THE FATE OF DNA-CONTAINING PARTICLES PHAGOCYTIZED BY MAMMALIAN CELLS

KLAUS BENSCH, M.D., GERALD GORDON, M.D., and LEONARD MILLER, M.D.

From Yale University School of Medicine, Department of Pathology, New Haven

ABSTRACT

Particulate DNA protein coacervates were digested immediately after being phagocytized by L strain fibroblasts in suspension culture. Enlargement of the phagocytotic vacuoles occurred simultaneously with a loss of the electron opacity of the phagocytized particles. Cytochemical reactions positive for non-specific esterase, acid phosphatase, and nucleoside phosphatase in the phagocytotic vacuoles provided additional evidence for the probability of complete hydrolysis of the phagocytized nucleoprotein.

It has been demonstrated that mammalian cells in suspension cultures will phagocytize DNA-protein coacervates (1). Failure to achieve transformation of these cells by incorporation of coacervated DNA from cells with specific biochemical properties prompted the authors to review the experimental system. Electron microscope observations of the morphologic alterations of the ingested DNA-protein particles and the associated cytochemical reactions are described in this report.

MATERIALS AND METHOD

L strain fibroblasts in suspension culture were permitted to phagocytize DNA-containing particles as reported previously (1). These particles were formed by coacervation of gelatin with DNA of high molecular weight. The stability of these particles was increased by brief exposure to 0.05 to 0.20 per cent cold glutaraldehyde. The coacervates were found to consist of 55 to 70 per cent DNA and appeared as spheres with diameters averaging from 1000 to 5000 A. The ratio of cells to particles at the start of each experiment was about 1:10. Addition of this number of particles had no ill-effect on cell growth. Cells were harvested at the start of each experiment and at intervals of ¼, ½, 2, 4, 8, 24, and 48 hours after the addition of the coacervate to the culture medium. Fixation was carried out with glutaraldehyde (2). This consisted of resuspending centrifuged cells in 1.8 to 2.0 per cent glutaraldehyde in 0.08 to 0.1 M cacodylate buffer for 4 to 10 minutes at 4°C. The osmolality of the buffered fixative was 290 milliosmols which was isotonic with the culture medium. The fixed cells were then transferred to an isoosmotic wash solution (3). Most cytochemical reactions were carried out within 1 to 2 days after the initial fixation.

The acid phosphatase reaction was carried out by incubating the cells in a Gomori medium (4, 5) at pH 5.0 to 5.3 for 15 to 25 minutes at 25°C. The incubation was followed with a rinse of 4 per cent acetic acid. This procedure did not affect the reaction product in the phagocytotic vacuoles or lysosomes (6). Controls either lacked glycerophosphate or contained 0.01 M sodium fluoride.

Adenosine-3′-phosphate (3-AMP) and adenosine-5′-phosphate (5′-AMP) were used as substrates for the nucleotidase reactions. The method employed was that of Naidoo and Pratt (7). Reactions were carried out at pH 6.8 and 7.1. Controls contained 0.01 M sodium fluoride or 0.05 M cysteine or lacked the substrate. Adenosinetriphosphatase (ATPase) activity was demonstrated by the method of Wachstein and Meisel (8). Non-specific esterase was demonstrated with thiolacetic acid as substrate in the presence of lead ions and diethyl-para-nitrophenyl phosphate (E600) (10^{-5} M) (9). The incubation medium was ad-

justed to 290 milliosmols in each instance. The cytochemical reactions were carried out on minute, loosely packed cell aggregates (less than 1 mm^3).

The cells from all experiments were postfixed with osmium tetroxide (10). Maraglas (11) or Epon (12) was used as embedding medium. The tissues were sectioned with an LKB microtome, mounted on either Formvar film-supported grids or naked grids and examined in an Elmiskop I. Sections were stained with lead hydroxide (13), except those of tissues incubated for cytochemical reactions.

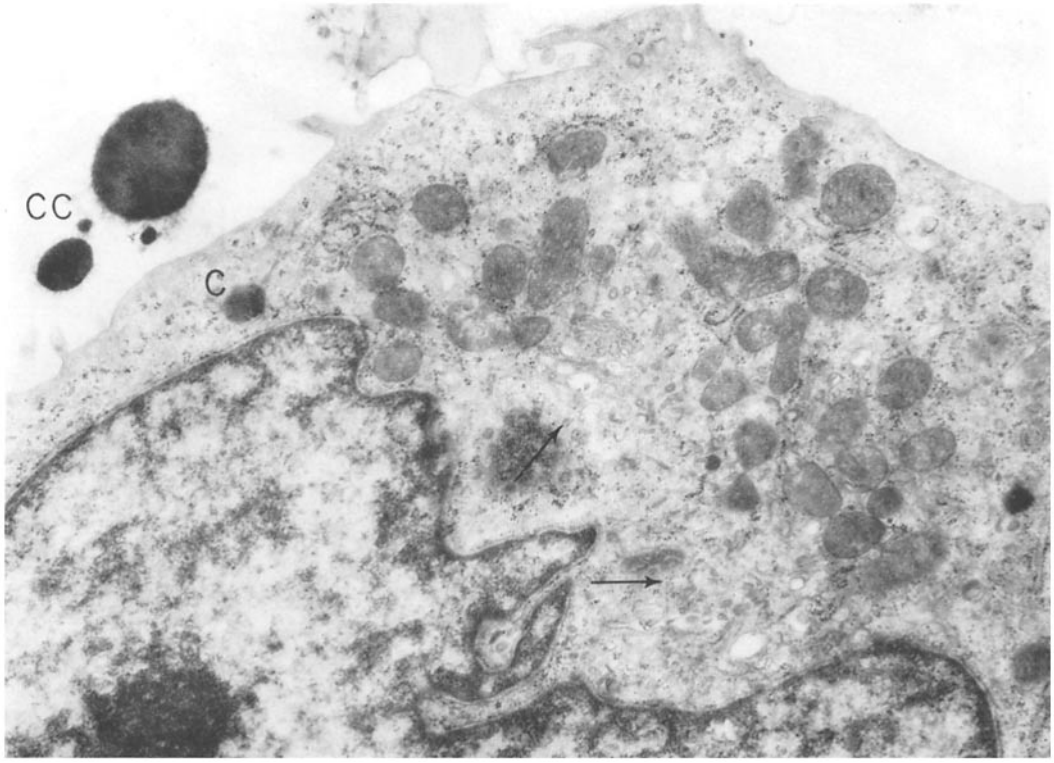


FIGURE 1 Portion of a cell immediately after initiation of a phagocytosis experiment. Arrows point to Golgi region. DNA-protein particles are seen (*CC*), with one particle already in cytoplasm (*C*). × 18,000.

RESULTS

The DNA-protein coacervates are, upon electron microscopic examination, well circumscribed spheres about as electron opaque as chromatin masses (Figs. 1 and 2). Phagocytosis of the coacervates occurs immediately after their addition to the culture medium. In the micrograph shown in Fig. 1, a phagocytized particle is seen in a cell harvested within seconds after the start of an experiment. The tissue culture cells are 20 to 30 microns in maximal dimension and usually oval or nearly spherical in suspension culture. The ratio of the volumes of cytoplasm and nucleus is about 4:1. The cells usually have a single indented nucleus. A large Golgi zone largely fills this nuclear indentation. Mitochondria, also present throughout the cytoplasm, tend to accumulate near the Golgi zone. Ribosomes are present as clusters and short straight chains in the hyaloplasm. They were also found along a scant endoplasmic reticulum. The lumen of the latter is frequently dilated and filled with a homogeneous material of greater electron opacity than the hyaloplasm (Fig. 9). Particularly in the Golgi zone, but also scattered throughout the cytoplasm are nearly spherical, moderately electron-opaque bodies measuring 2000 to 4000 A in diameter (Fig. 1). Cytochemical studies showed the presence of an E600-resistant esterase (? cathepsin C) and acid phosphatase within these structures (3), indicating that they are consistent

with the definition of lyosomes (14, 15). Short narrow microvilli are normally present on the cell surface, but larger pseudopodia and invaginations of the cytoplasm occur when the cell comes in contact with the DNA-protein particles (Figs. 1 and 12). An increase in the electron opacity of the peripheral cytoplasm occurs at the points of contact (Fig. 3), similar to the findings of Essner (16).

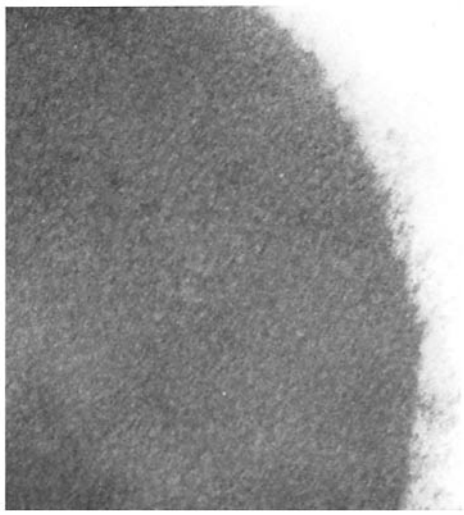


FIGURE 2 Part of a DNA-protein coacervate showing granular composition. The DNA is the more electron-opaque material. × 130,000.

After engulfment, one or more DNA-protein particles are found within each phagocytotic vacuole (Fig. 8, 11, and 12). These vacuoles appear to be present in larger numbers in the Golgi region than any other part of the cells (Figs. 4 and 9), except when cells have ingested excess numbers of DNA particles. The fluid in these vacuoles is rapidly absorbed so that a narrow space of uniform width remains between the vacuole membrane and the particle (Figs. 4 and 8). Within 15 minutes the particles appear less electron opaque (Figs. 7 and 8). This loss of opacity is present in all parts of a particle to almost the same degree (Fig. 7), except for the larger coacervates which often have a scalloped appearance (Fig. 5). Loss of density progresses until the vacuoles become almost completely electron transparent (Figs. 6 and 7). This change is observed within 30 minutes. Concomitant with the loss of density there is a marked increase in vacuole size (Fig. 7). Occasionally two of the enlarged vacuoles become confluent. Eventually the vacuoles decrease in size although they remain electron transparent (Fig. 7). These small vacuoles are often found to contain small myelin figures (Fig. 7). Rarely the vacuoles are found in

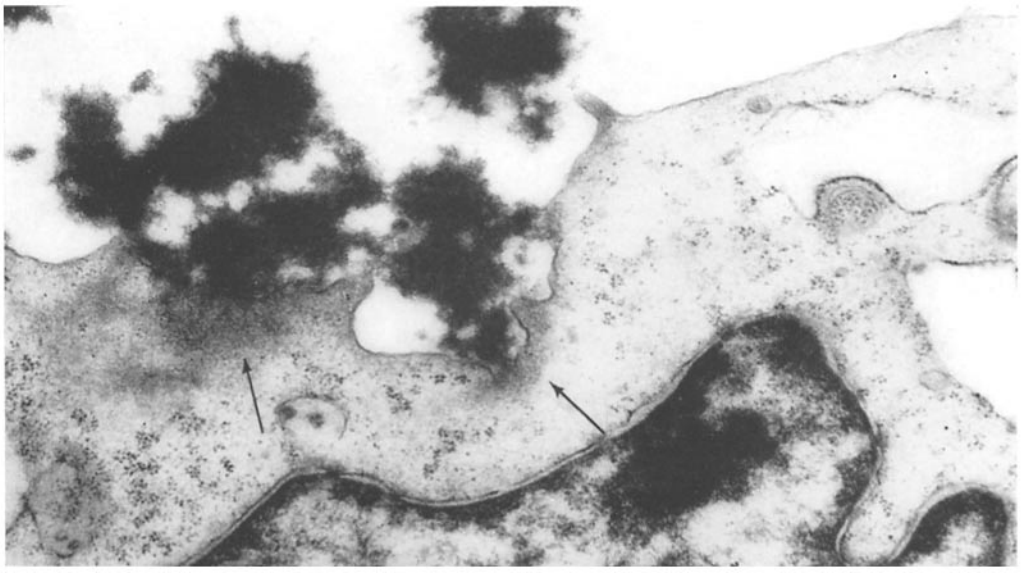


FIGURE 3 Early stage of phagocytosis of coacervate particles. The arrows point to region where the cytoplasm is of increased electron opacity. × 22,500.

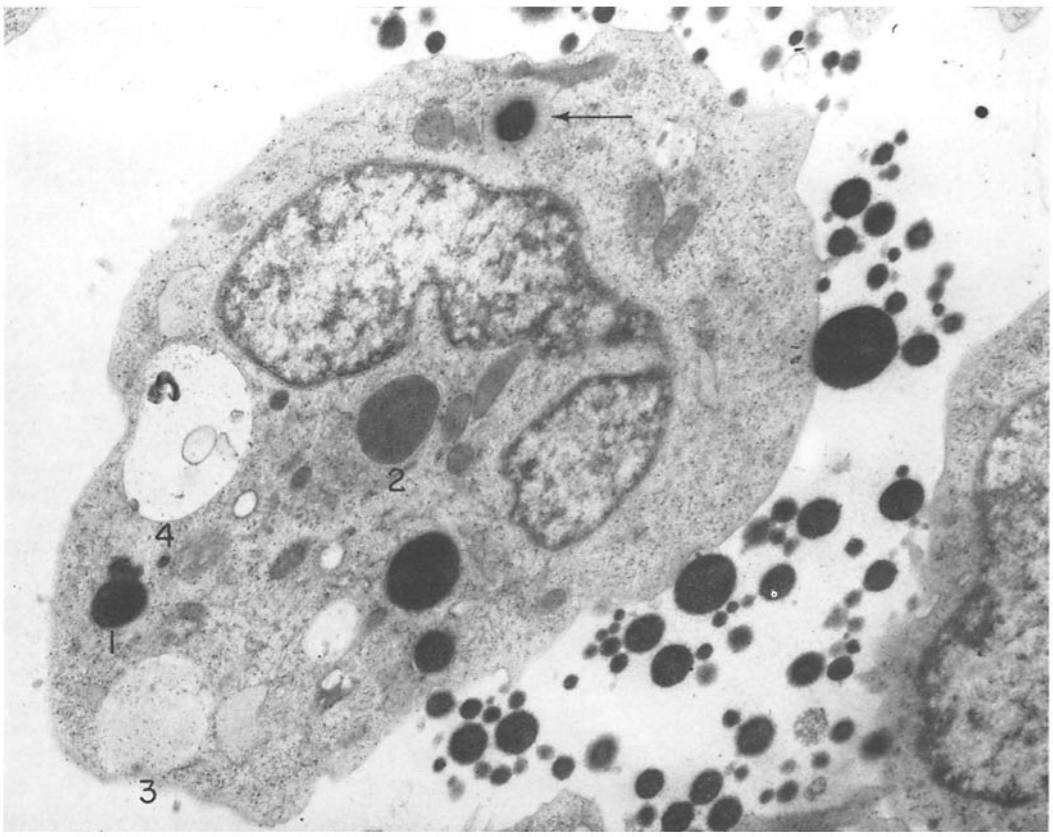


FIGURE 4 Early stages of digestion of particles. Halo of increased density of cytoplasm surrounds particle shortly after phagocytosis (arrow); later stages of digestion are present (*1*, *2*) as well as two digestive vacuoles (*3*, *4*) where the particles appear completely digested. × 14,000.

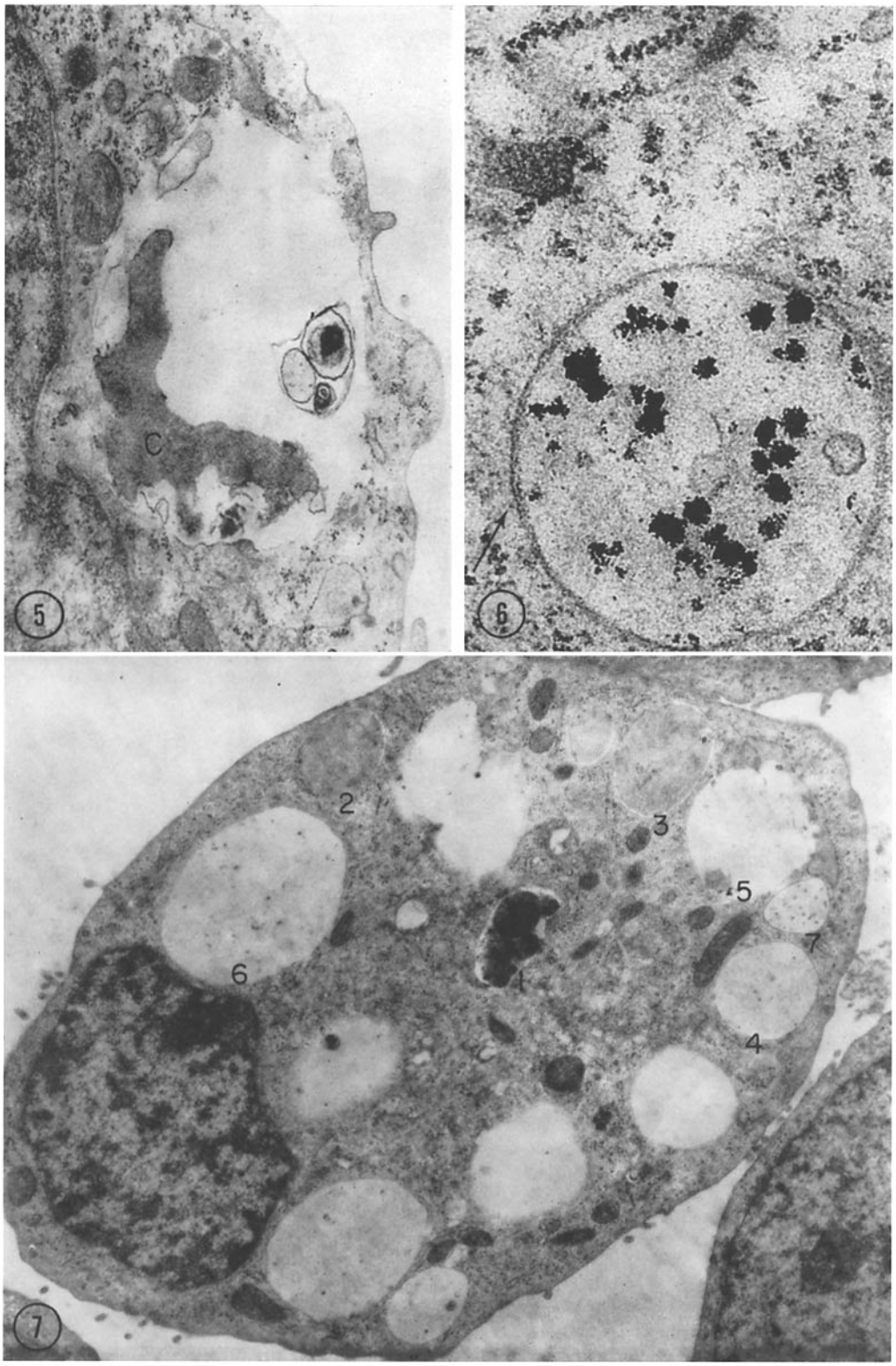
the process of discharging their contents into the surrounding medium.

When cells were incubated with glycerophosphate, the reaction product of acid phosphatase appears initially within the narrow space surrounding the phagocytized particle (Fig. 8) and later, throughout the phagocytotic vacuole (Fig. 9). The same distribution and type of reaction product was found when the cells were incubated (at pH 7.1) with either 5′-AMP or 3′-AMP (Figs. 6, and 10 to 12). An almost identical pattern of distribution of the lead phosphate was present when adenosine-5′-triphosphate (ATP) was used in the incubation medium, except the lead phosphate grains were usually not so coarse (Fig. 13). Controls for the cytochemical experiments were cells incubated without the appropriate substrate; all of these experiments yielded no precipitates. Incubations for acid phosphatase or nucleotidase with 0.01 M sodium fluoride resulted in no reaction products. Control media for 3′-nucleotidase also contained cysteine (2×10^{-3} M) and again no lead phosphate

FIGURE 5 Irregular remnant of partially digested large particle (*C*). × 27,000.

FIGURE 6 Part of digestive vacuole which contains reaction products of 5′-nucleotidase. The wall of the vacuole is partially lined by a double membrane (arrow), possibly the precursor of the intravacuolar myelin figures. This section was stained with lead hydroxide. × 80,000.

FIGURE 7 Cell showing all stages of digestion labeled in their chronological order (*1* to *7*). × 10,000.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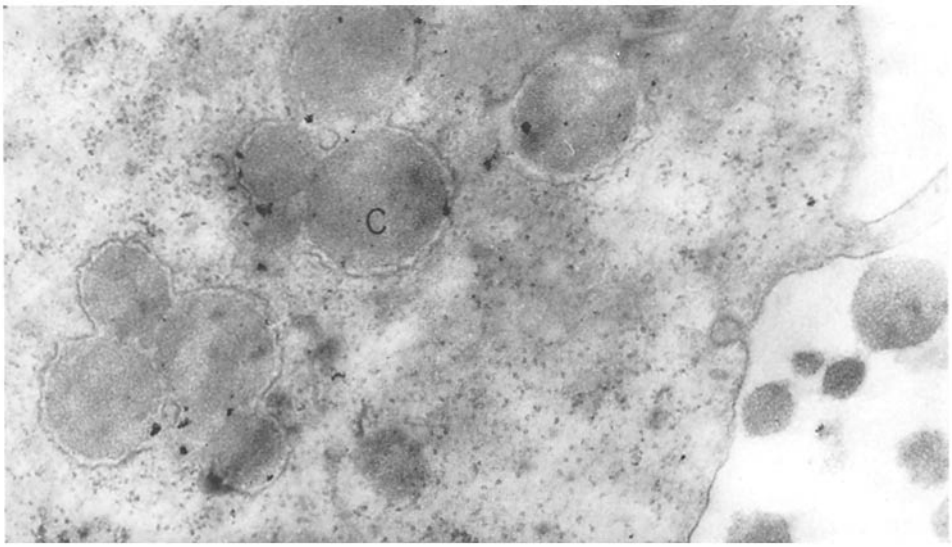

FIGURE 8 Reaction product of acid phosphatase surrounding undigested particles (*C*). × 38,500.

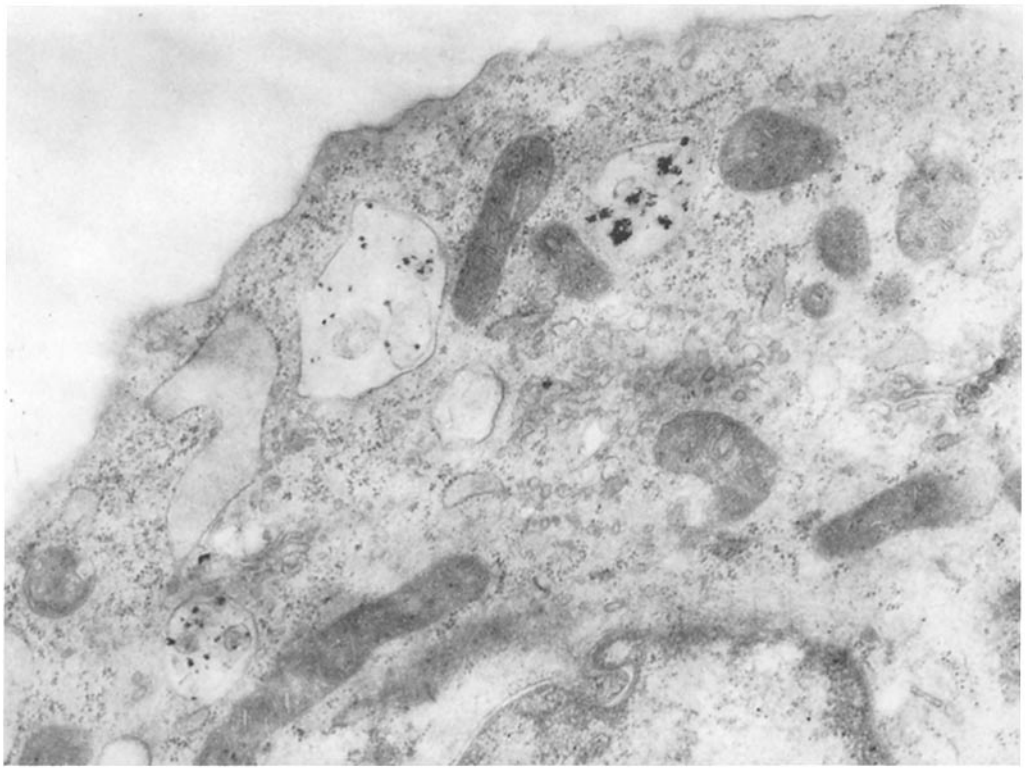

FIGURE 9 Acid phosphatase reaction product is scattered throughout three digestive vacuoles. × 24,000.

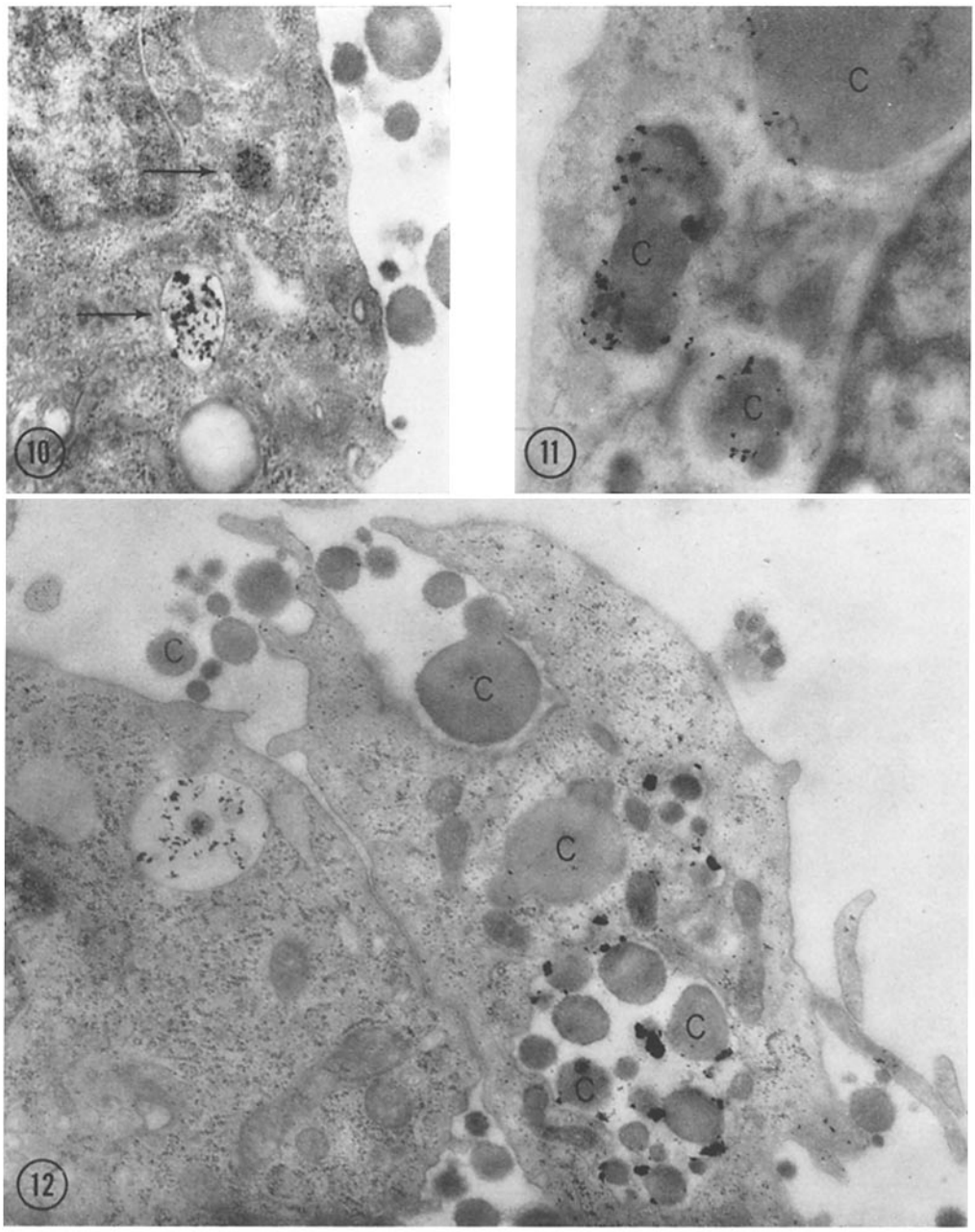

FIGURE 10 Reaction product of 5′-nucleotidase in two digestive vacuoles (arrows). × 25,000.

FIGURES 11 and 12 Deposits of lead phosphate due to 3′-nucleotidase activity. DNA-protein particles are marked *C*. × 20,000, × 16,000.

deposits were observed. 0.01 M $CaCl_2$ did not inhibit the 5′-nucleotidase reaction. With thiolacetic acid, the predominant localization of the reaction product was in the phagocytotic vacuole (Fig. 14).

Aside from the digestive vacuoles, reaction products of acid phosphatase and thiolacetic acid esterase were present in the previously described lysosomes of the control cultures. A marked de-

crease in the number of these structures was observed in phagocytosis experiments of 30 minutes' duration or longer. Reaction products of thiolacetic acid hydrolysis were occasionally found over mitochondria.

DISCUSSION

The progressive morphologic changes that take place in the particles and the phagocytotic vacuoles are indicative of the solubilization of the coacervates and the breakdown of the individual protein and DNA molecules. The molecular degradation apparently is accompanied by a hydropic enlargement of the digestive vacuoles due to the resultant osmotic alterations. The frequently observed threefold increase in diameter of a vacuole indicates a twenty-fivefold increase in volume. The eventual decrease in size of these vacuoles without change in the electron opacity of their contents is suggestive of transport of the metabolites across the vacuole membrane. Utilization of at least a portion of the phagocytized material was indeed shown in previous work with tritiated DNA coacervates (1).

The cytochemical experiments provide further evidence for the molecular degradation of the DNA-protein coacervates. The DNA is apparently hydrolyzed to nucleoside units. Accepted cytochemical methods were used to demonstrate the presence of nucleoside phosphatase activity and thiolacetic acid esterase. The hydrolysis of 3′-nucleotides in these vacuoles is of special interest because 3′-nucleotides are the predominant intermediates of DNA hydrolysis in mammalian tissues (17, 18). The esterolytic activity in the presence of E600 is considered by Hess and Pearse (19) and Wachstein *et al.* (20) to indicate cathepsin C activity. The hydrolysis of ATP in the phagocytotic vacuoles could be "non-specific," in so far as a non-specific phosphatase at pH 7.2 could have hydrolyzed this compound. Commercial preparations of ATP are known to contain varying amounts of ADP, and the reaction product in part could be due to ADPase activity. However, Marchesi and Barrnett recently demonstrated the presence of nucleoside phosphatase activity in pinocytotic vesicles (21), and this enzyme may also be present in phagocytotic vacuoles.

The relationship of the lysosomes to the phagocytotic vacuoles is not completely clear. Lysosomes are known to contain a variety of enzymes, including desoxyribonuclease (15). Aggregates of these structures were frequently observed in the immedi-

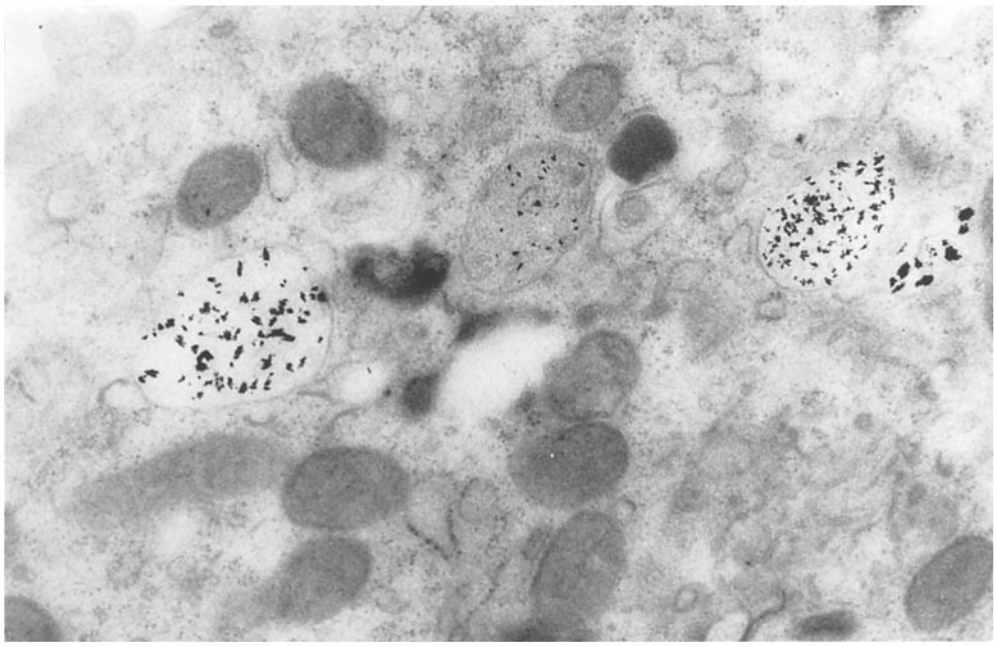

FIGURE 13 Products of ATPase activity in four digestive vacuoles. × 34,000.

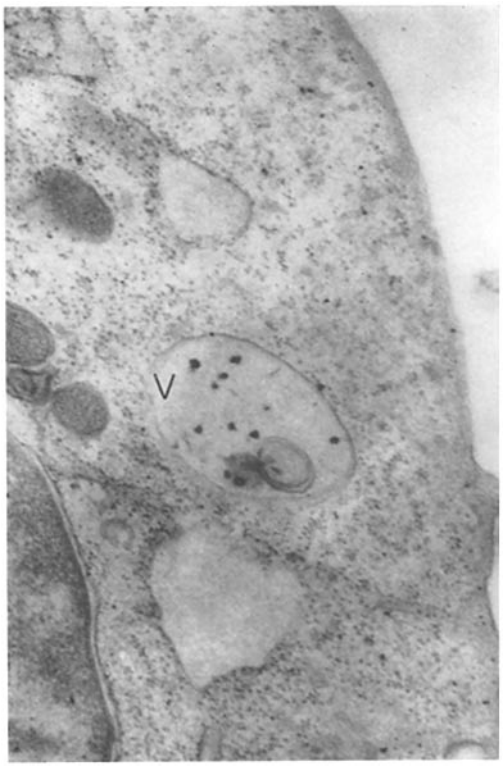

FIGURE 14 Reaction product of non-specific esterase in a digestive vacuole (*V*). The other two vacuolar structures are dilated endoplasmic reticulum bordered by ribosomes. × 32,000.

ate vicinity of vacuoles containing undigested coacervates. A merger of lysosomes and phagocytotic vacuoles may occur. Evidence for such a process was provided by Straus who observed with the light microscope the confluence of lysosomes and phagosomes in renal tubular cells (22). This merging process is now under further study.

The observed process of phagocytosis is similar to that described by Karrer (23, 24) who instilled indigestible material (India ink) into rat lungs. Essner (16) followed the phagocytosis of protein (erythrocyte fragments) by ascites tumor cells. He observed loss of electron opacity of the material in the phagocytotic vacuoles as well as vacuole enlargement. Similar findings were reported by Novikoff *et al.* who also studied the "endocytosis" of proteins (peroxidase etc.) by Kupffer cells (25, 26). In experiments by Dales and Siminovitch (27) in which tissue culture cells were exposed to a DNA virus (vaccinia), electron microscopic findings showed an initial phagocytosis resembling that observed with the DNA-protein coacervates.

The evidence presented in this report demonstrates that phagocytized coacervates of DNA-protein are hydrolyzed. In order to obtain transformation of mammalian cells with this system, it would be necessary to protect the DNA from enzymatic degradation and find a means of effecting its transport across the vacuole membrane.

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