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The Role of ATP in the Cytostructure of the Hepatocytes

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We have previously described the preparation of hepatocytes from which the plasm membrane was removed by digitonin treatment. Such "nude" cells were found to be very stable in sucrose media containing above 50 mM NaCl or KCl, but they disintegrate near instantly in salt-free media, liberating nuclei, mitochondria, and other organelles. We show here that disintegration occurs at a physiologic pH and in the presence of oxygen. Disintegration was blocked by rotenone, oligomycin, KCN, and carboxyatractyloside, establishing that oxidative phosphorylation and ATP generation is essential for disintegration to occur. The addition of ATP, GTP, ITP, or ADP (but not AMP) in the presence of the inhibitors, induced breakdown.

Taxol, an inhibitor of tubulin depolymerization and phalloidin, a drug that stabilizes actin fibers, prevented disintegration in salt-free media. The effect of these drugs was counteracted by the addition of ATP.

Our results show that two conditions are essential to induce the disintegration of the nude cell: media of low ionic strength, and ATP generation. The ATP effect is likely to be of physiological significance, suggesting role for ATP generation in affecting polymerization of cytoskeletal elements.

Key words: hepatocytes, cytostructure, digitonin, ATP, taxol, phalloidin

We have described the stripping of the plasma membrane from isolated rat hepatocytes by digitonin treatment [1]. Such "nude" cells show no vestiges of the plasma membrane. They contain about 60% of the hepatocyte protein and retain the nucleus and virtually all of the mitochondria, peroxisomes, and 80% of the lysosomes without damage to the outer mitochondrial membrane and membranes of the endoplasmic reticulum. Electron microscopy revealed a dense network of fibers which are attached to the nuclear and mitochondrial outer membranes and probably to other organelles. These "nude" cells are very stable in media of high ionic strength.

We have previously shown [2] that in liver homogenates prepared in media of high ionic strength, most of the mitochondria are present as branched chains held together by a network composed largely from endoplasmic reticulum. In low salt media, these chains break up, liberating free mitochondria. Likewise, we found [1]

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that digitonin-treated "nude" cells, which retain their shape for many hours in 0.25 M sucrose containing above 25 mM NaCl or KCl, disintegrate nearly explosively in salt-free sucrose. Breakdown occurs only at above pH 7 and only when the media are oxygenated. These observations suggested that the breakup of the nude cells was associated with an active aerobic metabolic process. Indeed, we show here that breakdown requires ATP generation. The breakup is prevented by inhibitors of oxidative phosphorylation (rotenone, oligomycin, and KCN), inhibitors of ATP transfer (carboxyatractyloside), and an uncoupler (dinitrophenol). Breakdown is restored under these conditions by the addition of ATP and several other nucleotides.

We also report that taxol and phalloidin, drugs which respectively prevent the depolymerization of tubulin and actin, block the disintegration of the cells in salt-free media, implicating these fibers in maintaining the integrity of the hepatocyte cytostructure.

The relevance of these observations to the nature of the cytostructure of the liver cell is discussed.

MATERIALS AND METHODS

The preparation of hepatocytes and digitonin treatment was previously described [1]. In short, isolated washed hepatocytes (40 mg cells protein per ml were suspended in 0.25 M sucrose, 50 mM NaHepes, pH 7.4) were treated for 2 min with 1 mg/ml of digitonin at room temperature. The medium was aerated with 100% oxygen. The suspension was then promptly diluted with a 10-fold excess of digitonin-free medium; the cells were collected by short centrifugation and suspended in 0.25 M sucrose, 5 mM NaHepes, pH 7.4, with additions as described in the text. In some experiments, the medium contained 0.01 mM sodium phosphate, which enhanced the rate of breakdown. When 1 mM ATP or other nucleotides were used, the controls contained 3 mM NaCl to compensate for the cations introduced with ATP. The salt retards the rate of breakdown.

Cell disintegration was followed by observation by phase contrast micrography at a magnification of ×400 and routinely recorded by photography at this magnification. Due to the need for rapid work, fluid movement, and vigorous Brownian movement of the organelles, the photographic representation is blurred and does not show the details seen visually. However, such photographs indicate clearly the extent of disintegration by the rupture and disappearance of the cells and appearance of free nuclei. Detailed examination of the process is provided by scanning electron micrographs, prepared as previously described [1].

Rotenone, atractyloside, oligomycin, colchicine, podophyllotoxin, cytochalasine B, and phalloidin were purchased from Sigma (St. Louis, MO). Taxol was a gift from the Drug Synthesis Branch, National Cancer Institute, NIH. The compounds were added either in an aqueous solution or in dimethylsulfoxide (10 μl/ml cell suspension). Control experiments established the solvent to be without effect.

RESULTS

Scanning electron micrographs (SEM) of the digitonin-treated nude cells suspended in 0.25 M sucrose, 5 mM NaHepes, 50 mM KCl, are shown at a magnification of ×800 and ×2000 in Figures 1A and 1B. The cells consist of a dense network of

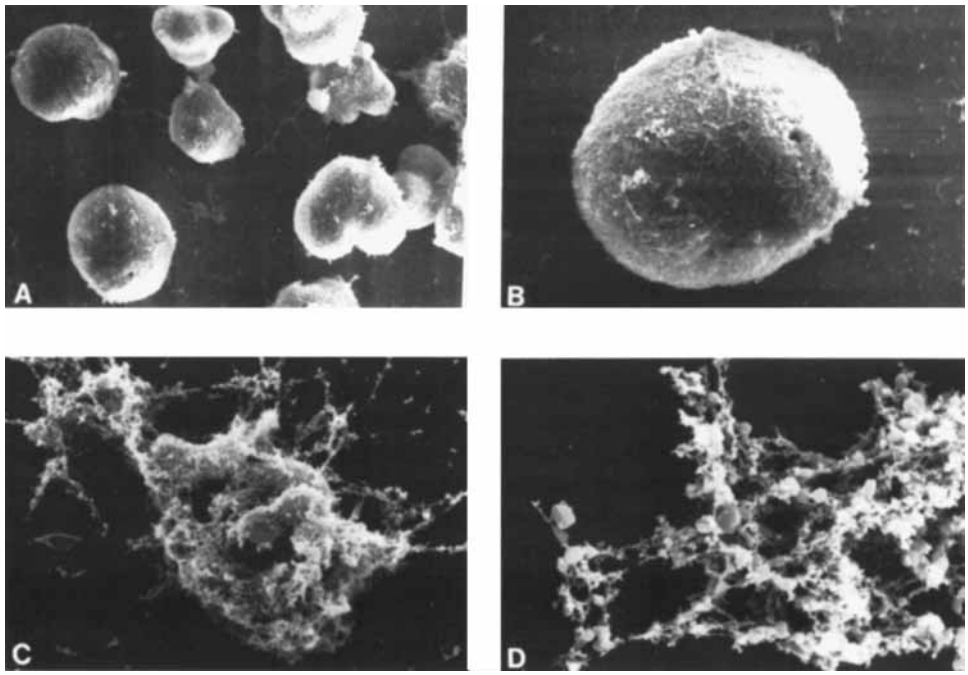

Fig. 1. Disintegration of nude cells in the absence of salt. SEM micrographs of digitonin-treated hepatocytes. The cells are suspended in 0.25 M sucrose, 5 mM NaHepes, pH 7.4, 0.1 mM sodium phosphate and either no KCl or 50 mM KCl. A) 50 mM KCl, 60 min after preparation, ×800. Cells nearly all intact. B) as A) but ×4000. Note dense network in which a nucleus (dark circle) is embedded, and several mitochondria (small light circles) appear on the surface of the network. C) no KCl, 2 min, ×800. Note several nuclei with fragments of the network still adhering to them. D) as in C) but ×4,000. Note numerous mitochondria still attached to fragments of disintegrating network.

fibers of different diameters in which the nucleus, mitochondria, and other organelles are imbedded. When prepared in 0.25 M sucrose containing over 25 mM sodium or potassium ions, the cells retain their round shape as long as overnight with little visual evidence of damage. When transferred into salt-free sucrose, the cells disintegrated completely within minutes, liberating nuclei and other organelles, as shown by SEM in Figure 1C and 1D (for detailed description of the nude cells and their disintegration (1). Figure 2 shows phase contrast micrographs at a magnification of ×400 of the same preparation and at the same time as shown in Figure 1. After 2 min, no intact cells were present, and numerous free nuclei were prominent.

Since rapid breakdown occurred only in aerobic conditions and was nearly absent when the cells were aerated with nitrogen [1], and since low concentrations of phosphate increased the rate of breakdown in many preparations, we suspected that the breakdown was associated with endogenous oxidative phosphorylation. The capacity for oxidative phosphorylation of the nude cells is equal or higher than that of isolated mitochondria. We thus examined the effect of inhibitors of ATP generation. The addition of inhibitors of oxidative phosphorylation, rotenone and oligomycin at concentrations of 50–100 μg/ml, or 0.5mM KCN, completely blocked disintegration in salt-free media. Carboxyatractyloside, an inhibitor of mitochondrial ATP transfer,

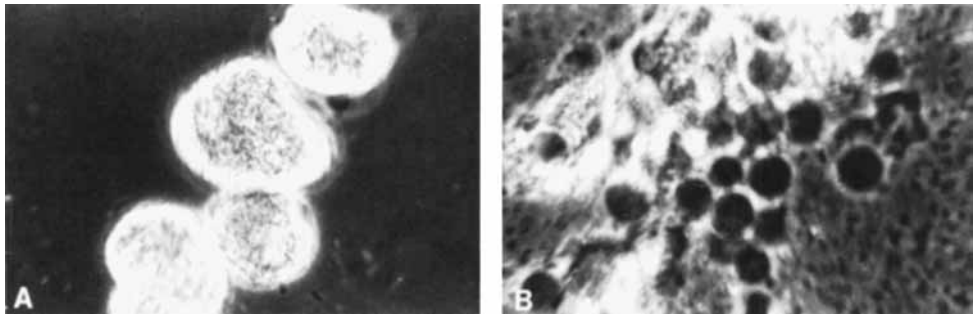

Fig. 2. Phase contrast micrographs of the same preparation as in Figure 1. A) Figure 2A, 50 mM KCl as in Figure 1A, × 400. Nearly all cells intact. Figure 2B, no KCl, as in Figure 1C, ×400. Numerous nuclei (black circles), cell debris and (indistinct) free mitochondria (small black dots).

at 50 μg/ml, also completely blocked disintegration with the cells remaining nearly all intact for periods of at least 1 hr. When ATP at a concentration of 1 mM was added, the cells disintegrated rapidly with complete breakdown within 10 min. The inhibition of breakdown by 0.5 mM KCN and its release by ATP is illustrated by SEM in Figures 3A and 3B. Inhibition by atractyloside and its release by ATP is illustrated by SEM in Figures 4A and 4B. Identical results were seen with rotenone and oligomycin (not shown).

At a concentration of 0.1 mM, ATP also induced disintegration, somewhat slower than 1 mM, but the effect of 0.01 mM ATP was rather limited. The nude cells contain an active ATPase. At 20 min after the addition of 1mM ATP, only 10% was detected in the cells. The endogenous ATP level of perchloric acid extracted cells was low, 0.15 μmol/ml packed cells.

Dinitrophenol, an uncoupler of oxidative phosphorylation, also blocked cell disintegration, but this was only slowly reversed by the addition of ATP. Possibly ATP breakdown was enhanced in the presence of the uncoupler. It should be stressed that in sucrose media containing above 25 mM sodium or potassium ions, ATP was without any apparent effect, with the cells remaining stable for hours. At lower concentrations of cations, the disintegration rate was markedly increased by the addition of ATP.

As described previously, cells suspended in salt-free sucrose aerated with nitrogen remained stable for hours [1]. When the period of anaerobiosis was a few minutes, the addition of ATP induced breakdown, but its effect was limited or absent after longer periods of oxygen deprivation. Thus cells kept for half an hour in 0.25 mM sucrose, 50 mM NaHepes without shaking and aeration, remained stable when transferred to aerated salt-free medium, even in the presence of ATP. Control cells shaken and aerated disintegrated promptly. As described previously, cells kept at pH 6.8 remain stable for many hours in salt-free media, and addition of ATP did not induce disintegration.

The action of ATP was compared to that of other nucleotides. In cells treated with KCN and other inhibitors, ADP caused disintegration at a rate somewhat slower than that of ATP, but AMP was without any effect (Fig. 5) at 1 mM, GTP and ITP

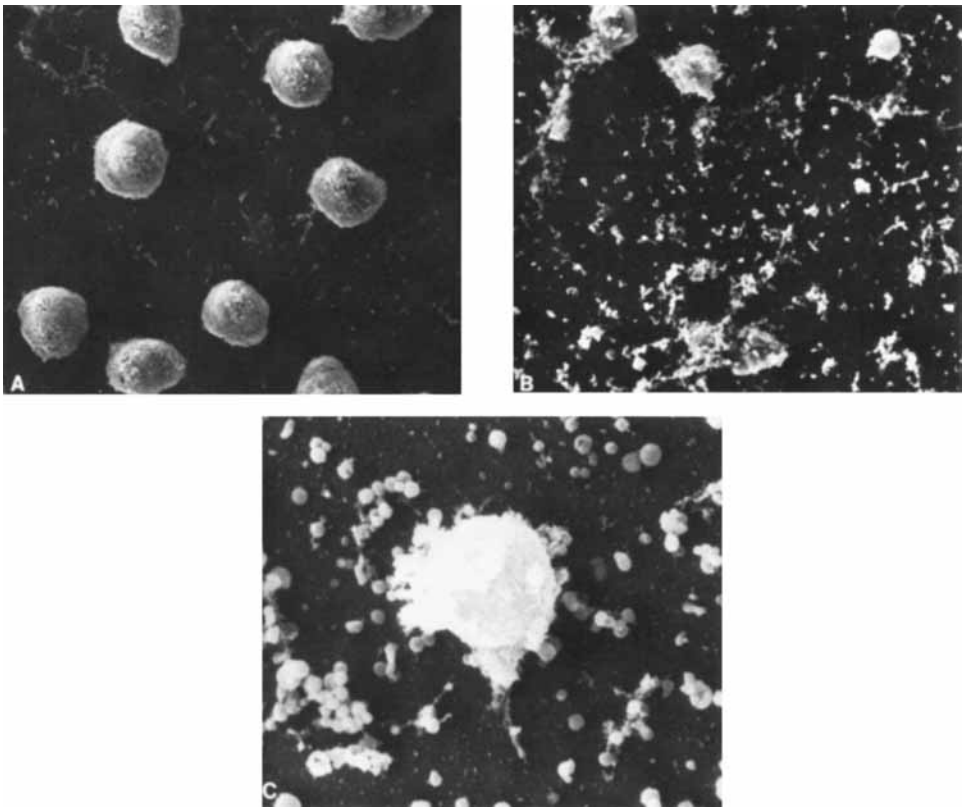

Fig. 3. Effect of KCN on cell disintegration. SEM of nude cells suspended in 0.25 M sucrose, 5 mM NaHepes, pH 7.4, 3mM NaCl. Figure 3A, 0.5 mM KCN, ×800, 30 min after preparation. No disintegration and normal appearance, as in Figure 1A. Same preparation but without KCN, 30 min after preparation, ×800. Note several nuclei, numerous mitochondria, and small organelles. The fibrous network nearly completely disintegrated to vesicles not detected at this magnification (compare with Figure 1C at 2 min). Figure 3C, as in Figure 3B, ×4,000. Note large nucleus, numerous free mitochondria, and unidentified small organelles or vesicles.

induced disintegration in cells whose oxidative phosphorylation was inhibited by KCN and oligomycin, at rates similar to that by ATP (Fig. 5C and 5D). It is not known whether these nucleotides exerted their effects as such or served as precursors to ATP via myokinase or transphosphorylation.

Our results suggest that ATP and possibly several other nucleotides bind to some elements of the cytostructure and induce depolymerization or bond breakage, which in non-physiologic conditions in a salt-free medium renders the whole structure unstable, causing dissolution. Binding of ATP and other nucleotides to actin [3] and tubulin [4] is well known. Bershadsky and Gelfand [5] have shown that in cultured fibroblasts, the addition of ATP causes depolymerization of tubulin fibers. Azide, oligomycin, and dinitrophenol, agents that deplete the ATP content of the cells, inhibited tubulin depolymerization [5].

Liver cells contain tubulin fibers and actin microfilaments [6]. We thus examined their involvement in cell disintegration by using drugs either to induce depoly-

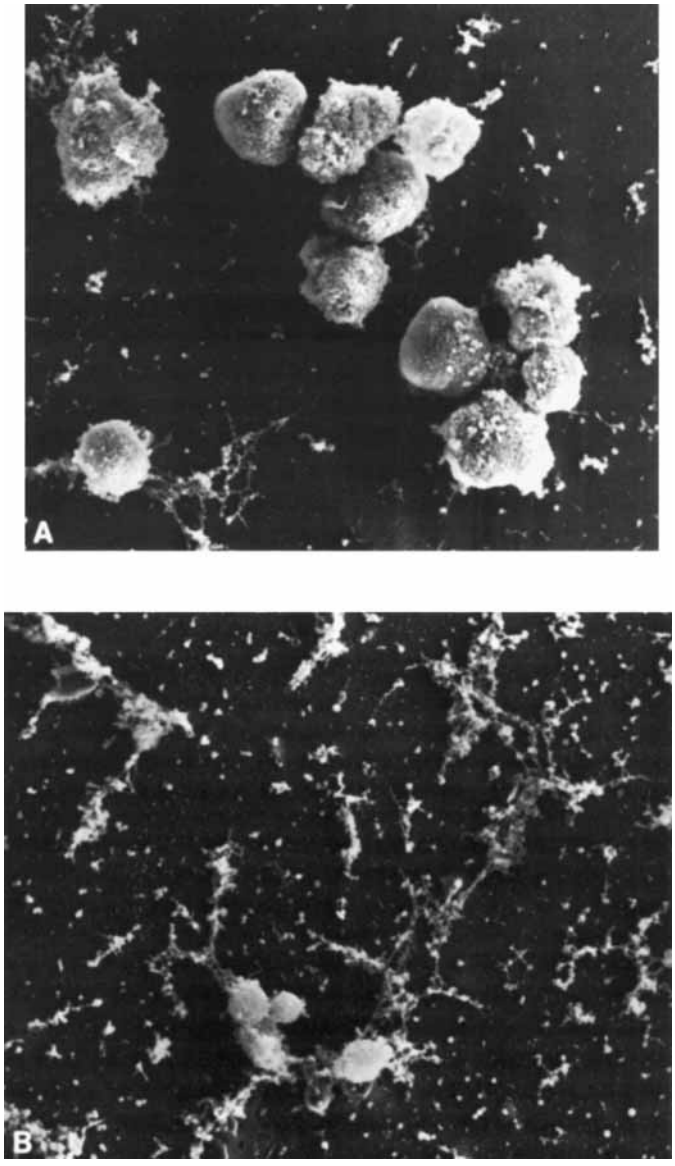

Fig. 4. The effect of carboxyatractyloside and ATP on cell disintegration. SEM of cells suspended in same medium as in Figure 3, with and without carboxyatractyloside. Figure 4A, 100 μg carboxyatractyloside per milliliter, 10 min, ×800. Disintegration inhibited and cells largely intact. Figure 4B, as in Figure 4A, with atractyloside plus 1 mM ATP, 10 min, ×800. Nuclei, free mitochondria, and few remants of the network. Appearance similar to that in Figure 3B. ATP addition completely overcomes the effect of inhibition.

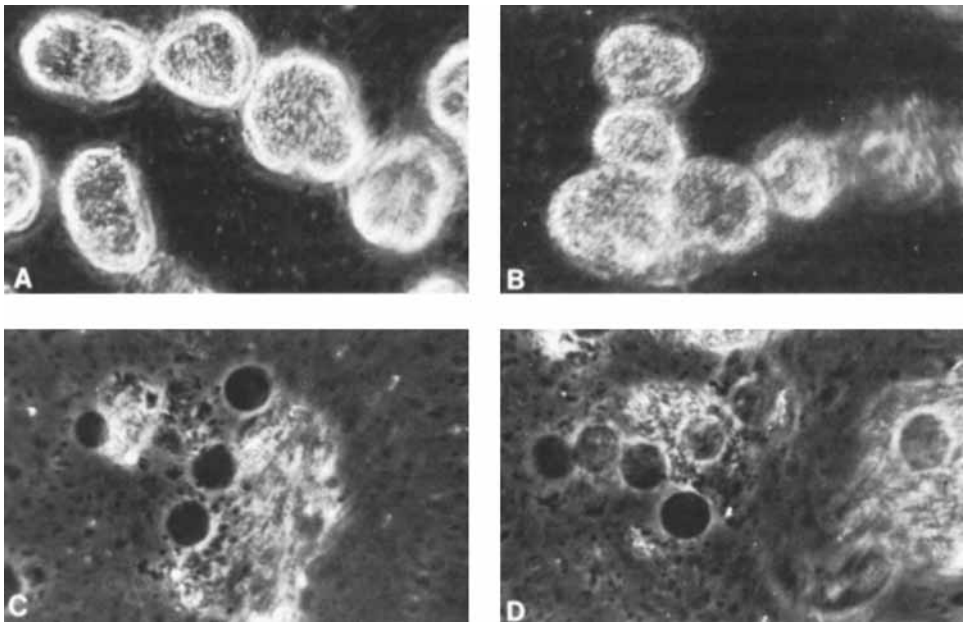

Fig. 5. Effect of nucleotides in cell disintegration. Phase contrast micrographs of cells suspended in 0.25 M sucrose, 5 mM NaHepes, 3 mM NaCl, and 0.5 mM KCN, all at ×400. Figure 5A, no nucleotides, 30 min after preparation. The cells are intact. Figure 5B, as in Figure 3A, plus 1 mM AMP, cells largely intact. Figure 5C, as in Figure 1A plus 1 mM ITP, Figure 1D, as in 5A, but with 1 mM GTP. Figure 5C and 5D, 3 min after nucleotide addition. Note extensive disintegration in Figure 5C and 5D, numerous free nuclei and fragments of cytostructural network, compared to Figure 2B.

merization or to stabilize tubulin and actin structure. Colchicine, vinblastin, and podophyllotoxin, drugs that induce a depolymerization of tubulin [2,7] were at concentrations as high as 100 μg/ml without any visible effect on the appearance or the disintegration of the cells. Taxol is a drug that promotes tubulin assembly in vitro [9] and stabilized microtubules in fibroblasts. Taxol at concentrations of 10 μM inhibited nearly completely the disintegration of the nude cells in salt-free media and greatly retarded the breakdown induced by ATP (Fig. 6A, B, C).

Cytochalasin B is well known to induce the depolymerization of F-actin to G-actin monomers [3]. This drug was without any visible effect on the cells or on cell disintegration. On the other hand, the hepatotoxin phalloidin binds avidly to actin and prevents its depolymerization in many cells, including hepatocytes [10]. There was no disintegration with phalloidin at concentrations of 10 μM in salt-free media (Fig. 6D and E), and it greatly retarded disintegration induced by ATP. These experiments establish that depolymerization of both microtubules and microfilaments has a role in the disintegration of the cytostructure of the hepatocyte.

DISCUSSION

The Cytostructure of the Nude Cell

We have previously described the proximate composition of the nude hepatocyte [1]. The nucleus, mitochondria, and small organelles account for at least one-half of the cell protein, and endoplasmic reticulum for 30 to 35%. The remainder of the

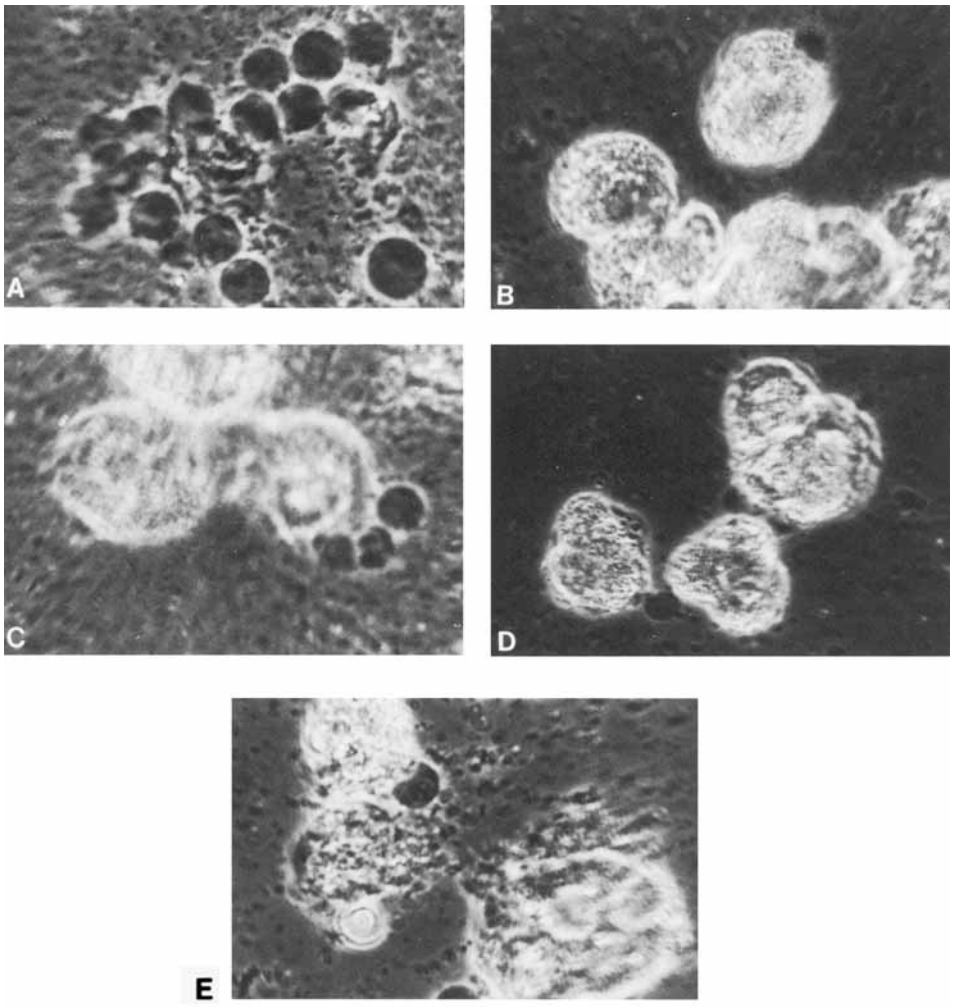

Fig. 6. Effect of taxol and phalloidin. Phase contrast micrographs of cells in 0.25 M sucrose, 5 mM NaHepes, 3 mM NaCl. All ×400. Figure 6A plus 1 mM ATP, 30 min. Figure 6B, no ATP plus 10 μM taxol, 30 min, cells largely intact, disintegration blocked by taxol (compare to Figure 2B). Figure 6C, plus ATP and taxol, 18 min, cells swollen, several free nuclei, but most cells still intact (Fig. 5C). Figure 6D with 40 μM phalloidin, 40 min, cells mainly intact. Figure 6E plus 1 mM ATP and 40 μM phalloidin, 15 min, some disintegration but marked inhbition as compared to cells with ATP.

protein, at most 20%, is composed from a great variety of elements, cytosolic enzymes partially bound to membranes as acetylCoA carboxylase [11], Golgi, a variety of vesicles, and many other known and unknown proteins. The classic components of the cytoskeleton, microtubules, microfilaments, and intermediate fibers ocur in liver [6], but no quantitative estimates are available. Altogether, the amount is not likely to exceed 5% of the protein of the nude cell.

Scanning electron micrographs shown by us previously [1] and here reveal a dense complex network of fibers of different diameters enmeshing the nucleus, mitochondria, and probably other organelles. It is clear from the composition detailed

above that the bulk of the network is composed of lamellae of the endoplasmic reticulum. It is likely that other fiber types and proteins are associated with the network. There are a number of reports of the association of fiber types with each other and to organelles. Thus, Hegeness, Simon, and Singer [12] reported association of microtubules with mitochondria, and Larsen et al [13] of intermediate fibers with mitochondria in fibroblasts. Connection between actin and microtubules was reported by Schliwa and Blerkom [14] in kidney cells and by Geiger and Singer [15] of microtubules and intermediate filaments in chicken gizzard cells. Griffith and Pollard [16] described the formation in vitro of an actin-microtubule complex in the presence of accessory protein (MAP). In isolated hepatocytes extracted with Triton, the existence of a three-dimensional network of microtubules and microfilaments and intermediate fibers was described by Okanoue et al [17] in liver cells. A continuity of the endoplasmic reticulum with the outer nuclear and outer mitochondrial membranes was described by several investigators, most recently Meier et al [18] and Montisano et al [19], and it is supported by our work (see [1], for review of earlier studies). However, the nature of the bonds between membranes of the organelles and the lamellae of the endoplasmic reticulum is unknown.

The Disintegration of the Nude Cell

Our observation that the nude cell, which is remarkably stable in the presence of solutions of moderate ionic strength (50 mM KCl or NaCl), decomposes explosively in salt-free media, but only at physiologic pH and in the presence of oxygen, is without precedent. Two conditions are essential for this phenomenon: solutions of low ionic strength, and the presence of ATP generation. We suggest that these conditions involve two independent mechanisms of some type of bond cleavage, and only when both occur does the cell disintegrate. Our previous studies [1] indicate that the endoplasmic network ruptures at low ionic strength. The mechanism is obscure. The depolymerization in vitro of G-actin to F-actin monomers in solutions of low ionic strength [3] suggests a similar type of dissociation.

The role of oxidative phosphorylation and of ATP generation in the disintegration is intriguing, suggesting a process of physiologic significance. Bershadsky and Gelfand [5] have shown that the depolymerization of tubulin requires oxidative phosphorylation and ATP generation and that depolymerization was blocked by inhibitors of oxidative phosphorylation. It is possible that a depolymerization of some elements connecting organelles to the network in the nude cell are dependent on the presence of ATP. With the high ionic strength in the cytosol, this would not affect cell integrity, but would cause disintegration in vitro in the absence of salt.

Our results with taxol and phalloidin suggest that both microtubules and actin filaments play a role in the integrity of the cytostructure, but the relation of these elements to the fiber network of the hepatocyte is obscure.

Physiological Implications

Tentatively, the ATP effect may be related to intracellular organelle movement. The position of mitochondria is not fixed and their saltation and that of other organelles occurs in a great variety of cells. Most significantly ATP plays a role in this movement [20]. Foreman et al [21] have shown that saltation of mitochondria in the nerve axon is activated by ATP, and at higher concentrations by ITP and GTP. It may be speculated that the binding of ATP or of other nucleotides induces somehow

a detachment of fibers of the network from organelles, permitting translocation. Such a process in vitro may account for the ATP effect observed here. If ATP generation is reduced or abolished in the absence of air or in the presence of poisons, no detachment occurs.

We believe that our results establish the nude hepatocyte as a valuable system for the study of cytostructure and the cytoskeleton of the liver cell. The mechanism of cell disintegration is yet rather obscure, but it is likely that the in vitro observations reveal some fundamental properties of the cytostructure. Much further work is required to elucidate the nature of the affected bonds and the mechanism of disintegration.

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