DYNAMICS OF ACRIDINE ORANGE-CELL INTERACTION

II. Dye-Induced Ultrastructural Changes in Multivesicular Bodies (Acridine Orange Particles)

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ABSTRACT

The brilliantly fluorescent cytoplasmic particles that accumulate in HeLa cells treated with acridine orange, previously referred to as acridine orange particles, are shown to represent acid phosphatase–positive multivesicular bodies (MVB). Dynamic changes in the ultrastructure of these organelles may be induced by varying the concentration of extracellular dye and the length of exposure to the dye. Low concentrations of dye for long intervals of time lead to marked hypertrophy of the MVB and accumulation of myelin figures within them, the acid phosphatase activity being retained. High concentrations of dye for short time intervals lead initially to a diffuse distribution of dye through out the cytoplasm (cytoplasmic reddening) as viewed in the fluorescence microscope. When cells are stained in this way and incubated in a dye-free medium, the diffusely distributed dye is segregated into MVB within 1 hour. Ultrastructurally, these MVB show dilatation but no myelin figures. The process of dye segregation is energy dependent and will not occur in starved cells. This energy dependence and the occurrence of segregation *via* dilatation of the MVB rather than ultrastructural transformation, *i.e.* formation of new binding sites, suggests that the process involves an active transport mechanism. Of the various energy sources supplied to starved cells, only glucose, mannose, and pyruvate are fully effective in supporting dye segregation. Blockage of the tricarboxylic acid cycle with malonate inhibits the effects of pyruvate but not of glucose, demonstrating the efficacy of both the tricarboxylic acid and glycolytic cycles in supplying energy for the process.

In an earlier report, the dynamics of the *in vitro* intracellular accumulation of acridine orange (AO) were discussed in detail (1). It was noted that the ultimate disposition of the stain within the cell was predictably contingent upon interactions among several mutually interdependent parameters, including pH, time, temperature, dye concentration, and cell energy expenditure. We, as well as others, have found the accumulation of the dye in brilliantly fluorescent juxtanuclear granules to be the most striking characteristic of the vital staining pattern. These granules have been designated acridine orange particles (AOP). During the past decade, many and conflicting interpretations of them have been offered (1–6). Their red fluorescence has inspired the suggestion by some that they contain RNA since RNA stains red in the fixed cell (2). Others

have variously assumed that they are mitochondria (3), a cellular response to injury (4), coacervates (5), or ingested aggregates of dye (6). Numerous other interpretations not included here have been offered, and much of this literature is reviewed by Wittekind (5). We have previously affirmed that these granules are a manifestation of normally present *multivesicular bodies* (MVB) (1). The present report establishes the equivalence of AOP and multivesicular bodies. In addition, the dynamic effects of AO on the morphological and histochemical aspects of the intracellular organelles in which the dye accumulates are described, from the points of view of both light and electron microscopy. Since energy-yielding reactions are required to induce some of the structural changes observed in the AOP (1), the energy sources capable of supporting these changes also have been investigated.

METHODS AND MATERIALS

HeLa cells (S3 strain) cultured as monolayers were used. The culture medium was Parker's M-199 (Microbiological Associates, Bethesda, Maryland) supplemented with 15 per cent fetal calf serum.

CELL MORPHOLOGY: Cells were grown in specially designed perfusion chambers (7) until a moderate cell density was reached ($4 \times 10^4/cm^2$). The chamber was then placed on a warming stage thermostatically set at 37°C, following which a specific field was photographed with Zeiss phase contrast optics. Acridine orange diluted 1:1 million was slowly perfused through the chamber at a rate of about 3 ml/hour and photographs were taken at 1 hour intervals for 5 hours. A green filter was interposed between cells and light source to minimize photodynamic effects. The effects of AO on general morphology of the cell were noted. At the conclusion of a run a fluorescence micrograph was taken. Exposure to the intense, near UV wave lengths ordinarily killed the cell, and therefore this last micrograph in the series was not taken until the cell was to be discarded.

HISTOCHEMISTRY: Monolayers spread on 15 mm coverslips (10^5 cells) were placed in a large volume of AO (about 50 ml). In this way the AO dilution, initially adjusted to 1:1 million, remained constant over the course of the experiment, notwithstanding extraction of the dye from the medium by the cells. After 48 hours the cells were fixed in calcium-formalin (8) for 30 minutes, and acid phosphatase localization was determined with the modified Gomori technique (8). The cells were incubated in the β-glycerolphosphate substrate plus lead nitrate for 5 hours at pH 5 and then "developed" with 2 per cent ammonium sulfide. They were then counterstained with methylene blue (1 per cent) for 10 minutes. Cells unexposed to AO were treated similarly for comparative purposes.

ELECTRON MICROSCOPY: Spread monolayers, exposed to AO as described above for intervals of 30 minutes to 10 days, were fixed according to the following schedule: 5 per cent glutaraldehyde in isotonic Tyrode's solution for 5 minutes at pH 6.5, followed by 1 per cent OsO_4 in ½ isotonic Tyrode's solution for 5 minutes at pH 6.5. Finally, 1 per cent OsO_4 in ½ isotonic Tyrode's solution plus 1 per cent formalin and 2 per cent ethyl alcohol for 35 minutes at pH 8.4. This solution is prepared fresh, and appears to improve the preservation of tissue culture cells. Dehydration in methyl-Cellosolve was followed by embedding in epoxy resin. Sections were cut on the Porter-Blum microtome and double-stained, first with saturated aqueous uranyl acetate in 40 per cent ethanol for 1 hour and then with lead hydroxide for 15 minutes. A Siemens Elmiskop I was used for the micrography.

ENERGY SOURCES FOR AOP TRANSFORMATIONS: Previous work has shown that segregation of AO in AOP following dye-induced cytoplasmic reddening (CR) requires an external energy source (1). The energy sources capable of supporting this process of dye segregation with its consequent AOP (MVB) transformation were studied by inducing cytoplasmic reddening, in cells starved for 24 hours, by exposing them to a high concentration of AO (1:20,000) for 1½ minutes. This treatment was followed by incubation in a dye-free, glucose-free, balanced salt solution to which the test substance was added. The presence or absence of dye segregation in AOP was studied as a function of time and of the concentration of the substance tested.

RESULTS

CELL MORPHOLOGY: Cells grown in AO (1:1 million) and examined with phase contrast optics show the changes illustrated in Figs. 1 *a* and 1 *b*. After 15 minutes the cells appear exactly as the normal control in Fig. 1 *a* and the figure is therefore omitted. However, fluorescence microscopy reveals that the cell contains considerable AO at this time (1), with most of the dye being localized in the juxtanuclear AOP. After 5 hours in AO the nuclei have swollen and the juxtanuclear granules, previously invisible in phase contrast, have become visible as black dots. Of particular interest is the decrease in the size of the nucleoli. This occurrence is reminiscent of changes noted when cells are treated with the RNA inhibitor, actinomycin D, and is consistent with the finding that cells in dilute AO do not incorporate tritiated uridine (9). Fig. 1

c is a fluorescence micrograph of the same cells, showing the identity relationship between the black dots seen in the previous figure and the intensely fluorescent AOP. Although these cells were not followed for longer than 5 hours, extrapolation from Fig. 1 *b* to Fig. 3 *a* (see below) is not difficult; *i.e.*, the changes shown after 5 hours are exaggerated after 2 days and the AOP occupy a large fraction of the cytoplasm. It is noteworthy that no further changes occur during ensuing days in AO. Even after 2 weeks the cells are hardly distinguishable from those shown in Fig. 3 *a*, with neither cell division nor degeneration taking place.

HISTOCHEMISTRY: Fig. 2 *a* illustrates the distribution of acid phosphatase-positive granules in the normal HeLa cell. These are small, approximately 1 micron particles with a polarized juxtanuclear distribution. The remainder of the cell is essentially negative for the reaction. These granules are not visible in phase contrast in the living cell but their distribution coincides with AOP seen in the fluorescence microscope following transient vital staining with dilute AO (*cf.* Fig. 2 *b*). On the other hand, the AOP seen after 2 days in AO are large, 1 to 3 micron particles clearly visible in phase contrast (Fig. 3 *a*). Fig. 3 *b* is a fluorescence micrograph of the same cells and Fig. 3 *c* pictures these cells stained histochemically for acid phosphatase. It is clear that the AOP and the acid phosphatase-positive granules may be superimposed exactly. While we have not made accurate counts of the AOP, we have obtained the impression that their number does not increase greatly during lengthy exposure to AO (compare Figs. 2 *b* and 3 *b*). The striking changes are in their size, fluorescence intensity and, as discussed below, their ultrastructure.

ELECTRON MICROSCOPY: Fig. 4 is an electron micrograph of an *untreated* HeLa cell. Mitochondria, Golgi vesicles and other cell organelles are apparent. The MVB are seen bounded by unit membranes and containing from one to several relatively uniform vesicles whose bounding membranes also appear of the same construction. Lysosomes as originally described by Novikoff *et al.* (10) were not observed.[1]

Fig. 5 is an electron micrograph of a cell exposed to AO (1 : 1 million dilution) for *2 days*. Most striking is the mass of cytoplasmic inclusions which undoubtedly are the electron microscopic representations of the AOP seen in phase contrast. Although a dynamic interpretation of electron micrographs is necessarily subject to ambiguity, it would appear that Fig. 5 contains MVB in several stages of transformation. Thus, at *a* is seen a typical MVB as described for the untreated cell; at *b* an MVB is seen which is surrounded by a unit membrane and displays a suggestion of internal organization interspersed with vesicles; at *c* some of the vesicles appear to have expanded markedly, acquiring thickened osmiophilic walls in the process, and the MVB itself has also undergone hypertrophy. While the MVB are always surrounded by a unit membrane when they are in an "advanced" stage, at *d* one sees the often-described (13) discontinuity in the membrane of a MVB and the representation of what may be either a breakdown or formation of a new membrane. No other organelles in these AO-treated cells, including the endoplasmic reticulum, Golgi vesicles, nuclei, or mitochondria, show any remarkable changes in structure.

In Fig. 6 it is established that the thickened walls of the vesicles in the MVB are typical myelin figures composed of lamellae with a periodicity of 38 A° and thus presumably containing largely phospholipid (11).

It is of interest that after 7 days in AO the cytoplasmic inclusions undergo no major additional transformations, and the changes that are seen are a matter of degree rather than kind, as shown in Fig. 7. The walls of the expanded vesicles within MVB frequently show increased thickness. Intermediate forms of the MVB are less in evidence than in the 2-day sample, although untransformed examples of MVB are still seen in some cells (arrows). Aside from the altered MVB, no recognizable disorganization or deterioration in cell ultrastructure is visible. The absence of division in these cells with no ultrastructural degeneration is considered unusual in *in vitro* cell culture, although its significance in this case requires further study.[2]

[1] If a lysosome is defined by its content of acid phosphatase, then, as suggested by Novikoff (19), the MVB are a type of lysosome. We have also found these bodies to contain esterases.

[2] Most fields did not contain the large number of MVB shown in Fig. 7; however, the explanation for those that did lies in the increase in size of the MVB during exposure to AO. Thus, each individual MVB will be present in a greater proportion of the total sections through the cell than is the case for the MVB in the untreated cell. The end result is that any one section of the AO-treated cell is more likely to have a large number of MVB than a com-

In Fig. 8 is illustrated the ultrastructure of a cell which was initially exposed to a high concentration of AO resulting in diffuse cytoplasmic reddening and was then incubated in fresh, dye-free medium for 1 hour. This sequence of treatments leads to rapid segregation of most of the dye in the AOP (see Figs. 9 *a* and 9 *b* and remarks below). It is emphasized that the fluorescence microscopic picture of the AOP in this cell does not differ markedly from that noted in Fig. 3 *b* (1). The rapid accumulation of AO in AOP stimulated by cytoplasmic reddening results in an ultrastructural response totally different from that obtained when the cell is exposed to dilute AO and accumulation takes place over several days. In the former case there is no transformation of fine structure of the MVB, but rather a marked dilatation of MVB probably representating engorgement with dye. Vesicles which normally are dispersed throughout the MVB frequently are juxtaposed to the bounding membrane of the expanded strucutre (Fig. 8 *b*).

ENERGY SOURCES FOR AOP TRANSFORMATIONS: When starved HeLa cells are stained with AO in high concentration (1:20,000) for 1½ minutes, the entire cytoplasm stains red, and there is no preferential localization of dye in specific cellular organelles (Fig. 9 *a*). Incubation of these starved cells for 1 hour in dye-free balanced salt solution containing glucose results in segregation of the dye in AOP (Fig. 9 *b*), while omission of the glucose leads to complete cellular degeneration within a short time.[3] This result is consistent with that obtained by Weissman (16) on ascites tumor cells, and this earlier paper should be consulted for a detailed discussion. This technique of establishing which compounds could induce dye segregation was employed as a convenient and rapid means of evaluating the efficiency of external energy sources which the cell might utilize to induce the AOP transformations discussed above. Table I lists the compounds tested for their ability to support the dye segregation process; *i.e.*, the transformation of the starved cell from the state shown in Fig. 9 *a* to that shown in Fig. 9 *b*. Few compounds are able to substitute for glucose. These include mannose, pyruvate, and, to a lesser extent, galactose. It has been found that fructose is ineffective even in a concentration three times the usual glucose concentration and 25 times the minimum concentration at which glucose is effective.

(*continued from preceding page*)

parable section of an untreated cell. This is obviously true even if the total number of MVB per cell is the same in both the treated and untreated samples.

[3] The same sequence of CR and dye concentration in AOP is seen in the normal unstarved cell, except that in the latter the omission of glucose from the incubation medium immediately after staining does not inhibit dye segregation.

FIGURE 1 *a* Untreated HeLa cells. Phase contrast. × 360.

FIGURE 1 *b* Same cells exposed to acridine orange, 1:1 million dilution, pH 7, 37°C for 5 hours. Nucleoli have decreased in size; nuclei have swollen and juxtanuclear dense granules have made their appearance. Phase contrast. × 360.

FIGURE 1 *c* Fluorescence micrograph of same cells that appear in Figs. 1 *a* and *b*, showing identity relationship between AOP and black granules appearing in phase contrast. × 360.

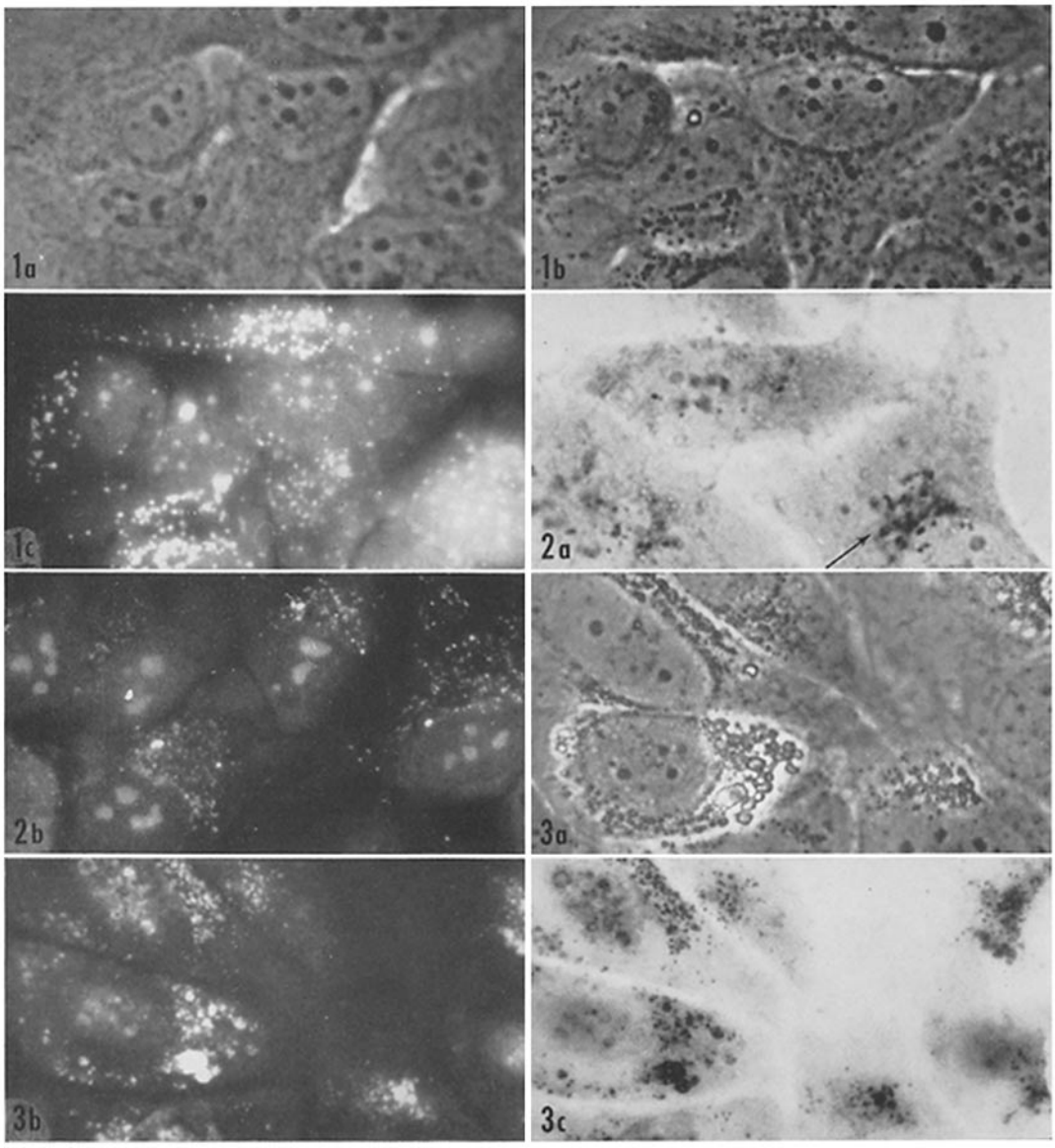
FIGURE 2 *a* Light micrograph of untreated HeLa cells stained for acid phosphatase and counterstained with methylene blue. Juxtanuclear black dots represent sites of enzyme activity (arrow). × 500.

FIGURE 2 *b* Fluorescence micrograph of HeLa cells exposed to acridine orange, 1:200,000 dilution, for 45 seconds at 3°C, pH 7. × 500.

FIGURE 3 *a* HeLa cells exposed to acridine orange in growth medium, 1:1 million dilution, for 2 days, pH 7, at 37°C. AOP have markedly increased in size; nucleolar and nuclear changes noted in Fig. 1 *b* are more exaggerated here. Phase contrast. × 500.

FIGURE 3 *b* Fluorescence micrograph of same cells that are shown in Fig. 3 *a*. × 500.

FIGURE 3 *c* Same cells stained for acid phosphatase. Note coincidence of AOP and acid phosphatase-positive granules. × 500.


1a
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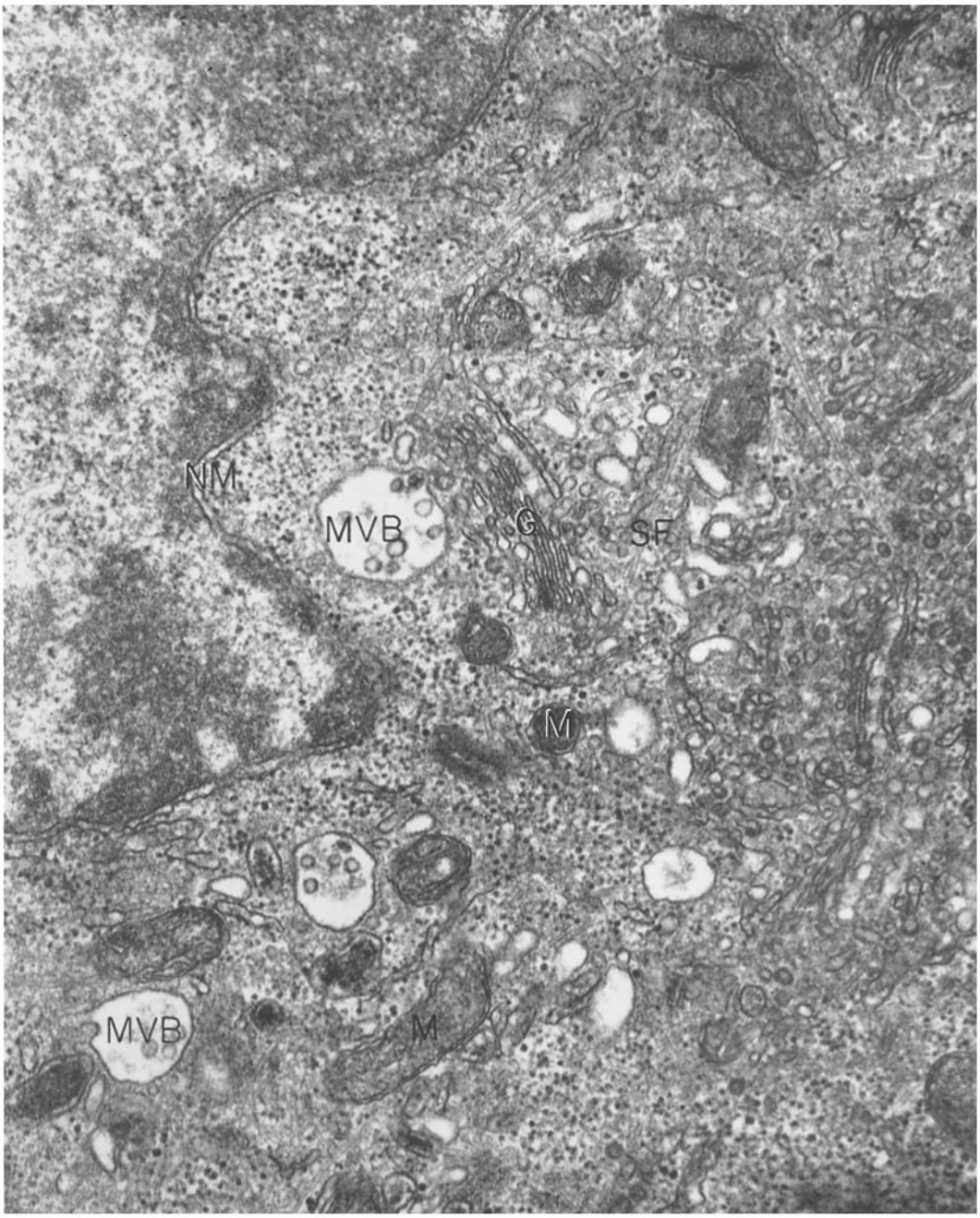

FIGURE 4 Electron micrograph of untreated HeLa cell. MVB = multivesicular body; *M*, Mitochondrion (note double membrane); *G*, Golgi complex; *NM*, nuclear membrane. × 45,000.

Addition of 6 mM malonate to equimolar glucose has no inhibitory effect on the dye segregation; however, segregation is prevented if malonate is added to a salt solution containing 12 mM pyruvate as the energy source. This suggsts that either the glycolytic or the Krebs cycle alone can supply the energy necessary for dye segregation. The inefficacy of cycle intermediates except for pyruvate

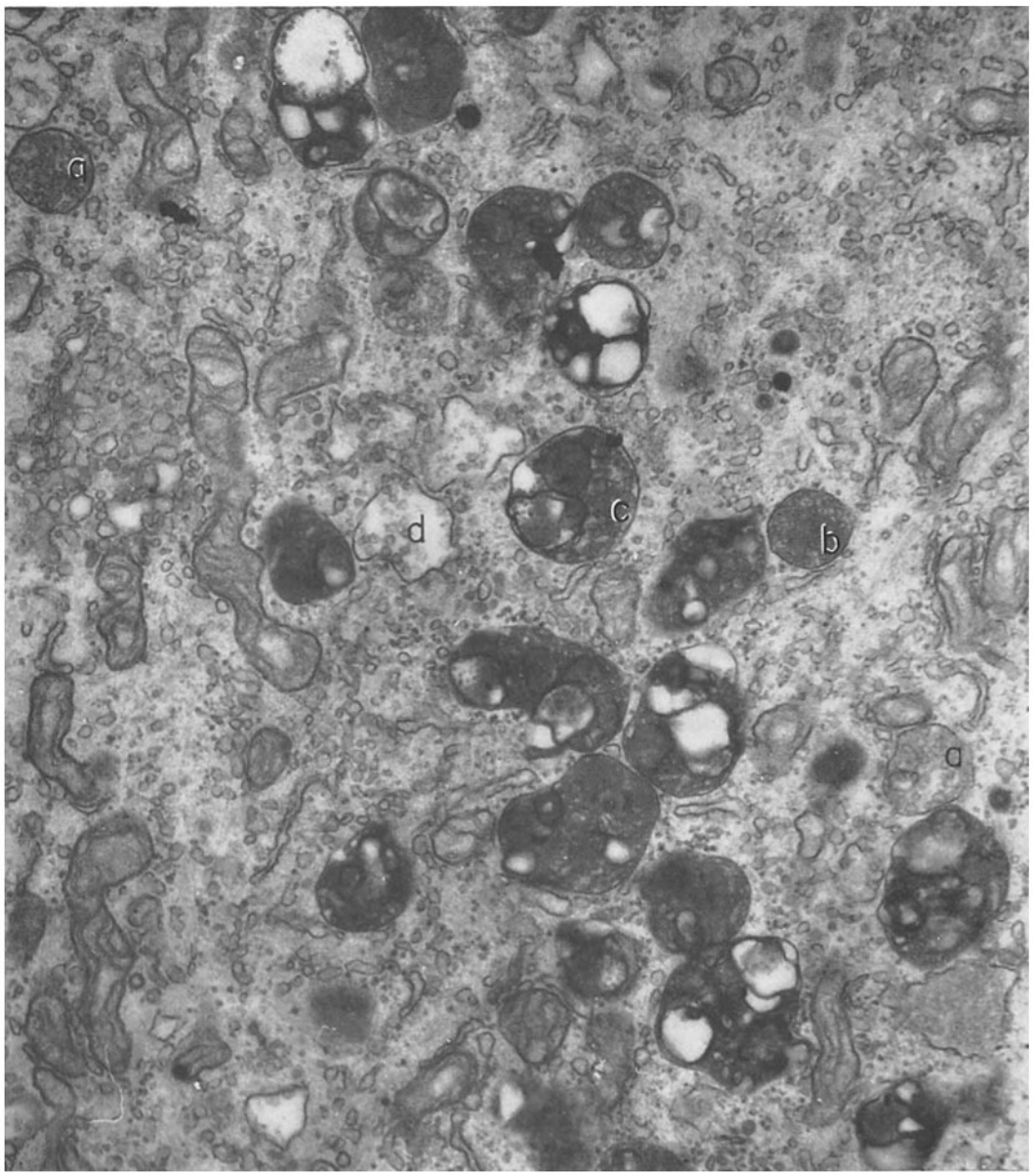

FIGURE 5 Electron micrograph of cell exposed to acridine orange, 1:1 million, for 2 days. Various transitional phases of AOP are seen (*a* to *d*). See text. × 40,000.

may be due to the cells' relative impermeability to them. However, it is necessary to stress the aspect of *relative* impermeability since glutamate and glutamine, among others which can enter the cell and which should find their way into the Krebs cycle, give negative results.

These results bear comparison with those of Eagle *et al.* (12). This group observed that several sugars and phosphorylated derivatives of glucose will substitute for glucose in supporting HeLa cell growth, although their efficiency in this role varied. Mannose and glucose were equally effective; the efficiency of fructose was, in some cases, only 5 per cent of that of glucose, and the response of galac-

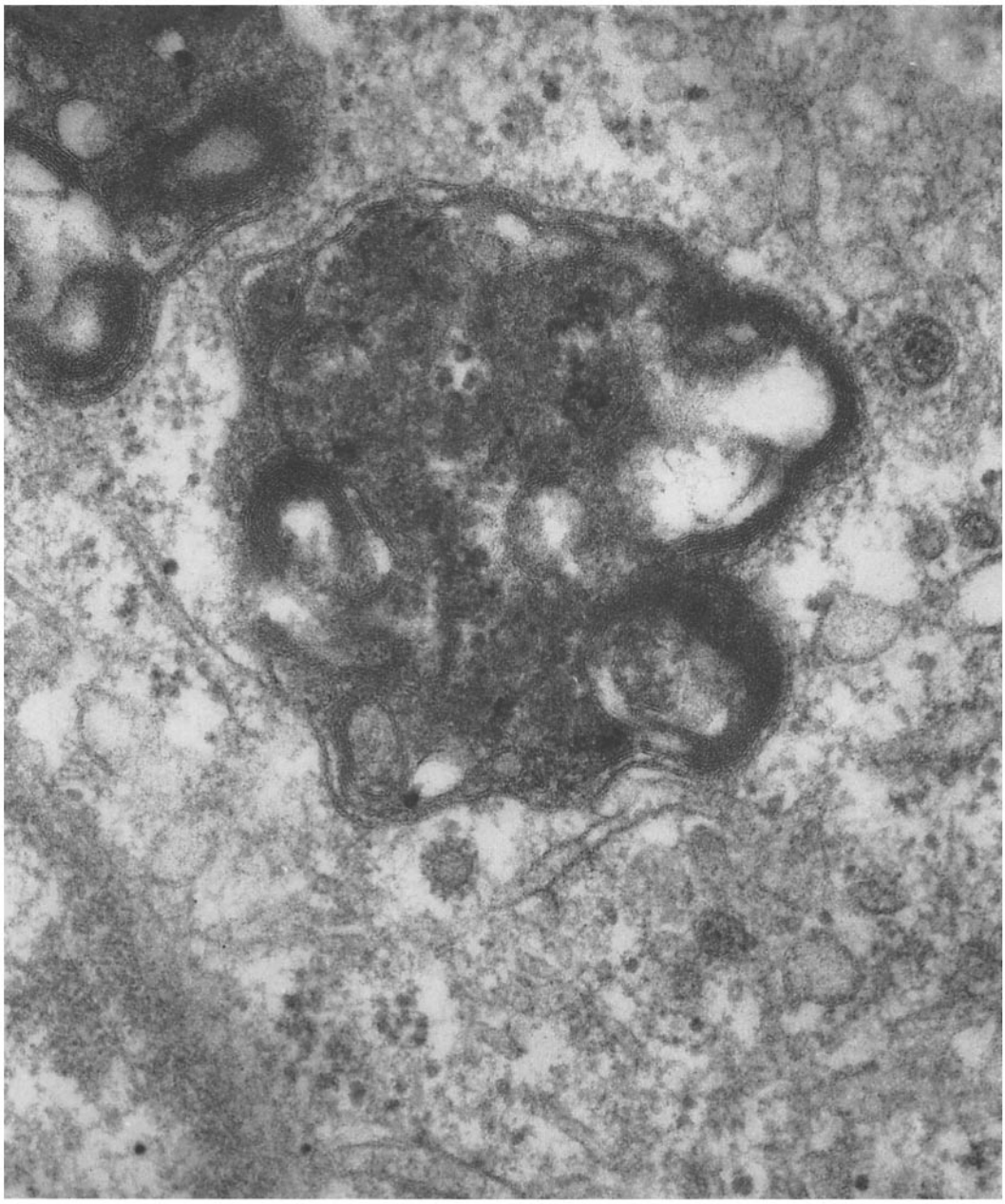

FIGURE 6 Electron micrograph of transformed MVB showing multilaminated myelin figures. × 131,000.

tose was variable. Pyruvate was found to act synergistically with galactose. It is interesting that qualitatively these results are consistent with those we have obtained employing the segregation of AO in AOP as a criterion of carbohydrate utilization. The single inconsistency lies in the absence of glucose-6-phosphate utilization by AO-stained cells. The reasons for this is not clear, although it may indicate that the transport mechanism of this compound is damaged by the test procedure. In general, however, it would seem that energy sources used by the AO-segregating system quite accurately reflect those found capable of supporting cell growth.

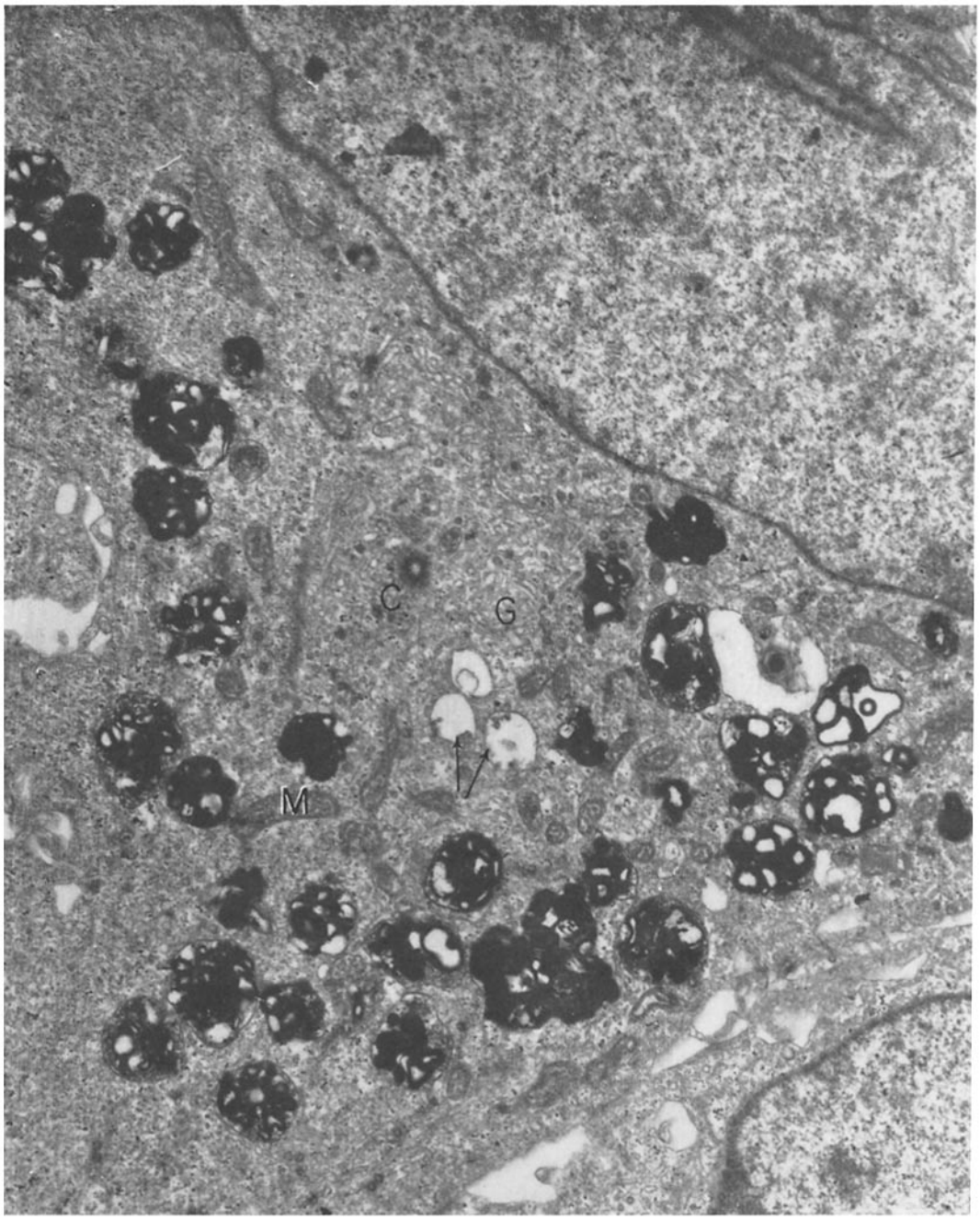

FIGURE 7 Electron micrograph of cell exposed to acridine orange, 1:1 million, for 7 days. Transformed AOP have thicker walls than after 2 days in AO, indicating increase in phospholipid content. *G*, Golgi complex; *M*, mitochondrion; *C*, centriole. Arrows indicated untransformed MVB. × 9,700.

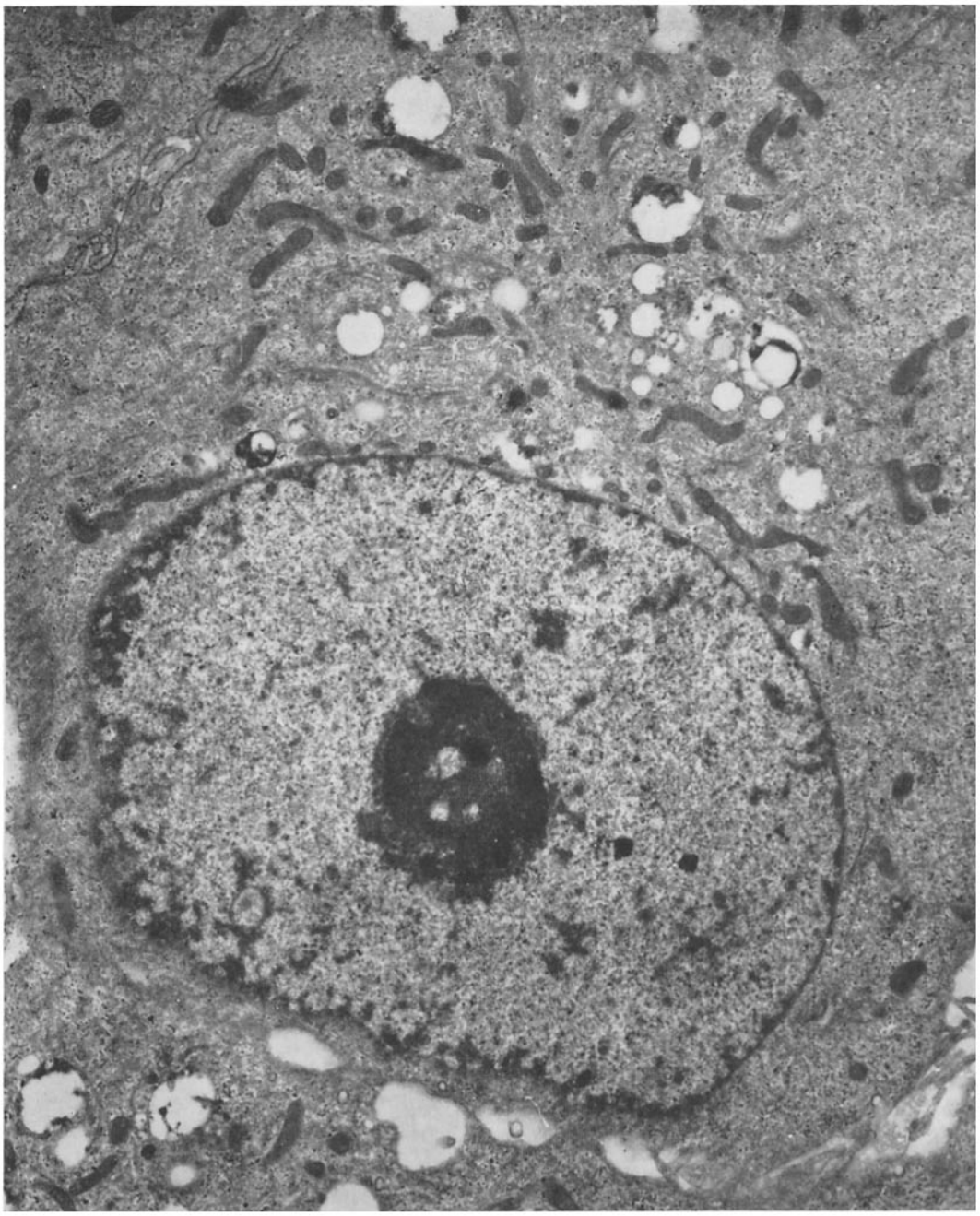

FIGURE 8 *a* Electron micrograph of cell exposed to acridine orange 1:20,000 dilution, for 1.5 minutes, followed by incubation in dye-free medium for 1 hour. Large numbers of "empty" MVB are seen, although some of them show vesicles within. No myelin figures have appeared. × 9,700.

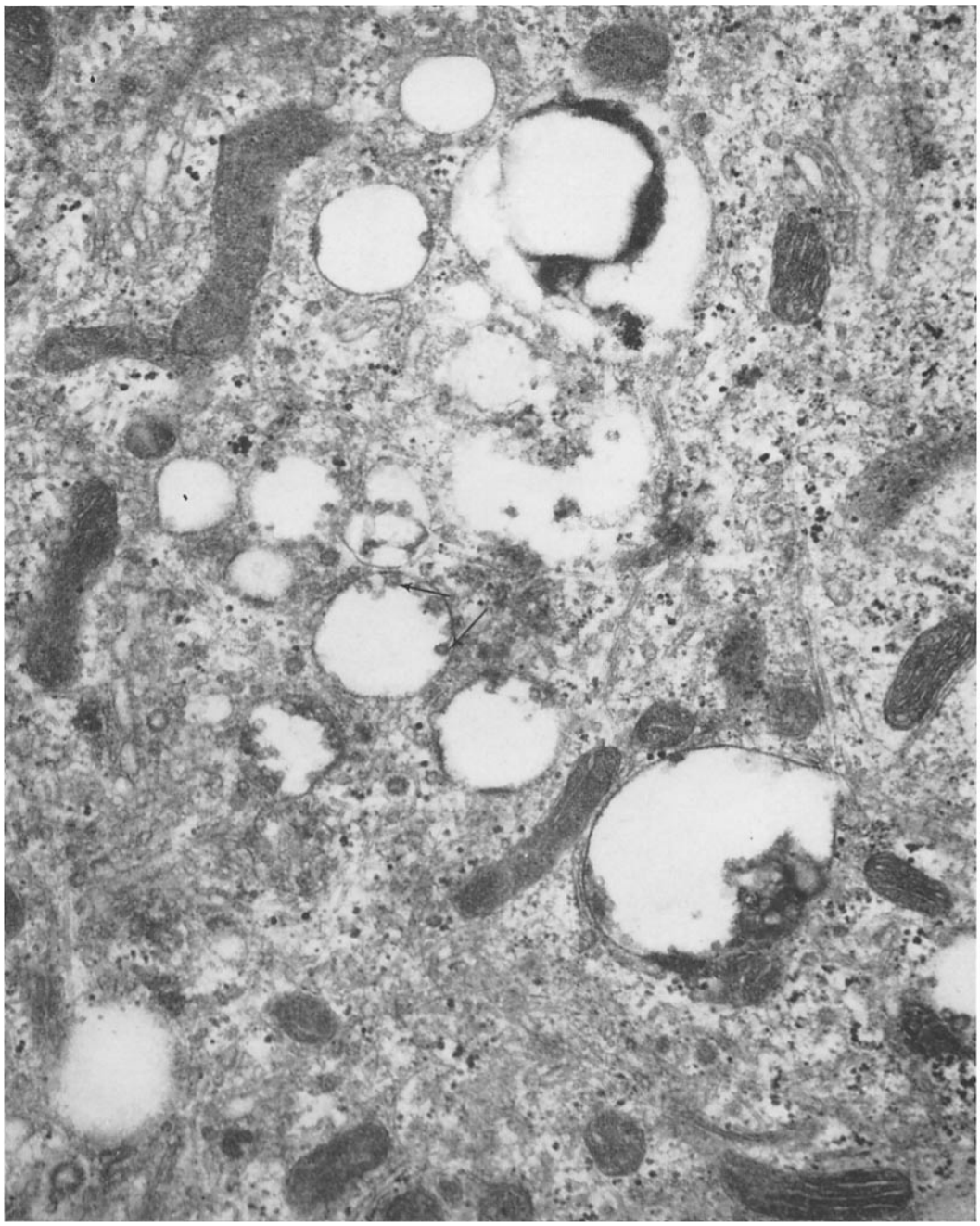

FIGURE 8 *b* Higher magnification of part of Fig. 8 *a*, showing occasional vesicles (arrows) still present in engorged MVB. × 31,500.

TABLE I

*The Effects of Various Substances on the Segregation of AO into AOP in Starved HeLa Cells**

Sugars		*Glycolytic intermediates*	
Glucose	+‡	Glucose-6-PO_4	−
Mannose	+	3-Phosphoglyceric Acid	−
Fructose	−		
Arabinose	−	3-Phosphoglyceraldehyde	−
Ribose	−		
Maltose	−	Fructose 1,6 $DiPO_4$	−
Sucrose	−	Pyruvate	+
Lactose	−	Lactate	−
Galactose	±		
Amino acids		*Krebs intermediates*	
Lysine	−	Citrate	−
Glutamic Acid	−	Oxaloacetate	−
Aspartic Acid	−	Succinate	−
Serine	−	Keto glutarate	−
Glycine	−	Fumarate	−
		Malate	−
		Miscellaneous	
		Glutamine	±
		Asparagine	−
		ATP	−

* Results in this table are for concentrations of 6 mM. Glucose and mannose were effective at concentrations as low as 0.8 mM. Fructose was ineffective at concentrations of 20 mM.

‡ + denotes utilization of substance to support dye segregation. − denotes negative effect.

DISCUSSION

The foregoing studies establish the acridine orange particles (AOP) in HeLa cells as acid phosphatase-positive multivesicular bodies (MVB). These findings are consistent with those obtained by other investigators using different cells and different vital dyes. Thus Rebhun (13) noted the accumulation of methylene blue in the MVB of oocytes, although no correlative histochemical evidence was presented. Ogawa (14) using *in vitro* hamster cultures found that neutral red accumulates in acid phosphatase-positive granules, which he designated lysosomes. This designation, however, was not supported by electron microscopy or by superposition of neutral-red granules and acid phosphatase positive-granules in the same cells.

Perhaps of greater interest than the fact of AO accumulation in the MVB are the multiple interactions between these bodies and the dye. In the previously unexposed cell the initial segregation of AO in the MVB is a passive process since it occurs rapidly at 3°C (1). This phase of intracellular staining with AO probably represents the binding of the dye by the phospholipid moiety of the MVB membranes following diffusion of dye into the cell. Since this process is passive, it is significant that the dye binds preferentially to the membranes of the MVB and not to those of the various other cytoplasmic organelles whose limiting membranes likewise contain structural phospholipid. As implied by de Duve (15), the lysosomal (MVB) membrane may be unique since it appears impervious to contact with a large repository of digestive enzymes, and this preferential binding of AO may be a hint of this structural uniqueness.

As described above, the energy-dependent transformations in the MVB that may be induced under specified environmental conditions are of two types: (*a*) the rapid segregation of AO into AOP following cytoplasmic reddening and leading to dilated MVB, and (*b*) induction of myelin figures within

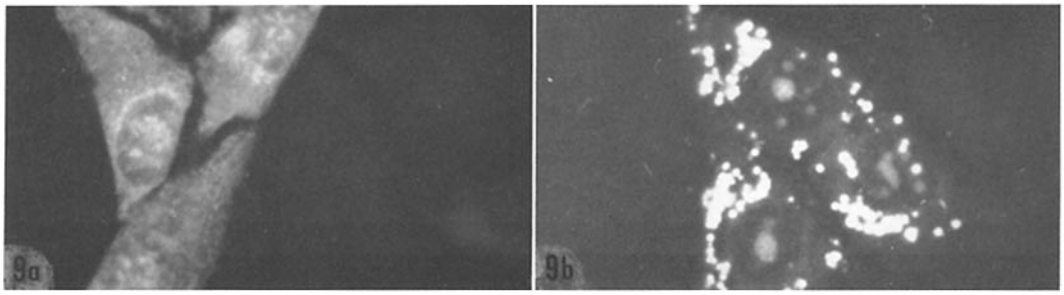

FIGURE 9 *a* Fluorescence micrograph of cells starved for 24 hours and stained with acridine orange, 1:20,000, for 1.5 minutes, pH 7. Cell stains diffusely. × 500.

FIGURE 9 *b* Fluorescence micrograph of cells treated as in Fig. 9 *a* except that following staining they were incubated in salt solution containing glucose for 1 hour. Almost all of the diffuse cytoplasmic stain has been segregated in AOP. × 500.

MVB following incubation of cells in a low concentration of AO for several days. While the formation of the myelin figures in the MVB obviously represents, in addition, the formation of increased binding sites, that this is the case in the rapid segregation of AO is less clear. There is, in fact, some evidence that the process is one of active transport. This follows from its energy dependence and the apparent absence of new fine structure in MVB during the 1 hour between cytoplasmic reddening and dye segregation. The absence of fine structure to which the AO can bind and the engorgement of the MVB may imply that the dye is free and, therefore, transported against a steep concentration gradient. Another possibility in keeping with the character of the MVB is that the dye is phagocytized and concentrated in the MVB. This would result in a decrease in the concentration of cytoplasmic AO in the immediate vicinity of the MVB, with the result that a diffusion of dye in their direction would occur and lead presumably to additional phagocytosis. Without further confirmatory evidence, however, we must defer a definite conclusion with regard to the question.

It thus appears that three distinct types of interaction occur between AO and cellular MVB. These are: (*a*) a passive, charge-mediated interaction—this takes place at 3°C; (*b*) an energy-dependent accumulation of AO in the MVB— this ensues when the concentration of cytoplasmic AO is rapidly raised to a high level by exposing the cell to a high concentration of dye for a short time and then removing it to a dye-free medium; (*c*) AO induction of phospholipid accumulation in MVB— this results from staining with AO at low concentrations for long periods of time.

The increase of phospholipid which appears in the MVB during this third type of AO-MVB interaction may be a cellular response to provide binding sites for the AO and thus increase the efficiency of the dye segregation, or it might conceivably be due to the disruption of the cell's capacity to metabolize phospholipid with a consequent accumulation of the phospholipid in the organelles that normally take part in this process. We cannot yet decide between these two possibilities. In either case, it seems that, while there is an initial passive attraction between MVB phospholipid and the AO cation, the presence of AO subsequently becomes the stimulus for the accumulation of large quantities of what is presumably phospholipid. Once formed, the myelin figures in the MVB appear to persist and, consequently, the cell accumulates large numbers of brilliantly fluorescent AOP. Under certain conditions this accumulation may be exaggerated. Preliminary experiments with HeLa cells and several types of virus suggest a hyperactivity of AOP development and accumulation in the virus-infected cell. These and other studies on the biochemical constituents of the transformed AOP may provide some answers to the significance of these dye-induced intracellular alterations.

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Note added in proof: Since this manuscript was submitted, two references have been brought to our attention which have a direct bearing on the subject matter under discussion. The first, (Koenig, 17) is an abstract suggesting that AOP are lysosomes, and the second, (Schmidt, 18), is an electron microscope study which presents evidence that various dyes and colloidal particles are segregated in "cytosomes."

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