

# Immune Cytolysis

## I. The Release of Ribonucleoprotein Particles

## II. Membrane-Bounded Structures Arising during Cell Fragmentation\*

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PLATES 322 to 325

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### ABSTRACT

It has been shown that Krebs ascites tumor cells incubated *in vitro* with immune gamma globulin and complement lose the bulk of their cytoplasmic RNA to the suspending medium, although the cell membrane remains visibly intact.

The present experiments show that about four-fifths of the lost RNA is sedimented by centrifugation of the cell-free medium at 105,000 *g*. Electron microscopic and chemical analyses of the pellets show them to consist of 150 A ribonucleoprotein particles. It is concluded that most of the RNA passes from the cells in this form.

Antibody-complement action causes osmotic swelling of the tumor cells and they become quite fragile. Fragmentation of such preparations yields large numbers of membrane-bounded spheres which may be separated from the heavier nuclei by differential centrifugation. Electron microscopic study of the spheres provides evidence that they can arise from segments of the cell surface as well as from mitochondria and the endoplasmic reticulum.

### INTRODUCTION

Krebs mouse ascites tumor cells, exposed *in vitro* to antitumor gamma globulin and complement (C') suffer changes in their cell membranes and become freely permeable to Na<sup>+</sup>, K<sup>+</sup>, and small molecules (1). As a result of disordered osmotic regulation, water enters the cells and as the cell membrane is stretched, macromolecules (RNA and protein) escape into the medium. All of these losses occur through a cell membrane which retains its integrity as judged by phase and electron microscopy (1-3).

Earlier studies (3) reported that a portion of the RNA lost from the cells could be recovered in the trichloroacetic acid (TCA)-insoluble fraction of the medium. The present report provides evidence that much of this RNA escapes from the

swollen but unbroken cells as intact 150 A ribonucleoprotein granules (ribosomes), for if the antibody-C'-treated cells are removed by low speed centrifugation, ultracentrifugation of the supernatant yields a pellet comprised for the most part of characteristic ribosomes.

After the cells have lost the bulk of their cytoplasmic macromolecules they are exceedingly fragile. A brief but vigorous manual shaking of the suspension disrupts the cells and membrane-bounded structures appear in the medium. Electron microscopic studies provide information on their mode of formation.

### Materials and Methods

The methods of preparation of the tumor cells, complement, and rabbit anti-mouse tumor gamma globulin have been previously described (1, 2). All incubations were performed with gentle shaking at 37° in a bicarbonate buffered balanced salt solution (BSS) (4) containing glucose, 1.0 mg./ml., under an atmosphere of 5 per cent CO<sub>2</sub> and 95 per cent oxygen.

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Isolation of RNA was carried out by the method of Schmidt and Thannhauser (5). Cell and ribosome pellets were suspended in 5 per cent TCA at 0° by sonic oscillation and centrifuged. The precipitates were digested with 1 N NaOH for 18 hours at 37°, chilled, acidified, and centrifuged. Aliquots of supernatant containing the hydrolyzed RNA were analyzed by either the orcinol method for ribose (6), or the molybdate method for phosphorus (7). Adenosinemonophosphate was the primary standard for the orcinol determinations but the results were expressed as RNA by multiplying measured values by 2.0. RNA was calculated from the phosphorus values, assuming that all the phosphorus was RNA phosphorus, by multiplying by 11.1 (RNA = 9.0 per cent P).

Fixation for electron microscopy was performed with 1 per cent osmium tetroxide buffered with veronal acetate to pH 7.2-7.4. Ribosome pellets were obtained from high speed centrifugations in lusteroid tubes whose walls had been previously coated with a thin uniform layer of dental plate wax. The pellets were overlaid directly with osmium tetroxide and fixed for 1½ hours. After the pellets were carried into graded alcohols for dehydration, they spontaneously detached or were easily stripped intact from the waxed walls of the centrifuge tubes. After methacrylate embedding and polymerization, the intact pellets were bisected and oriented for cutting in such a manner that the cut face paralleled the axis of centrifugation and sampled the entire population of centrifuged particles.

Fixation of cell fragments obtained by disruption of antibody-complement-treated cells was carried out by the method previously reported for intact cell suspensions (2). Thin sections were cut on a Porter-Blum microtome, mounted on copper grids, stained for 30 minutes in saturated uranyl acetate, and examined in an RCA EMU-3D electron microscope.

## RESULTS

### *1. The Liberation of Ribosomes from Cells Treated with Antibody and Complement*

When antibody and complement are added to a suspension of the tumor cells, the cells take in water and swell. As this proceeds, RNA passes from the cells into the medium (1, 2); in this way up to 75 per cent of the cell RNA may be released.<sup>1</sup> In order to determine whether any of this RNA passes through the cell membrane in the form of intact ribosomes, the swollen but intact cells were

<sup>1</sup> In earlier experiments (3) a considerable proportion of the RNA lost from antibody-C'-treated cells was recovered in the medium in the TCA-soluble fraction. In later experiments more nearly all of the RNA lost from the cells was recovered in the TCA-precipitable fraction of the medium. No explanation for this change can be given at present.

removed by low speed centrifugation (550 g for 10 minutes) and the opalescent supernatant was subjected to centrifugation at 105,000 g for 1 hour. The pellets obtained from the high speed centrifugation were examined in the electron microscope and analyzed chemically. The complete incubation conditions are given in the legend to Table I.

Thin sections revealed that the pellets consisted largely of dense granules, conforming in appearance and dimensions to the free ribonucleoprotein (RNP) granules of the cytoplasm of untreated cells. A small portion of the pellet, the fastest sedimenting one-fifth of the total packed volume, also contained swollen membrane-bounded structures consistent with forms derived from mitochondria, endoplasmic reticulum, and cell surface. Their presence presumably reflected a minimal degree of cell disruption during the experimental procedure. However, even in this zone, characteristic ribosomes were present in moderate numbers entrapped between the vesicular membranous components rather than attached to their surfaces. This is consistent with the fact that in the intact ascites tumor cell most of the particulate RNP exists as free cytoplasmic granules, independent of the membranes of the endoplasmic reticulum. The latter, moreover, is a rather poorly developed system in this cell. The remaining four-fifths of the pellet consisted of a slower centrifuging, homogeneous population of ribosomes, intermixed with amorphous debris. The latter presumably represented sedimented protein, either of cellular origin or from the gamma globulin and complement. Fig. 1 is representative of the densely packed ribosome zone of the pellet, and Fig. 2, a higher resolution micrograph of a less densely packed zone, provides a more detailed view of individual particles. They appeared morphologically identical with RNP particles studied in other cell types (8) and with ribosomes isolated from tissues by cell fractionation procedures (9-11). In our experiments the typical particles ranged from 100 to 230 Å in diameter with a mean diameter of 150 Å. The greatest density was characteristically at the center of the particle and diminished toward the periphery, so that the margins were not precisely defined. The particles were generally spherical, but some structural variation was noted. Not all of the particles displayed a similar avidity for osmium and uranyl stains. Some particles possessed slightly eccentric rather than central dense zones and appeared more ellipsoid than spherical. A few particles were noted to have a central pale zone.

TABLE I  
*Loss of Cellular RNA: Recovery from Medium as Ribosomes*

Incubation time, min.		Experiment 1	Experiment 2	Experiment 3
		30	30	25
Control (see legend)	Cells (a) RNA (orcinol), mg.	1.05	2.93	3.84
	"Ribosomes" RNA (orcinol), mg.	0.02	0	<0.20
Experimental (anti-body + C')	Cells (b) RNA (orcinol), mg.	0.32	1.01	1.76
	Ribosomes (c) { RNA (orcinol), mg.	0.52	1.87	1.58
	{ RNA (molybdate), mg.		1.62	
	Nitrogen (Kjeldahl), mg.		0.91	0.87
	Protein, mg.*		4.06	3.81
	RNA/Protein		0.43	0.45
Fraction of lost RNA recovered in ribosomes $\left[ \frac{(c)}{(a) - (b)} \right]$		0.71	0.91	0.83

\*  $(N_{\text{total}} - N_{\text{RNA}}) \times 6.25$ . See text.

Tumor cells were washed twice with BSS and resuspended to a concentration of  $4.0 \times 10^7$  cells per ml. To an experimental flask (250 ml. capacity), 30 ml. cell suspension, 30 ml. of rabbit immune gamma globulin (10 mg./ml.) and 10 ml. C' were added. To a control flask (125 ml. capacity) containing 9.0 ml. cell suspension, the following were added: 9.0 ml. of rabbit immune gamma globulin (10 mg./ml.) and 3.0 ml. of heat inactivated C' (Experiment 1), or 9.0 ml. human serum albumin (10 mg./ml.) and 3.0 ml. of C' (Experiments 2 and 3). After 25 to 30 minutes incubation with gentle shaking at 37° the suspensions were chilled and centrifuged at 550 g for 10 minutes to sediment the cells. 5.0 ml. aliquots of the supernatants were diluted to 11.5 ml. with BSS and centrifuged at 105,000 g for 1 hour to sediment the ribosomes. Several pellets were fixed for electron microscopy and others were analyzed chemically.

Present also were particles that lacked the characteristic osmiophilia and form of ribosomes, but whose appearance suggested that they might have represented degenerated forms of the latter. However, the majority of the ribosomes appeared morphologically intact, and it was from such particles that the above-cited diameters were measured.

The chemical analyses of the pellets supported the impressions gained from the electron microscopic data. Table I shows that after about 30 minutes' incubation in the presence of antibody and C', the cells lost half to three-quarters of their RNA, and that from 71 to 91 per cent of this RNA was sedimented by centrifugation at 105,000 g for 1 hour. The RNA recovered in control "ribosomes" was negligible and was ascribed to a small fraction of cells broken during the experimental procedure.

In the experimental ribosome fraction, measure-

ment of RNA by methods for ribose and for phosphorus gave values in fair agreement, indicating no great contamination by phospholipids. Substantially all the phosphorus present in the cold-TCA-precipitated ribosomes became soluble after alkaline hydrolysis and reacidification, indicating that no appreciable amount of DNA phosphorus was present in the original TCA precipitate.

Assuming that all the nitrogen was accounted for by protein and RNA, that the protein was 16 per cent nitrogen, and the RNA 15 per cent nitrogen, the ribosome fraction consisted of about one-third RNA and two-thirds protein. Direct determination of protein by the method of Lowry (12) gave values in good agreement with those obtained by calculation. Some of the protein was undoubtedly derived from the globulin and the complement, for these solutions yielded small quantities of precipitate when centrifuged at 105,000 g for 1 hour.

*II. Ultrastructural Forms Derived from  
Fragmented Antibody-Complement-  
Treated Cells*

Tumor cells incubated with antibody and complement for approximately 30 minutes became quite fragile and were efficiently broken by a brief but vigorous shaking. Examination of the suspension by phase microscopy now revealed clumped nuclei with attached cytoplasmic cuffs, amorphous debris, and a large number of spherical forms with a structureless, pale interior and a peripheral limiting membrane. These spheres varied in size from the limits of resolution to approximately  $10\ \mu$  in diameter. If the broken cell suspension was centrifuged at  $40\ g$  for 10 minutes to remove the nuclei, and the supernatant centrifuged at  $550\ g$  for 10 minutes, a pellet was obtained that contained almost a pure population of the membrane-bounded spheres. Such "sphere preparations" were examined in the electron microscope.

It was reported previously (2) that the secondary osmotic swelling resulting from antibody-complement action affected all the membrane-bounded organelles of the cytoplasm. Fig. 3 is typical of such a cell. The cell membrane appeared intact; the cytoplasmic matrix lacked density owing to the loss of ribosomes. The cavities of the endoplasmic reticulum and the perinuclear space were expanded. Mitochondria were swollen to varying degrees, those most severely affected displaying shortened vesicular cristae confined to the periphery. Since the number of spheres produced by breaking such cells as illustrated in Fig. 3 always exceeded the original cell count, it seemed likely that the spheres arose from: (a) swollen, membrane-bounded intracellular organelles, and/or (b) detached cytoplasmic fragments around which the surface membrane had resealed. Electron microscopic study of the sphere preparations supported both modes of formation.

Figs. 4 to 7 illustrate the variety of structures observed. Since under phase microscopy all the forms appeared spherical, some of the irregularity of outline present in the ultrathin sections may be ascribed to compression or shrinkage artifact. Fig. 4 represents a typical membrane-bounded structure judged to have arisen from the cell surface and the adjacent cytoplasm. Where clearly defined, the limiting membrane measured approximately  $70\ \text{A}$  in thickness. Along some segments of the circumference the membrane appeared less electron-opaque and of greater thickness. This

focal "smudging" of the membrane could be explained by oblique sectioning or perhaps by altered structure. Smaller satellite spheres ( $S_1$ ,  $S_2$ ,  $S_3$ ), also enclosed by  $70\ \text{A}$  membranes, were present on the external surface of the large sphere. The interior of the large sphere contained a vesicular, membrane-bounded space ( $V$ ) and  $150\ \text{A}$  granules; most of the latter were free, but a few were associated with the internal vesicle. A few such granules were also seen in one of the small surface spheres ( $S_1$ ). Assuming the granules were ribosomes and the small internal vesicle was derived from the endoplasmic reticulum, it seemed most likely that the large sphere arose by detachment of a fragment of cytoplasm from the main cell mass. The largest profiles contained in the pellets appeared to be so derived. The external satellite spheres, at least those containing ribosomes, could have arisen by a similar mechanism; namely, by a "pinching-off" of a smaller segment of surface membrane. The sphere designated  $S_3$  in Fig. 4 appeared to be arising by such a process.

The sphere  $S_2$  in Fig. 4 lacked internal granules and therefore might alternately have represented an element of smooth surfaced endoplasmic reticulum that was merely adherent to the larger sphere. Similar smooth surfaced forms existed independently within the pellets (Fig. 5); moreover spherical profiles of rough surfaced endoplasmic reticulum were definitely identified (Fig. 6).

In addition, spheres were found that were consistent with altered mitochondria (Fig. 7), for they retained to some degree a peripheral double membrane and remnants of cristae mitochondriales. Other spherical forms not illustrated were of less certain origin. Bounded by a single membrane, containing internal vesicles but lacking RNP granules, they might have also represented damaged mitochondria.

Thus, the ultrastructural studies indicated that the spheres observed by phase microscopy after cell fragmentation arose from the cell surface, endoplasmic reticulum, and mitochondria.

The behavior of the spheres in hypertonic sucrose solutions was observed by phase microscopy. Just as was the case with antibody-complement-treated whole cell preparations (1), the spheres did not shrink when placed in a solution of  $0.5\ M$  sucrose in BSS. In contrast, untreated tumor cells or tumor cells initially swollen in hypotonic salt solutions, rapidly shrank and became crenated at this sucrose concentration.

It is noteworthy that throughout the sphere preparations where 70 A membranes were observed, they were generally continuous structures establishing spherical profiles. Thus, a broken membranous fragment derived from the cell must have rounded up, resealed its free ends, and formed an enclosed space. It is also of interest that whenever two completely defined spherical forms were closely associated (Fig. 4), there was a minimal distance of approach measuring approximately 110 A.

Nuclei were rarely found in the sphere preparations (Fig. 8), and cuffs of cytoplasm were always attached to the nuclei even though the cell membrane in these fragments had not been re-established. The attachment was ascribed to the continuity of the endoplasmic reticulum with the nuclear membrane (13) and to the communication of nucleoplasm with cytoplasmic matrix through widened nuclear pores (13, 14). Many organelles were still retained in the cytoplasmic cuffs and occasionally the fragmented endoplasmic reticulum assumed concentric forms reminiscent of myelin figures (Fig. 9).

#### DISCUSSION

The experiments indicated that RNA was lost from antibody-complement-treated cells by passage of 150 A ribonucleoprotein particles across the cell membrane into the medium. Although the visibly intact cell membrane was permeable to this extent, it still prevented larger organelles from leaving the cell.

The exact mode of passage of the ribosomes through the cell membrane could not be established. The most likely means of escape would have been through small "holes" in the membrane. Although the cell membrane appeared intact by electron microscopy, the detection of such holes would have been unlikely if they were few in number, quickly resealed, or small in diameter with respect to the thickness of the sections. Further, whenever occasional small discontinuities in the membrane were observed, they were of such a character that they might also have represented shrinkage and cutting artifacts. Therefore, the data neither ruled out nor established the existence of minute holes in the cell membrane.

Both the microscopic and chemical data supported the view that the ribosome pellets were relatively uncontaminated by other cell organelles. No nuclei were identified in the thin sections and

DNA was not released from antibody-complement-treated cells (3). The degree of contamination by phospholipid-rich mitochondria and endoplasmic reticulum was estimated to be small, and the RNA/protein ratios compared favorably with values reported from other isolation procedures (9, 15-17). It remains to be tested whether ribosomes isolated from antibody-complement-treated cells will be biochemically active in protein synthesis. If so, this simple technique might be useful for the routine preparation of such particles.

The studies on spherical forms released upon cell disruption attested to the remarkable plastic properties of cellular membranes. The ability of cell membranes to stretch, pinch off, fragment, and reseal, has been noted in the erythrocyte and the erythrocyte ghost (18). Palade and Siekevitz noted that in microsomal fractions of tissue homogenates the characteristic structure was a hollow profile limited by a continuous membrane (10). The authors concluded that such structures arose from the endoplasmic reticulum by fragmentation and "healing" or by a spontaneous "pinching-off" process. Our experiments provide evidence that the cell surface can also form such closed profiles, and thus not all smooth surfaced profiles obtained by cell fractionation need necessarily originate from intracellular organelles. As our sphere preparations were obtained by low speed centrifugation (550 g) they were probably enriched with respect to the larger and heavier profiles.

Good analogies to the above described plastic behavior of cell membranes exist in model systems of lipoprotein films floated on water. Such artificial films, when partially collapsed, can pinch off tubular segments that detach from the parent film, float freely, and behave as osmometers (19). Artificially formed myelin figures possess a similar plastic capacity, for they have been shown to reconstitute when fragmented (20).

The failure of the spheres to shrink in hypertonic sucrose must mean that they were freely permeable to this molecule. To the extent that the spheres originated from the cell membrane this was to be predicted, for earlier studies demonstrated such sucrose permeability of whole cells after antibody-complement action. Since many of the spheres presumably also arose from intracellular organelles, it may be assumed that the intracellular membranes were also directly attacked by antibody and complement.

The observation that associated spheres main-

tained a minimal distance of approach of about 110 Å suggests that structure was interposed that was not visible in the electron microscope. A similar observation was made by Rhodin with regard to apposition of adjacent microvilli in the proximal nephron (21). In this instance, the closest distance of approach was 100 Å and the author proposed a less dense layer of 40 to 50 Å outside of each membrane.

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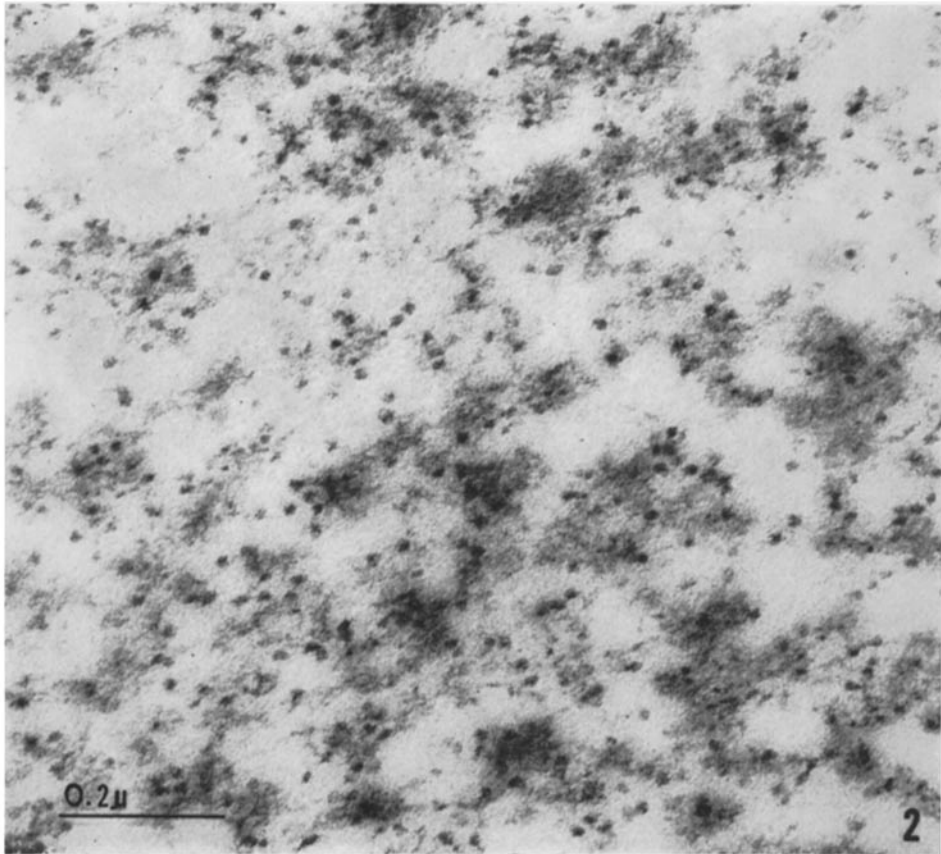
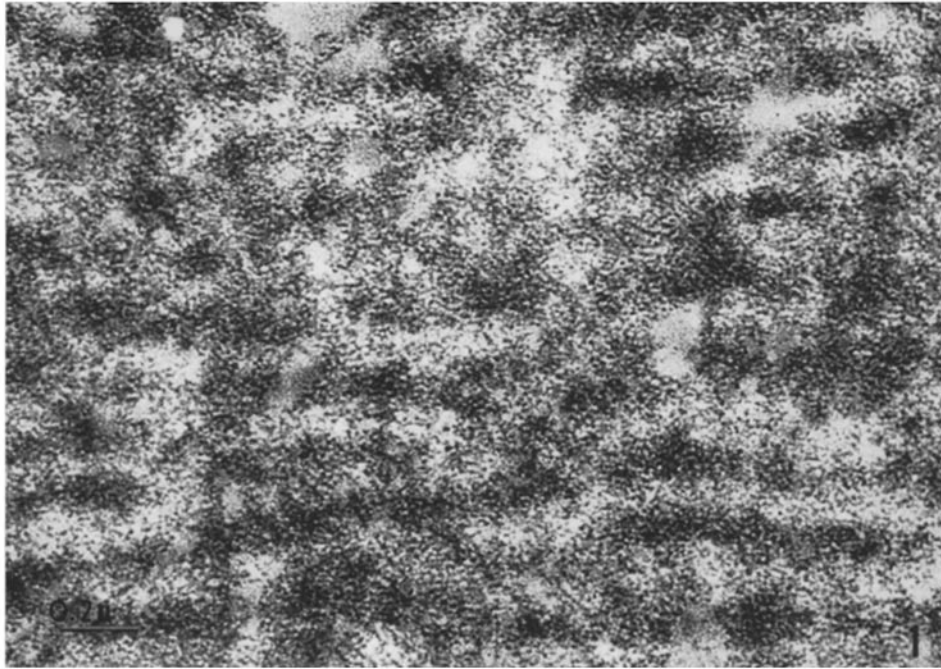
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## EXPLANATION OF PLATES

## PLATE 322

FIG. 1. Ribosome pellet. Zone of pellet containing almost pure population of RNP granules.  $\times 50,000$ .

FIG. 2. Ribosome pellet. More detailed view of individual RNP particles in slower centrifuging, less densely packed zone of the pellet. Amorphous debris represents sedimented protein.  $\times 110,000$ .

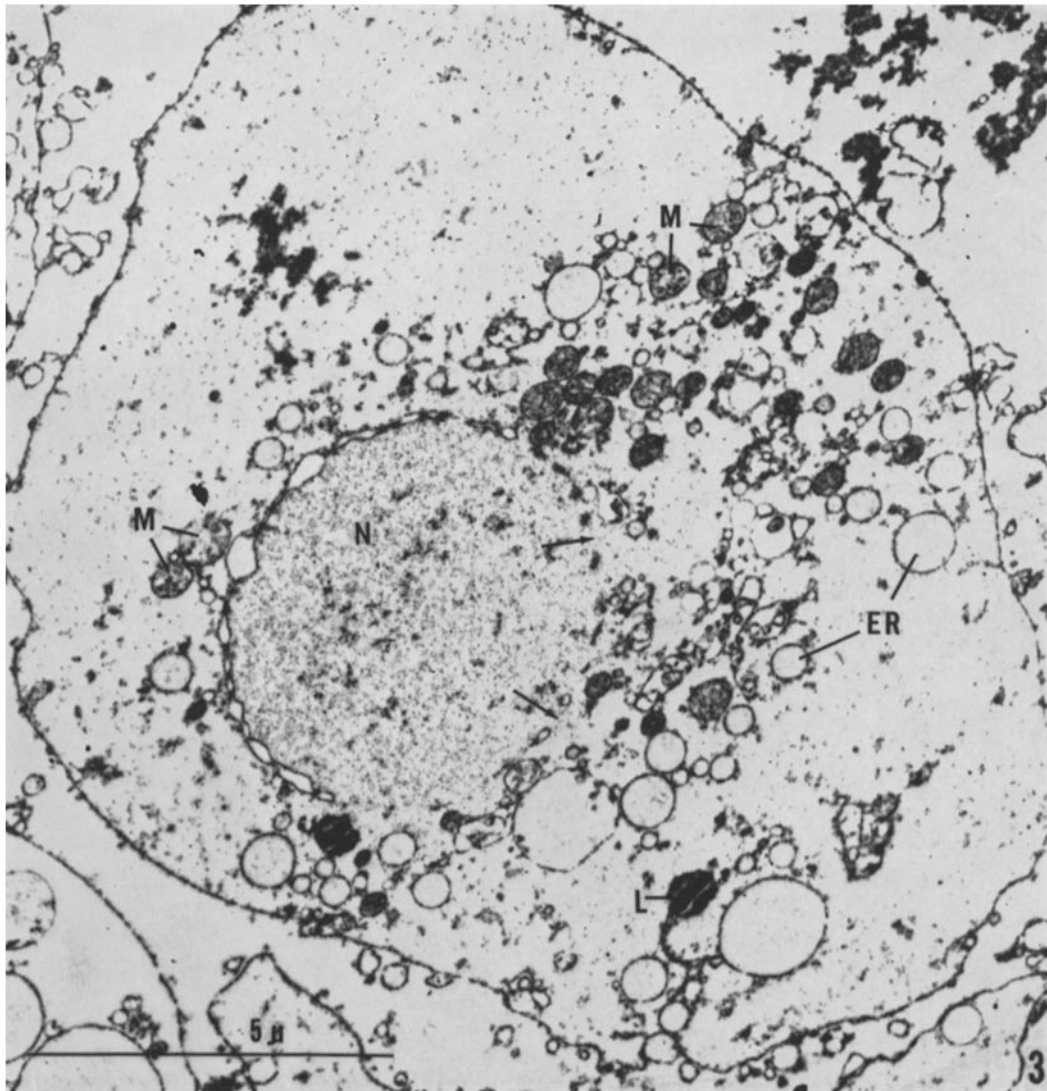


(Goldberg and Green: Immune cytotoxicity)

PLATE 323

FIG. 3. Antibody-complement-treated cell. The cavities of the endoplasmic reticulum (*ER*) and the perinuclear space are expanded. Arrows indicate widened nuclear pores. Mitochondria (*M*) are swollen to varying degrees. *N* denotes nucleus, *L* lipid.  $\times 10,000$ .





(Goldberg and Green: Immune cytotoxicity)

PLATE 324

FIG. 4. Sphere preparation. Large profile enclosed by 70 A membrane and containing cytoplasmic structures. Interior contains a vesicle ( $V$ ) and attached (arrow) and free 150 A granules. Similar granules are identified in the interior of a small surface sphere ( $S_1$ ), but are lacking in others ( $S_2$  and  $S_3$ ). A space of 110 A separates  $S_1$  and  $S_2$  from main sphere.  $S_3$  appears to be in process of "pinching off" from surface membrane.  $\times 40,000$ .

FIG. 5. Sphere preparation. Isolated smooth surfaced profile possibly derived from the endoplasmic reticulum.  $\times 35,000$ .

FIG. 6. Sphere preparation. Sphere with peripheral 150 A granules derived from rough surfaced endoplasmic reticulum.  $\times 41,000$ .

FIG. 7. Sphere preparation. Form consistent with altered mitochondrion. Peripheral double membrane and cristal remnants (arrows) can be identified.  $\times 41,000$ .

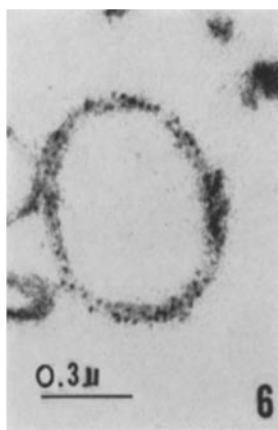
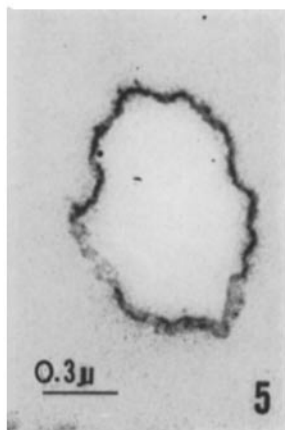
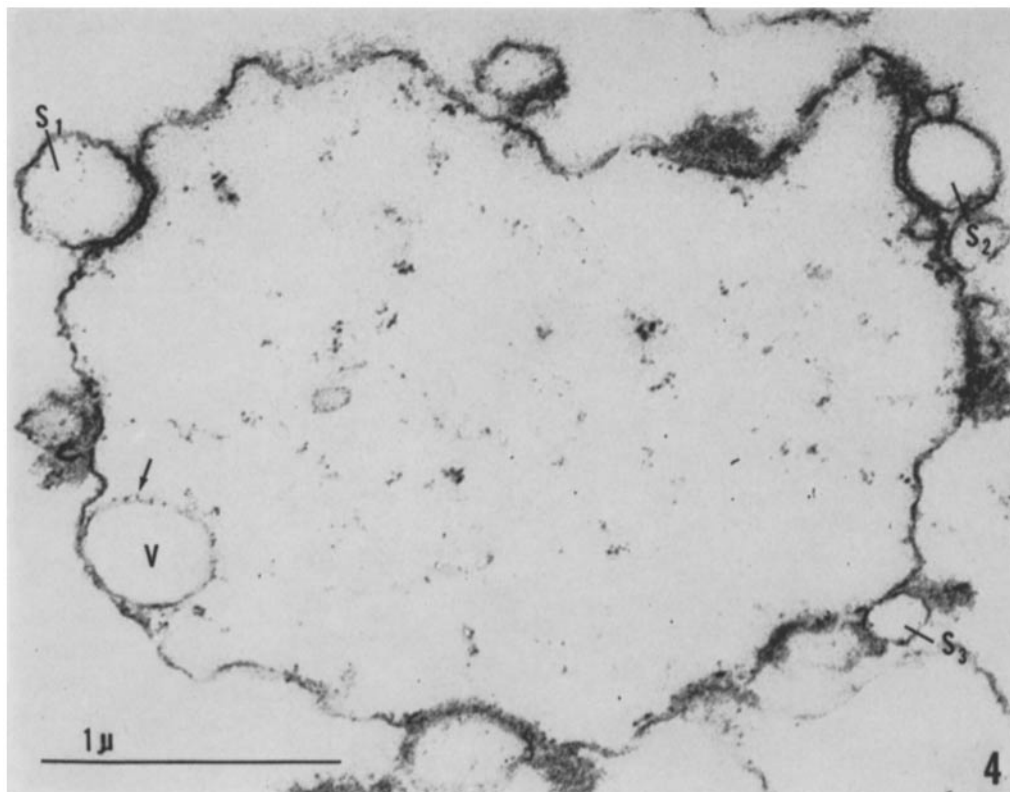
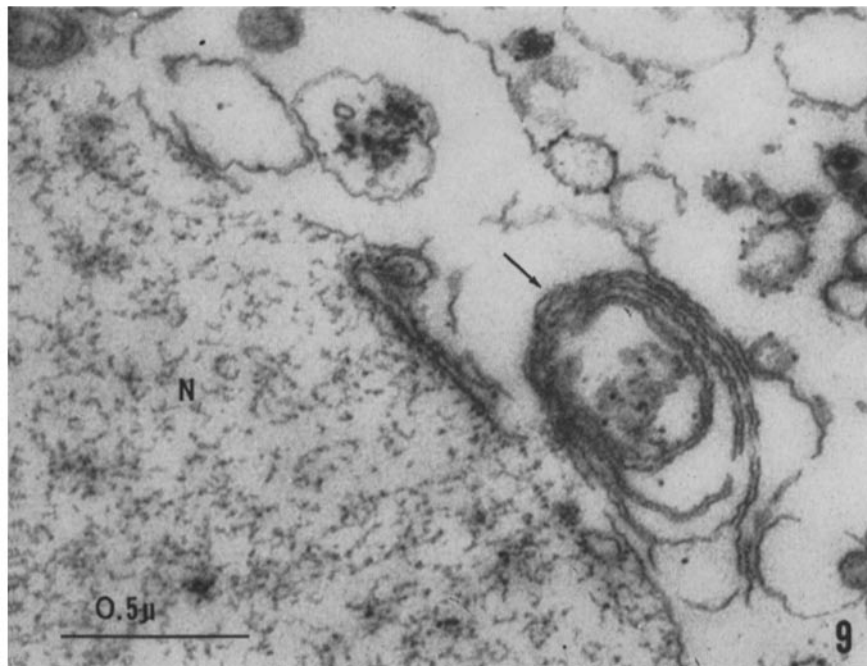
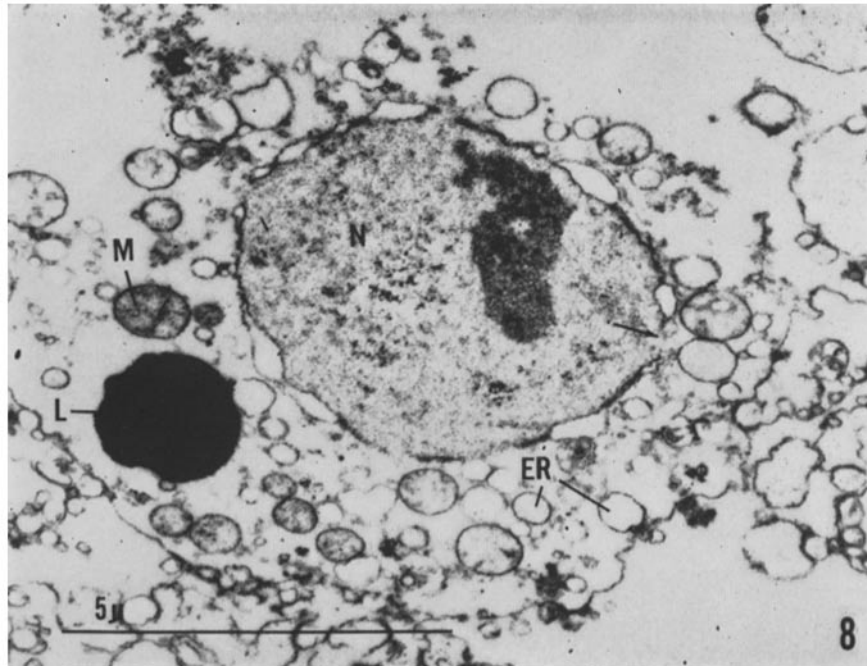


PLATE 325

FIG. 8. Nucleated cell fragment found in sphere preparation. Cytoplasmic cuff containing lipid (*L*), mitochondria (*M*), and endoplasmic reticulum (*ER*), is still attached to nucleus (*N*). Arrow indicates widened nuclear pore.  $\times 10,000$ .

FIG. 9. Concentric arrangement of endoplasmic reticulum in cytoplasmic cuff (arrow). *N* indicates nucleus.  $\times 50,000$ .



(Goldberg and Green: Immune cytotoxicity)