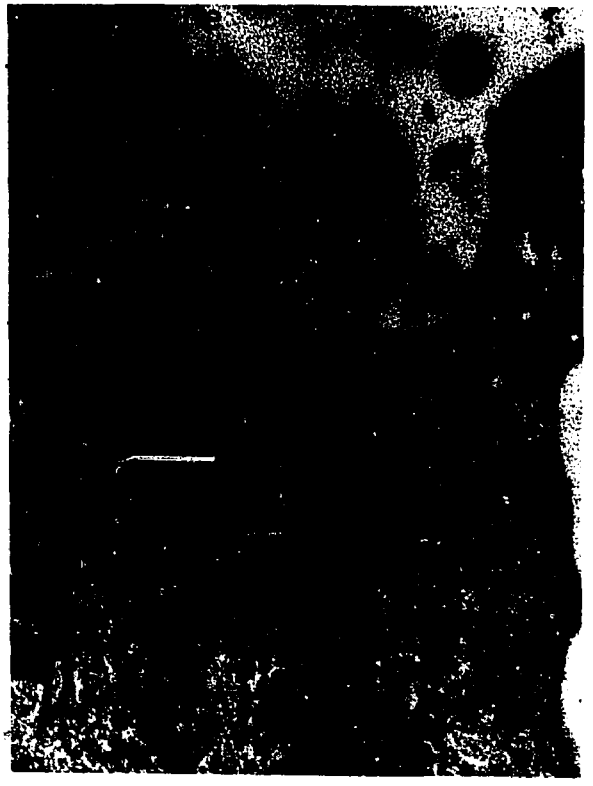
Fig. 1. EM micrograph of a membranous whorl attached to the nuclear membrane. Several vesicles are also shown. A membranous attachment to the whorl is suggested at a. A whorl contacts the vesicle at b.

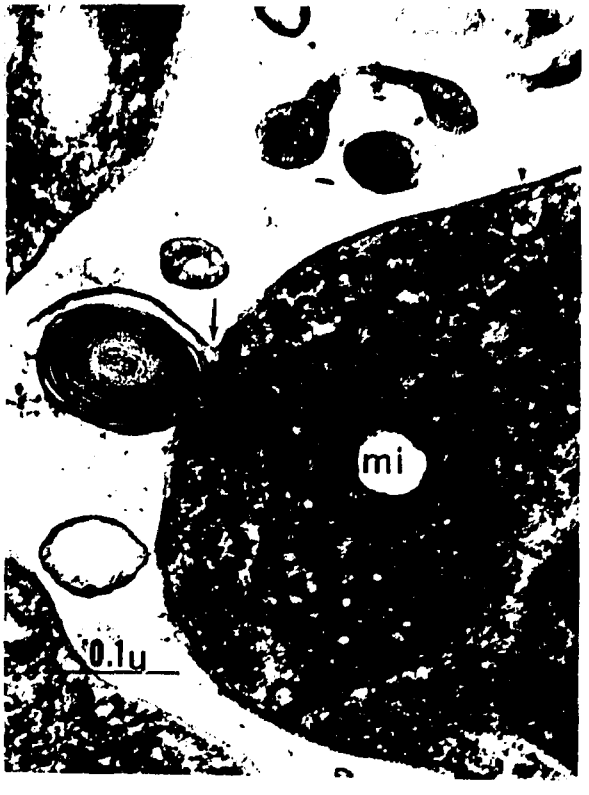
Fig. 2. EM micrograph of a cytoplasmic whorl. Note the unit membrane structure of its constituents.

Fig. 3. EM micrograph of a membranous whorl in association with a mitochondrion.

Fig. 4. EM micrograph of a whorl associated with a fragmentary mitochondrion and two structured vesicles.

Plate XI





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