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Ouabain-Resistant Mechanism of Volume Control and the Ultrastructural Organization of Liver Slices Recovering from Swelling *in vitro*

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Summary. We have studied the net extrusion of water by liver slices recovering from swelling at 1 °C and have attempted to relate this to ultrastructural alterations. Special attention was paid to the ouabain-resistant extrusion of water. The restoration of many details of intracellular architecture was dissociated from the net loss of water, since an osmotic stimulus (produced by 5% inulin) caused a passive withdrawal of water with little recovery of structure. Also, a similar recovery of structure was produced during active extrusion of water in the absence and presence of ouabain, even though ouabain reduced the water extrusion by 50%. The time-course of water extrusion in the presence of ouabain was correlated with the formation of cytoplasmic vesicles. Incubation without K^+ in the medium had similar effects to those caused by ouabain. Colchicine had little effect on water extrusion in presence or absence of ouabain except at concentrations which reduced tissue ATP levels and caused much necrosis. Cytochalasin B alone had little effect on water extrusion, but led to the accumulation of many vesicles in the cytoplasm and appeared to abolish the access of such vesicles to the canaliculi. In the presence of ouabain, cytochalasin B had a similar effect on ultrastructure, and totally prevented the ouabain-resistant water extrusion. Ni^{2+} had rather similar effects to cytochalasin B both in the presence and absence of ouabain, although to a smaller degree. The results support our previous suggestion that the ouabain-resistant water extrusion proceeds by secretion of water into cytoplasmic vesicles, followed by the exocytotic expulsion of the vesicular contents into the bile canaliculi. Microfilaments appear to play an important role in this process.

Previous work has established that the recovery of liver-cell volume after a period of swelling at low temperatures has two components, distinguishable by their sensitivity to ouabain (Macknight, Pilgrim & Robinson, 1974; Claret, Mazet & Poggioli, 1976; Russo, van Rossum & Galeotti, 1977). The component inhibited by ouabain accounts, on the average, for 50% of the water extrusion (although varying from approximately 25 to 75% in different experiments), and is apparently due to the activity of the coupled Na^+ and K^+ transport system, as first suggested by Leaf (1956). The component not inhibited by ouabain is associated with transport of Na^+ and Cl^-, and we have provided evidence from electron microscopy that it involves passage of water into cytoplasmic vesicles, with the subsequent elimination of the vesicular contents into the bile canaliculi by exocytosis (Russo, Galeotti & van Rossum, 1976; Russo et al., 1977).

In seeking to test our hypothesis for the ouabain-resistant mechanism, we have made further electron-microscopic studies of the cytoplasmic vesicles which, we suggest, form its essential manifestation. In particular, we have studied the time-course of their formation and the effects on their distribution and exocytotic expulsion of agents known to interfere with cytoskeletal structures. The ultrastructural observations have been correlated with measurements of the extrusion of water and net movements of ions.

The results are consistent with the proposed mechanism for ouabain-resistant elimination of water and indicate the importance of microfilaments for its activity. Some of the results have been published in abstract form (van Rossum & Russo, 1979).

Materials and Methods

Male albino rats, fed *ad libitum*, were used throughout. The methods for preparation, incubation and analysis of liver slices were as described previously (Elshove & van Rossum, 1963; van Ros-

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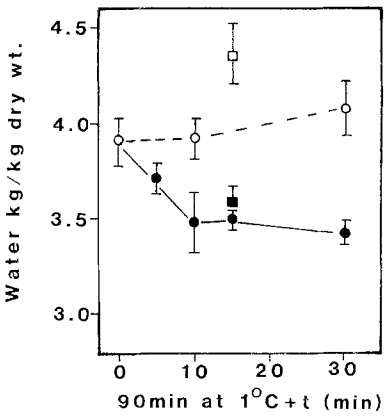


Fig. 1. Reversal of swelling by addition of inulin (5%) to medium. Slices were incubated for 90 min at 1 °C in the medium described in Materials and Methods, to allow swelling. They were then transferred to fresh samples of medium, as follows: ○ unmodified medium at 1 °C, $n=7$ at each point; ● medium containing 5% (wt/vol) inulin at 1 °C, $n=9$; □ medium containing 2 mM CN^- and warmed to 38 °C, $n=6$; ■ medium containing 2 mM CN^- and 5% inulin, warmed to 38 °C, $n=6$

sum, 1972; Russo et al., 1977). Briefly, the slices were cut free-hand, pre-incubated for 90 min at 1 °C to permit tissue swelling, and then allowed to recover during incubation at 38 °C in oxygenated medium. Oxygen uptake was determined manometrically. Slices were collected at appropriate intervals, dried at 105 °C and ions extracted with 0.1 N-HNO_3. For analysis of adenine nucleotides, samples of the slices were homogenized in perchloric acid/ethanol (8%/40%). After neutralization, the nucleotides were assayed by enzymic techniques in a fluorimeter. The incubation medium for the slices contained (in mM): 161 Na^+, 5 K^+, 1.2 Ca^{2+}, 1.0 Mg^{2+}, 153 Cl^-, 1.0 SO_4^{2-} and 10 Tris (pH 7.4); inulin (0.5%) was used as extracellular marker for determination of water spaces. All inhibitors used were added to the incubation medium containing the slices during the first 30 min of pre-incubation at 1 °C. The methods used in the preparation of samples for histology and electron microscopy were as described previously (Russo et al., 1977). Values given in the text, Tables and Figures are expressed as mean ± standard error of the mean (number of observations). Tests for statistical significance were done by "Student's" t test.

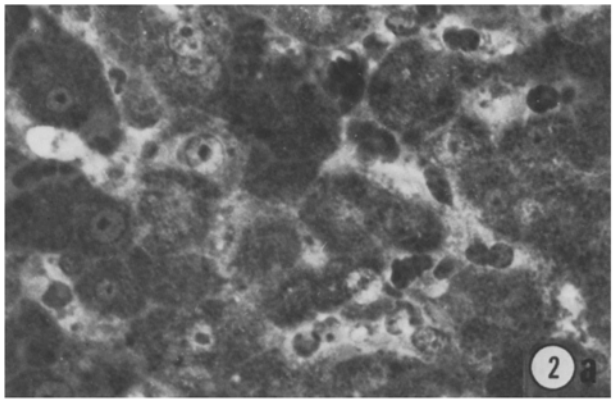
Results

Osmotic Reversal of Swelling

We have previously shown that when slices of liver, swollen by incubation at 1 °C, are restored to metabolic activity at 38 °C they show both a reversal of tissue swelling, as measured by water extrusion, and a recovery of ultrastructural organization (Russo et al., 1977). Conversely, both water extrusion and structural recovery were inhibited when the slices were incubated at 38 °C with cyanide and, to a lesser extent, oligomycin. This suggested that metabolic energy was required for both aspects of recovery, but it remained a moot point as to whether or not they were obligatorily coupled. In order to see whether the water removal could be dissociated from structural recovery, we have used the nonpenetrating molecule, inulin (5% wt/vol, equivalent to 23 mosmol/kg water), to induce a reduction of tissue water by passive, osmotic forces (Parsons & van Rossum, 1962).

Fig. 1 shows that liver slices which had been allowed to swell for 90 min at 1 °C in the normal, Tris-buffered Ringer's solution lost 0.5 kg water per kg dry wt when they were transferred to medium containing 5% inulin at 1 °C, the bulk of the water loss occurring in 10 min. The net loss of water was similar to that brought about by metabolic activity in the presence of ouabain (e.g., *see* Figs. 4 and 11). The appearance in the light and electron microscopes of the swollen slices after 90 min at 1 °C (Figs. 2*a* and 3*a*) has been described in detail previously (Russo et al., 1977). After 15 min in the presence of inulin, when the water content was nearly at its minimum (Fig. 1), the size of the cells was reduced, indicating a reduction of cell volume, while the cell boundaries and intercellular spaces were clearly defined (Fig. 2*b*); in general, the manifestations of "cloudy swelling" observed in the swollen slices were no longer apparent. As seen in the electron microscope (Fig. 3*b*), the electron density of the cytoplasm increased somewhat upon osmotic reversal of swelling, but not to the degree observed after metabolism-dependent extrusion of water (*see below*). Mitochondria were predominantly in swollen configurations after 90 min at 1 °C but were uniformly in the condensed configuration after the further 15 min with 5% inulin. After a total of 30 min in the presence of inulin the changes of cell size, intercellular spaces and cytoplasmic density were all somewhat further advanced (not illustrated). The above alterations all appear to be readily explicable as consequences of the removal of water from mitochondrial and cytosolic compartments. However, there was no evidence of any recovery of the architecture of intracellular organelles such as the endoplasmic reticulum, Golgi apparatus, polysomes and nucle-

Fig. 2. Optical micrographs of the effect of 5% inulin on the swelling of liver slices. (*a*) Slices incubated for 90 min at 1 °C in the absence of inulin, followed by (*b*) 15 min at 1 °C with 5% inulin in the medium. (*c*) Slices incubated for 90 min at 1 °C followed by 15 min at 38 °C with 2 mM cyanide in the absence of inulin; (*d*) as for (*c*), except that 5% inulin was present during the incubation at 38 °C. For descriptive details, *see text*. 600× (reproduced at 95%). **Fig. 3.** Electron micrographs of the effect of 5% inulin on the swelling of liver slices. (*a*)–(*d*) as for Fig. 2. For descriptive details, *see text*. 10,000× (reproduced at 95%)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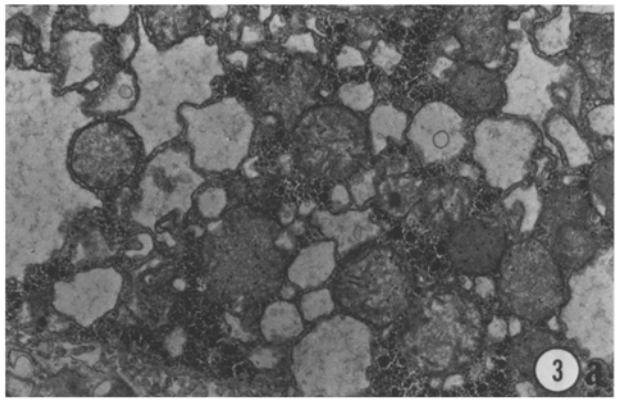
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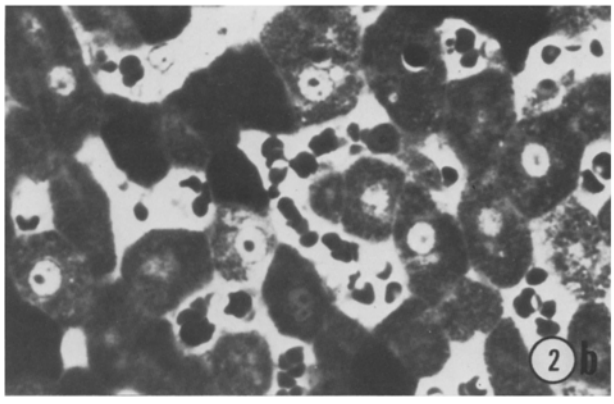
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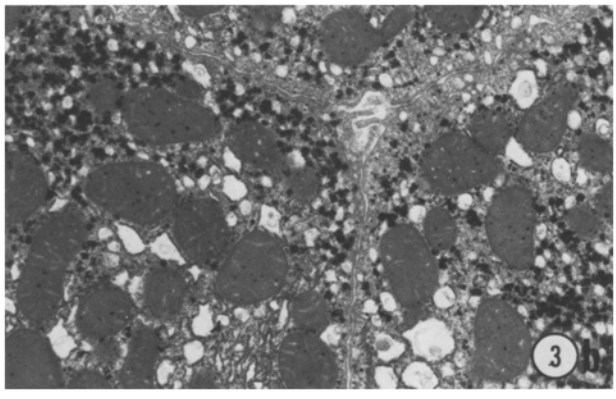
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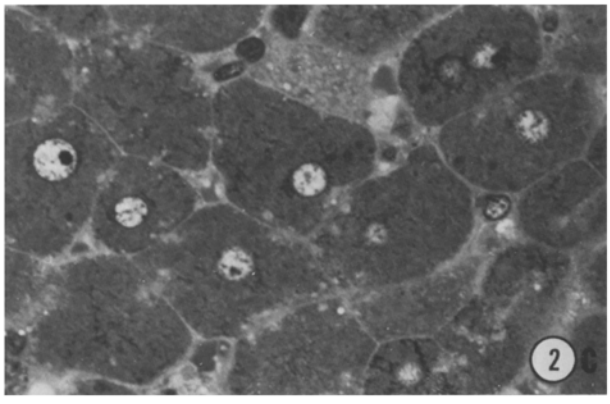
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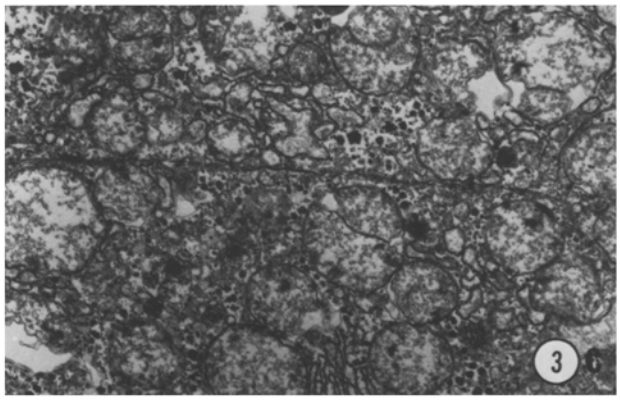
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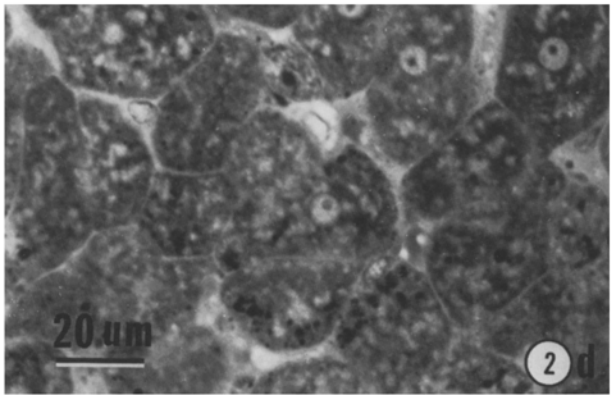
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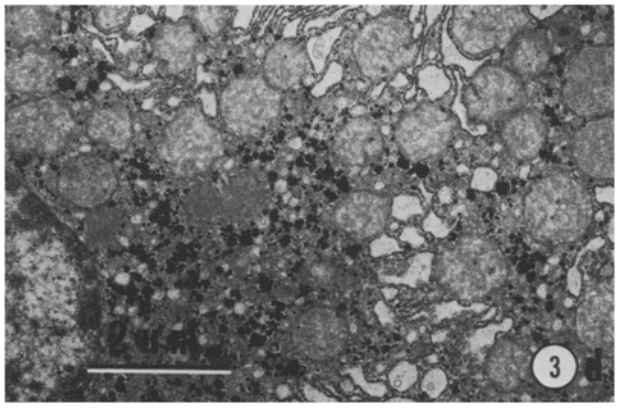
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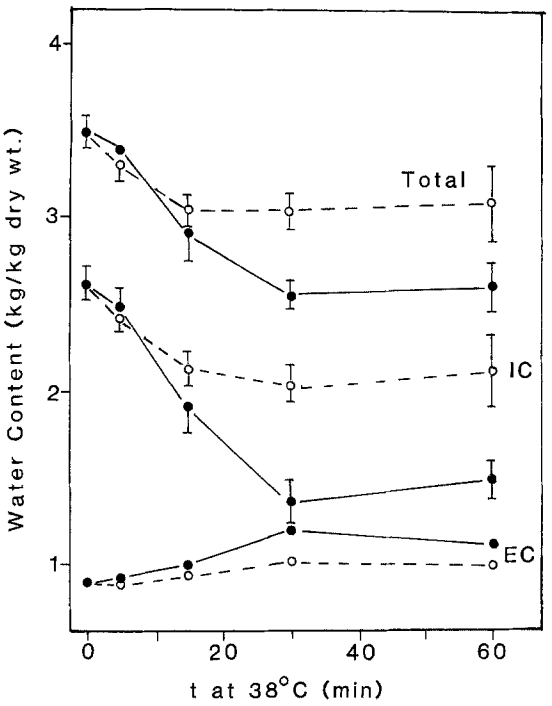


Fig. 4. Time-course of the extrusion of water in the absence and presence of ouabain. Liver slices were incubated for 90 min at 1 °C followed by incubation at 38 °C in oxygenated medium for the times indicated. Time nought minutes at 38 °C is equivalent to 90 min at 1 °C. Composition of the incubation medium as in Materials and Methods, with 0.5% (wt/vol) inulin as extracellular marker. Ouabain (2 mM) was added after the first 30 min at 1 °C. • Control slices; o slices with 2 mM ouabain. Each point is the mean ± SEM of 10 observations. *IC*, intracellular, *EC*, extracellular

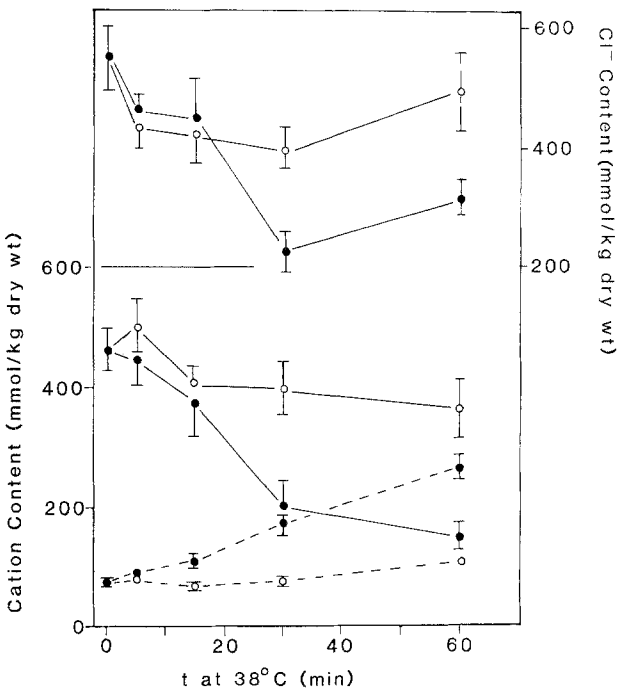


Fig. 5. Time-course of the change in intracellular ion contents of liver slices incubated in the absence and presence of ouabain. Results are from the same slices as Fig. 4. Intracellular ion contents were calculated on the assumption that the inulin-containing water was extracellular and had the same ionic composition as the bulk medium. • Control slices; o 2 mM ouabain. The upper two lines show the Cl^- content. For the lower portion of the graph, – – – – K^+ content, —— Na^+ content

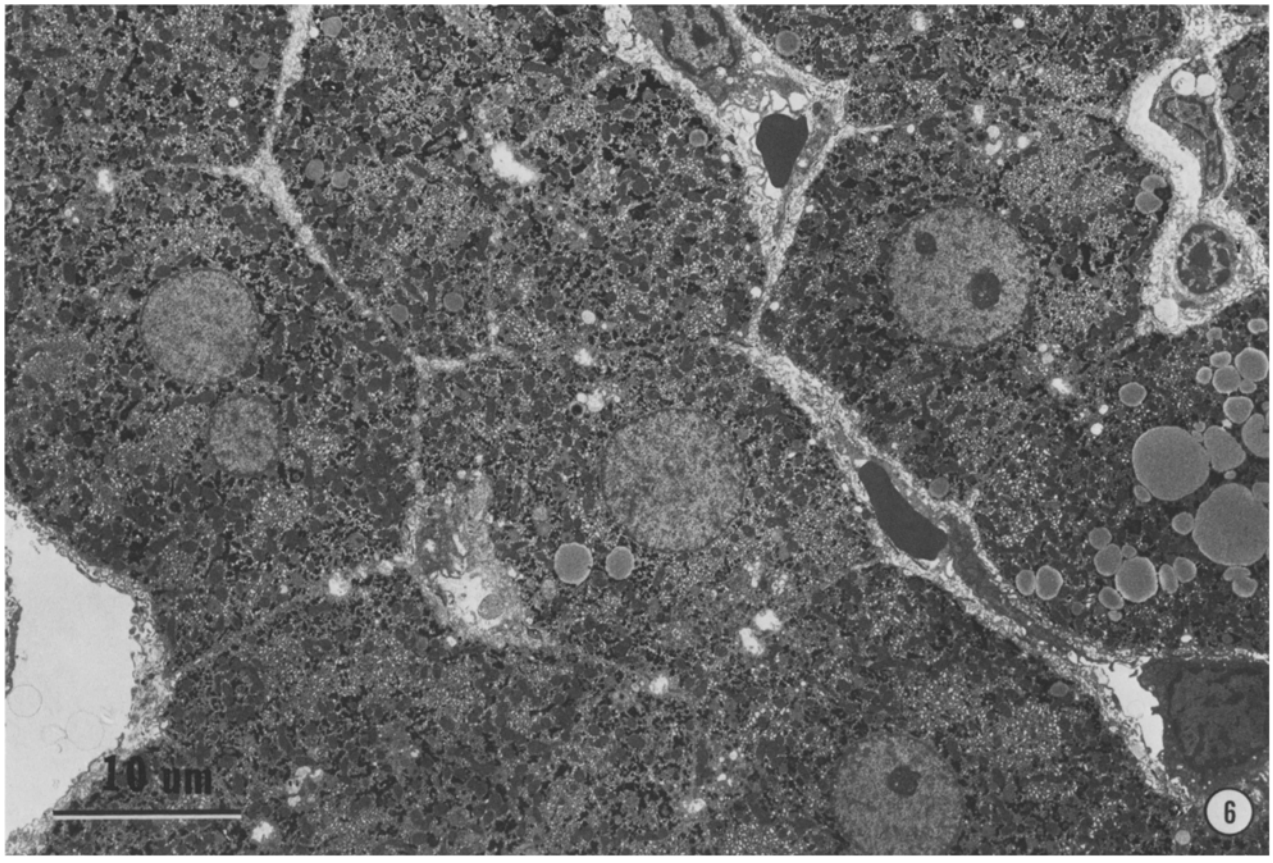
oli, in marked contrast to the situation in metabolism-dependent reversal of swelling.

It seemed possible that the temperature of 1 °C might be too low to permit structural recovery of the various intracellular organelles upon osmotic removal of water, and we therefore also examined the effect of 5% inulin when swollen slices were incubated at 38 °C, using CN^- (2 mM) to prevent a metabolism-dependent extrusion of water (Russo et al., 1977). Slices which had been allowed to swell for 90 min at 1 °C showed a further uptake of water during subsequent incubation for 15 min at 38 °C with CN^-, but this was prevented by 5% inulin which, in fact, reduced the water content to the same level as it had done at 1 °C (Fig. 1). Samples of the slices incubated without inulin had clear cytoplasmic ground substance, cellular boundaries which were barely distinguishable and mitochondria which were either in the intermediately condensed configuration or swollen (Figs. 2*c* and 3*c*). In the slices incubated with inulin, the appearance was consistent with the reduced water content in that cell size was reduced (Fig. 2*d*), the cytoplasm was of medium density and cell boundaries were well-defined (Fig. 3*d*). However, as at 1 °C, 5% inulin induced no recovery of other organelles. In contrast to the reduced swelling of the cells as a whole in the presence of inulin, the mitochondria were identical to those treated with CN^- in the absence of inulin, so that the inhibitor apparently had an adverse effect on the mitochondria which prevented osmotic reversal of their swelling.

These results indicate that an osmotically induced

Fig. 6. Time-course of the ultrastructural recovery in the presence of ouabain (2 mM). Low magnification of slices incubated for 5 min at 38 °C, after 90 min at 1 °C. Note the wide distribution of clusters of small vesicles associated with endoplasmic reticulum. Few large vesicles are seen in the region of the bile canaliculi. (For further descriptive details, *see text.*) 2,500× (reproduced at 99%)

Fig. 7. Time course of the ultrastructural recovery in the presence of ouabain (2 mM). Low magnification of slices incubated for 15 min at 38 °C, after 90 min at 1 °C. Compared to the appearance after 5 min at 38 °C (Fig. 6), there are greater numbers of large vesicles and these are closely associated with small vesicles identical to those noted in Fig. 6 and with the bile canaliculi. Glycogen is found in large aggregates instead of being dispersed as in Fig. 6. (For further descriptive details, *see text.*) 2,500× (reproduced at 99%)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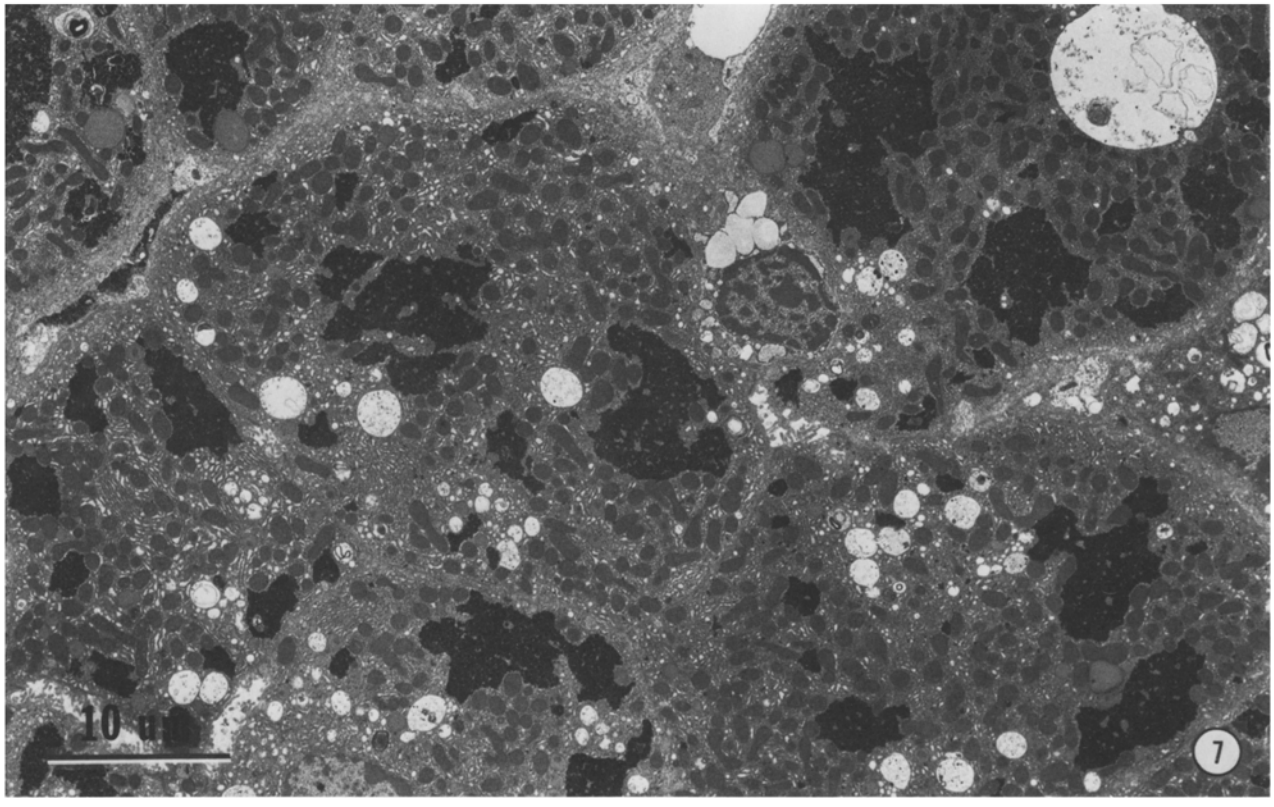
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reversal of swelling, whether at 1 °C or at 38 °C in the absence of mitochondrial respiration, only results in aspects of structural recovery which are clearly related to the cell water volume, recovery of more specific aspects of intracellular architecture being absent.

Time-Course of Ouabain-Resistant Recovery

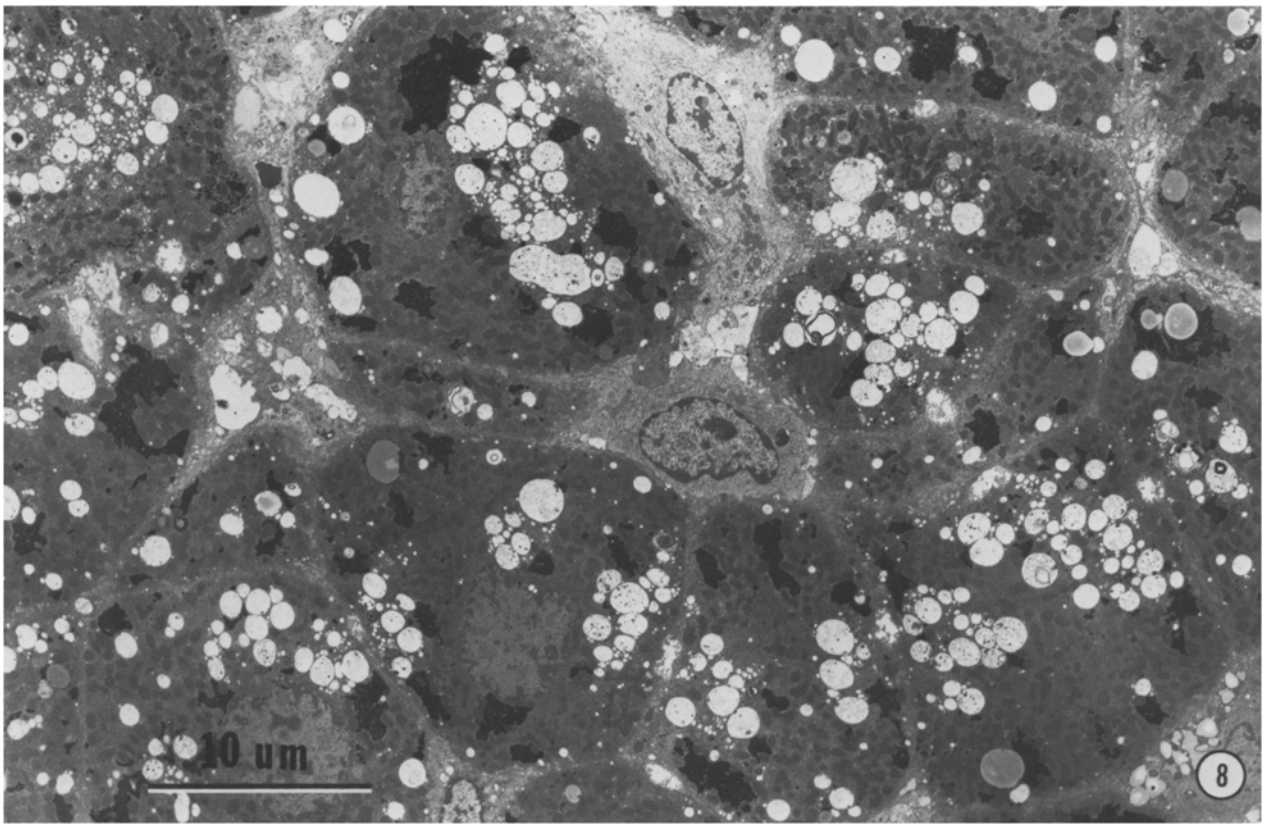
In earlier work we noted that the main difference in appearance between liver slices which had extruded water for 60 min at 38 °C in the presence and absence of ouabain lay in the occurrence in the latter of a large number of vesicles of varying sizes, roughly circular in outline, in the regions of the Golgi apparatus and canaliculi (Russo et al., 1977). It was postulated that these vesicles represented the vehicle for the ouabain-resistant water extrusion. In order to examine this possibility further, we have made a detailed study of the appearance of cytoplasmic vesicles during the course of incubation at 38 °C in the presence of ouabain (2 mM), in comparison to the extrusion of water.

The time-course of the change in composition is illustrated in Fig. 4 (water compartments) and Fig. 5 (intracellular ions). After 5 min at 38 °C there was an indication of some water extrusion from the intracellular compartment which was of a similar extent both with and without ouabain, although neither change was statistically significant. This initial loss of water was accompanied by an extrusion of intracellular Cl^-, but not of Na^+. Microscopic studies of the slices in the presence of ouabain (for studies without ouabain, *see* Russo et al., 1977) showed that a measure of structural recovery had already occurred by 5 min but, in agreement with the analytical results, many features of swelling persisted (*compare* Figs. 3*a* and 6). Thus, only partial recovery of cell shape and reduction of size were apparent and cell boundaries remained indistinct. The cytoplasmic ground substance remained electron clear, indicating continued dilution with intracellular water, while the rough endoplasmic reticulum, which at 1 °C was swollen into large, irregular vesicles (Fig. 3*a*), was now represented by large numbers of smaller vesicles (Fig. 9*a*). In many parts of the slices the Golgi apparatus showed a degree of recovery, forming clusters of vesicles but no classical dictyosomes. Most marked, however, was the occurrence at this stage of only very few of the large, rounded vesicles characteristic of longer incubation with ouabain, and these few were smaller than those found later (Fig. 9*a*). We suggest that the small vesicles of the endoplasmic reticulum, and the clusters of small Golgi vesicles, represent an early stage in the formation of the larger vesicles. Unlike the swollen tissue, the slices incubated for 5 min at 38 °C showed open canaliculi with well-organized microvilli.

After 15 min at 38 °C, the intracellular water was reduced further, and to the same extent in the presence and absence of ouabain. While this level (i.e. an extrusion of about 0.5 kg water/kg dry wt) was nearly the maximum extrusion observed with ouabain, water continued to be extruded from the control slices up to 30 min with a total loss of 1.13 kg/kg dry wt (Fig. 4). The changes of intracellular water were paralleled by the extrusion of both Na^+ and Cl^- (Fig. 5). The structural recovery at 15 min (Fig. 7) was also well advanced towards that seen after 60 min (Fig. 8; *see also* Russo et al., 1977). The intercellular spaces were clearly visible, the ground substance had an electron opacity similar to that usually seen *in vivo* and the rough endoplasmic reticulum was also restored to its state *in vivo*, with no indication of dilated cisternae. There were now many large, rounded vesicles in the region of the Golgi apparatus and near the canaliculi (Fig. 9*b*), while smaller numbers of such vesicles were sometimes also seen near the sinusoids. After 30 and 60 min at 38 °C in the presence of ouabain there was little difference in appearance from that at 15 min (Fig. 8), although the rounded vesicles were somewhat more numerous and the intercellular spaces more widely open, indicating a somewhat more advanced degree of recovery.

In general, during the course of incubation with ouabain at 38 °C there was a good correlation between the recovery of cell water content, the increase in electron density of the cytoplasmic ground substance and the increase in the number and, especially, the size of rounded vesicles in the region of the Golgi

Fig. 8. Time-course of the ultrastructural recovery in the presence of ouabain (2 mM). Low magnification of slices incubated for 60 min at 38 °C, after 90 min at 1 °C. This is typical of the appearance of slices incubated in the presence of ouabain for 60 min, as described by Russo et al., (1977). Large numbers of large vesicles are present in the region between nuclei and canaliculi. (For further descriptive details, *see text*.) 3,000× (reproduced at 99%). **Fig. 9.** Time-course of the ultrastructural recovery in the presence of ouabain (2 mM). Details of Figs. 6 and 7. (*a*) Slices incubated for 5 min at 38 °C, after 90 min at 1 °C. In the peri-nuclear region are seen typical clusters of small vesicles which are clearly associated with endoplasmic reticulum. Mitochondria are mostly in intermediately condensed forms. (*b*) Slices incubated for 15 min at 38 °C, after 90 min at 1 °C. Large vesicles are associated with small ones. It will be noted that there is a continuous gradation in size between small vesicles of the type seen in (*a*) and the largest vesicles. In many instances (arrows) a process of fusion between vesicles of the same or different sizes is suggested, and in some cases is clearly seen. Many of the vesicles are associated with elements of the Golgi apparatus (*G*). Mitochondria are in the orthodox form and well preserved. 9,000× (reproduced at 98%)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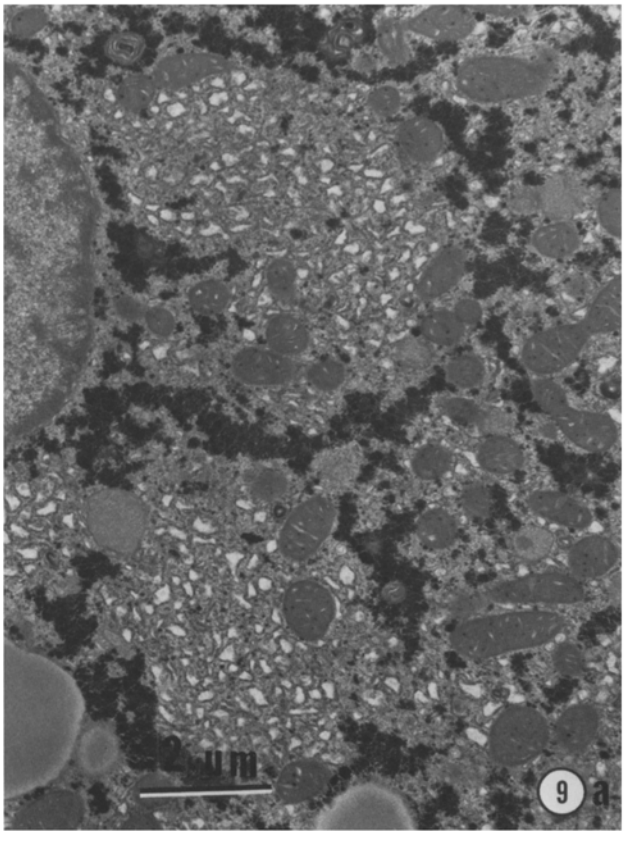
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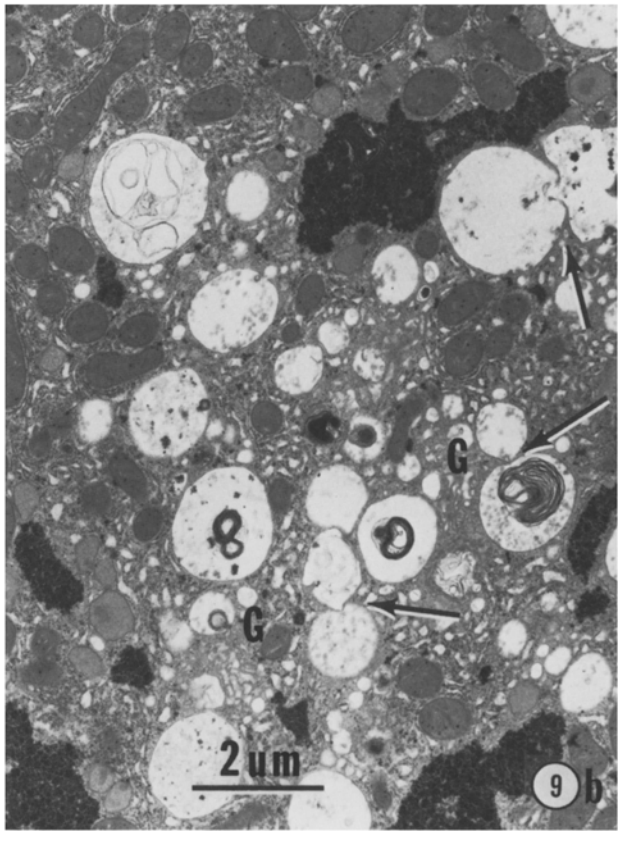
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apparatus and bile canaliculi. It was noteworthy that very little necrosis was evident in the slices at any stage of the recovery.

The mitochondria showed a progressive alteration during the course of incubation. After 5 min they had changed from being almost exclusively in the swollen form (Fig. 3*a*) to either condensed or intermediate configurations (i.e. intermediate between condensed and orthodox forms, Figs. 6 and 9*a*), with very few in the swollen form. By 15 min they were present only in intermediate and orthodox configurations (Figs. 7 and 9*b*), and this state persisted throughout the rest of the 60 min (Fig. 8).

Potassium-Free Medium

In many respects the incubation of liver slices in K^+-free medium mimics the effects of ouabain (Elshove & van Rossum, 1963) and we have shown previously that this is also true for water extrusion, which is inhibited by about 50% (Russo et al., 1977). Since it may be supposed that both ouabain and the absence of K^+ act by inhibition of $(Na^+ + K^+)$-ATPase, and the associated extrusion of water, it was important that the degree and nature of the structural recovery in K^+-free medium should be similar to that seen with ouabain. This was found to be the case, especially in that the cytoplasm of the slices contained abundant numbers of rounded vesicles in the Golgi region, while the canaliculi were dilated and lined with microvilli (Fig. 10). Once again, there was little or no necrosis in the slices.

Colchicine

Microtubules have been implicated in a number of exocytotic processes of liver cells (Orci, Le Marchand, Singh, Assimacopoulos-Jeannet, Rouiller & Jeanrenaud, 1973; Redman, Banerjee, Howell & Palade, 1975) so that they might also be important for the exocytotic step of the proposed ouabain-resistant mechanism of volume control. The conditions pertaining in our slices at the end of the swelling phase of incubation (i.e. 1 °C, high cell Ca^{2+}) were such as to promote microtubular disaggregation (Morré, Kartenbeck & Franke, 1979) and we therefore examined the effects of colchicine, which inhibits reaggregation and function of microtubules.

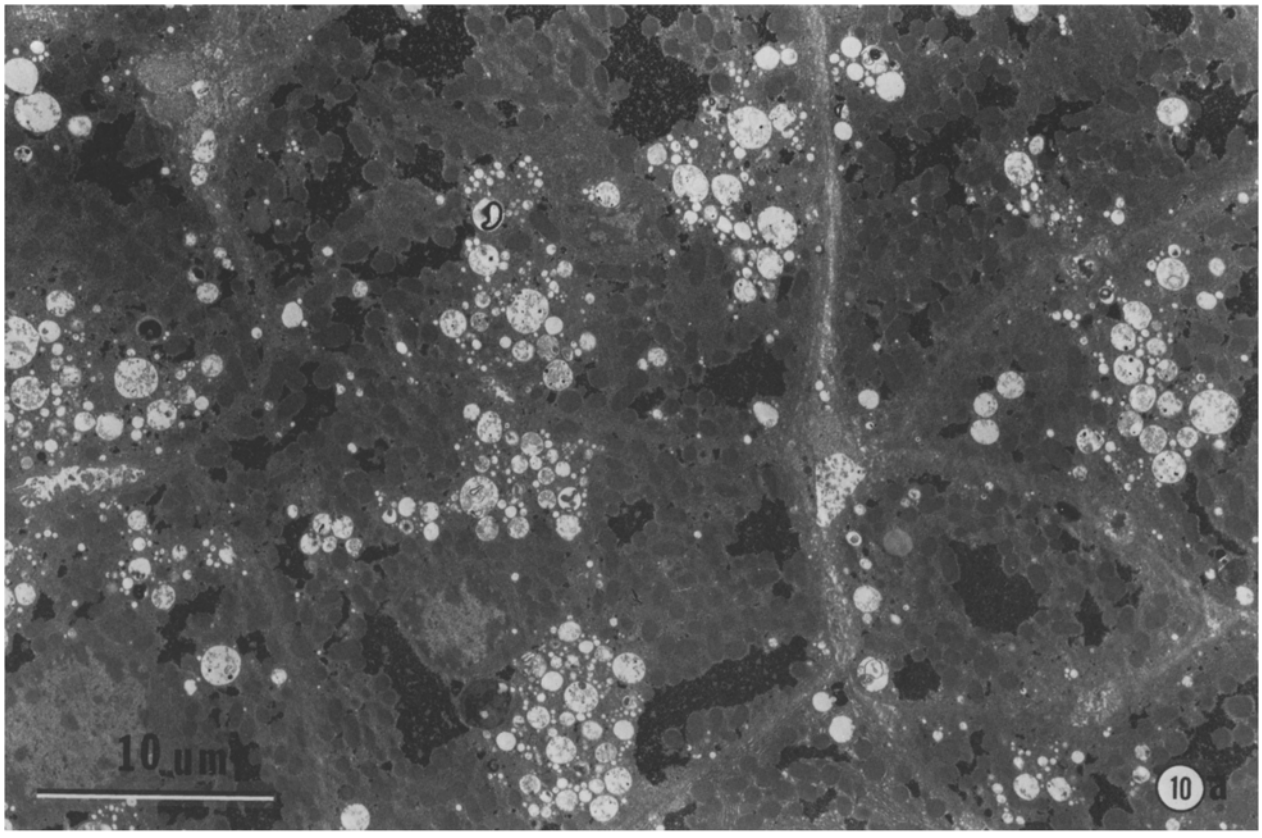
Colchicine alone had no effect on water or ion movements at any concentration from 10^{-5} to 10^{-2} M (Table 1), although 10^{-5} M was enough to inhibit plasma protein secretion in liver slices (Redman et al., 1975) and 10^{-4} M inhibited lipoprotein secretion by perfused mouse liver (Orci et al., 1973). In agreement with the other workers just quoted, we found that concentrations up to 10^{-3} M had no significant effect on slice energy metabolism, although 10^{-2} M colchicine reduced both the rate of respiration ($p<0.01$) and the ATP content ($p<0.02$) of slices by about 25% (Table 1). That such an inhibition was not accompanied by a fall of ion transport is in accordance with previous findings that respiration and ATP levels of liver slices had to be reduced by 50% or more before the limitation of the energy supply leads to an effect on ion transport (van Rossum, 1972).

Morphological observations were made on slices incubated with the two highest concentrations of colchicine. After 60 min at 38 °C, there was a substantial degree of apparent necrosis, as indicated by light staining with azure II, which amounted to some 40% of the slice volume at 1 mM colchicine and up to 60% at 10 mM. However, the appearance of the 'necrotic' cells was quite different from the necrosis produced by cyanide (Fig. 11), being characterized by the presence of very large vesicles in the cytoplasm. It will be noted from Table 1 that the slices containing such large proportions of 'necrotic' cells transported ions and water as efficiently as control slices, and we conclude that the lightly staining cells were still able to transport materials, and to maintain ionic gradients, even though they had undergone some damage which would eventually result in their death.

In the remaining, clearly viable cells of the same slices, 1 and 10 mM colchicine caused rather small changes of ultrastructure which were reminiscent of the effect of ouabain, namely an agglomeration of vesicles around the canaliculi (Fig. 12*a*), the presence of many microvilli in the canaliculi and (at 10 mM) the sinusoidal spaces. However, the microvilli were arranged in a random or convoluted fashion, instead of perpendicularly to the canalicular surface as in control cells (Fig. 12*b*, *c*).

When 1 and 10 mM colchicine were added to the slices in the presence of ouabain, a marked inhibition of water, Na^+ and Cl^- extrusion was seen (Table 1) together with a 25% reduction of respiration. The proportion of cells staining with azure II rose to 65–70%, and these contained large vesicles similar to those seen with colchicine alone. The ultrastructure

Fig. 10. Electron micrographs of liver slices incubated for 60 min at 38 °C in K^+-free medium, after 90 min at 1 °C in the same medium. (*a*) Low magnification of the general appearance of the tissue, which is identical to that of slices incubated for 60 min at 38 °C with ouabain (Fig. 8). 3,300×. (*b*) Detail of region between nuclei and canaliculi, showing the numerous vesicles of various sizes which are seen to be associated with elements of the Golgi apparatus (*G*). The appearance is very similar to that in the presence of K^+ *plus* ouabain (e.g. Fig. 9*b*). 15,000×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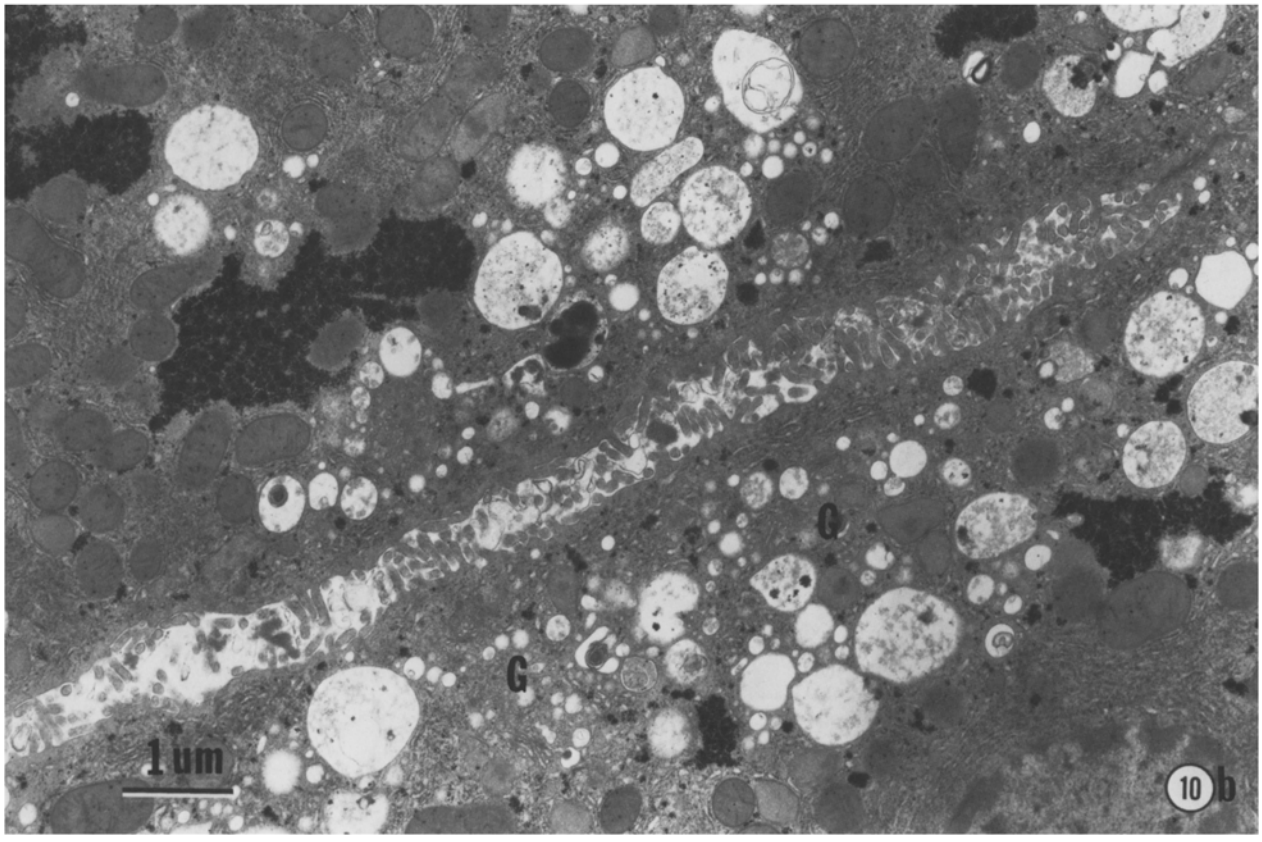
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Table 1. Effects of colchicine on water and ion movements and energy metabolism of liver slices incubated in the presence or absence of ouabain

Incubation	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mmol/kg dry wt)			Respiration (μl/mg dry wt·hr^{-1})	ATP content (mmol/kg protein)
		Na^+	Cl^-	K^+		
90 min at 1 °C	2.73±0.10 (26)	482±30 (26)	447±19 (18)	93±7 (23)	–	–
Then at 38 °C for 60 min:						
Control	1.68±0.11 (22)	214±34 (23)	301±29 (19)	189± 7 (19)	8.4±0.5 (9)	5.0±0.4 (6)
plus Colchicine 1 mM	1.61±0.08 (7)	181±32 (7)	314±35 (6)	188±16 (7)	8.3±0.7 (6)	5.1±0.5 (4)
plus Colchicine 10 mM	1.65±0.12 (7)	160±21 (7)	297±21 (7)	164± 8 (7)	6.6±0.3 (8)	3.7±0.2 (5)
Ouabain (2 mM)	1.98±0.17 (13)	318±38 (13)	349±52 (10)	86±10 (13)	5.8±0.4 (7)	–
plus Colchicine 1 mM	2.15±0.25 (4)	354±45 (4)	496±24 (4)	39± 3 (4)	5.3 (2)	–
plus Colchicine 10 mM	2.41±0.11 (6)	410±18 (6)	486±33 (6)	50± 3 (6)	4.7±0.3 (4)	–

[a] Slices were incubated for 90 min at 1 °C followed by 60 min at 38 °C in oxygenated medium; colchicine and ouabain were added to the medium at the concentrations indicated during the first 30 min at 1 °C. Experiments were also done with colchicine at 10^{-5} and 10^{-4} M both in the presence and absence of ouabain, but no significant effect of the colchicine was noted at these levels. Neither ouabain nor colchicine (1–10 mM) affected slice composition during the incubation at 1 °C, and the results at this temperature have therefore been pooled. The extracellular (inulin-containing) water compartment was not significantly affected by either agent at the concentrations used.

of the viable cells differed little from the appearance with colchicine alone, described above, except that the cytoplasmic vesicles were larger and more numerous, as would be expected from the usual effect of ouabain (not illustrated). A remarkable effect of colchicine, the significance of which is not clear, was a large increase in the quantity of rough endoplasmic reticulum, especially when ouabain was also present (not illustrated).

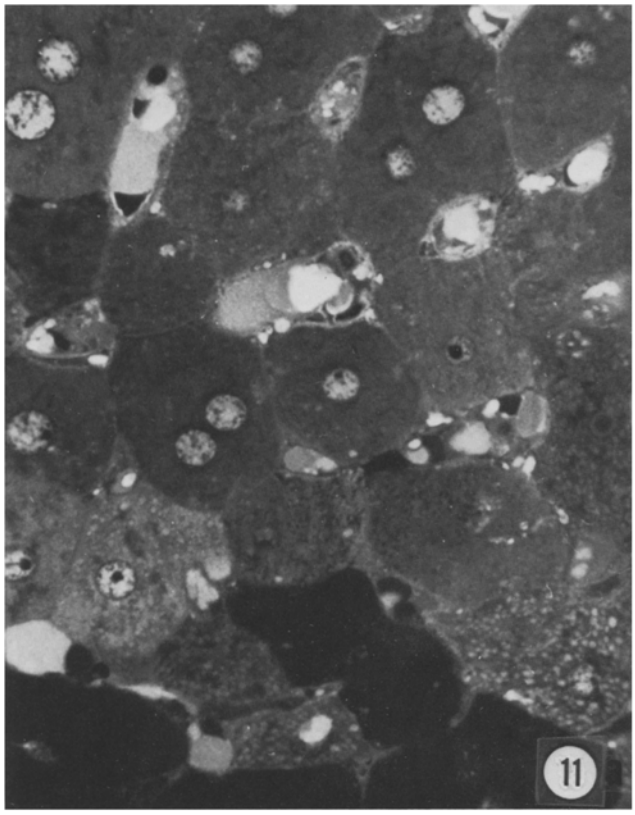
Thus, while colchicine greatly inhibited the ouabain-resistant extrusion of water from the liver cells, it did so only at concentrations 10–1,000 times greater than those required to inhibit other exocytotic processes in rat-liver slices (Redman et al., 1975). It therefore seems unlikely that its effect is due to a specific requirement of microtubules for the ouabain-resistant volume control, and more probably results from a less specific effect on mitochondrial metabolism or cell membrane permeability.

Cytochalasin B

This agent inhibits the activity of microfilaments and has been found to prevent bile flow both *in vivo* and *in vitro*, the latter at concentrations of 100 μg/ml (Phillips, Oda, Mak, Fisher & Jeejeebhoy, 1975), suggesting that it might block the ouabain-resistant extrusion of water if this indeed involves exocytosis into the bile canaliculi. Fig. 13 shows that cytochalasin alone, at 10–100 μg/ml, caused a small reduction in the extrusion of total and intracellular water, the effect only being significant at the highest concentration. It had no significant effect on respiration (Fig. 13) or on the net movements of any ions (not shown).

While its effect on tissue activity were small, cytochalasin B (100 μg/ml) had marked morphological effects on slices incubated for 60 min at 38 °C. In the light and electron microscope some 10–20% of the cells still appeared swollen, possibly accounting for the partial inhibition of water extrusion from the whole slices. In the remaining 80–90% of the cells the ultrastructural recovery was good (Fig. 14), but showed an important difference from control slices in containing numerous vesicles of many sizes (Fig. 15). The larger vesicles were similar to those induced by ouabain in that *i*) they were roughly spherical, *ii*) their contents were similar, and *iii*) they tended

Fig. 11. Comparison of the necrotic appearance of liver slices incubated with (*a*) colchicine (1 mM) and (*b*) cyanide (2 mM) for 60 min at 38 °C, after 90 min at 1 °C. Light micrographs. (*a*) The dark cells in the lower part of the field are cells which have recovered ultrastructurally (as in Fig. 12*a*). The cells in the remaining part of the field show varying degrees of ultrastructural disorganization, despite a fairly good appearance in the light microscope. Numerous intracellular vesicles are present. Mitochondria show various conformations, ranging from condensed to low amplitude swelling. (*b*) The necrosis induced by cyanide is characterized by high amplitude swelling of the mitochondria. In contrast to the situation with colchicine, this appearance is uniform throughout the slices. 600× (reproduced at 97%). **Fig. 12.** Electron micrographs illustrating the effects of colchicine on liver slices incubated for 60 min at 38 °C, after 90 min at 1 °C. These pictures represent only the non-necrotic portions of the slices. (*a*) Vesicles of various sizes associated with Golgi apparatus and endoplasmic reticulum which are similar to those seen with ouabain in Fig. 9*b*; 1 mM colchicine. 16,000×. (*b*) Convoluted microvilli in the canalicular lumen; 1 mM colchicine. 30,000×. (*c*) Convoluted microvilli in space of Disse; 10 mM colchicine. 19,000× (reproduced at 97%)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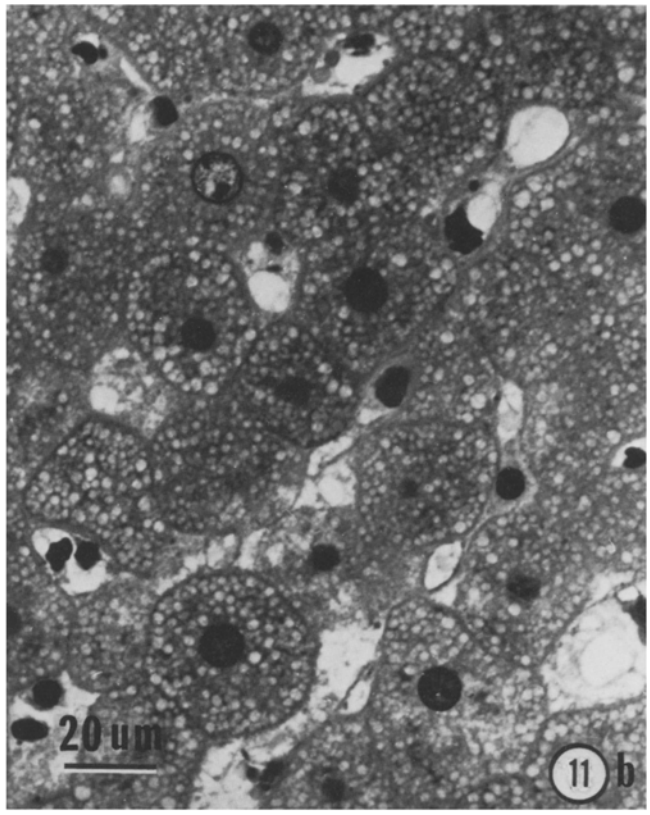
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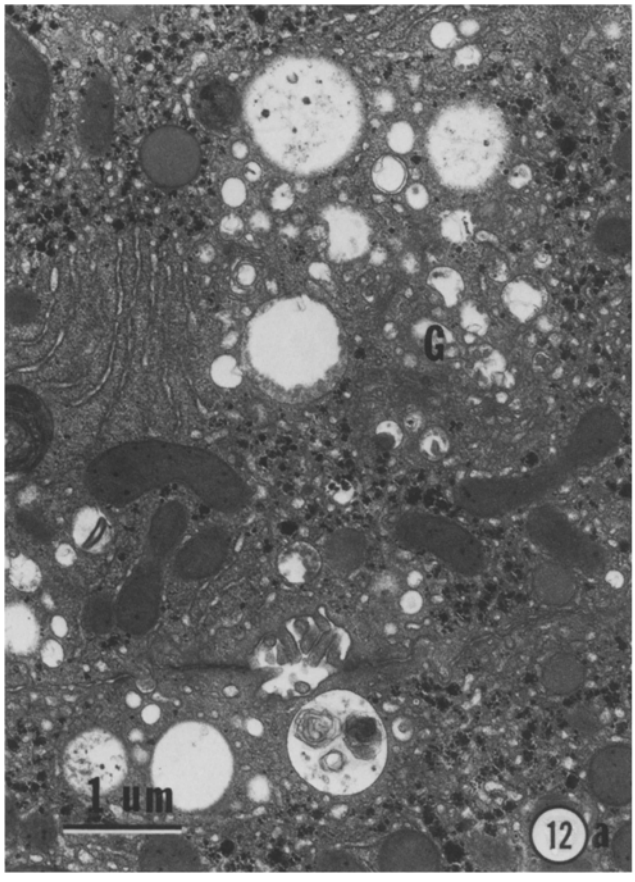
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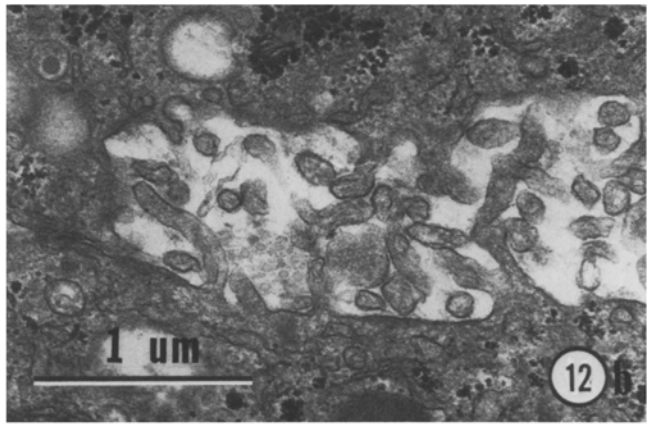
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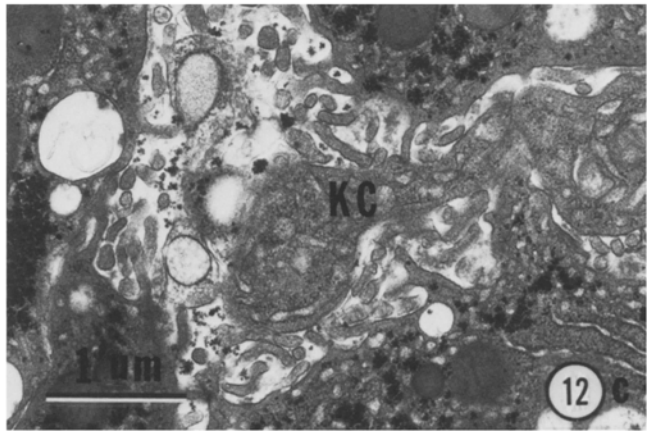
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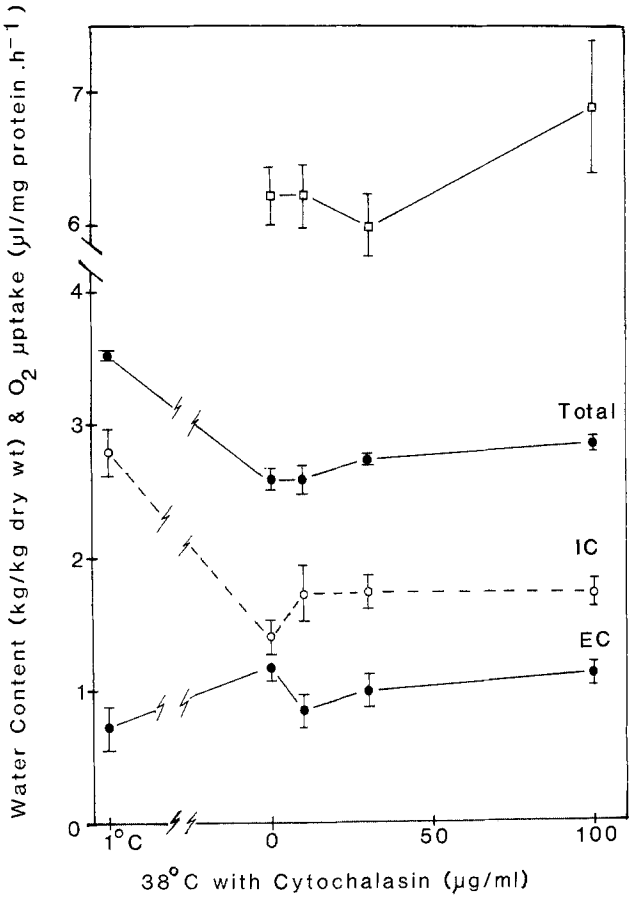


Fig. 13. Effects of cytochalasin B on water content and respiration of liver slices. The slices were incubated for 90 min at 1 °C followed by 60 min at 38 °C in oxygenated medium in the presence of the indicated concentrations of cytochalasin B. All flasks contained 0.1% (vol/vol) dimethyl sulfoxide, this being the solvent used for cytochalasin B. Each point is the mean ± SEM of 6 observations. *IC*, intracellular; *EC*, extracellular

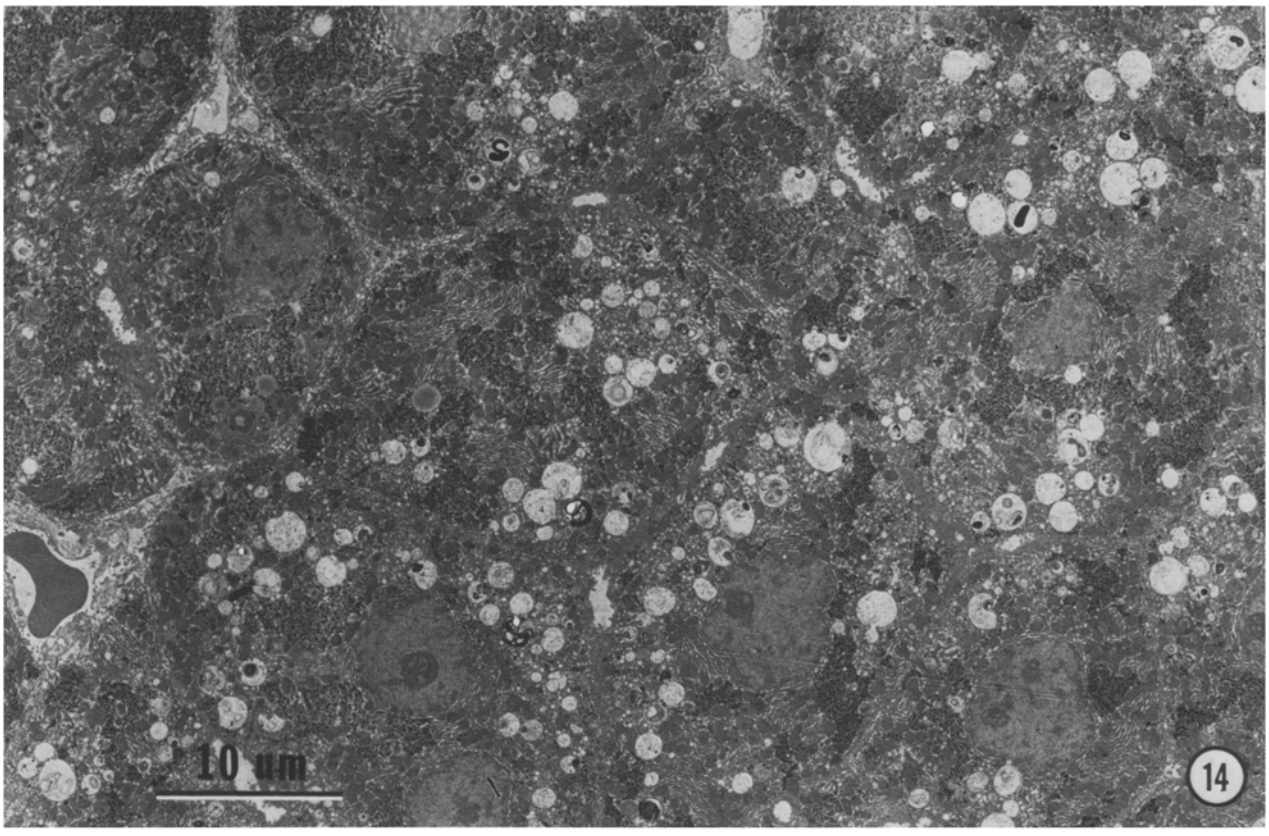
to be more numerous in the region of the Golgi apparatus (which could itself be readily distinguished). However, it was very noticeable that the vesicles were not restricted only to the regions of the Golgi and the canaliculi, but were scattered throughout the cells. On occasion, they were observed to fuse with one another. A second difference from control, recovered slices lay in the appearance of the bile canaliculi which were open and almost totally devoid of microvilli (Fig. 16*a*), an observation also made by Phillips et al., (1975). The cytoplasm surrounding the canalicular lumen took the form of a homogeneous zonula, containing material of medium density which could represent depolymerized constituents of the microfilaments (i.e. contractile proteins). At higher magnification, this zonula appeared to consist of finely granular or filamentous material (Fig. 16*b*). Microvilli were also much less numerous in the spaces of Disse, compared to controls.

In marked contrast to the small effect of cytochalasin B by itself, when added in the presence of ouabain this agent caused an almost total inhibition of water extrusion (Table 2). There was a corresponding inhibition of the ouabain-resistant extrusion of Na^+ and Cl^-, but respiration was, again, not affected. The ultrastructural appearance of these slices was generally similar to that with cytochalasin B alone (Fig. 17) with the vesicles being rather larger and more widespread. Characteristic was the occurrence of large vesicles in the immediate vicinity of the smooth-surfaced bile canaliculi, with no indication of their passage into the homogeneous zonula or their fusion with the limiting membrane (Fig. 15). We interpret these observations to mean that a polarized contractile system, normally working to move vesicles from the Golgi region to the canaliculi (as in bile secretion), was not functional in the presence of cytochalasin. As a result, the vesicles accumulated in the cell and spread throughout the cytoplasm in a nonpolarized fashion, while those that did approach the canaliculi were unable to enter into contact with the membranes. The inability of the cells to extrude water at a time when fusion of the vesicles with the canalicular membranes was prevented, strongly supports our suggestion that the exocytosis of vesicular contents into the canaliculi is an important part of the ouabain-resistant water extrusion, and the effectiveness of cytochalasin indicates the importance of microfilaments in this mechanism. That cytochalasin B had similar morphological effects in the absence of ouabain, while having little influence on water extrusion, can be explained if the water extrusion coupled to the ouabain-sensitive, Na-K transport system had sufficient reserve capacity to compensate for inhibition of the vesicular extrusion system.

Nickel

This transition metal has been found markedly to inhibit protein secretion by a number of glands, either by direct effects on exocytosis or by antagonizing Ca^{2+} (Dormer, Kerbey, McPherson, Manley, Ashcroft, Schofield & Randle, 1974). At concentrations of 0.5–5.0 mM, the effective range noted by Dormer

Fig. 14. Effect of cytochalasin B on liver slices incubated for 60 min at 38 °C, after 90 min at 1 °C. Low magnification. 100 μg/ml cytochalasin B. Vesicles of various sizes were distributed in all parts of the hepatocytes, in contrast to the polarization seen in the presence of ouabain (*see* Fig. 8). (For further descriptive details, *see text.*) 2,500× (reproduced at 99%) **Fig. 15.** Effect of cytochalasin B on liver slices incubated for 60 min at 38 °C, after 90 min at 1 °C; 100 μg/ml cytochalasin B. Detail showing the wide and uniform distribution of vesicles and the alterations of bile canaliculi (*BC*) which are devoid of microvilli. 8,000× (reproduced at 99%)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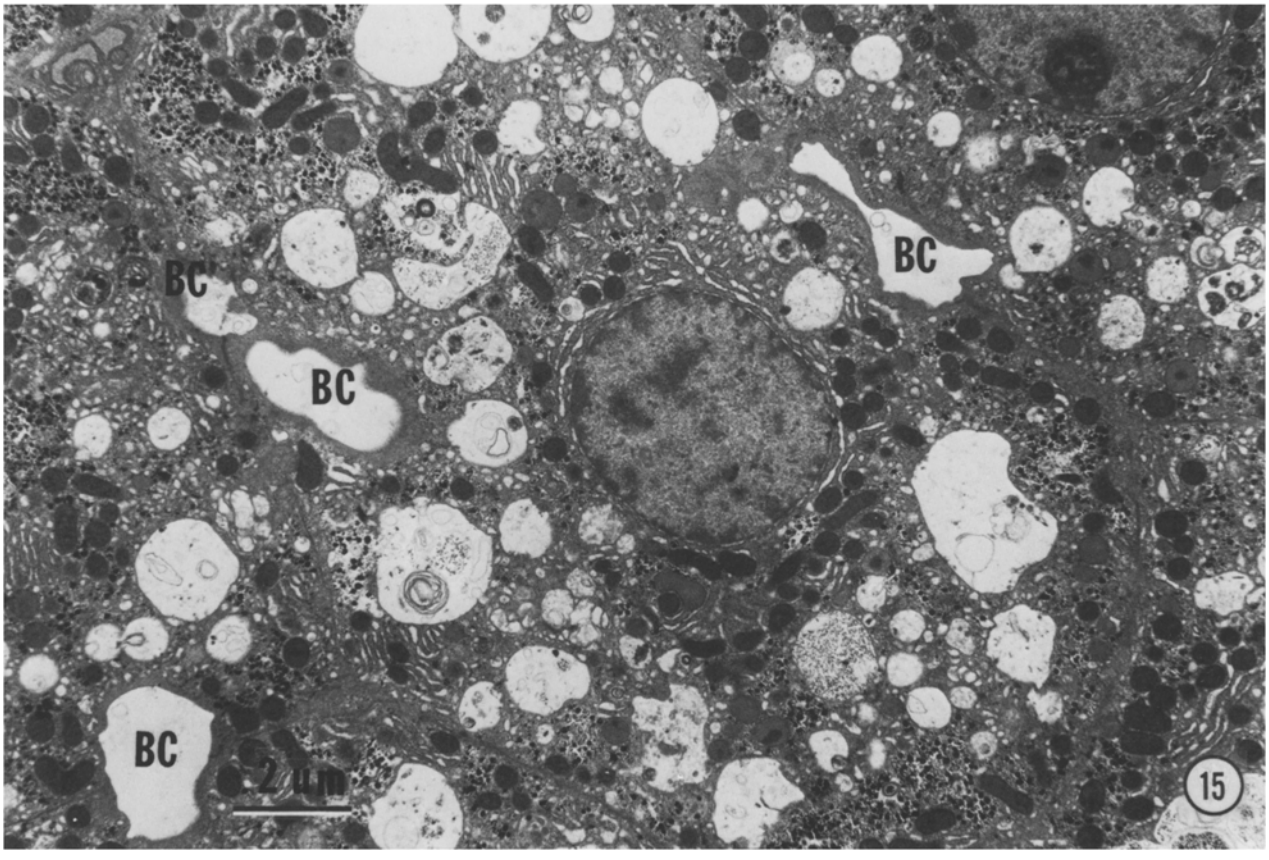
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Table 2. Effects of cytochalasin B and ouabain on liver slices (Details as in Materials and Methods and Fig. 13; all flasks contained 0.1% dimethyl sulphoxide, the solvent for cytochalasin B)

	After 90 min at 1 °C	Then 60 min at 38 °C			
		Controls	Cytochalasin (100 µg/ml)	Ouabain (2 mM)	Cytochalasin +ouabain
Total water	3.63±0.08	2.37± 0.10	2.73± 0.05	2.63± 0.05	3.30± 0.11
Extracell. H_2O	1.21±0.39	0.89± 0.20	1.14± 0.21	0.86± 0.09	1.11± 0.06
Intracell. H_2O	2.42±0.33	1.48± 0.10	1.60± 0.17	1.72± 0.10	2.19± 0.11
K^+ Content	59 ±3	203 ±13	208 ± 6	60 ± 4	42 ± 3
Change of Na^+	–	−292 ±32	−324 ±26	−123 ±19	−77 ±44
Change of Cl^-	–	−205 ±27	−257 ±17	−191 ±20	−93 ±23
Rate of respiration	–	8.9 ± 0.17	8.5 ± 0.27	5.6 ± 0.38	6.6 ± 0.24
(*n*)	(4)	(4)	(4)	(8)	(8)

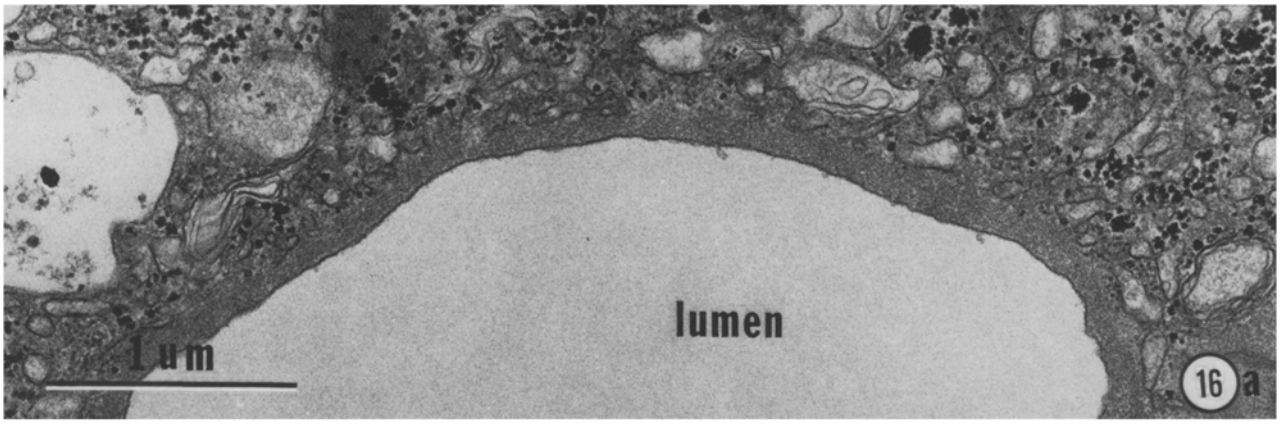
Water contents expressed as kg water/kg dry wt; ions as mmol/kg dry wt and respiration as µl O_2/mg dry wt·h^{-1}.

and co-workers, Ni^{2+} had no significant effect on the net extrusion of water, Na^+ or Cl^- during recovery at 38 °C (not illustrated). However, 5 mM Ni^{2+} did reduce K^+ reaccumulation by 30% ($p=0.05$) and caused a 40% reduction in the rate of respiration which was unaccompanied by any effect on ATP content (Table 3; *see also* Dormer et al., 1974).

Fig. 18 shows that that 5 mM Ni^{2+}, but not lower concentrations, caused a partial inhibition of the ouabain-resistant extrusion of intracellular water. Ouabain itself reduced water extrusion from 1.1±0.2 kg/kg dry wt to 0.8±0.1, while the further presence of 5 mM Ni^{2+} caused a reduction to 0.5±0.1 kg/kg ($n=24$, in each case). The rate of respiration in the presence of Ni^{2+} was not further reduced by addition of ouabain (Table 3), suggesting that a part of the inhibition of O_2 consumption caused by Ni^{2+} alone may have arisen indirectly from an inhibition of the ouabain-sensitive transport of cations (note the inhibition of K^+ uptake by Ni^{2+}). In the electron microscope there was a generally good degree of ultrastructural recovery in the presence of Ni^{2+} alone (not illustrated) and in the presence of Ni^{2+} and ouabain together (Fig. 19). Cytoplasmic vesicles of the type normally seen with ouabain were present in large numbers when the slices were treated with ouabain *plus* Ni^{2+}, while many bile canaliculi had an appearance very similar to that seen with cytochalasin, namely wide open lumina with few or no microvilli and a surrounding, homogeneous zonula (Figs. 19 and 20). However, these effects of Ni^{2+} on the canaliculi were less consistent than with cytochalasin B, since adjacent cells often showed canaliculi with numerous microvilli. Among the cytoplasmic vesicles close to the canaliculi could be seen large clusters of small, homogeneous vesicles, possibly pinocytotic vesicles (Fig. 20). Nickel thus had effects which were qualitatively similar, although quantitatively less in degree, to those of cytochalasin B, giving a partial inhibition of water extrusion only in the presence of ouabain, while causing accumulation of cytoplasmic vesicles and an appearance of inactivity in many bile canaliculi both in the presence and absence of ouabain.

An additional effect of Ni^{2+}, which may be related to its mechanism of action, was on tissue Ca^{2+} (Table 3). The Ca^{2+} content of the liver slices after preincubation at 1 °C was about 30% lower in the presence of Ni^{2+}, while during subsequent incubation at 38 °C the net extrusion was reduced from 8.4 mmol/kg dry wt to 5.9 mmol/kg. Similar results were obtained in the presence of ouabain. It should be noted that the calcium-extruding mechanism of liver cells is insensitive to ouabain (van Rossum, 1970; Cittadini & van Rossum, 1978). These results could result from the displacement of Ca^{2+} from binding sites by Ni^{2+} during incubation at 1 °C. A similar pattern has been seen in liver and kidney slices treated with Pb^{2+} *in vitro* (Kapoor & van Rossum, 1977).

Fig. 16. Effect of cytochalasin B on liver slices incubated for 60 min at 38 °C, after 90 min at 1 °C; 100 µg/ml cytochalasin B. (*a*) Detail of bile canaliculus which is without microvilli and has a sub-membrane zonula of uniform thickness and finely granular appearance. 44,000×. (*b*) Greater magnification of the sub-membrane zonula. 80,000× (reproduced at 99%). **Fig. 17.** Effect of cytochalasin B in the presence of ouabain on liver slices incubated for 60 min at 38 °C, after 90 min at 1 °C; 100 µg/ml cytochalasin B, 2 mM ouabain. A peri-canalicular region is shown. The general appearance is similar to that seen with cytochalsin B alone (Figs. 14 and 15). There are many vesicles of various sizes and they are associated with both Golgi apparatus (*G*) and endoplasmic reticulum. However, fewer of these vesicles show the indications of fusion with each other than do the vesicles found in the presence of ouabain alone (*cf.* Fig. 9*b*). 16,000× (reproduced at 99%)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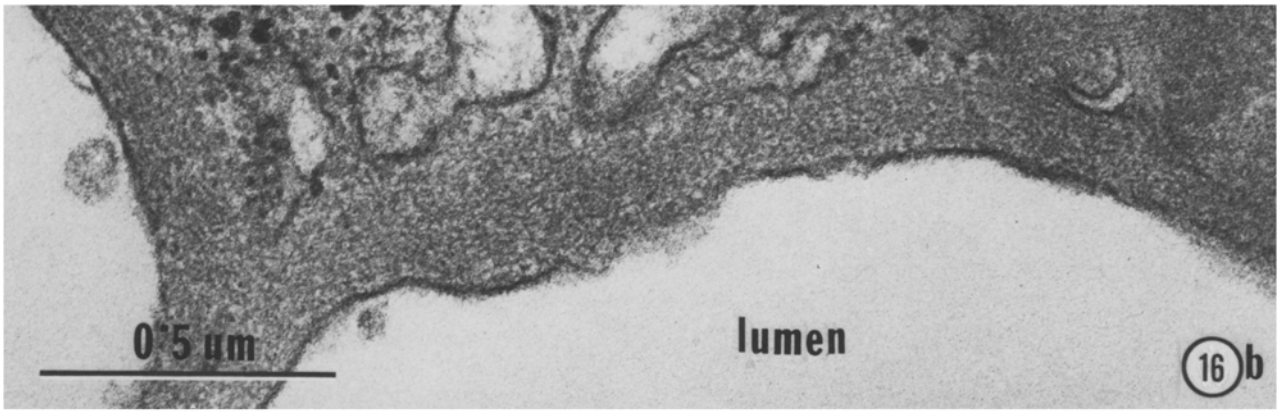
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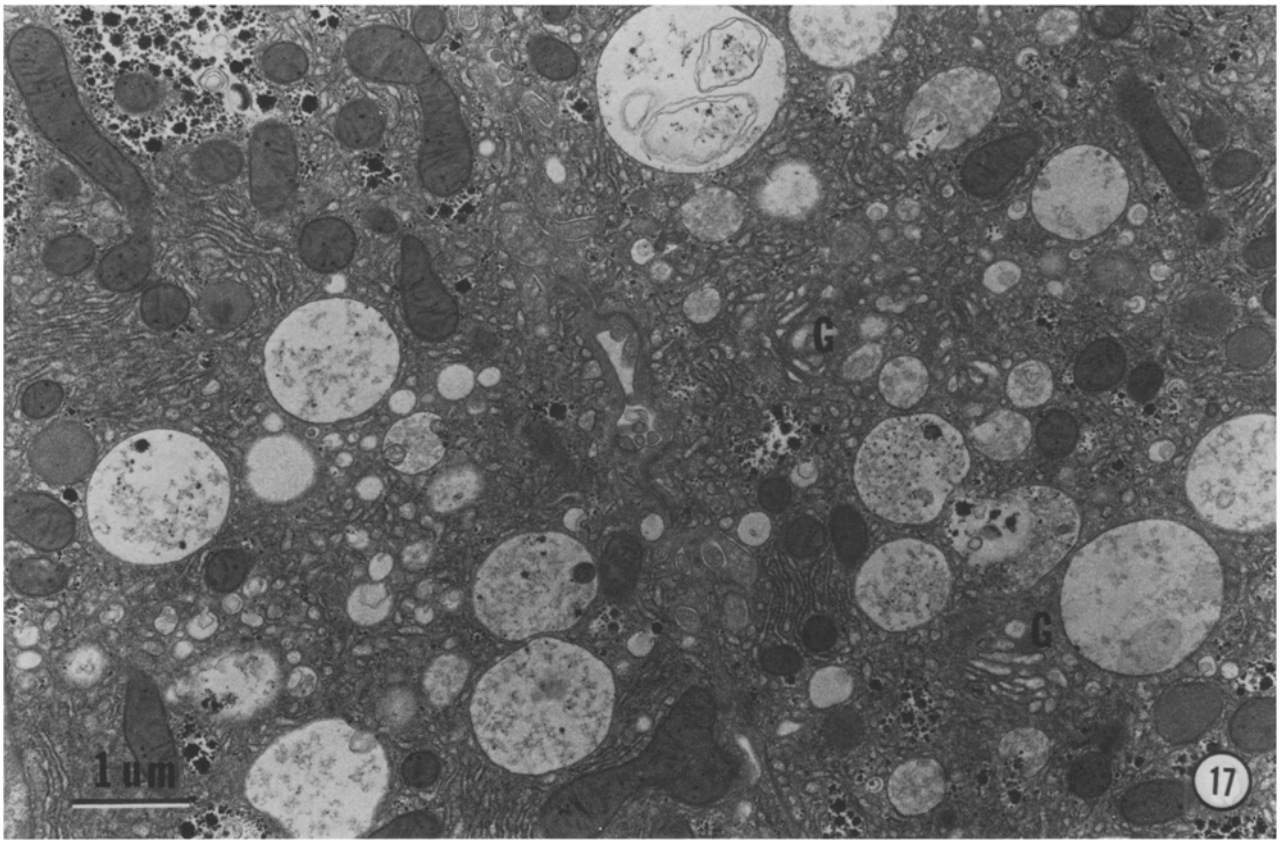
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Table 3. Effects of Ni^{2+} on ion movements and energy metabolism of liver slices incubated in the presence or absence of ouabain (Experimental details as in Materials and Methods and Fig. 18)

Incubation	Ionic contents (mmol/kg dry wt)		Respiration (μl/mg dry wt·hr^{-1})	ATP content (mmol/kg protein)
	K^+	Ca^{2+}		
90 min at 1 °C				
Control	82± 5 (6)	20.1±2.0 (4)	–	4.4±0.6 (4)
plus Ni^{2+} (5 mM)	74± 6 (6)	14.2±2.5 (3)	–	–
Then at 38 °C for 60 min:				
Control	197±10 (11)	11.7±1.3 (8)	8.4±0.5 (9)	5.0±0.4 (6)
plus Ni^{2+} (5 mM)	157±12 (11)	8.3±0.6 (8)	4.3±0.3 (7)	5.7±0.5 (4)
Ouabain (2 mM)	84±11 (8)	11.9±1.0 (8)	5.4±0.6 (8)	–
plus Ni^{2+} (5 mM)	110± 9 (8)	8.0±0.7 (8)	4.1±0.4 (8)	–

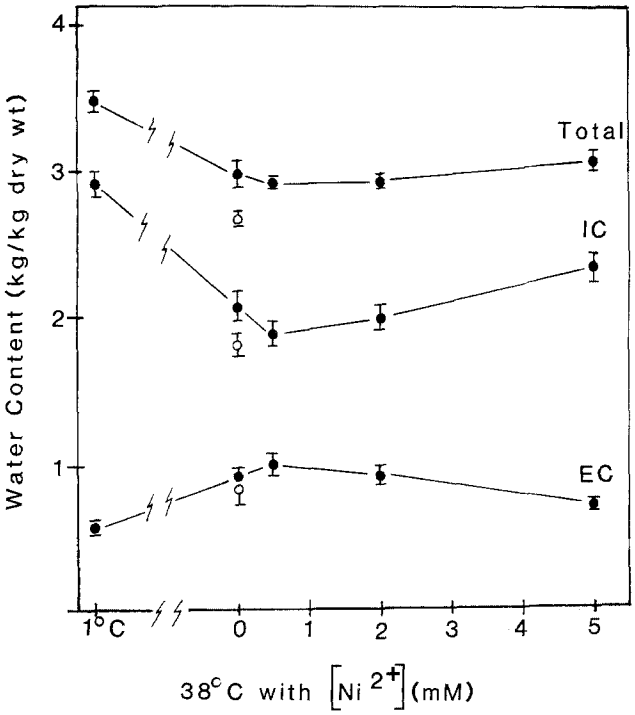
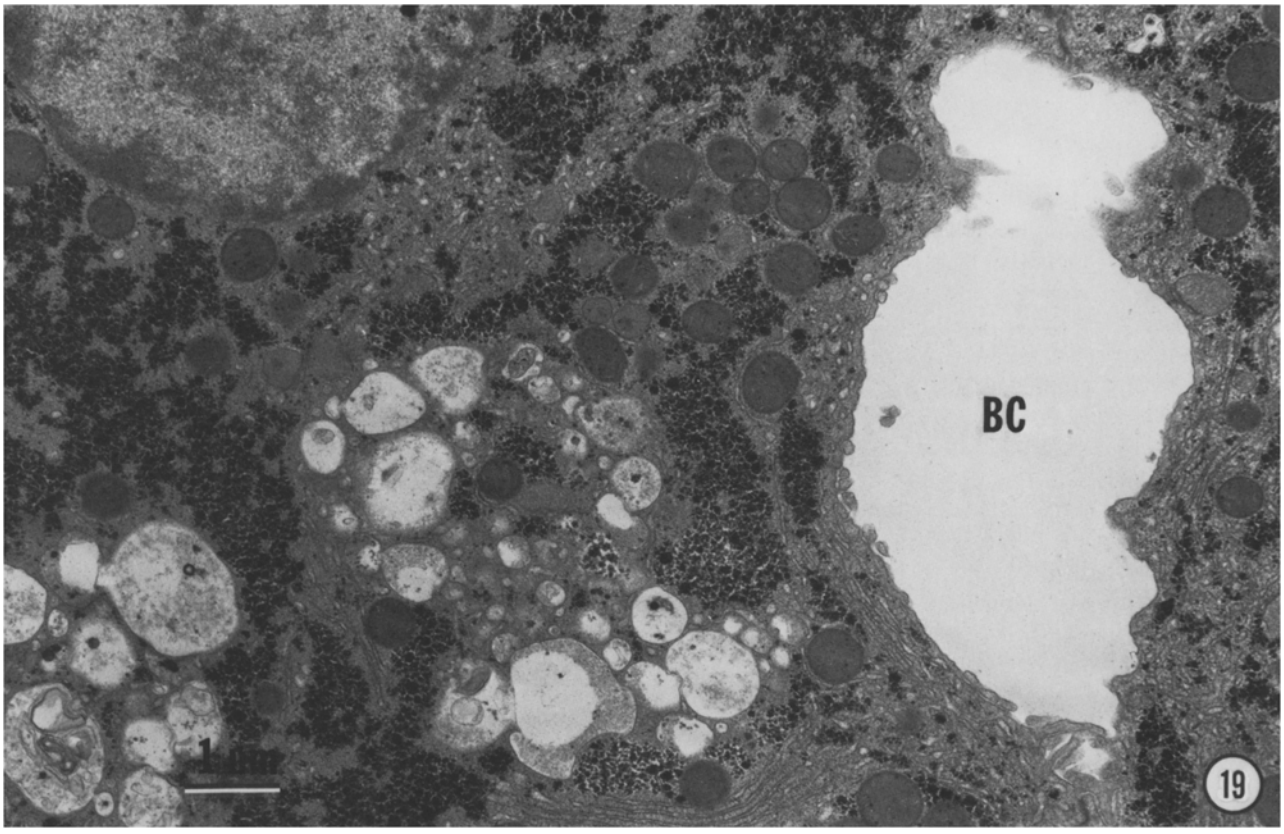


Fig. 18. Effects of Ni^{2+} on the water content of liver slices incubated in the presence of ouabain. Slices were incubated for 90 min at 1 °C followed by 60 min at 38 °C in oxygenated medium. Ni^{2+} was added as $NiSO_4$ during the first 30 min at 1 °C. o Incubation throughout without inhibitors. • Incubation with 1 mM ouabain *plus* the indicated concentrations of Ni^{2+}. Each point is the mean ± SEM of 24 observations. *IC*, intracellular; *EC*, extracellular

Discussion

Our results confirm that the ability of liver slices to extrude water after a period of swelling is to a considerable extent, but not entirely, resistant to ouabain (*cf.* Macknight et al., 1974; Russo et al., 1977). The extrusion is accompanied by recovery of the overall form of the cells and of their cytological architecture, both of which were much distorted during swelling at 1 °C. However, the structural recovery was not solely a direct consequence of a primary reduction of cell water since the intracellular architecture was not markedly restored upon removal of water in response to a simple osmotic stimulus. Conversely, most aspects of cell structure recovered completely in the presence of ouabain, even though water extrusion was only half that in control slices showing similar structural recovery. Rather, continuation of respiratory metabolism was a more important requirement for structural recovery. In general, while architectural recovery had several common requirements, and a similar time-course, to water extrusion, it was largely a separate process. An important factor in the recovery of structure may be the changes of cellular ionic contents occurring during incubation at 38 °C, in which case Ca^{2+} is the most likely candidate since its extrusion is the only one not affected by ouabain (*cf.* van Rossum, 1970; Cittadini & van Rossum, 1978). The failure of colchicine and cytochalasin B, when applied

Fig. 19. Combined effects of Ni^{2+} (5 mM) and ouabain (2 mM) on liver slices incubated for 60 min at 38 °C, after 90 min at 1 °C. General appearance of the region between nucleus and canaliculus. The canalicular lumen is almost devoid of microvilli, and is surrounded by an apparently amorphous zonula which is similar, although more narrow, to that seen with cytochalasin B (Fig. 16). Other features, and especially the numerous characteristic cytoplasmic vesicles, are as seen with ouabain alone (e.g., Fig. 8). 13,000× (reproduced at 99%). **Fig. 20.** Combined effects of Ni^{2+} (5 mM) and ouabain (2 mM) on liver slices incubated for 60 min at 38 °C, after 90 min at 1 °C. Canalicular region, showing large clusters of very small vesicles, of uniform diameter and of an appearance indistinguishable from normal pinocytotic vesicles; they appear not to fuse together. Other features as in Fig. 8. 27,000× (reproduced at 99%)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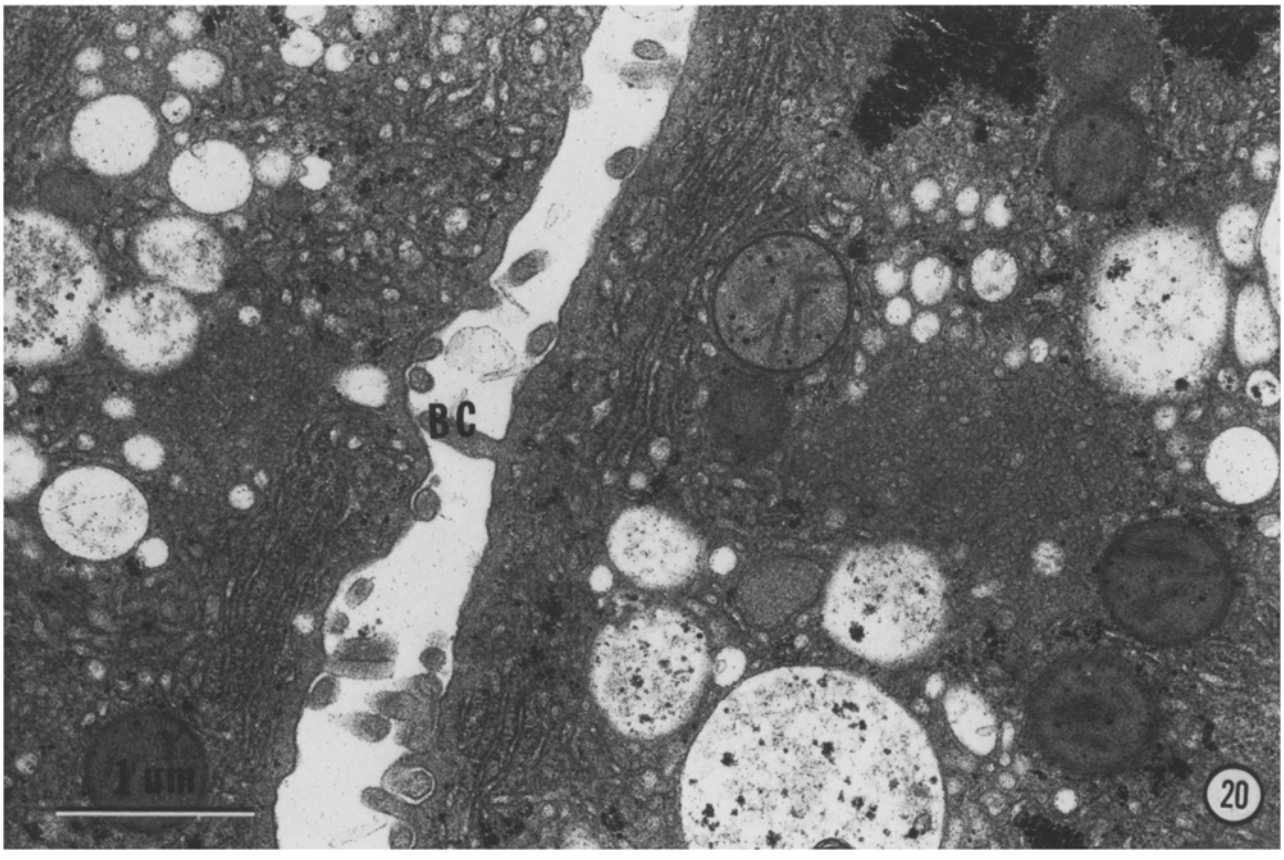
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alone, to cause marked inhibition of the structural recovery indicated that microtubules and microfilaments were not obligatorily required for the recovery of the cell architecture of the type seen here.

With regard to the mechanism of water extrusion, we have previously used the differential effects of oligomycin and ouabain as evidence that two distinct mechanisms exist in the liver cells, and that each mechanism can increase its capacity to compensate, in some measure, for the inhibition of the other. The ouabain-sensitive portion of the water extrusion, which can be inhibited by oligomycin (Russo et al., 1977) proceeds, we suggest, by the mechanism postulated by Leaf (1956) and uses the driving force of the $(Na^+ + K^+)$-ATPase. Recent histochemical evidence suggests that this enzyme is confined to the sinusoidal and lateral borders of the hepatocyte (Blitzer & Boyer, 1978), and these regions of the cell would be the site of this part of water extrusion.

We further postulated that the rounded vesicles so characteristic of the slices treated with ouabain were intimately concerned in the ouabain-resistant mechanism of water extrusion (Russo et al., 1977). The mechanism would proceed in three stages: *i*) Secretion of water accompanied by, or possibly driven by, transport of Na^+ and Cl^- into cytoplasmic vesicles; continued secretion leads to an increase in size of some of the vesicles. *ii*) Passage of the vesicles via the Golgi region to the canaliculi. *iii*) Exocytotic extrusion of the vesicular contents into the bile canaliculi. Cytoplasmic vesicles were much more apparent, both numerically and in size, in the presence of ouabain than in control slices, and we suggest that this is because the "load" placed upon the vesicular system during recovery from swelling is much increased when the alternative mechanism has been inhibited by ouabain. It is of interest that vesicles of a rather similar appearance have been observed in kidney cortical slices incubated in medium low in Na^+, especially at 37 °C (Evan, Park & Solomon, 1978) or during recovery at 37 °C from swelling at 1 °C (S.A. Ernst and G.D.V. van Rossum, *unpublished observations*). The present work provides evidence to support a number of our proposals, especially with regard to steps *ii*) and *iii*) of the ouabain-resistant mechanism:

i) The similarity of effects of potassium-free incubation medium and of ouabain corroborate the suggestion that the rounded vesicles are associated with inhibition of the $(Na^+ + K^+)$-ATPase.

ii) An indication of the close correlation between cytoplasmic vesicles and water extrusion in the presence of ouabain is given by the finding that only small vesicles are scattered through the cytoplasm after 5 min at 38 °C – at a time when water extrusion had barely started. During the next 10 min, larger, rounded vesicles typical of ouabain treatment started to appear. Their formation could be accounted for by an enlargement of the earlier, smaller vesicles as a result of the secretion of water into their lumina and/or by fusion. At the same time, water extrusion was in its most rapid phase.

iii) Cytochalasin B, and to a smaller extent Ni^{2+}, both inhibited ouabain-resistant water extrusion and led to a marked inactivity of exocytotic processes at the canaliculi; they clearly prevented approach of the cytoplasmic vesicles to the canalicular membranes. This we consider to be strong evidence of an association between exocytotic activity and the ouabain-resistant water extrusion.

iv) Cytochalasin B in particular led to a wide dispersion of vesicles of many sizes through the cytoplasm, and this is consistent with our postulate that water is secreted into the vesicles before extrusion. Inhibition of exocytosis, noted above, would then lead to an accumulation within the cytoplasm of vesicles unable to release their contents. We previously used a similar argument to account for the accumulation of vesicles in the presence of oligomycin (Russo et al., 1977), but those vesicles were of a markedly different form from those seen with cytochalasin, and the canaliculi, instead of being open as with cytochalasin, were tightly occluded in the presence of oligomycin. While in both cases the vesicular mechanism of water extrusion was inhibited, we believe that the basis for the inhibition is different, being due to disorganization of microfilaments in the presence of cytochalasin B, and to partial depletion of cell energy levels in the presence of oligomycin. These differences may underly the different appearance of the inhibited system in the two cases.

Our results with cytochalasin also provide further evidence that the ouabain-resistant and -sensitive portions of the water extrusion are due to separate functions. Application of cytochalasin B alone (i.e. in the absence of ouabain) had no significant effect on water extrusion, despite producing a picture of cytoplasmic vesicles and inactive canaliculi very similar to that seen with cytochalasin plus ouabain. Thus, if the vesicular exocytosis is indeed the basis for ouabain-resistant water extrusion, this mechanism has apparently been inhibited by cytochalasin in both cases. It would then follow that the water extrusion depending on the $(Na^+ + K^+)$-ATPase has been able totally to compensate for the absence of the vesicular mechanism. Conversely, in the presence of ouabain, K^+ transport, and so presumably the $(Na^+ + K^+)$-ATPase, was completely inhibited, but cytochalasin B caused a total inhibition of the persisting system, which thus must have proceeded by a different means from the cytochalasin-resistant system in the absence of ouabain.

The relative sensitivities of the ouabain-resistant water extrusion to cytochalasin B and to colchicine indicate that microfilaments, rather than microtubules, are cytoskeletal elements closely associated with the ouabain-resistant water transport. While microtubules have been shown to be involved in secretory processes of hepatocytes, and to be inhibited by much smaller concentrations of colchicine than those used here (Orci et al., 1973; Redman et al., 1975), the particular processes studied involved exocytosis into the sinusoids rather than the canaliculi, and this may provide a basis for different effects of colchicine on the water extrusion. In contrast, cytochalasin B clearly led to a marked alteration of the peri-canalicular zone of the cytoplasm, possibly indicative of a disintegration of the microfibrillar contractile proteins. The importance of microfilaments in bile secretion has been emphasized, its inhibition by cytochalasin B at concentrations similar to those used here having been reported (Phillips et al., 1975).

The inhibition of water extrusion in the presence of ouabain by cyanide or oligomycin indicates the great sensitivity of the ouabain-resistant mechanism to energy depletion, since oligomycin only causes partial reduction of cell ATP levels (van Rossum, 1976; Russo et al., 1977). The energy requirement may be due to the need for microfilament activity in exocytosis. In addition, the secretion of water into the cytoplasmic vesicles is also likely to be an energy-requiring process. However, our present work provides no direct evidence on the nature of the mechanism which might underly this postulated secretion.

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