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Studies With Digitonin-Treated Rat Hepatocytes (Nude Cells)

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Isolated rat hepatocytes were treated with digitonin to strip the plasma membrane. The effect of digitonin concentration and exposure time on the recovery of marker enzymes for cell organelles was examined. Hepatocytes treated at room temperature for 1–2 min with 1 mg/ml of digitonin lose some 40% of their protein but retain over 95% of their intact mitochondria and peroxisomes, 90–95% of their endoplasmic reticulum, and about 80% of their lysosomal enzymes. There is little loss of the mitochondrial intermembrane content, and both oxygen uptake and phosphorylation are unimpaired by the treatment.

Electron microscopy reveals a complete loss of the plasma membrane, in spite of limited loss of marker enzymes for this membrane. Scanning electron microscopy revealed the interior of the cells to be made up of a dense network of fibers and lamellae attached to the nucleus, mitochondria, and small organelles. The treated cells were stable for many hours when kept in 0.25 M sucrose containing 25 mM monovalent salts. In salt-free sucrose the cells broke up very rapidly into nuclei and other single organelles. Addition of 5 mM NaCl or KCl retards breakup, and 15–20 min were required for dissolution. Intermediate stages, illustrated by scanning electron micrographs, show structure and chains made up mainly of mitochondria held together by a lamellar network. The rapid breakdown occurred at a pH above 7.5 in an oxygen atmosphere and in the presence of phosphate and apparently is an energy-requiring process. It is slow below a pH of 7.2, and at a pH of 6.8 the treated cells remain completely stable in salt-free sucrose. Our results suggest that endoplastic reticulum is a major component of the cytostructure holding together nuclei and organelles.

Key words: hepatocytes, rat liver, digitonin, mitochondria, cell structure, organelles, endoplasmic reticulum

In a previous paper [1] we have shown that in homogenates of rat and quail liver prepared in a sucrose medium containing above 25 mM sodium or potassium ions, the mitochondria are attached to a network composed mainly of the endoplasmic reticulum. We found this network to be stable in media of high ionic strength, but to disintegrate at low ionic strength. Siess and Wieland [2], Fiskum et al [3], and Cook et al [4] have shown by electron microscopy that isolated rat hepatocytes treated with digitonin lose their plasma membrane, but remain intact when prepared in buffered media. Such cells could be a valuable system for the study of cytostructure, and

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scanning electron microscopy should permit observation of the interior of the cell. We reasoned from our previous studies that the reticular network of such treated "nude" cells would remain stable in the presence of salts but would disintegrate in salt-free sucrose. As shown here, our prediction was borne out, and these cells promptly disintegrate in salt-free sucrose media. We describe here our observations on the effect of digitonin and on some properties and structure of the treated cells.

METHODS

Preparation of Cells

Isolated rat hepatocytes were prepared by perfusion with collagenase of livers from fed rats, as described elsewhere [5]. The cells were washed three times in Krebs-Henseleit bicarbonate buffer equilibrated with 95% O_2-5% CO_2, and then twice with 0.25 M sucrose-50 mM sodium Hepes, pH 7.4. The sodium concentration in this buffer is about 45% that of Hepes. The sucrose media were aerated with 100% O_2. To a volume of packed cells (80–100 $\times$ 10^6 cells/ml, about 200 mg dry wt), an equal volume of this medium was added. One milliliter of this suspension was delivered into 50-ml Erlenmeyer flasks, and an equal volume of medium containing 2 mg/ml of digitonin was added. Flasks were shaken gently for 2 min at room temperature (22–25°C) while being gassed with oxygen. Ten volumes of 0.25 M sucrose-1 mM NaHepes were then added and the cells were centrifuged for 1 min (total time of run), attaining a maximal acceleration of 800g. The pellet was suspended in 5 ml 0.25 M sucrose-5 mM NaHepes, with varying concentrations of KCl. The cell suspensions were examined by phase contrast microscopy. For electron microscopy the cells were fixed in 2% glutaraldehyde. For scanning electron microscopy, the cells were mounted on glass coverslips with poly-L-lysine, as described by Sanders et al [6] or on Nuclepore filters (1 μm, 13 mm; purchased from Nuclepore, Pleasanton, CA). The samples were dehydrated in ethanol, critical-point dried with CO_2, spattered with 50% gold-50% palladium, and examined with an Elton Instrument. For transmission, the samples were stained with osmium tetraoxide, dehydrated with acetone, embedded in epoxy resin, and 600-angstrom sections were cut.

Assays

For the assay of enzymes Lubrol was added to 0.2%. The assay for protein, glucose 6-phosphatase and glutamic dehydrogenase was as previously described [1]. Glycogen was assayed according to [5], monoamino oxidase according to [7], RNA according to [8], urate oxidase and acid phosphatase as described in [9], NADPH cytochrome c reductase as in [10], Mg and (Na,K)-ATPase as in [11], 5-nucleotidase as in [12], and adenylate kinase as in [13]. The enzymes were assayed in untreated cells and after digitonin treatment in both pellet and supernatants and recoveries expressed as percent of the activity in control cells.

Iodination of Hepatocytes

The reaction was conducted in a sealed 25-ml Erlenmeyer flask. Hepatocytes, 0.25 ml of packed cells, were suspended in 3 ml of a Krebs-bicarbonate buffer containing 5 mM of glucose, 15 units of lactoperoxidase, and 18 units of glucose oxidase. After gassing with 95% O_2-5% CO_2, 60 Ci of $Na^{125}I$ was injected through the rubber stopper. After 10 min of gentle shaking, 0.5 ml of 0.1 M sodium

hydrosulfite was added, and the cells were washed five times with 20 ml each of buffer. The cells contained 1–2% of the added radioactivity, but less than 10% of this was insoluble in trichloroacetic acid (TCA).

Separation of Organelles

These were separated by centrifugation for 10 min in a Sorvall RC-3 (swinging head) centrifuge to obtain a pellet sedimenting at 300g, according to Lewis and Tata [14], followed by centrifugation for 10 min in a Sorvall RC-5B with a conical SS-34 rotor, to obtain a 1,500g and a 10,000g pellet, and the supernatant thereof, as described by Katz et al [1].

Oxygen Uptake

This was measured with a Clark electrode. Mitochondria or digitonin-treated cells were suspended in a mannitol-sucrose medium, as described in [1]. The protein content and the activity of glutamate dehydrogenase of these preparations was determined.

Supplies

Collagenase was purchased from Worthington (Freehold, NJ); digitonin from ICN (Irvine, CA), and enzymes and other reagents from Sigma (St. Louis, MO).

RESULTS AND DISCUSSION

The Effects of Digitonin

The extraction of marker enzymes for mitochondria, endoplasmic reticulum, peroxisomes, and lysosomes by digitonin was examined when using 1 mg of digitonin and 40 mg cell protein per ml, for 2 min at room temperature. The treatment released 35–40% of the protein. The recovery of enzymes and protein in supernatant plus pellet averaged near 100% of untreated controls, with a range of 93–108% for a large series of determinations. Most of the cytosolic enzymes were found in the supernatants, with only traces to less than 3% of lactic dehydrogenase, glucose-6-P isomerase, glycogen synthase, and glycogen phosphorylase in the pellet. As shown in Table I, there was virtually no loss of glutamic dehydrogenase and monoamine oxidase. Loss of only 6% of adenylate kinase, a marker for the intramembrane space, indicates that there was little damage to the outer mitochondrial membrane. Two marker enzymes for the endoplasmic reticulum were employed, glucose-6-phosphatase and NADPH cytochrome c reductase. The loss of the former was under 5%, and that of reductase was about twice as high. Thus, at least 90% of the endoplasmic reticulum was retained in the treated cells. There was a 25% loss in acid phosphatase, a lysosomal enzyme. There was a loss of 30% of cell RNA; in the presence of 5 mM EDTA during digitonin treatment the loss of RNA was increased to 60% (Table I). The loss is probably predominantly of ribosomes, and EDTA may have caused their detachment from the reticulum or a dissociation of polyribosomal chains.

A stringent test for damage to the reticular membrane is the latency to mannose-6-P. The transporter for glucose-6-P is located at the cytosolic side of the reticular membrane, and it is impermeable to mannose-6-P, which is completely inert with intact endoplasmic reticulum [14a]. When the membrane is permeabilized by detergents, mannose-6-P and glucose-6-P are hydrolyzed at near-equal rates [14a]. When

TABLE I. The Composition of Digitonin-Treated Cells*

	n[a]	Units[b]	Content	No EDTA		5mM EDTA		Main marker for—
				Supernate (%)	Pellet (%)	Supernate (%)	Pellet (%)	
Protein	10	mg	142 ± 5	39 ± 1.6	59	43	54	
RNA	4	mg	4.2 ± 0.4	28 ± 2.2	67	55	38	
Glutamic dehydrogenase	10	μmol/min	178 ± 16	3.2 ± 0.5	97	1.0	97	
Monoamine oxidase	5	μmol/min	0.26 ± 0.05	2.3 ± 0.6	95	1.5	95	Mitochondria
Adenylate kinase	4	μmol/min	48 ± 1.0	6.0 ± 0.4	93	—	—	
Uricase	5	μmol/min	1.1 ± 0.1	2.8 ± 0.3	102	0	95	Peroxidase
Acid phosphatase	7	μmol/min	6.4 ± 0.4	21 ± 2.4	80	24	80	Lysosomes
Glucose 6-phosphatase	8	μmol/min	9.9 ± 1.0	7.1 ± 0.6	101	3	94	
Cytochrome c NADPH reductase	5	μmol/min	2.9 ± 0.2	8.5 ± 1.3	94	—	—	Endoplasmic Reticulum

*Hepatocytes were treated with 1 mg/ml of digitonin for 2 min, and cells and supernate separated as described in Methods. The recovery in cell pellet and supernate is reported as % of the total activity in untreated cells.

[a]Number of experiments without EDTA (there were two experiments with EDTA).

[b]mg or enzyme units (μmol/min) per ml of untreated packed cells.

TABLE II. Effect of Digitonin Concentration on the Extraction of Protein and Enzymes from Hepatocytes*

	Percent extracted at digitonin level (mg/ml) of—							Average recovery[a]
	0	0.25	0.50	1.0	2.5	5	10	
Protein	2	12	27	35	46	53	57	94
Glutamic dehydrogenase	0.8	1	1	2	3	5	10	96
Monoamine oxidase	0	0	0	0.4	1	10	54	[b]
Adenylkinase	1	2	4	5	9	38	49	101
Uricase	0.3	1	2	2	3	6	13	100
Glucose 6-phosphatase	0	0.5	2	5	15	32	56	91
Cytochrome c NADPH reductase	3	4	5	10	28	55	68	97
Acid phosphatase	6	8	12	26	56	60	75	98

*Cells were extracted for 2 min as described in Methods with increasing concentrations of digitonin.
[a]Sum of the activity measured in supernate and pellet as % that in untreated cells.
[b]Recovery of monoamine oxidase was 97–99% with up to 2.5 mg of digitonin. At higher concentrations recovery (sum of supernate and pellet) was 70–80%. Higher concentrations of digitonin appear to interfere with the assay.

the digitonin-treated cells were incubated with 5 mM glucose-6-P, it was rapidly hydrolyzed to glucose at a rate of 0.18 μmol/min/mg protein. The hydrolysis of mannose-6-P under the same conditions 0.004 was μmol/min/mg protein, or the latency was about 98%. Thus, although, as seen by electron microscopy (see below), the appearance of the endoplasmic reticulum is altered, it remains intact with little damage to the permeability of the membrane.

Other investigators [13,15], using high concentrations of digitonin, reported a much more extensive loss of marker enzymes, and thus, we studied the effect of digitonin concentration and time of exposure. Incubation was at room temperature for 2 min, and protein concentration was 40 mg/ml, whereas digitonin concentration was varied from 0.25 to 10 mg/ml (Table II). At the lowest concentration, the loss of cytosolic protein was incomplete. Glutamic dehydrogenase, monoamine oxidase, and urate oxidase are extracted to a rather limited extent even at 5 mg/ml, but at this concentration there is an appreciable loss of adenylate kinase, indicating disruption of the outer mitochondrial membrane. The extraction of NADPH cytochrome c reductase was always larger than that of glucose 6-phosphatase, the loss of the former being substantial at a concentration of digitonin of 2.5 mg/ml.

In Figure 1 the effect of time of exposure is shown. Beyond 2 min, extraction increased with time. After 15 min, 45% of the protein, 10% of the mitochondrial, and peroxisomal enzymes and 20–30% of the enzymes of the endoplasmic reticulum were solubilized. The loss of acid phosphatase was greater than any other enzyme and was about 10% even at 0.5 mg/ml of digitonin.

Recently Cook et al [16] reported with a digitonin concentration of 0.67 mg/ml a much larger extraction of adenylate kinase than that seen by us, and they claimed extensive damage to mitochondria and endoplasmic reticulum occurred after 2 min of incubation. In their procedure they had a digitonin-protein ratio of 1 to 6, whereas our ratio was 1 to 40. Comparing our conditions to those of Cook, we found (using our procedure for cell separation) little difference in the extraction of protein, glutamic dehydrogenase, glucose 6-phosphatase, NADPH cytochrome c reductase,

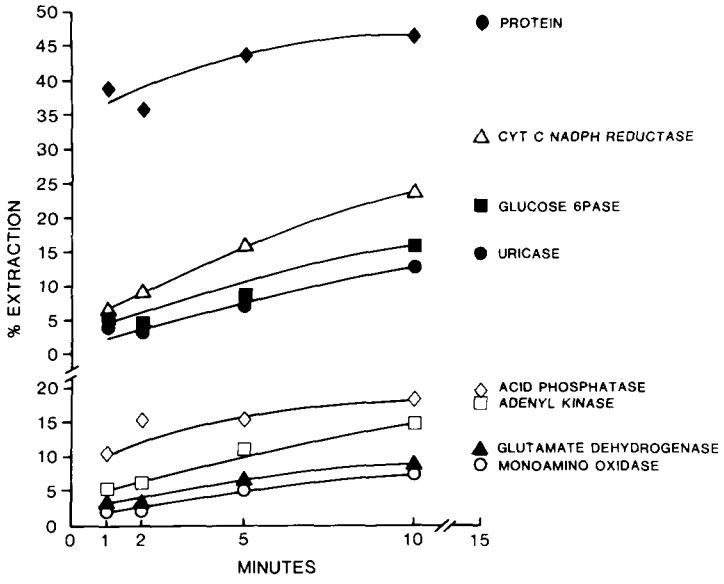


Fig. 1. Effect of duration of digitonin treatment on extraction. Rat hepatocytes were treated with 1 mg/ml digitonin as described in Methods. Results are protein and enzyme activities extracted as percent of the content of untreated cells.

and uricase. Our procedure extracted 6% adenylate kinase and 15% lysosomal acid phosphatase, compared to 18% and 59%, respectively, in their method.*

Oxygen Uptake

The oxygen uptake with succinate with digitonin-treated cells was about 60 and 300 natoms/min per mg of protein without and with ADP, respectively. The activity of glutamic dehydrogenase was 2.9–3.3 units/mg protein, and thus, maximal oxygen uptake was about 100 nmol/min per unit of glutamic dehydrogenase. In isolated mitochondria the oxygen uptake was in state 3 from 80–100 natoms/min per unit of glutamic dehydrogenase [1], and the respiratory control ratio was about 5. Also the oxidation of glutamate and malate + pyruvate was much the same (per units of glutamate dehydrogenase) in treated cells as in isolated mitochondria.

Composition

A rough estimate of the composition of the treated cells may be made from the specific activities of glutamic dehydrogenase and glucose 6-phosphatase in mitochondrial and microsomal fractions [1]. The treated cells have lost most of the cytosol and

*Our results were at great variance with the loss reported by Cook et al [16] of 28% and 65% glucose 6-phosphatase at 0.5 and 2 min, respectively. These workers separated the cells from the supernatant by centrifugation through bromododecane-bromodecane oil. We therefore repeated their treatment, adhering closely to their procedures and using the same tubes and centrifuge employed by these workers. We found at 2 min of incubation 10% of the glucose 6-phosphatase and 16% of adenylate kinase in the supernatant (as % of the enzyme content of untreated cells). However, the apparent enzyme content of the pellet was very low, with total recovery (supernatant plus pellet) of about 40% for glucose 6-phosphatase and 50% for adenylate kinase. Apparently the oil interferes in the enzyme assay. We note that the total activity of glucose 6-phosphatase reported by Cook et al is only 30% of that observed by us.

contain about 60% of the hepatocyte protein. About 45% of this protein is mitochondrial, 30% endoplasmic reticulum, and 7–10% nuclear protein. A few percent are due to the small organelles and to retained cytosolic enzymes. For example, Cook et al [4,16] observed very little loss of acetyl coenzyme A (CoA) carboxylase even after prolonged digitonin treatment. Cytoskeletal elements such as actin, tubulin, intermediate filaments, and the numerous accessory proteins (for a listing, see Birchmeier [17] are not likely to contribute over 10% to the protein mass of the treated cell.

To sum up, digitonin-treated cells prepared by our procedure contain the nucleus, nearly all the mitochondria and peroxisomes, at least 90% of the endoplasmic reticulum, and most of the lysosomes. The mitochondria are intact and retain their oxidative function. While there may be some structural alterations of the endoplasmic reticulum (see below), the endoplasmic membrane is intact. These results suggest that the treated cell represents a stable organelle complex that is likely to have retained most of its enzymatic and metabolic capacity associated with the organelles. In reconstitution experiments, when supplemented with cytosol and cofactors, the system was capable of extensive ATP and NADH generation and formed glucose at substantial rates from a number of substrates, including lactate. This preparation should be of potential interest for the study of cell function and of regulation.

VISUAL AND ELECTRON MICROSCOPY

Digitonin-treated cells increase in volume as shown by doubling of the packed cell. Figure 2A,B shows phase contrast micrographs of treated and untreated cells. The main apparent difference is the loss of refractivity at the cell periphery. The treated cells are very stable in 0.25 M sucrose-50 mM NaHepes, with no apparent damage or change in shape after hours of standing. On the other hand, untreated cells develop blebs within half an hour, and within an hour or two become highly distorted.

Hepatocytes suspended in KHB buffer or in sucrose media appear much the same in transmission electron micrographs, except that those in sucrose are more compact. The appearance of mitochondria in isolated hepatocytes differs from that of liver in situ, with the mitochondria highly condensed (Fig. 3A). Transmission electron micrographs of treated cells are shown in Figure 3B–D. The background is much ligher than in Figure 3A, owing to the loss of cytosolic protein and the doubling of cell volume. There is no evidence for the plasma membrane, and the surface of the cell appears frayed. The mitochondria appear much the same as in untreated cells. An occasional cell shows a swelling of the intramitochondrial space (Fig. 3C). The outer mitochondrial membrane appears with rare exceptions intact, as shown at high magnification (Fig. 3D). The nuclei appear larger and lighter but the nuclear membrane appeared to be intact (Fig. 3D).

Digitonin treatment caused a major change in the appearance of rough endoplasmic reticulum. There was a reduction in the number of parallel stacks, and in some sections few of them were seen. This is consistent with the loss in RNA, shown in Table I. An association and contact between reticulum and mitochondria is shown in Figure 3D. Reticulum appears as thin-walled vesicles with some ribosomes attached at the surface.

Scanning electron micrographs of the cell interior reveal an image greatly different from that conveyed by transmission of the 0.5-μm section. As shown in Figure 4A–C, the surface revealed by the removal of the plasma membrane is

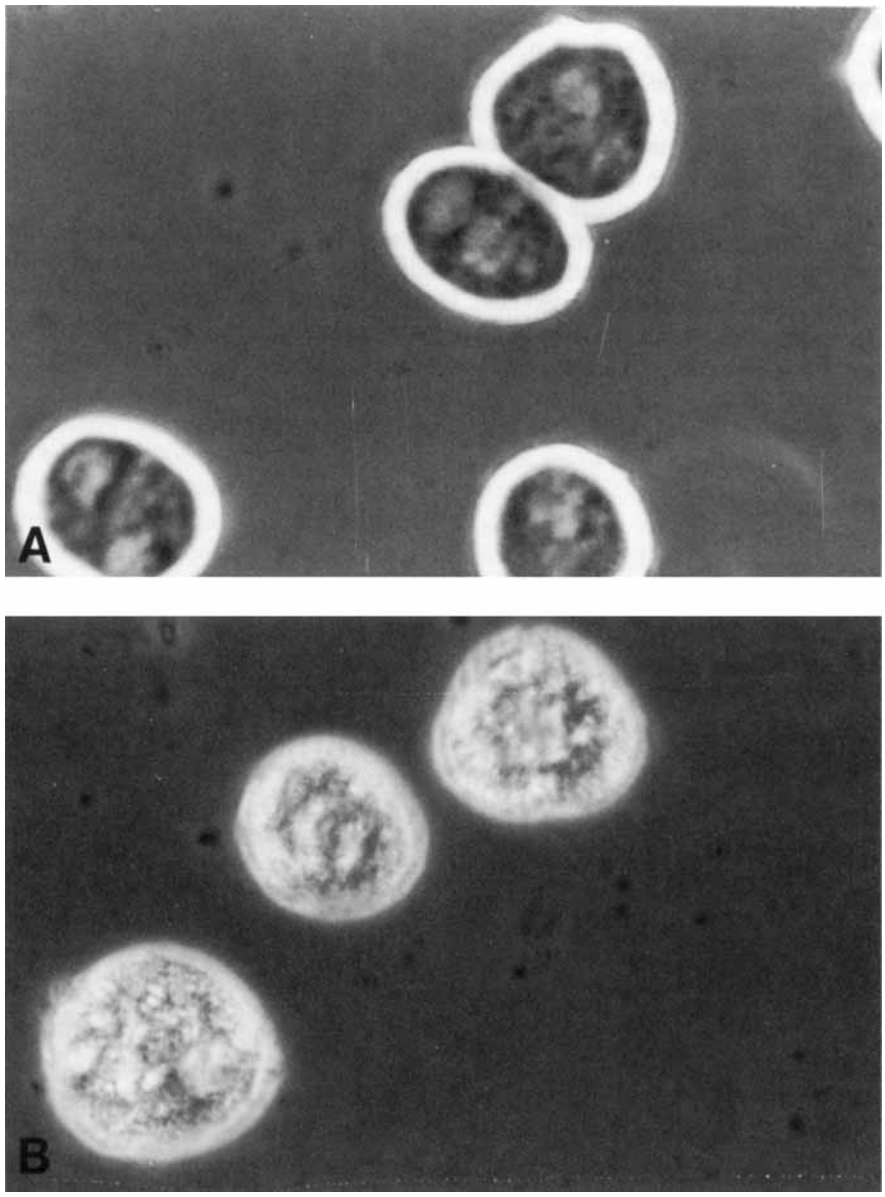


Fig. 2. Microphotographs of control and digitonin-treated cells. Hepatocytes suspended in 0.25 M sucrose-50 mM NaHepes, pH 7.4 A) Untreated control cells. B) Digitonin-treated cells in same medium, 1 hr after treatment. Note the loss of birefringence at periphery. Phase contrast, ×400.

composed of a dense network of lamellae and fibers continuous with the outer membrane of the nucleus and mitochondria. The enlargement of Figure 4C shows a complex structure made up of several types of membrane sheets and filaments. We cannot identify the nature of these elements in these photographs, but clearly from correspondence with transmission micrographs and chemical composition, the major

part of the network is made up of endoplasmic reticulum. Figure 4A–C reveals only the appearance of the surface of the treated cell. As will be shown below, the cell interior is also filled with a dense filamentous network.

The Plasma Membrane

Our transmission and scanning electron micrographs show no trace of the plasma membrane, even when examined at a magnification of 100,000. Siess and Wieland [2] noted only small fragments of the membrane after 15 sec of digitonin treatment, and they and others [3,16] could not detect this membrane after longer treatment. Cook et al observed no membrane after 1 min of treatment. After 10 min the cells were extensively degraded, and the cell periphery eroded and was irregular. However, the loss of 5-nucleotidase, a common marker for plasma membrane, was only 15%, whether the treatment was for 1 or 10 min. This enzyme is predominantly located in the canalicular domain of the plasma membrane [18], which comprises only a small fraction of the surface area. We thus assayed for another marker enzyme, Mg and (Na,K)-ATPase. The latter is a marker for the sinusoidal domain [19]. We also iodinated the hepatocytes with a procedure that introduces the label only in the protein extending into the exterior space. Table III shows a uniform loss of some 20% of the markers upon digitonin treatment. In one experiment (performed by Dr. Mayer Davidson at Cedars-Sinai Research Institute) the binding of insulin to hepatocytes before and after digitonin treatment was determined. The percent specific binding decreased from 10.3% to 8.3%, about a 20% loss.

The contrast between visual observation and the retention of marker enzymes is very puzzling. An attachment of fiber elements to the plasma membrane has been proposed in hepatocytes by French et al [20], and we speculate that membrane fragments remain attached to such fibers and are possibly internalized.

Disintegration of the Nude Cells

When the nude cells were suspended in sucrose containing above 25 mM sodium or potassium ions, there was no change in shape for many hours. However, when the nude cells were suspended in 0.25 M sucrose containing no or only 2 mM sodium ions, the cells rapidly disintegrated. The breakdown was nearly explosive. Within 2 min no intact cells remained. Breakdown was nearly complete at 4–6 min, as shown by the phase micrograph of Figure 5A. The suspension contained only nuclei and free organelles. As a result of Brownian movement, these photographs are blurred, but the absence of cells is apparent by comparison with Figure 2B. Scanning electron micrographs (Fig. 5B–D) of such preparations showed nuclei, mitochondria, and small unidentified organelles, many of them with attached short fibers or lamellae.

In the presence of KCl or NaCl, disintegration was retarded. With 5 mM KCl, the course of disintegration could be conveniently followed under the microscope. First, there were heavy streams of organelles streaking from the cells; later there appeared cell fragments consisting of nuclei surrounded by masses of organelles and chains made up mainly of mitochondria. Disintegration was usually complete by 20–25 min. The process of the disintegration in the presence of 5 mM KCl is illustrated by the scanning electron micrographs of Figure 6A–C. The area around the nucleus was slowest to erode. Chains of mitochondria held together by fibers and reticular strands were present and finally disintegrated; some chains of smaller organelles were also present. The appearance of chains (Fig. 6C) resemble strikingly the structures

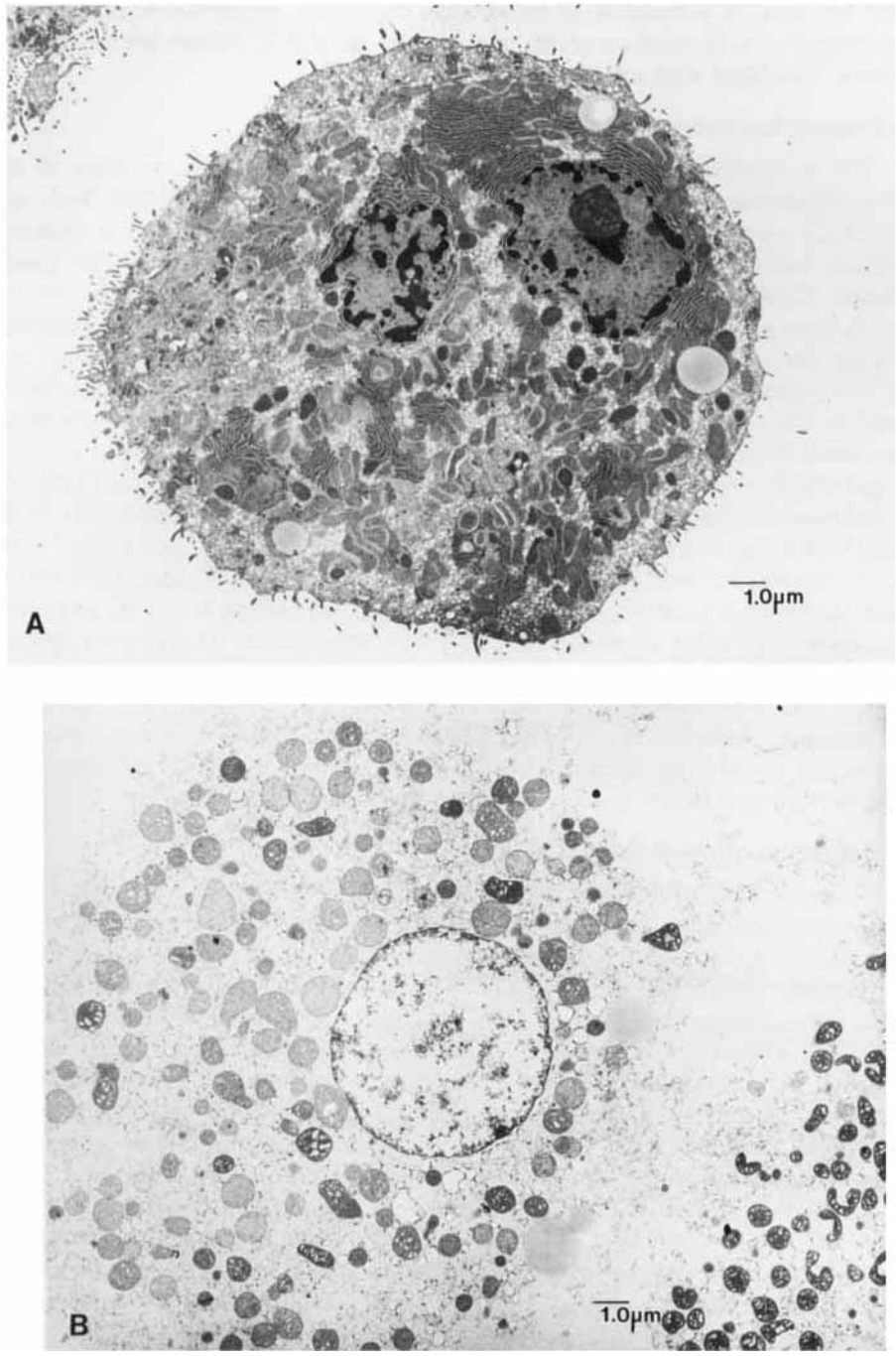
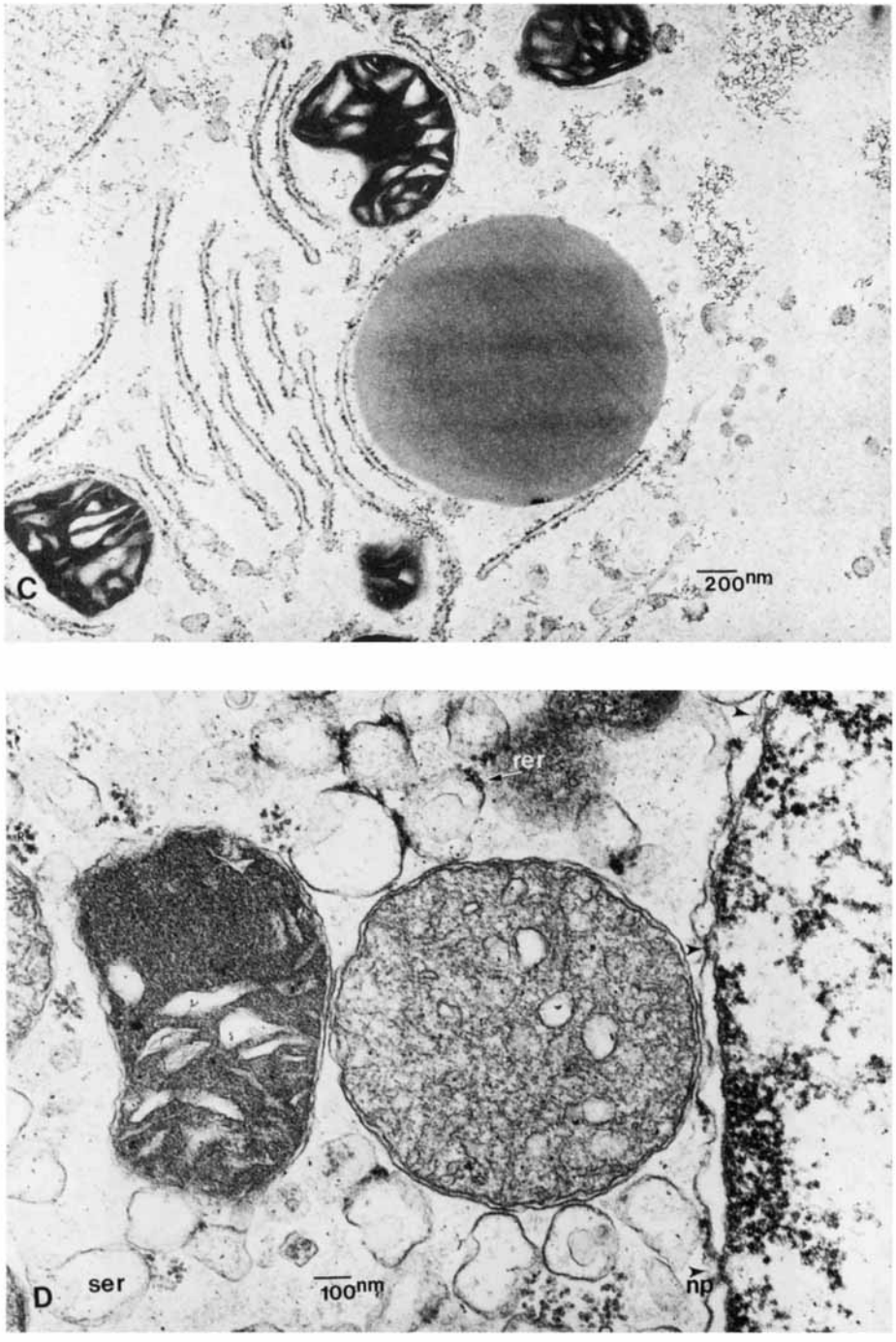


Fig. 3. Transmission electron micrographs of hepatocytes. A) Untreated cells in 0.25 M sucrose-50 mM NaHepes. ×10,000. Note contraction of cell and nucleus (see text) and highly condensed mitochondria. B–D) Digitonin-treated cells in same medium as A. B) Note absence of plasma membrane, increase in volume of cell and nucleus, scarcity of rough endoplasmic reticulum. ×10,000 C) Lipid droplet and



mitochondrion with intact outer membrane and (rarely seen) enlargement of intramitochondrial space. ×50,000. D) Note mitochondrion with intact membrane, nucleus with intact double membrane and nucleopores (np), vesicles of rough endoplasmic reticulum (rer), with attached ribosomes, free ribosomes (R), and smooth endoplasmic reticulum (ser). ×100,000.

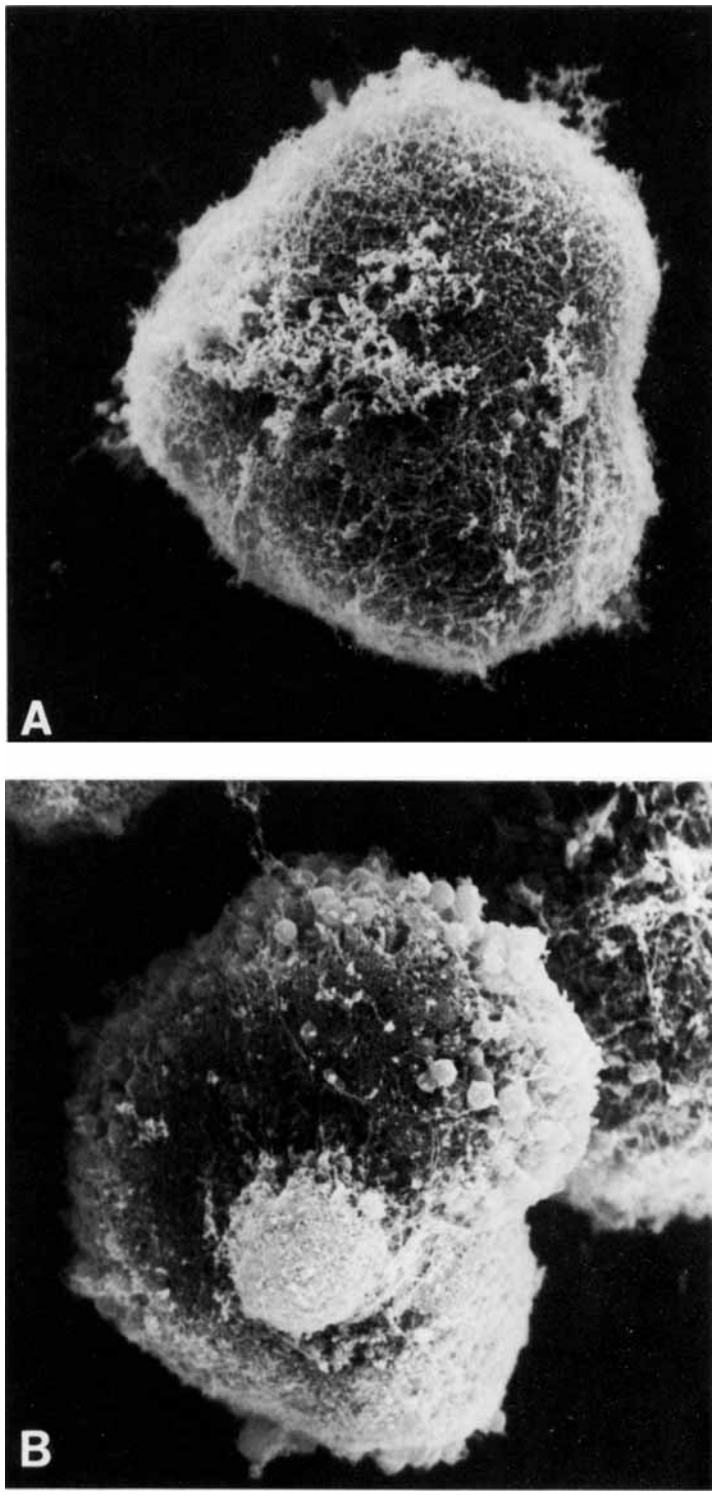


Fig. 4. Scanning electron micrographs of digitonin-treated cells. A, B. View of two cells. Note heavy fiber network in the interior at the surface of the nude cell, with intimate contact between network elements and outer membranes of nucleus and mitochondria. ×4,000. C. Enlarged view. ×8,000.

TABLE III. Extraction of Plasma Membrane Proteins with Digitionin*

		Content[a]	Supernatant (%)	Pellet (%)
Protein	mg/ml	174	45	61
5-nucleotidase	Units	6.2	19	84
Mg-ATPase	Units	5.3	20	60
(NaK^+)-ATPase	Units	1.4	21	73
TCA insoluble	Untreated cells	—	8	92
^{125}I	Ditigonin-treated	—	15	77

*Cells were treated with digitonin (1 mg/ml per 0.25-ml packet cells) for 2 min as described in Methods. Recovery in supernatant and pellet after digitonin treatment is expressed as % of that in untreated cells. Mean of two experiments.

[a]mg or μmol/min per ml of packed cells.

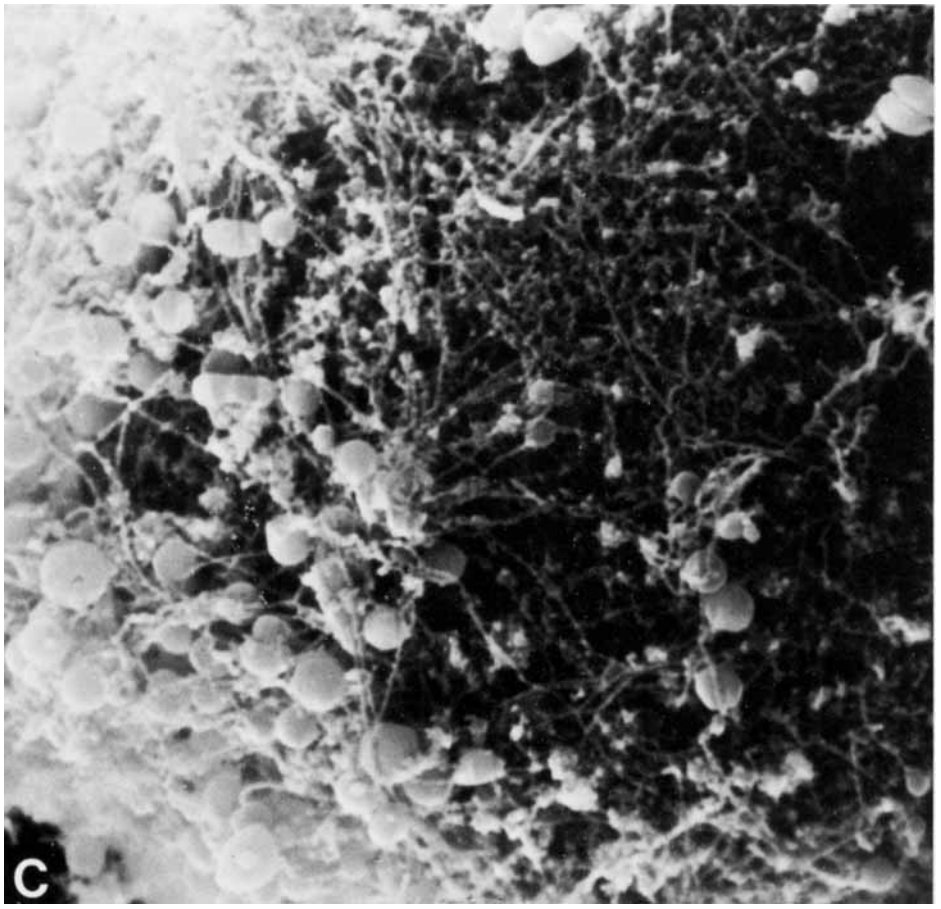


Fig. 4C

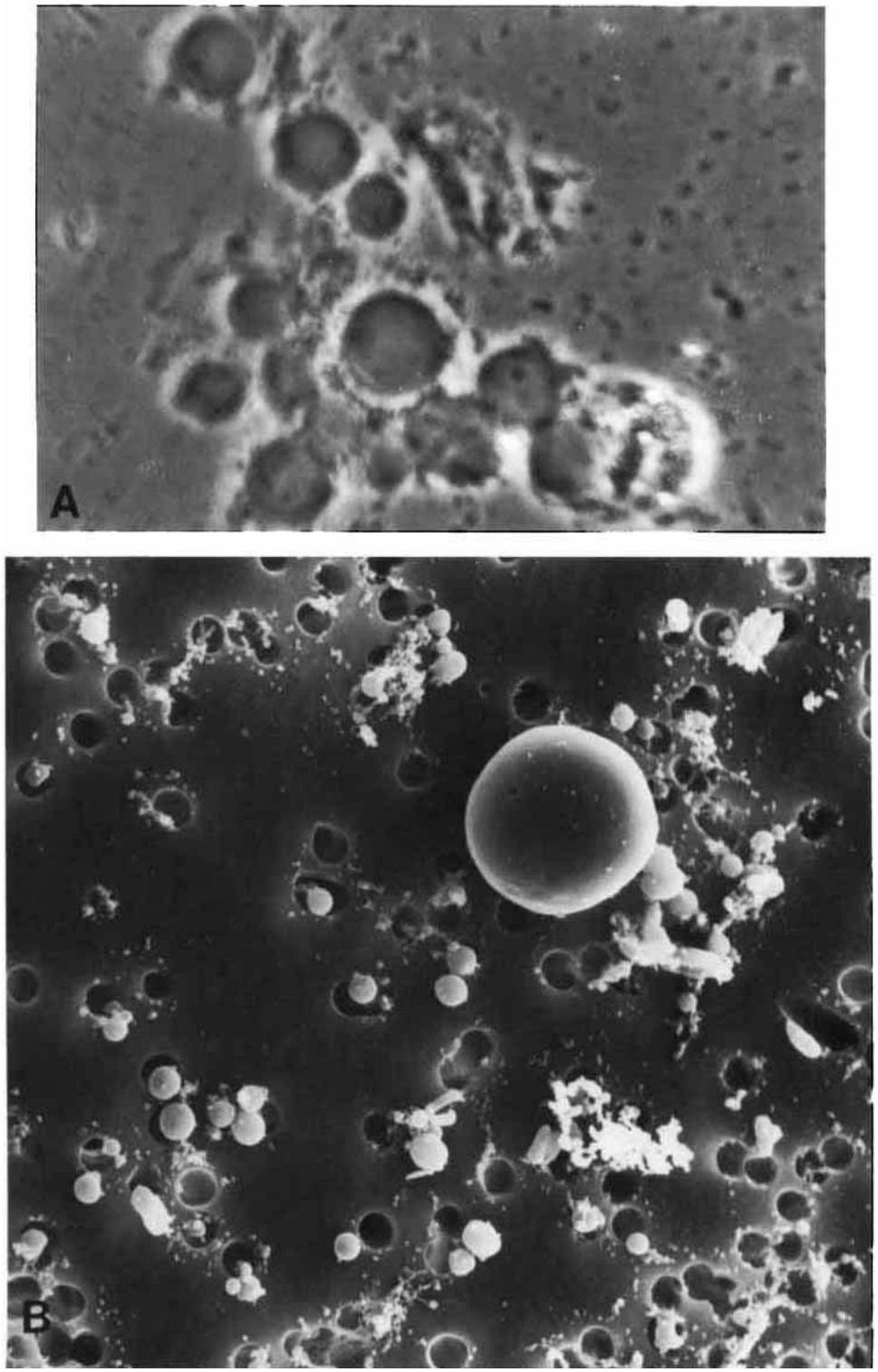


Fig. 5.

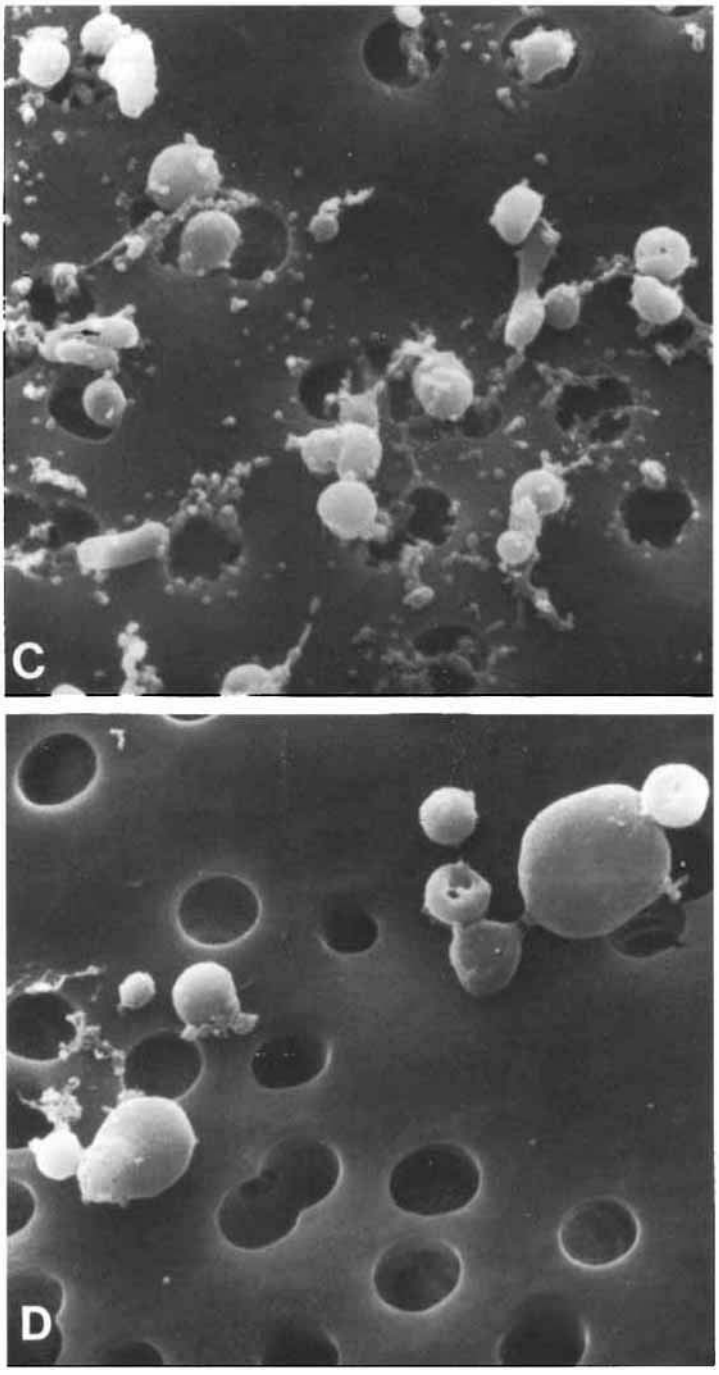


Fig. 5. Dispersed nude cells. Cells were suspended in 0.25 M sucrose-5 mM NaHepes following digitonin treatment. A) Phase contrast micrograph taken after 4 min. As a result of heavy Brownian movement, the appearance of the organelles is rather blurred. Note (by comparison with Fig. 2B) the complete absence of intact cells with the suspensions consisting of nuclei and organelles, mainly mitochondria. ×400. B–D) Scanning electron micrographs of several preparations as in A. The suspensions were mounted on Nucleopore filter paper. The pores (dark circles) are 1 μm in diameter. B) Nucleus, mitochondria, and unidentified small organelles. ×4,000. C) Enlargement of B. The larger organelles are mitochondria. Note attachment of fibers or lamellae to mitochondria and small organelles. ×10,000. D) Same preparations as in C. Note different size of mitochondria. × 10,000.

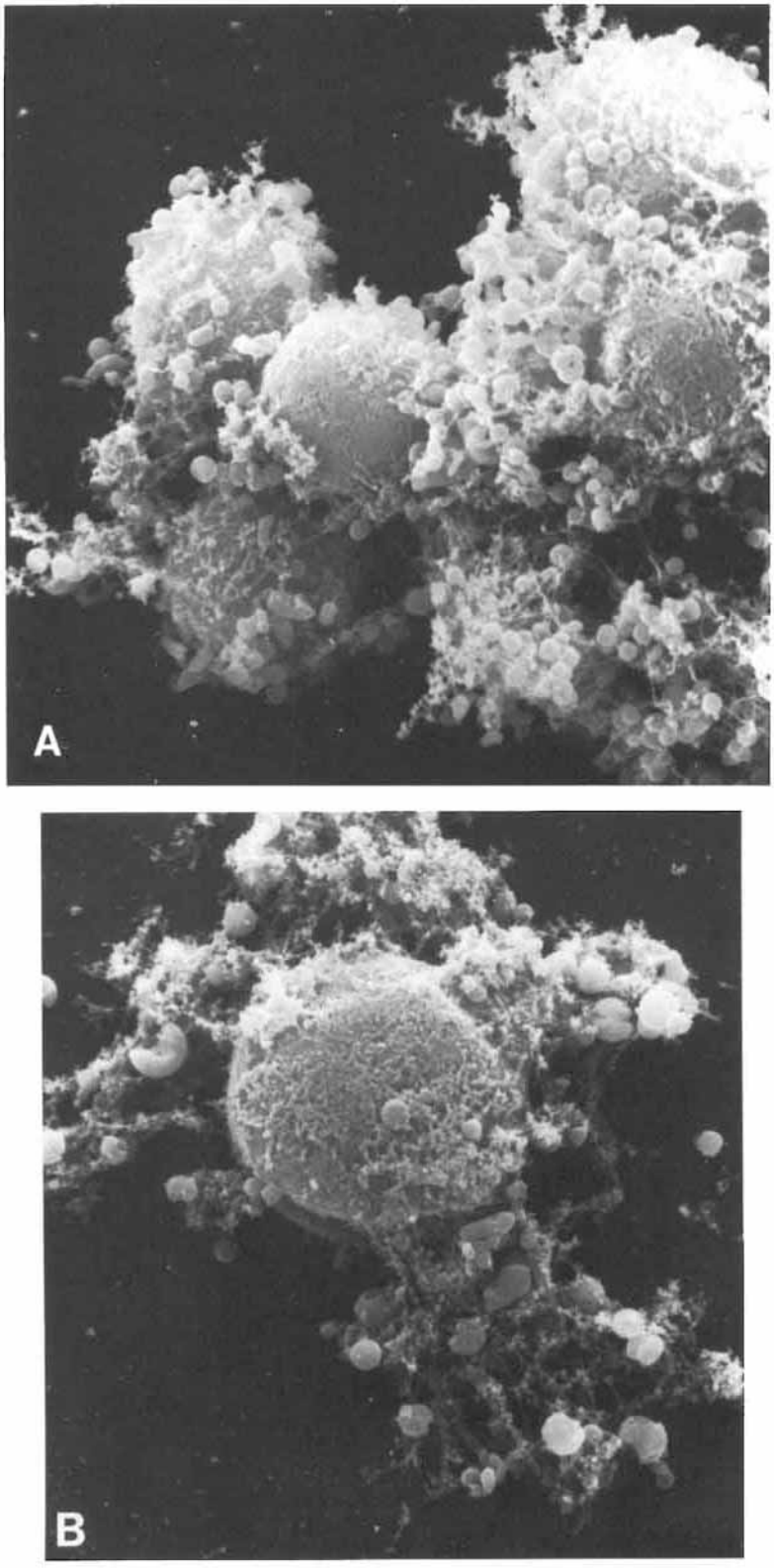


Fig. 6.

shown by us with negative staining of mitochondrial preparations from rat liver homogenates [1]. At the end the suspension consisted of nuclei and single organelles. Some mitochondria had attached "tails" of membranes. Upon standing the nuclei tended to aggregate and to stick to each other, forming large clumps in about 30–40 min.

When the cells were suspended in 0.25 M sucrose containing 10–15 mM NaCl or KCl, the time of breakdown was extended to 1–2 hr. The cells were stable overnight at 50 mM KCl. When KCl was added during breakdown, the disintegration was stopped.

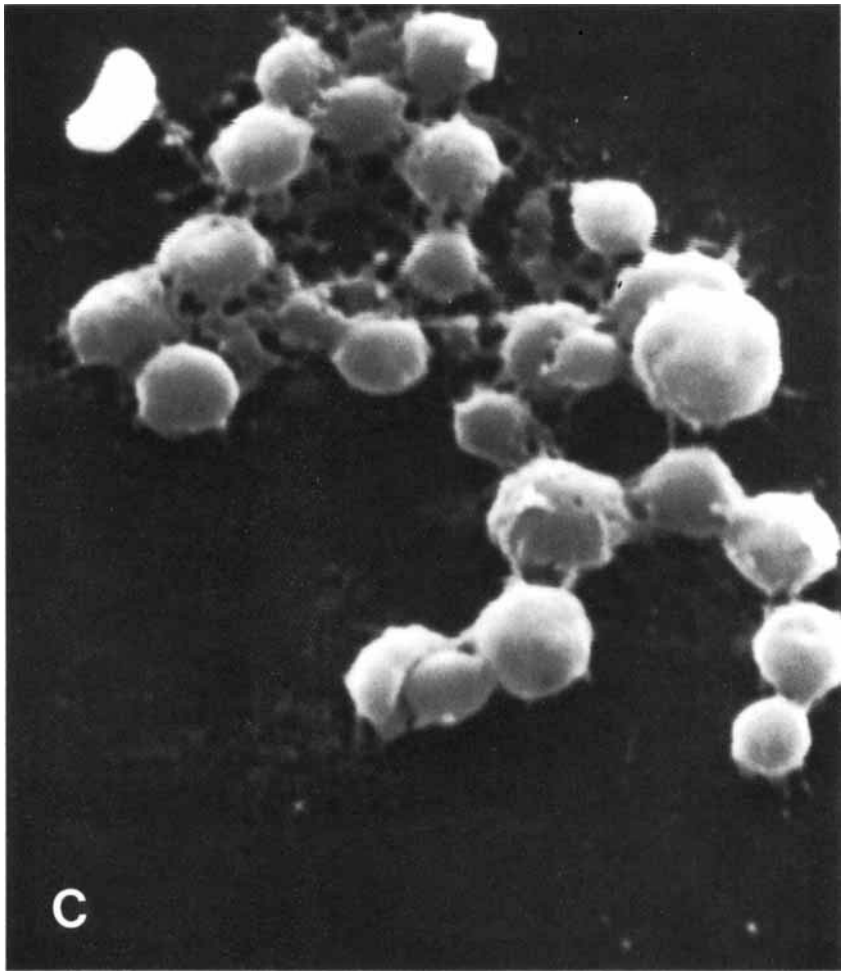


Fig. 6. States in disintegration. Scanning electron micrographs of nude cells suspended in 0.25 M sucrose 5 mM NaHepes, 5 mM KCl. A, fixed in glutaraldehyde at 6 min; B and C, at 12 min. A) The area around the nucleus is the last to disintegrate (see text). Note fibers attached to mitochondria and nuclei. ×4,000. B) Further stage of disintegration. Note network continuous between nucleus and mitochondria. ×5,000. C) Detail; note multibranched network of mitochondria and attachment by thin fibers and lamellae. ×16,000.

The composition of the disintegrated cell suspension was examined by gravity sedimentation as used with liver homogenates. Nude cells were suspended in 0.25 M sucrose-5 mM NaHepes, and after 30 min, KCl was added to one-half of the preparation, to a concentration of 50 mM. The two suspensions were centrifuged to separate sequentially the 300g, 1,500g, and 10,000g pellets and a supernatant fraction. Microscopic examination showed the nuclear fraction to consist of clumps of nuclei that entrapped numerous mitochondria, especially in 50 mM KCl. The 1,500g and the 10,000g pellets at phase contrast microscopy appeared to consist of mitochondria, single and in short chains. The chains were more prominent in the presence of 50 mM KCl. The distribution of the enzyme markers is shown in Table IV. Except for the higher yield in the nuclear fraction, the distribution of glutamic dehydrogenase and glucose 6-phosphatase resembles that in liver homogenates prepared in 0.25 M sucrose with either 5 or 50 mM NaHepes [1]. The presence of salt shifted the sedimentation pattern to lower centrifugal force and increased the association of glucose 6-phosphatase with mitochondria. About one-third of this enzyme remained associated with mitochondria in the low-salt and two-thirds in the high-salt medium. In the medium with 50 mM KCl one-half of the mitochondria and 30% of the endoplasmic reticulum and about 30% of the small organelles came down at 300g in the nuclear pellet, probably by trapping of chains of the organelles by the large nuclear aggregates. Mitochondrial chains were apparent in liver homogenates when these were made 50 mM in KCl [1].

The large difference in the ratio of glucose-6-P to glutamic dehydrogenase between the 1,500g and the 10,000g pellet in the absence of KCl indicates that the majority of mitochondria in the 1,500g fraction contain very little endoplasmic reticulum, whereas those centrifuging at higher speed contain appreciable amounts. These may correspond to the mitochondria in the electron micrograph pictures containing "tails" of attached membranes. In Table IV, the distribution of peroxisomal and lysosomal marker enzymes is also shown. Both of these organelles appear in the three particulate fractions, and their sedimentation shifts to lower centrifugal force in higher salt medium, as with mitochondria.

pH, Oxygen, Phosphate, and the Stability of the Nude Cells

When inadvertently the digitonin medium was gassed with 95% O_2-5% CO_2 rather than with 100% O_2, there was no breakdown of the treated cells. This was traced to the acidification of the medium. The breakdown is rapid between pH 7.4 to 8.2, slows down below pH 7.2, and the cells are completely stable in 0.25 M sucrose at a pH below 6.8. When the media were not gassed with O_2, breakdown was retarded, and it was very slow or did not occur at all when the media were gassed with nitrogen.

Most of the results described here were obtained with hepatocytes prepared with one batch of collagenase. Subsequently, upon its exhaustion, we found, with several other batches of this enzyme, that the rate of disintegration of the cell was somewhat variable and slower. Thus, in the presence of 5 mM KCl, complete disintegration required from 40 to 60 min. The addition of 0.1 mM phosphate during the preparation of the cells increased the rate of breakdown severalfold, to an equal or faster level than that described here.

The Cytostructure of the Hepatocyte

An association of cytoskeletal elements with organelles has been described in numerous studies. Correspondence in the location of mitochondria and tubulin fibers

TABLE IV. Fractionation of Disintegrated Digitonin-Treated Nude Cells*

	Protein		Glutamic Dehydrogenase		Glucose 6-phosphatase		Uricase		Acid phosphatase		Relative glucose 6-phosphate Glutamic dehydrogenase ratio[b]	
Content[a]	71		220		9.8		0.95		4.3		0.045	
KCl (mM)	0	50	0	50	0	50	0	50	0	50	0	50
300g pellet [c]	18	37	15	50	14	31	12	31	10	27	0.93	0.62
1,500g pellet[c]	21	26	52	35	5	14	17	22	30	19	0.096	0.40
10,000g pellet[c]	22	12	31	15	14	19	62	33	31	27	0.45	1.27
Supernate[c]	37	30	2	1	69	34	10	11	30	27	—	—
Recovery[c]	98	105	100	101	102	98	101	97	101	100		

*Hepatocytes were treated with digitonin as described in Methods. The protein content of the supernatant and treated cells was determined. (Digitonin extracted 41% of the protein, 3% of the glutamic dehydrogenase, 5% of glucose 6-phosphatase, no uricase, and 28% of acid phosphatase.) The pellet of nude cells was taken up in 0.25 M sucrose-5 mM NaHepes, 20 ml per ml of untreated hepatocytes, and incubated for 30 min. One-half of the suspension of nuclei and organelles was made 50 mM in KCl, and both suspensions were centrifuged and fractions separated, as described by Katz et al [1]. The protein and enzyme content of each fraction was determined and expressed as % of the content of the digitonin-treated cells. Average of two experiments.

[a]Protein content (mg) and enzyme activities (μmol/min) remaining in the cells after treating 1 ml of packed hepatocytes with digitonin. This amount was set as 100%.

[b]The ratio in the preparation (9.8/220 = 0.045) was set to 1.00.

[c]Percent in fraction.

has been demonstrated by immunofluorescence in fibroblasts [21], cultured cell lines [22,23], and nerve cells of insects [24]. Cross-bridges connecting tubulin to the outer mitochondrial membrane and to other organelles have been described in epithelial cells and neurons [25].

There are relatively few studies on the cytoskeleton of liver cells (reviewed by Fisher and Phillips [26]. Fiskum et al [3] suggested an association of mitochondria with intermediate filaments. French et al [20] proposed that mitochondria are attached to intermediate filaments, which are anchored in the plasma membrane. A number of studies support a role for endoplasmic reticulum (ER) in the cytostructure of the liver cell. Continuity between the ER and the outer nuclear membrane is well documented ([27] and references therein). A continuity between ER and the outer mitochondrial membrane was described in liver cells by Morrè in 1971 [28] and has been extensively documented by Cascarano and coworkers [29,30]. Such connections have also been described in neurons [31], Tetrahymena [32], onion root tips [28], and in fungi [33]. Novikoff and Shin [34] described in hepatocytes a continuity between ER and lysosomes. Biochemical studies by Tata and coworkers [14,35], Meier et al [36], and Cascarano and coworkers [29,30] provide evidence for the attachment of the ER to mitochondria. Our own work [1] supports and extends these observations. Our negatively stained electron micrographs of liver homogenate preparation show the existence of a network of mitochondria held together by fibers and strands of material, which, judging from the enzymic composition of the fraction, is mainly endoplasmic reticulum. These structures resemble those in Figure 5, which were obtained by scanning during cell disintegration. Claims that these structures are artifacts resulting from coprecipitation at high salt concentrations or artifacts of dehydration in preparations for electron microscopy are untenable. We show that these structures are present in the absence of salt at low pH, and there is no dehydration in negative staining.

The nature of the network revealed by scanning the hepatocyte interior is unknown. It resembles somewhat the network in the cells and neurons obtained by Heuser and coworkers [37,38] and by Hirokawa [25] by quick-freeze, deep-etch electron microscopy. Most of the elements of this dense network were not identified by them. The hepatocyte network may contain tubulin, intermediate filaments, and actin, as well as other uncharacterized fibers. However, since about 30% of the protein of the nude cell is that of ER, which greatly exceeds the contribution of any other cytoskeletal element, most of the network must consist of the lamellae of the ER. A major role of ER in the mitochondrial network is supported by the fractionation studies. ER may be connected directly, or through some accessory proteins to the nucleus and mitochondria, and probably to other cell organelles.

The disintegration of the nude cells in low-salt isotonic sucrose medium is a novel and striking observation. The very narrow physiological pH range and the requirement for oxygen and phosphate suggest that the disintegration is somehow coupled to oxidative phosphorylation. In studies in progress, to be reported elsewhere, we found that cyanide, carboxyatractyloside, and oligomycin greatly retard or abolish disintegration in salt-free sucrose, and this inhibition is promptly released, with immediate breakup, by addition of ATP or several other nucleotides. We speculate that the disintegration depends on two separate independent events. A prerequisite is a state of depolymerization of some protein, which may have a role in the attachment of some cytoskeletal elements to mitochondria (or other organelles) or to each other.

This requires oxygen and presumably oxidative phosphorylation. Disintegration occurs then by breakage of some bond unstable in the absence of salt. Mitochondrial movement (saltation) in cells occurs [39], and, possibly, partial depolymerization of some proteins in the cytostructure may be a normal state under aerobic conditions. In this state, breakage of some bonds at (unphysiological) low salt concentrations would lead to disintegration.

This working hypothesis is at present highly speculative. Much further work using labeled antibodies against known elements of the cytoskeleton will be required for the identification of the components of the cytostructure and the nature of the labile bonds.

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