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Observations on the Regulation of Cell Volume and Metabolic Control *in Vitro*; Changes in the Composition and Ultrastructure of Liver Slices under Conditions of Varying Metabolic and Transporting Activity

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Summary. Liver slices incubated at 1 °C underwent swelling of both cellular and intercellular compartments, as judged by electronmicroscopy. The ultrastructure showed marked changes, including disorganization of the cytocavitary network and plasma membrane and alterations of mitochondria. Restoration of metabolically favorable conditions (oxygenated medium at 38 °C) caused a nearly complete recovery of ultrastructure closely associated with extrusion of water; measurements of inulin space and electronmicroscopy both indicate a recovery of cell volume, with intercellular spaces remaining somewhat expended. The fluid lost was a roughly isotonic solution of Na^+ and Cl^-, while K^+ was reaccumulated in exchange for Na^+. Cyanide prevented recovery. Ouabain and oligomycin each partially prevented fluid extrusion, but had little effect on ultrastructural recovery except to induce intracellular vesicles containing particles of thorium dioxide derived from sinusoidal spaces. The vesicles were, however, markedly different in form with each inhibitor. There are, thus ouabain-sensitive and -insensitive components of volume regulation; the former appears to depend on the coupled transport of Na^+ and K^+ and the latter, we suggest, on a secretion of Na^+ and Cl^- into vesicles which release their contents into the bile canaliculi by an oligomycin-sensitive mechanism. Mitochondria showed conformational changes between orthodox and condensed forms, but these could not be directly related to tissue energy states; the numbers of mitochondrial dense granules bore a closer relation to tissue ATP.

In common with other isolated, mammalian tissues [4, 21], liver slices swell when incubated in an isosmotic, saline medium under conditions of reduced metabolic activity [11, 19], but regain the ability to control

their volume when metabolically favorable conditions are restored [5, 20]. Measurements with extracellular marker compounds indicate that the swelling is due to an increase of both intra- and extracellular water, while the recovery results mainly from the loss of cell water [5, 20]. Ultrastructural studies of these compartmental changes and their correlation with the accompanying organellar changes should throw light on the mechanism of volume control, but apart from work on swelling processes in flounder nephrons [25] such studies are lacking. The movements of ions during swelling led Wilson [35] and Leaf [16] to suggest that the regulation of cell volume was a consequence of the ouabain-sensitive mechanism for the coupled transport of Na^+ and K^+, but recent observations have led to the postulation of a second, ouabain-insensitive, component of volume regulation [15, 34]. Evidence for this latter component has also been presented for liver slices [17] although the absence of measurements of extracellular spaces in this case prevented more precise assessment of changes in cellular volume and composition.

The factors which adversely affect the regulation of cell volume in liver include low temperatures, inhibitors of respiration and ATP synthesis [26, 32] and inhibitors of the Na^+–K^+ transport system [5]. Each of these affects the metabolic state of the mitochondria, the first two directly, and the last apparently indirectly by virtue of the changes in extramitochondrial utilization of ATP and of cell K^+ content [5, 27, 28]. Hackenbrock [9, 10] has shown that isolated mitochondria undergo conformational changes which may be able to act as indicators of their metabolic state *in situ*, and ultrastructural studies may thus throw light on metabolic control phenomena in cells during changing volume-regulating activity.

We have now attempted to correlate transport activity and energy levels with the changes of ultrastructure in liver slices undergoing swelling and its reversal. The use of inhibitory agents provides structural and analytical evidence which further supports the conclusion [17] that there exists more than one component of volume-regulating activity in the liver cell and, in addition, provides an indication of a possible cytological basis for the ouabain-insensitive mechanism.

Materials and Methods

Male albino rats of Wistar strain weighing 300–400 g were used. They were fed *ad libitum*. The preparation of liver slices and the analytical techniques used have been described [5, 32]. Incubation was continued for 90 min at 1 °C, followed by up to 60 min at 38 °C

either in the absence ("controls") or presence of inhibitors. Samples of slices were taken at intervals during the incubation for study by electronmicroscope. Thorium dioxide, when used, was added to the incubation medium (3 ml) as 0.1 ml of "Thorotrast."

The slices for microscopy were fixed at 4 °C with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) with $CaCl_2$, and then post-fixed for 2 hr in a solution of OsO_4 (1.33%) in phosphate buffer. After dehydration in ethanol, the tissue was placed in toluol for 1 hr and then embedded in Epon-812. Sections were prepared with a diamond knife, using an ultramicrotome (Porter-Blum MT2), mounted on 150 mesh copper grids without supports, and stained with lead hydroxide [18]. Each original tissue slice was studied in its entirety by phase-contrast light microscopy; three representative samples were studied by electronmicroscope (Philips EM-300), five ultra-sections being prepared from each.

Results

Swelling at 1 °C

During incubation at 1 °C in a phosphate-buffered Ringer's medium, liver slices gained water and Na^+ and lost K^+, the changes being largely completed by 60 min (Table 1; *see also* refs. 11, 19). The retention of a relatively high ATP content (6.7 ± 0.8 (6) mmoles/kg protein in this series of experiments) after 90 min indicates that the low temperature effectively inhibited the enzymes utilizing and catabolizing ATP. In agreement with the increase of total tissue water, the size of the cells and extracellular spaces seen at low magnification in the electronmicroscope

Table 1. Composition of liver slices during the course of swelling and its reversal[a]

Treatment	Water (kg/kg dry wt)	Na^+ (mmoles/kg dry wt)	K^+
Fresh tissue	2.31 ± 0.03 (4)	132 ± 16 (4)	293 ± 18 (4)
Incubated at 1 °C:			
60 min	3.63 (2)	554 (2)	76 (2)
90 min	3.70 ± 0.13 (6)	583 ± 18 (6)	69 ± 5 (6)
Then at 38 °C for:			
10 min	3.27 ± 0.44 (4)	468 ± 49 (4)	97 ± 6 (4)
30 min	2.91 ± 0.14 (4)	375 ± 41 (4)	157 ± 17 (4)
70 min	2.98 ± 0.12 (7)	338 ± 25 (7)	223 ± 14 (7)

[a] Samples of the slices from these experiments were taken for study in the electronmicroscope (Figs. 1–5 and Fig. 7). The incubation medium contained (in mM): 161 Na^+, 5 K^+, 1.2 Ca^{2+}, 1.0 Mg^{2+}, 153 Cl^-, 1.0 SO_4^{2-} and 10 phosphate (pH 7.4); it was gassed with O_2. Slices were incubated for 90 min at 1 °C and the vessels were then transferred to a bath maintained at 38 °C. Samples were taken for analysis at the times indicated. Values are mean ± standard error of the mean (number of observations).

increased markedly (*cf.* Figs. 1 and 4), although the latter came to contain structures which, at higher magnification, were seen to be largely fine microvilli and possibly some membrane debris. These alterations were accompanied by ultrastructural changes which, after 60 and 90 min, respectively, were rather similar to stages 3 and 4 of the cell swelling as defined by Trump and Bulger [25]. The nuclei and nucleoli underwent relatively minor changes but other cell components showed much more marked changes of form. The endoplasmic reticulum became much dilated and fragmented and the irregular vesicles thus formed came to occupy much of the space between other intracellular organelles (Fig. 3). The Golgi apparatus was well organized in the fresh tissue (Fig.2), but was no longer clearly distinguishable at 1 °C, although it was presumably represented by some of the smaller vesicles. The swollen slices showed a great increase in the number of free ribosomes (Figs. 3, 5*b* and 5*c*) apparently due to dissociation of the polysomes seen in the fresh tissue. The plasma membranes of adjacent cells in the fresh tissue were closely apposed, showed junctional complexes (Fig. 2), but formed microvilli (0.1–0.2 μm diameter) only in the regions of the bile canaliculi and spaces of Disse (Figs. 1 and 2). After incubation at 1 °C, the cells became widely separated by a broad band of smaller microvilli (0.03–0.05 μm diameter) which covered the entire cell surface (Figs. 4, 5*a*). The junctional complexes could not be observed after cold incubation (compare Fig. 5*a* with Fig. 2). The general picture of a large degree of intracellular vacuolization, convolution of the cell surface, and expansion of intercellular spaces agrees well with the observed increase of total tissue water and cell size.

The mitochondria underwent a marked conformational transition from the "orthodox" configuration in the fresh tissue (Fig. 2) to the mixture of intermediately "condensed" (Figs. 3 and 5*c*), swollen, and degenerative forms (Figs. 4, 5*a* and *b*) which is characteristic of stage 4 swelling (as defined in ref. 25).

The macrophages observed in some sections underwent similar changes to the hepatocytes during incubation at 1 °C (Fig. 4).

Incubation at 38 °C without Inhibitors

(a) Time-course. Table 1 and Fig. 6*a* show that much of the extra water that entered the slices at 1 °C was lost within 10 min of the onset of incubation at 38 °C in oxygenated medium, and that a minimum value was attained by 30 min. Fig. 6*a* shows, further, that the water loss was

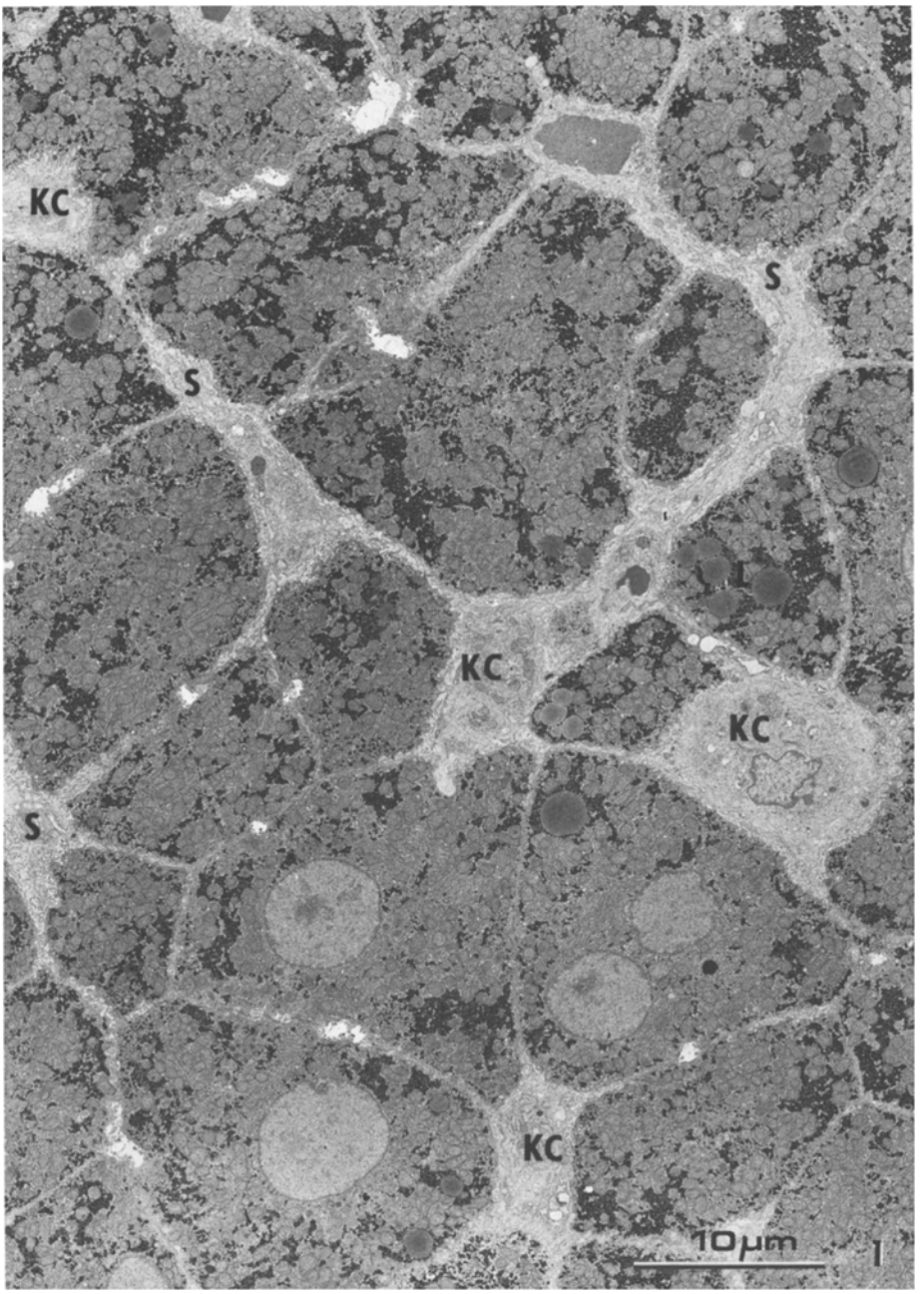
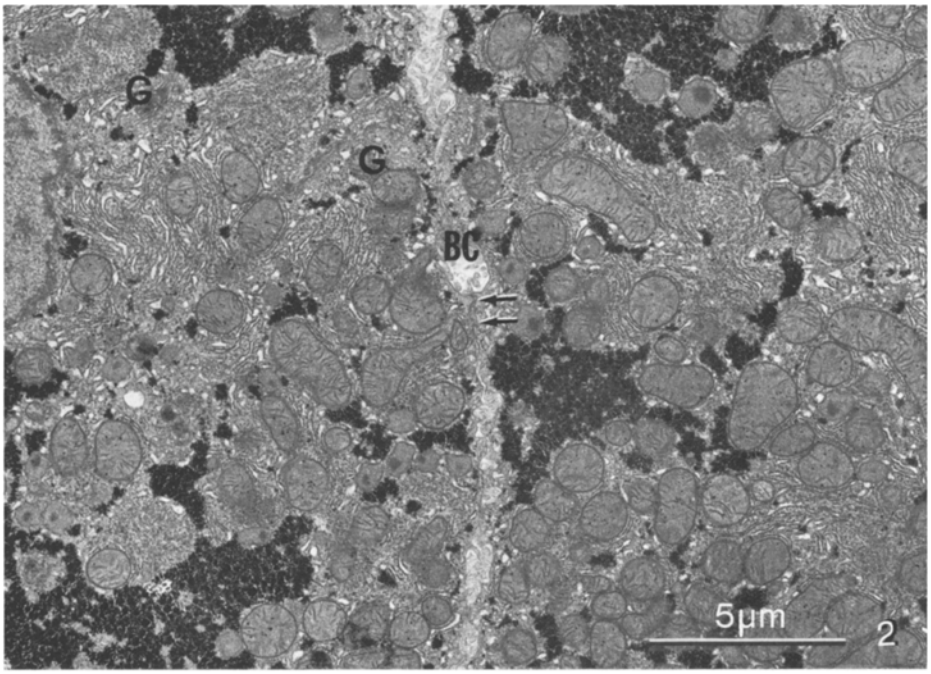


Fig. 1. Low magnification of fresh rat-liver prepared from a sample slice fixed immediately before the start of incubation at 1 °C. This illustrates the good preservation of structure throughout the slice-cutting procedure and provides a basis for comparison of the changes occurring during subsequent incubation under various conditions. Nuclei, mitochondria, cytocavitary network and glycogen are well preserved. The Kupfer cells (*KC*) appear electron-clear due to the use of lead hydroxide only as the stain. Sinusoid, *S*; ×3,000



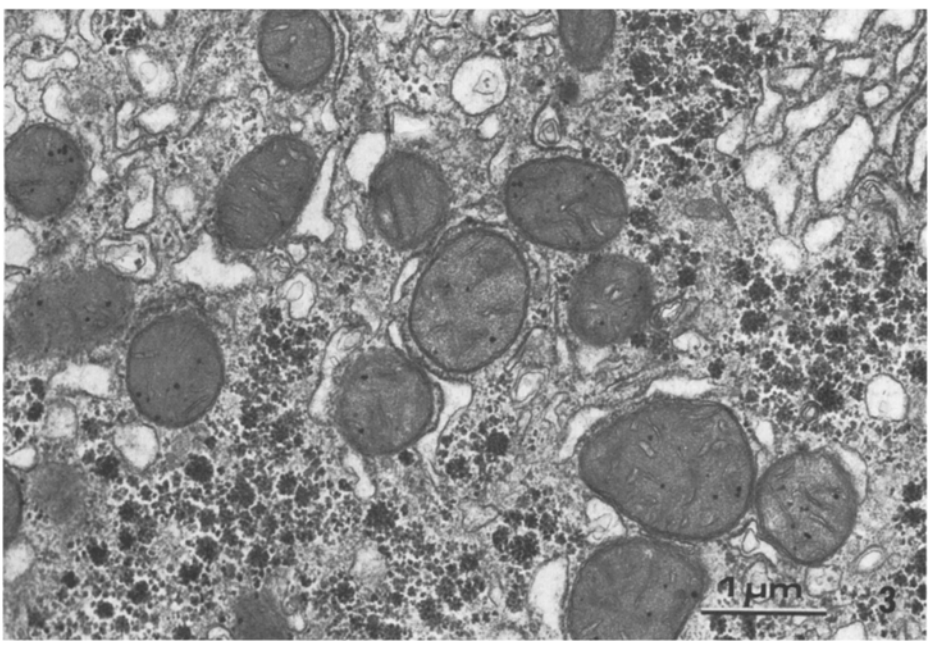


Fig. 2. Parts of two fresh-tissue cells. Note bile canaliculus (*BC*) and related junctional complexes (arrows). Mitochondria are in the orthodox configuration [9] and show many dense granules; Golgi apparatus, *G*. ×6,000

Fig. 3. Detail of slice incubated for 60 min at 1 °C. Compared to the fresh tissue, the mitochondria have undergone condensation of the matrix and an enlargement of the intracristal space, resulting in the appearance of the intermediate configuration of Hackenbrock [9, 10]. The presence of dilated cisternae, dilution of cell sap and some dispersion of the glycogen are indicative of the early cell swelling seen in Stage 3 as defined by Trump and Bulger [25]. ×20,000

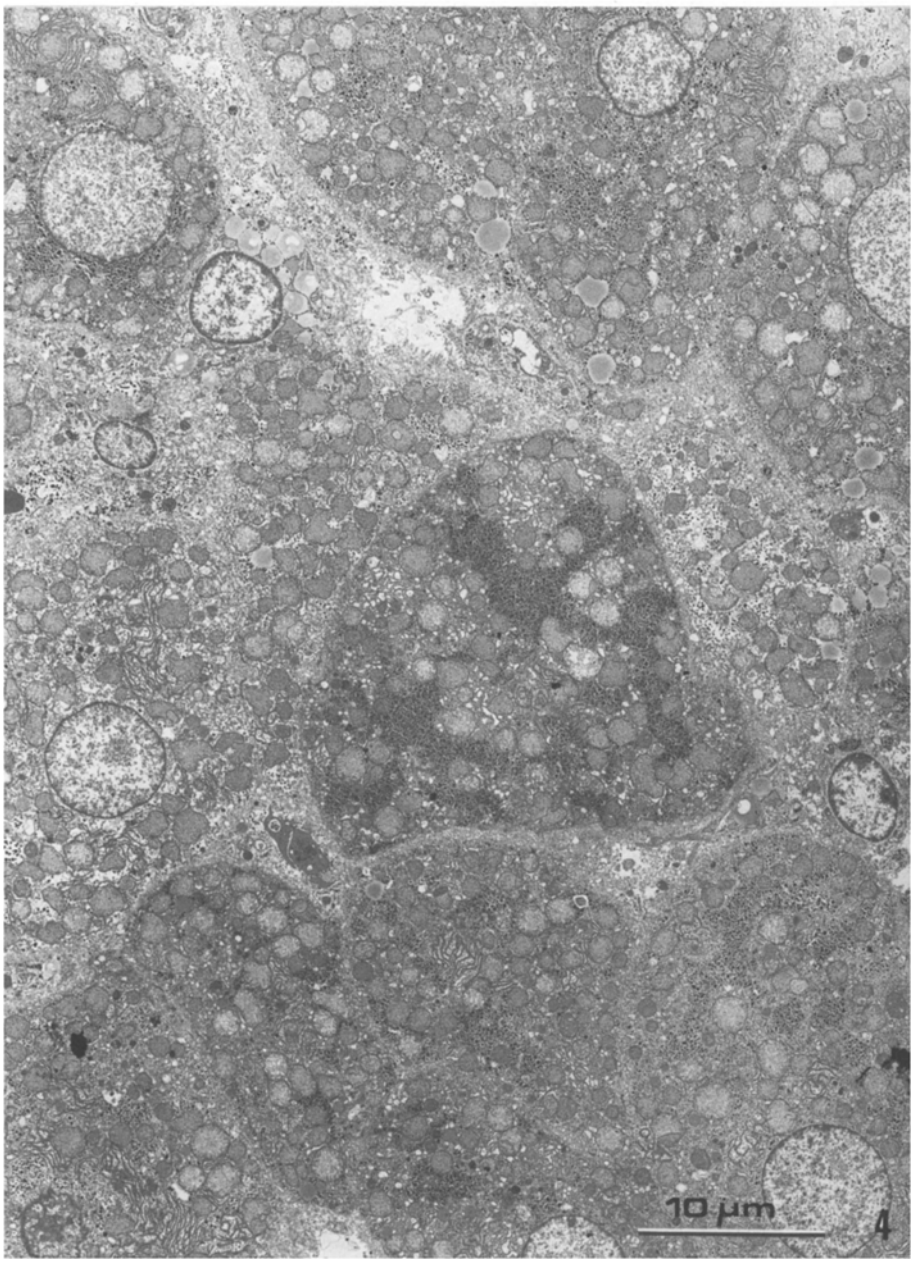


Fig. 4. General view of liver slice after incubation at 1 °C for 90 min (for comparison with Fig. 1, which is at the same magnification). The number of cells per unit area is less than in the fresh tissue, due to evident swelling of all the cells and of the extracellular spaces. The spaces adjacent to the lateral cell surfaces are especially enlarged. The general impression is that of stage 4 swelling [25]. × 3,000

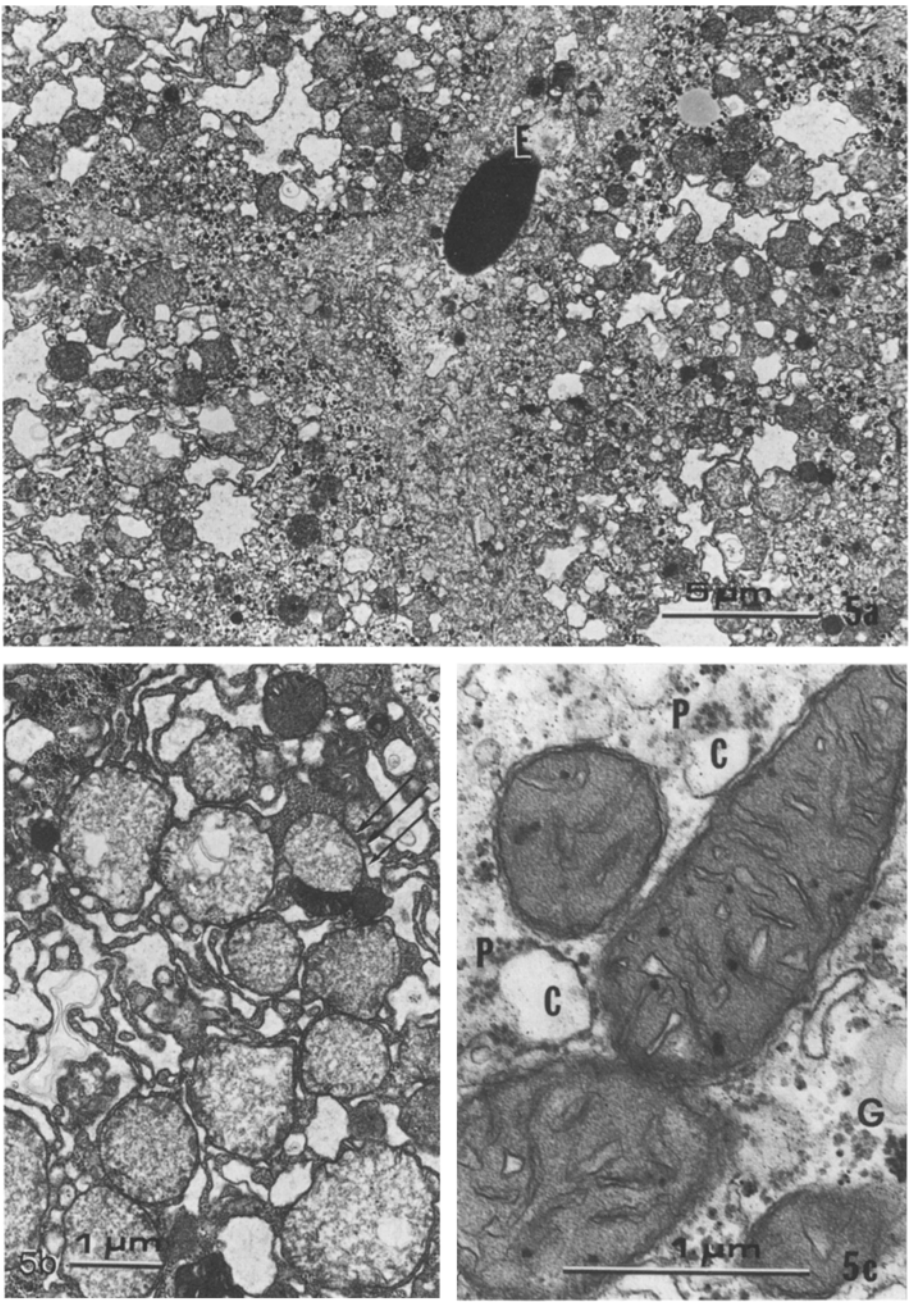


Fig. 5. Greater magnification of slices after incubation at 1 °C for 90 min. (*a*) Detail of cellular boundaries between three cells. The boundaries are not sharp, due to the presence of numerous, fine microvilli. × 5,000. (*b*) Swollen, vesiculated and condensed mitochondria in the same cell. In one instance (three arrows) it is possible to see swollen and condensed regions within the same mitochondrion. Some ribosomes are seen to be attached to the reticular membranes and others to be free in the cytosol. × 15,000. (*c*) Detail of intermediately condensed mitochondria. The matrix is condensed and intracristal spaces are somewhat dilated; dense granules are present. Cisternae of dilated rough endoplasmic reticulum, *C*; Polysomes, *P*; Rosettes and α-particles of glycogen, *G*. × 40,000

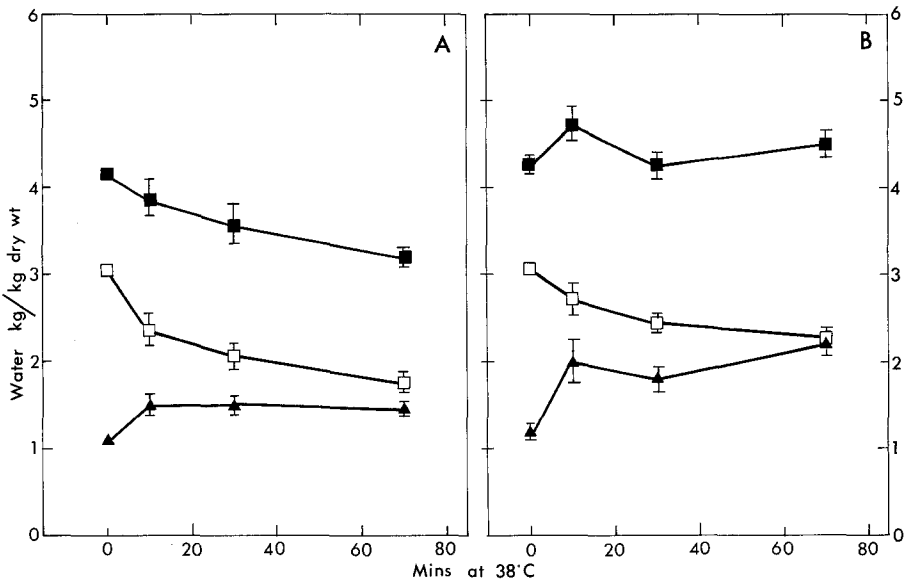


Fig. 6. Time-course of changes in tissue water compartments during incubation at 38 °C in (A) control and (B) cyanide-treated liver slices. ■ Total tissue water content, $(H_2O)_T$. ▲ Inulin-containing ("extracellular") water, $(H_2O)_{In}$; calculated as,

$$(H_2O)_{In} = (H_2O)_T \times \frac{\text{inulin concentration in tissue water}}{\text{inulin concentration in medium}}.$$

□ Inulin-free ("intracellular") water, calculated as,

$$(H_2O)_{In\text{-}free} = (H_2O)_T - (H_2O)_{In}.$$

Each point represents the mean ± SEM of 8 observations; in some cases the standard errors are smaller then the size of the symbols used

exclusively from the inulin-inaccessible compartment, with the inulin-containing water volume becoming slightly expanded. The loss of Na^+ (Table 1) and Cl^- (not shown; but *see* [32]) initially followed a time-course similar to that of water, but the net accumulation of K^+ was preceded by a lag period of about 10 min (*see also* refs. 14, 29 and 32). Further, the Na^+ extrusion continued after the loss of water had been completed, and at a time when K^+ was still being accumulated. Thus, the loss of Na^+ may formally be envisaged to occur partly as a net extrusion together with Cl^- and water, and partly by exchange for K^+, the former predominating in the first 10–30 min (*see also* refs. 5 and 32). However, this does not necessarily imply two independent mechanisms for Na^+ transport. The ATP content increased slightly, attaining a value of 8.0 ± 0.8 (8) mmoles/kg protein at 20 min. The accompa-

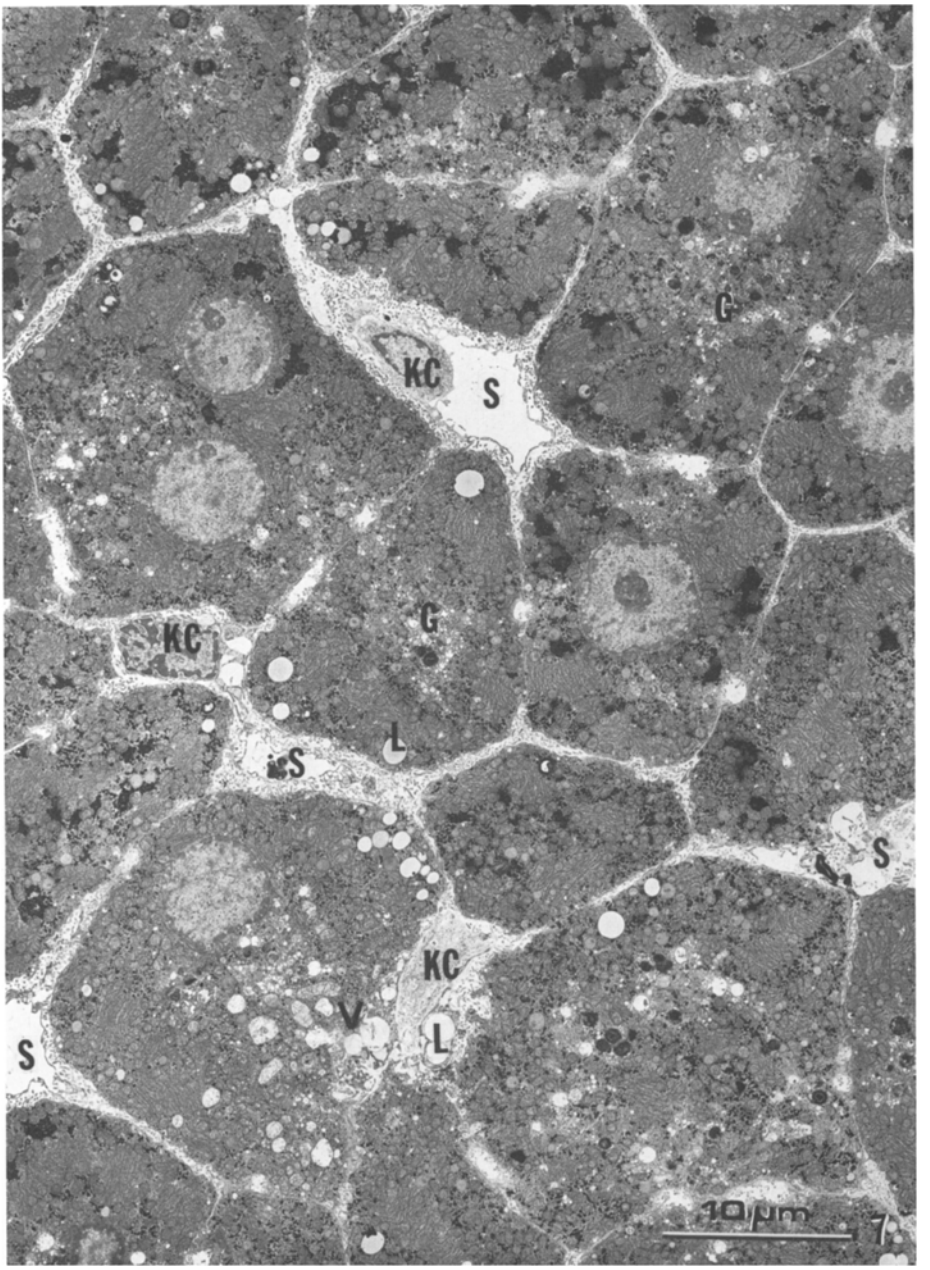


Fig. 7. General view illustrating recovery of most cell structures and cell volume after incubation for 70 min at 38 °C in oxygenated medium (for comparison with Figs. 1 and 4). The number of cells per unit area is similar to that in fresh tissue (Fig. 1) and greater than in the cold-incubated slices (Fig. 4). The spaces of Disse and lateral intercellular spaces remained somewhat expanded, and the latter were largely cleared of microvilli. Intracellularly, the vesicles in the golgi region are slightly larger than those in the fresh tissue. Kupfer cell, *KC*; sinusoid, *S*; lipid droplet, *L*; vesicles, *V*. ×3,000

nying recovery of ultrastructure towards the steady state described in detail below was well advanced by 10 min (not illustrated), and thus preceded all but a small part of the recovery of K^+ content.

(b) Steady state. By 70 min at 38 °C the water compartments and ionic contents had attained a steady state (Fig. 6*a*) in which the K^+ content per unit dry wt was about 76% of that seen in fresh tissue (Table 1). A low magnification study of the tissue (Fig. 7) shows, in agreement with the loss of inulin-free water, that the cells had returned to a size approximately equal to that seen in the fresh tissue. The ATP content remained constant, showing a value of 8.3 ± 0.7 mmoles/kg protein after 60 min.

The calculated changes of intracellular ionic content accompanying the recovery of cell volume after 70 min in a larger series of experiments are shown in Table 2. Assuming part of the net loss of intracellular Na^+ to occur as an equimolar exchange for K^+ entering the cells, and

Table 2. Net changes of water and ion content during incubation at 38 °C in the presence of oligomycin and ouabain

		Additions			
		None	Oligomycin (10 µg/ml)	Ouabain (1 mM)	Oligomycin + Ouabain
Total water	kg/kg dry wt	-0.81 ± 0.06	-0.51 ± 0.08	-0.52 ± 0.07	-0.23 ± 0.09
Extracellular water	kg/kg dry wt	0.14 ± 0.06	0.36 ± 0.08	0.03 ± 0.05	0.45 ± 0.06
Intracellular water	kg/kg dry wt	-0.96 ± 0.09	-0.92 ± 0.10	-0.56 ± 0.08	-0.69 ± 0.08
Intracellular Na^+	mmole/kg dry wt	-306 ± 15	-245 ± 25	-99 ± 28	-110 ± 29
Intracellular Cl^-	mmole/kg dry wt	-196 ± 17	-172 ± 19	-126 ± 16	-134 ± 17
Intracellular K^+	mmole/kg dry wt	166 ± 9	131 ± 11	21 ± 5	13 ± 6
Sum of intracellular cations	mmole/kg dry wt	-150	-124	-88	-107

Liver slices were incubated for 90 min at 1 °C, followed by 60 min at 38 °C in the presence of the inhibitors shown. The medium contained (in mM): 161 Na^+, 5 K^+, 1.2 Ca^{2+}, 1.0 Mg^{2+}, 153 Cl^-, 1.0 SO_4^{2-} and 10 phosphate (pH 7.4); it was gassed with O_2. All flasks contained 0.5% ethanol (the solvent for oligomycin). The values represent the content of the slices after incubation at 38 °C *minus* the content after 90 min at 1 °C. Each value is the mean ± SEM, of 21–23 observations. Determination of intracellular contents is based on correction for the distribution of inulin and the ionic concentration of the medium. The sum of intracellular cation changes includes an allowance for a net extrusion of 10 mequiv Ca^{2+} per kg; cell Mg^{2+} contents did not change significantly during incubation (*see* text and ref. 30).

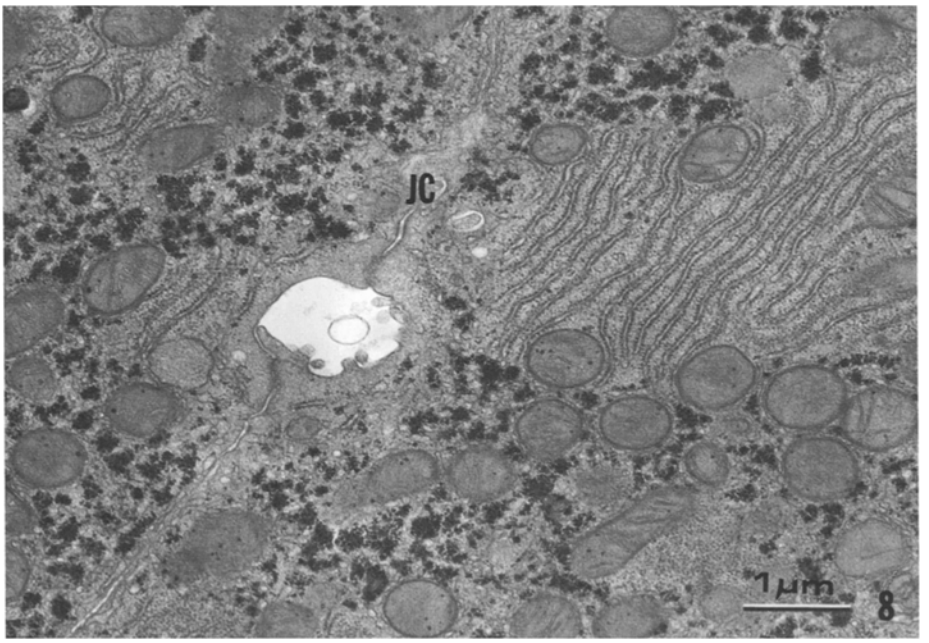


Fig. 8. Detail of control slices after incubation for 70 min at 38 °C. Manifestations of ultrastructural recovery include: electron-dense cell sap, well-organized cytocavitary network, restored junctional complexes (*JC*) delimiting the canaliculi, cellular boundaries similar to those of fresh tissue, and glycogen rosettes restored to their original arrangement. However, peroxisomes and mitochondria are smaller than in the fresh tissue (*cf.* Fig. 2; note different magnification). × 17,000

taking into account the net extrusion of some 10 mequiv Ca^{2+}/kg dry wt [30], a net loss of about 150 mequiv cations per kg dry wt can be calculated. This is rather similar to the loss of Cl^- (196 mmoles/kg). The ratio of the lost Na^+ (in excess of that exchanging for K^+) to the extruded cell water was 149 mmoles/kg water, while the apparent concentration of Na^+ in the intracellular water at the onset of incubation at 38 °C was 186 mmoles/kg water. The corresponding values for Cl^- were 200 mmoles per kg of extruded water and 163 mmoles per kg initial cell water. Thus, the loss of cell water occurred as a solution roughly isotonic with the intracellular fluid.

The ultrastructural appearance of the slices after 70 min at 38 °C showed a very complete degree of recovery. In particular, the rough endoplasmic reticulum, and such features of the cell surface as the bile canaliculi, junctional complexes, plasma membranes and microvilli all resumed the appearance they had in the fresh tissue (Figs. 7 and 8).

Recovery was somewhat incomplete in the regions of the sinusoids in that the microvilli remained rather numerous and the spaces of Disse partially expanded. This clearly contributed to the analytical finding of a failure of the inulin-containing water content to fall during recovery (Fig. 6*a*); a further contributory factor is that the membranes of adjacent cells often remained farther apart than in the fresh tissue, and the spaces between them became cleared of microvilli (Fig. 7). The Golgi apparatus reappeared to a large extent as the small vesicles seen in the peri-nuclear region (Fig. 7); when seen in larger magnification, the vesicles were always associated with parallel membrane complexes (not illustrated).

The degree of dispersion of glycogen, which increased during incubation at 1 °C, was much reduced. The mitochondria all returned to the "orthodox" configuration and swollen forms were no longer visible (Figs. 7 and 8). Compared to the fresh tissue, all the mitochondria were substantially reduced in size (about one-third the diameter; *cf.* Figs. 2 and 8, taking note of the different magnification), the matrix was more electron dense and the cristae less numerous.

Macrophages showed a similar structural recovery to the hepatocytes, and now contained many fine vesicles indicative of pinocytotic activity (Fig. 7).

Effects of Inhibitors after 70 min at 38 °C

(a) Cyanide (1 mM). This inhibitor reduced the rate of respiration from 8.0 ± 0.3 μl/mg dry wt/hr to 2.4 ± 0.2 μl/mg/hr, the latter being probably entirely due to extra-mitochondrial O_2 consumption [24, 32]. The net transport of ions was almost completely blocked [5, 32]. The tissue as a whole lost the ability to control its volume, the total water content remaining constant during incubation at 38 °C. The reduction in the volume of inulin-free water was much smaller than in the controls, and was roughly balanced by a doubling of the inulin-containing water compartment (Fig. 6*b*). Moreover, after 70 min the slices showed little ultrastructural recovery and, indeed, presented an appearance of considerable necrosis throughout (Fig. 9). The intercellular spaces, canaliculi and spaces of Disse were virtually completely obliterated, so that the increase of inulin-containing water volume can only have occurred by penetration of the inulin into a hitherto inaccessible compartment, i.e., by passage though the plasma membranes of the necrotic cells. Thus, the decrease of "inulin-free water" calculated from the determination of the apparent

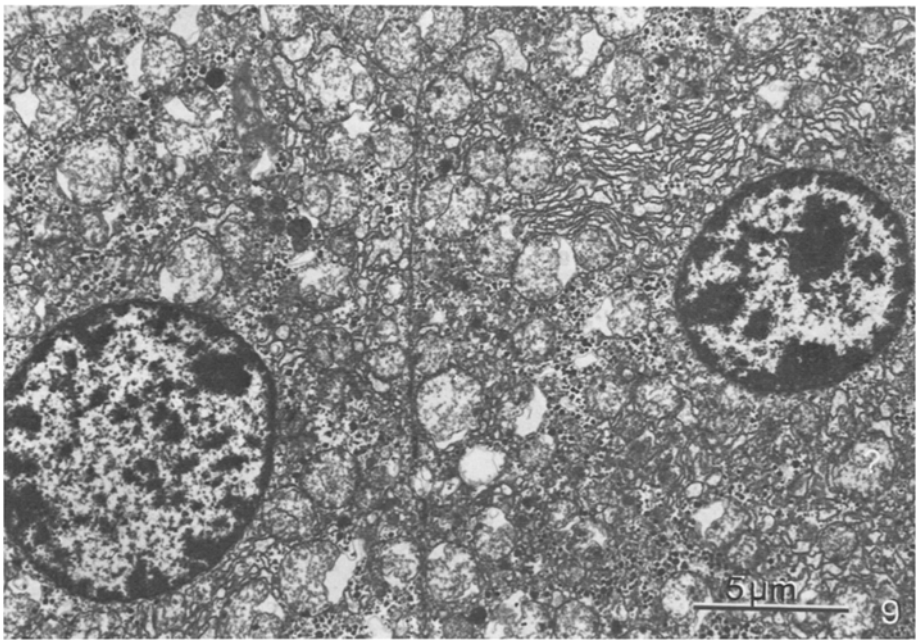


Fig. 9. Detail of three cells of a slice incubated for 70 min at 38 °C in the presence of cyanide (1 mM). A large degree of necrosis is evident, although the cell boundaries are intact and closely juxtaposed. × 5,000. The picture represented here is typical of all regions of the slices incubated with cyanide

volume of distribution of inulin does not provide evidence for a persisting extrusion of water in the presence of cyanide. A degree of ultrastructural reorganization was evidenced by the re-apposition of adjacent plasma membranes, a reduction in the numbers of microvilli and some diminution of the dilatation of the endoplasmic reticulum; but this was clearly not equatable with the normal recovery. The mitochondria appeared in degenerating forms; cristae were no longer visible, the matrix was greatly expanded and, in most cases, an irregular space appeared between the inner and outer membranes at one pole of the organelle (*cf.* ref. 8). The nuclei showed a typically pycnotic appearance, with fragmented nucleoli and clumped chromatin. The amount of glycogen was much lower than in slices incubated at 38 °C without cyanide, presumably reflecting the more rapid rate of glycolysis [31]. However, the ATP so formed was clearly insufficient to maintain more than a rudimentary degree of cell organization and transport activity.

(b) Oligomycin (10 µg/ml). The concentration of oligomycin used was one which causes maximal effects on the slices [26, 31]. It reduced

Table 3. Effects of oligomycin and ouabain on respiration and adenine nucleotides of liver slices

		90 min at 1 °C	Then 60 min at 38 °C with additions:			
			None	Ouabain (1 mM)	Oligomycin (10 µg/ml)	Oligomycin + Ouabain
Respiration	µl/mg dry wt/h	–	10.1±0.5	8.0±0.4	8.3±0.3	7.6±0.3
ATP	mmole/kg dry wt	5.5±0.5	3.9±0.3	5.5±0.2	2.7±0.1	3.1±0.2
ADP	mmole/kg dry wt	2.4±0.3	1.1±0.3	1.4±0.3	1.8±0.2	1.8±0.2
AMP	mmole/kg dry wt	2.5±0.2	1.7±0.1	1.8±0.1	2.0±0.04	1.9±0.1
ATP/ADP		2.5±0.4	4.2±0.7	5.5±1.5	1.7±0.3	1.9±0.3

Incubation procedure as for Table 2, the analyses in that Table and this one being carried out in the same experiments. O_2 consumption was determined for 50 min of the incubation at 38 °C (following 10 min equilibration); final adenine nucleotide contents were determined after incubation at 1 °C and after further incubation at 38 °C for 60 min. The number of observations was 23 for respiration and 6 for the adenine nucleotides.

ATP content by 40% and respiration by 20% (Table 3), and so inhibited energy provision much less than cyanide did [31]. The net loss of total water was reduced by 37%, but this was entirely due to an increase in the content of inulin-containing water, the reduction of inulin-free water being equal to that seen in controls (Table 2). The net movements of Na^+ and K^+ were each reduced by only 15–20%. The net loss of total cations was again somewhat exceeded by the loss of Cl^-, and the apparent concentrations of Cl^- and Na^+ (in excess of exchange for K^+) in the extruded water (namely, 187 mmoles Cl^- and 121 mmoles Na^+/kg water) were similar to those in the controls. The reduction of cell volume (*cf.* Figs. 1 and 10) and degree of ultrastructural recovery (Figs. 10–12) were also little affected by the rather marked changes in energy state caused by oligomycin, and were as complete in many respects as that of the control slices.

The most marked intracellular differences caused by oligomycin included an appearance of inactivity in the Golgi apparatus and the fact that, despite a well-organized endoplasmic reticulum, most cells contained a number of large, irregular vesicles (Figs. 10 and 11*a*). At a greater enlargement these are seen to be surrounded by ribosome-free membranes (Fig. 11*b* and *c*) and were thus clearly distinct from the vesicles seen at 1 °C. In many cases the lumen was traversed by membranes, suggesting their formation by confluence of several vesicles. In order to obtain more information on the origin of these vesicles, control and oligomycin-

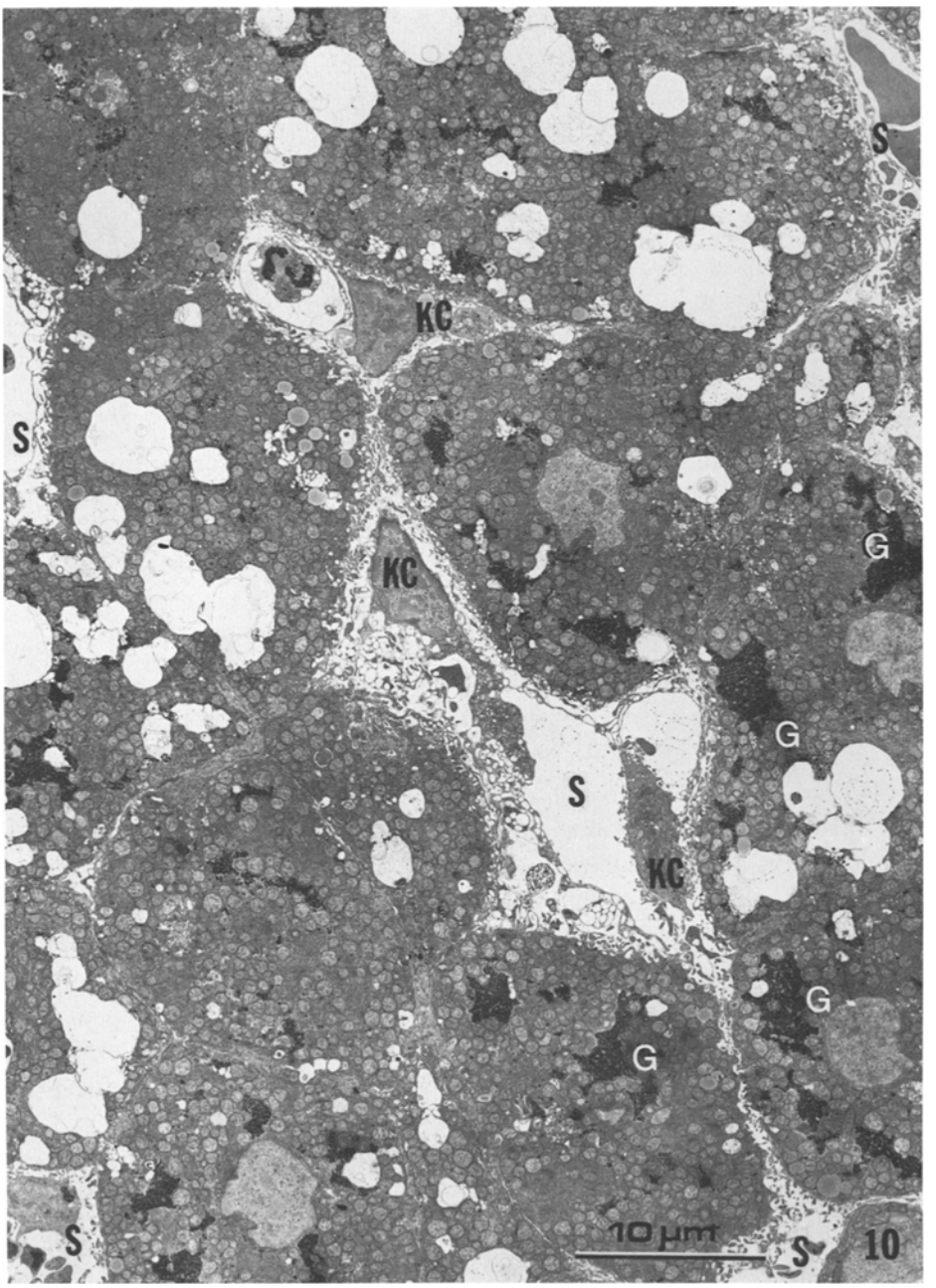


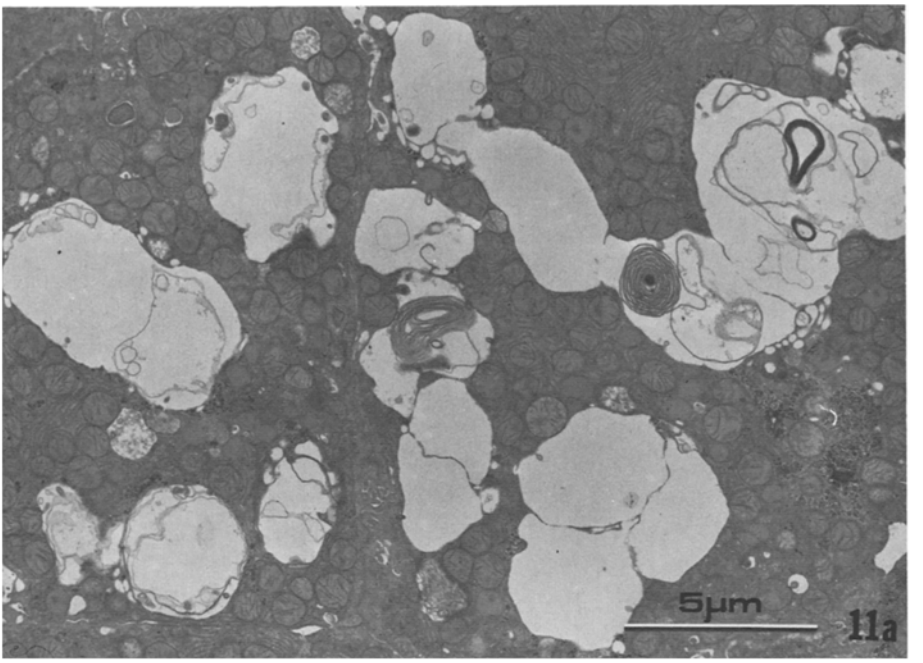
Fig. 10. General view of slice after incubation for 70 min at 38 °C in the presence of oligomycin (10 µg/ml). A generally good recovery is seen, but in comparison to Figs.1 and 7 note the large intracellular vesicles, larger sinusoids, irregular nuclei and larger mitochondria. Particles of thorium dioxide are clearly visible in the large, irregular vesicles and in the sinusoids (*S*). Kupfer cells (*KC*) are strongly vesiculated; glycogen (*G*) is often arranged in large clumps. × 3,000

treated slices were incubated at 38 °C in the presence of thorium dioxide. Particles of this material were found in the extracellular spaces of the sinusoidal regions, but were never observed in the open canalicular spaces of control slices. Since thorium particles were detected in the large intracellular vesicles of the oligomycin-treated slices (Fig. 10), the vesicular contents must have originated from the extracellular spaces of the sinusoidal region and will also have contained inulin. The increase of inulin-containing water (Table 2) can thus be partly attributed to the formation of these vesicles. Further, despite the reappearance of junctional complexes (Fig. 12*b*), the intercellular spaces remained considerably enlarged especially in the region of the sinusoids (Fig. 10) and will also have contributed to the larger volume of inulin-containing water. In contrast, the canalicular lumina were so reduced in diameter as to be completely obliterated by a few microvilli (Figs. 10 and 12*b*). The sinusoids retained a lining of microvilli which, however, were larger in size (1.0–1.5 μm diameter) than those observed either at 1 °C or in the fresh tissue (Fig. 12*a*). A striking observation was that of an apparently inverse relation between the number of intracellular vesicles and the size of the adjacent sinusoidal space. Thus, in the parts of Fig. 10 where sinusoidal spaces were large, the cells contained only small vesicles, and *vice versa.*

The effects of oligomycin on energy metabolism lead to the expectation of changes in mitochondrial configuration. However, as in control slices, the mitochondria were all restored to the orthodox configuration at 38 °C, although remaining rather larger in size than controls, and showed a complete absence of dense granules (Fig. 12*b*).

(c) Ouabain. Titration of liver slices with ouabain showed a maximal affect on respiration at 0.5–1.0 mM (Fig. 13). Although 1 mM ouabain reduced the rate of respiration by about 20%, it tended to retain the ATP content ($p<0.01$) and ratio, ATP/ADP, at levels somewhat higher than in controls (Table 3). These effects are consistent with a primary inhibition by ouabain of ATP utilization by K^+ and Na^+ transport, resulting in a secondary fall in the rate of respiration [27, 28, 33]. Nevertheless, the mitochondria underwent the same transition to the orthodox configuration as in control slices, and the only marked difference was an increase in the number of dense granules over that seen in mitochondria of control and, especially, oligomycin-treated slices.

Preparations of rat liver are known to be relatively insensitive to cardiac glycosides [1, 13] and reports of the concentrations required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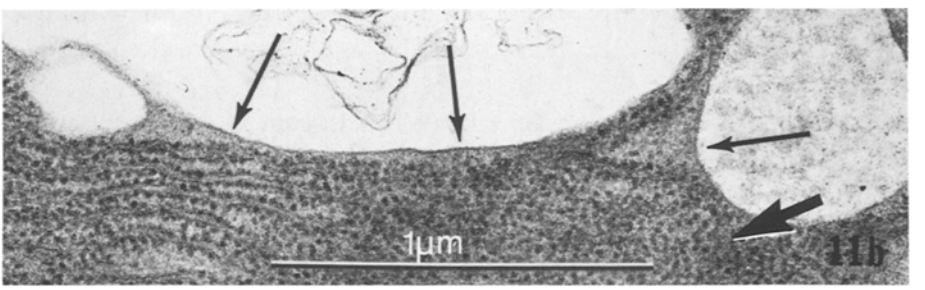
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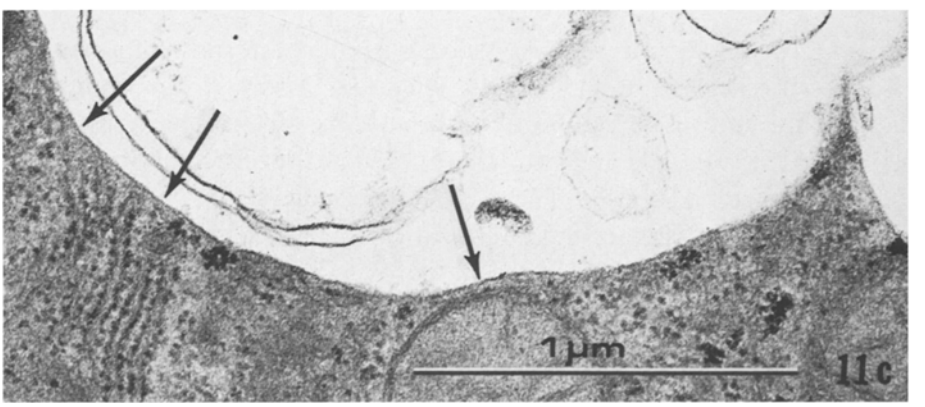
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for total inhibition of ion transport and related activities vary considerably. For example, total inhibition of the Na^+-K^+-sensitive adenosine triphosphatase activity of rat liver preparations has been variously reported to require ouabain at 0.05–0.1 mM [6, 7], 1 mM [23] and 10 mM [1]. The titration with ouabain illustrated in Fig. 13 gave a complete inhibition of the net accumulation of K^+ by liver slices at 0.5–1.0 mM ouabain. This agrees with the finding of Bakkeren and Bonting [2] that 1 mM ouabain completely inhibited the ability of rat-liver slices to accumulate ^{86}Rb to a level greater than the medium concentration (despite the higher concentration required to inhibit the Na^+-K^+ dependent ATPase in their work). The complete inhibition by 0.5 mM ouabain of the K^+-dependent component of ^{24}Na-efflux from liver slices may also be noted [27]. In subsequent experiments we therefore used ouabain at a concentration of 1 mM and found it to inhibit total water loss to the same extent as oligomycin (Table 2). In the case of ouabain, however, the effect was entirely accounted for by a 40% reduction of the loss of cellular (Inulin-free) water, and there was no increase in the quantity of water containing inulin. Accumulation of K^+ was greatly (although, in this series, not completely) prevented and extrusion of both Na^+ and Cl^- was substantially reduced, so that ouabain inhibited both the coupled transport of Na^+ and K^+, and a net movement of Na^+ and Cl^- accompanied by water. The latter may thus be a secondary consequence of the coupled transport of Na^+ and K^+, as suggested by Leaf [16]. However, it is also clear that about 60% of the water and Cl^- extrusion, and 30% of the Na^+ extrusion, were insensitive to 1 mM ouabain, the net loss of Na^+ taking place at an apparent concentration of 140 mmoles/kg extruded water and the loss of Cl^- at 225 mmoles/kg water.

Fig. 11. Details of slices after incubation for 70 min at 38 °C in the presence of oligomycin. (*a*) Large intracellular vesicles are seen, mainly disposed along the lateral cell surface. They show transverse membranes, and contents which are empty except for membrane debris in the form of myelin figures or whorls with double membranes. Note excellent recovery of the rough endoplasmic reticulum, and the complete absence of dense granules in the mitochondria. ×6,000. (*b*) Greater enlargement of the vesicles showing that their limiting membrane is free of ribosomes (small arrows), in contrast to the rough endoplasmic reticulum. The latter shows good recovery and is here seen to be parallel to the vesicular membrane. Free ribosomes are also seen in one region (large arrows), but they are absent from the homogeneous zone immediately adjacent to the vesicles. At the right, a small vesicle appears to be fusing with a large one. ×60,000. (*c*) As in (*b*); in this case the rough endoplasmic reticulum is orientated at right angles to the membrane of the vesicle. Again, the vesicle membrane appears completely free of ribosome (arrows). Mitochondria do not contain dense granules. ×60,000

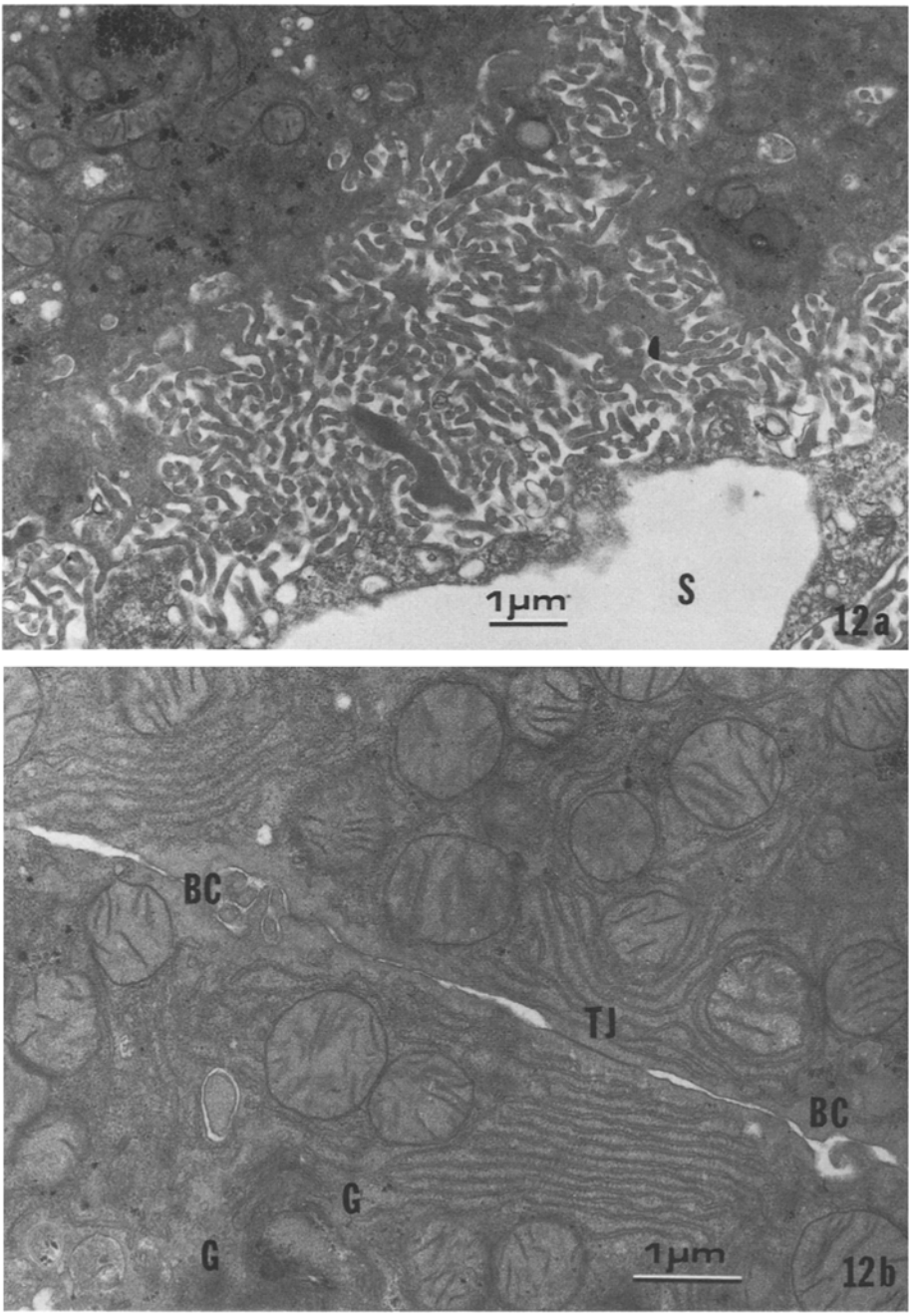

Fig. 12. Slice incubated for 70 min at 38 °C in the presence of oligomycin. (*a*) Detail of sinusoid (*S*) and space of Disse. The latter are densely lined with large microvilli. ×11,000. (*b*) Recovered junctional complexes and rough endoplasmic reticulum can be seen. Two bile canaliculi (*BC*) are shown; they are smaller than in the fresh tissue and their lumina are completely occupied by a few microvilli. Mitochondria are in the orthodox conformation and are one and a half times larger than in fresh tissue; dense granules are completely absent. Tight junction, *TJ*; Golgi apparatus, *G*. ×17,000

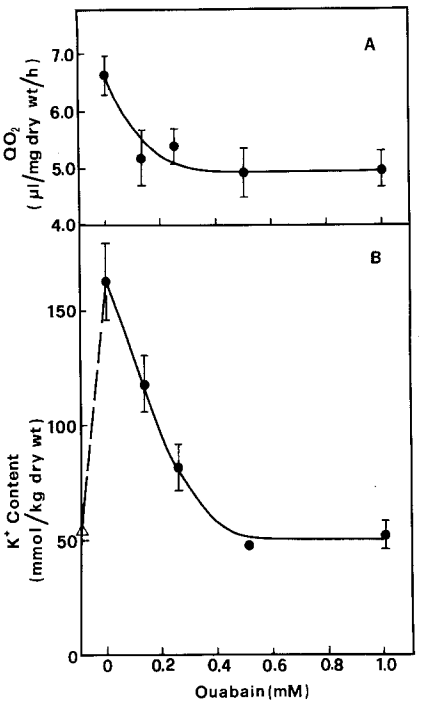

Fig.13. The effect of varying concentrations of ouabain on (*A*) Oxygen uptake and (*B*) K^+ content of liver slices. Experimental conditions were as in Table 1. △ Slices incubated for 90 min at 1 °C; ● Slices incubated for 90 min at 1 °C, followed by 70 min at 38 °C in the presence of the indicated concentrations of ouabain. Each symbol represents the mean ±SEM of 4 observations, except the K^+ content at 0.5 mM ouabain which is from 2 observations only

Although these results suggested that the latter portion of the NaCl and water extrusions proceeded by the ouabain-insensitive system noted by MacKnight *et al.* [17], the persistence of a small net uptake of K^+ (12% of controls) in this particular series of experiments prevents the results of Table 2 from completely excluding the possibility that the fluid loss in the presence of ouabain was due to a K^+-coupled mechanism. Experiments were therefore done with slices pre-incubated at 1 °C in a K^+-free medium, the slices being transferred to fresh medium six times during the course of the 90 min period in order to remove K^+ that was washed out of the slices. The slice K^+ content was thus reduced to 35±3 (8) mmoles/kg dry wt. Leakage of K^+ from liver slices at 1 °C is very slow after the first 60 min (Table 1; *see also* refs. 11 and 30) and it is probable that most of the remaining K^+ is bound to tissue components [12]. The slices were then transferred to a seventh portion

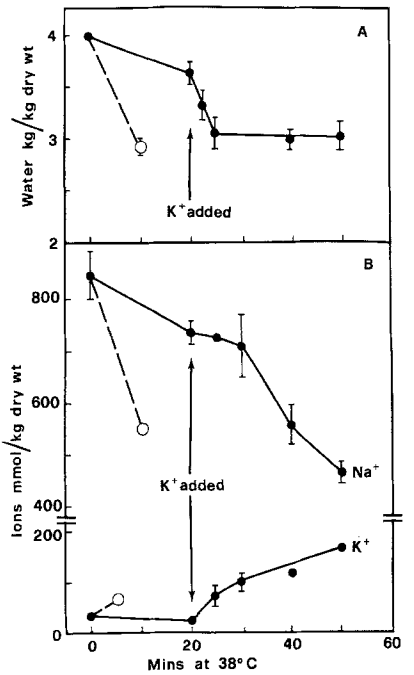

Fig. 14. Time course of (*A*) water content and (*B*) ion contents of liver slices incubated in media with and without 5 mM K^+. The K^+-free medium was identical to the medium described in Table 1, except for the omission of KCl. Slices were incubated in 6 changes of the K^+-free medium for a total of 90 min at 1 °C, before being transferred to a seventh portion and gassed with O_2 for 5 min. Zero-time samples were then taken for analysis and the vessels containing the remaining slices were placed in the bath at 38 °C. To control flasks (○) KCl was added to give a medium concentration of 5 mM K^+ at zero time. Others (●) were incubated at 38 °C for 20 min, at which time samples were taken and KCl then added to give 5 mM K^+ in the medium. Each point represents the mean ± SEM of 8 observations; in some cases the standard errors are smaller than the radii of the symbols

of K^+-free medium, gassed with O_2 and incubated at 38 °C. Fig. 14 shows that during the first 20 min at 38 °C the water content of the slices fell significantly ($p=0.001$, by Student's t test) from 4.00 ± 0.04 to 3.65 ± 0.10 kg/kg dry wt, and the Na^+ content fell from 840 ± 44 to 729 ± 22 mmoles/kg dry wt ($p=0.05$), while the K^+ content did not change. Addition of 5 mM K^+ to the medium after 20 min initiated an uptake to K^+, accompanied by a further extrusion of water and Na^+. Other slices transferred to 5 mM K^+ medium at the onset of incubation at 38 °C showed a loss of water and Na^+ within 10 min which was similar to that noted in Table 1. The inulin distribution was not measured in this series of experiments, but the reduction in total water content during 20 min in K^+-free medium at 38 °C (0.35 kg water/kg dry wt)

and the mean, net loss of Na^+ (111 mmoles/kg dry wt) were similar to the corresponding losses of total tissue content observed with slices incubated in 1 mM ouabain in the experiments of Table 2 (i.e., loss of 0.52 kg water and 101 mmoles Na^+/kg dry wt), particularly when the shorter incubation time in the former case is considered. We conclude that a major part of the volume regulation of the slices occurs by a mechanism that is not coupled to K^+ accumulation, and that at least the largest part of the water and NaCl extrusion observed in the presence of 1 mM ouabain (as in Table 2) probably proceeds by this mechanism.

Electronmicroscopic studies of the slices treated with 1 mM ouabain showed that, despite the partial inhibition of water and ion transport, the ultrastructural recovery was generally similar to that of the controls (Fig. 15); this finding is in contrast to results with flounder nephron [25]. However, the intercellular spaces were somewhat larger than in the controls and were lined with a larger number of microvilli. In addition, the ouabain-treated liver slices showed two characteristic features: (i) The Golgi apparatus was represented by vesicles of varying sizes, clearly arranged along a line running from the perinuclear region to the bile canaliculi (Figs. 15 and 16*a*, *b*), and on occasion the line apparently originated from the spaces of Disse. (ii) In marked contrast to the slices treated with oligomycin, the lumina of the canaliculi were open (compare Figs. 15 and 16*b* with Figs. 10, 11*a* and 12*b*), and had diameters greater than in either the fresh tissue or control slices at 38 °C.

In order to understand the nature and, possibly, function of the characteristic vesicles in these slices treated with ouabain, we examined them at greater magnifications. The vesicles were surrounded by a simple membrane, and in experiments with thorium dioxide were found to contain electron-dense particles of this material (Figs. 16*a*, *b*, and 17*d*). The quantity of thorium particles in the vesicles appeared to be similar to that in the sinusoidal spaces and was clearly greater than in the canaliculi, thus suggesting their origin by endocytosis at the sinusoidal membrane; this result appears to be similar to work with other tracers in perfused liver [22]. In the cytoplasm, smaller vesicles were often seen to be fusing with larger ones (Figs. 16*a* and 17*d*). There was a close spatial association of the vesicles to the canaliculi (Figs. 16*b* and 17*a*, *d*). Furthermore, there is direct evidence that small vesicles open into the canaliculi (Fig. 17*a*, *c*) and indirect evidence that the larger vesicles could do so as well. The latter is suggested by the following points: (i) the close association of large vesicles to the canaliculi (Figs. 16*b* and 17*d*); (ii) the presence in the canaliculi of material of the same

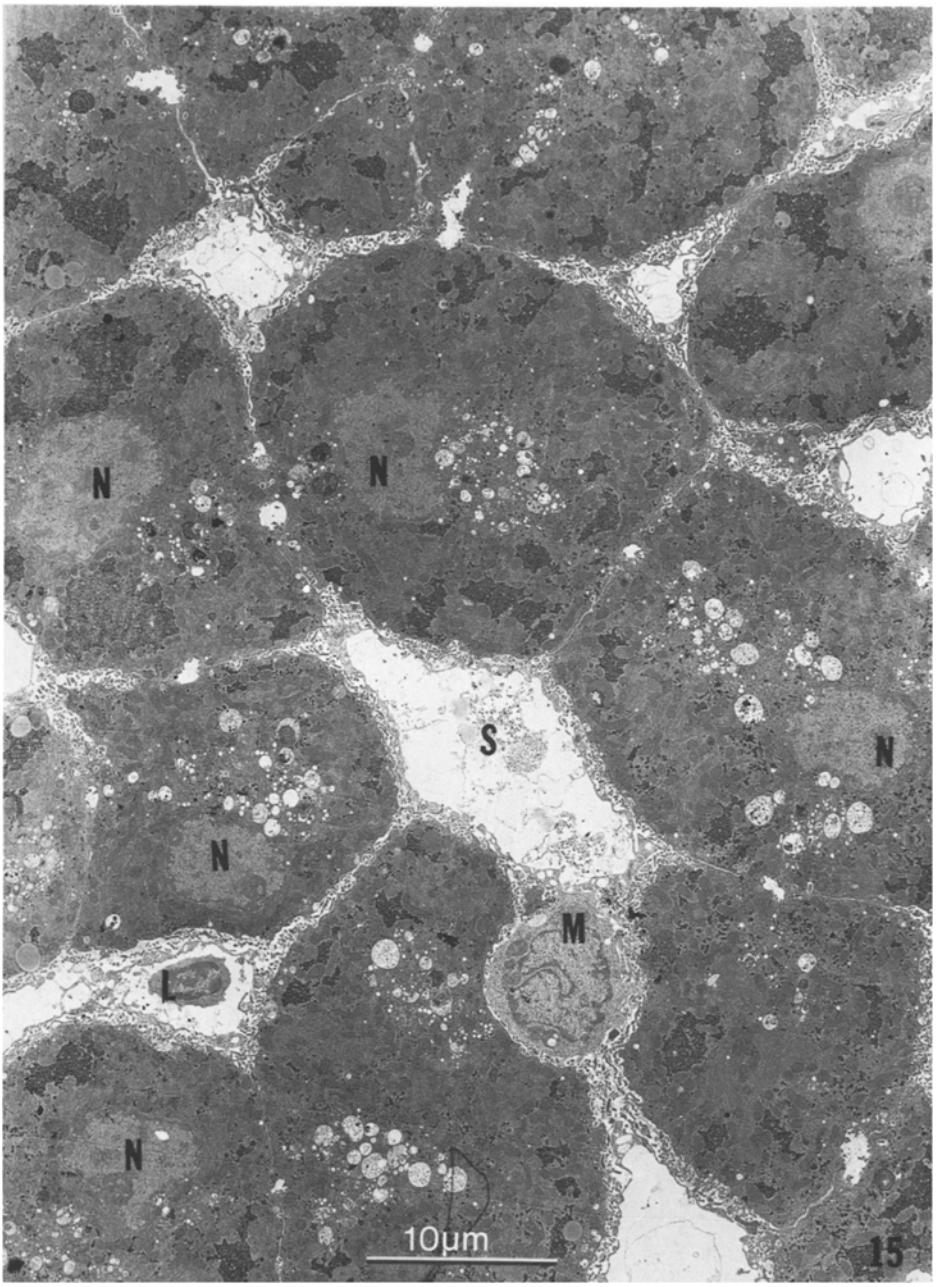

Fig. 15. General view of the effect of ouabain (1 mM) on a slice incubated for 70 min at 38 °C. In general, the recovery is similar to that of the control slices incubated at 38 °C (Fig. 7). However, there is a line of vesicles stretching from the Golgi region to the bile canaliculus. These vesicles contain thorium dioxide at a greater concentration than the vesicles formed in the presence of oligomycin. Bile canaliculi are wide open and clear. The sinusoidal surface is very densely covered with microvilli. Nucleus, *N*; sinusoid, *S*; macrophage, *M*; lymphocyte, *L*. ×3,000

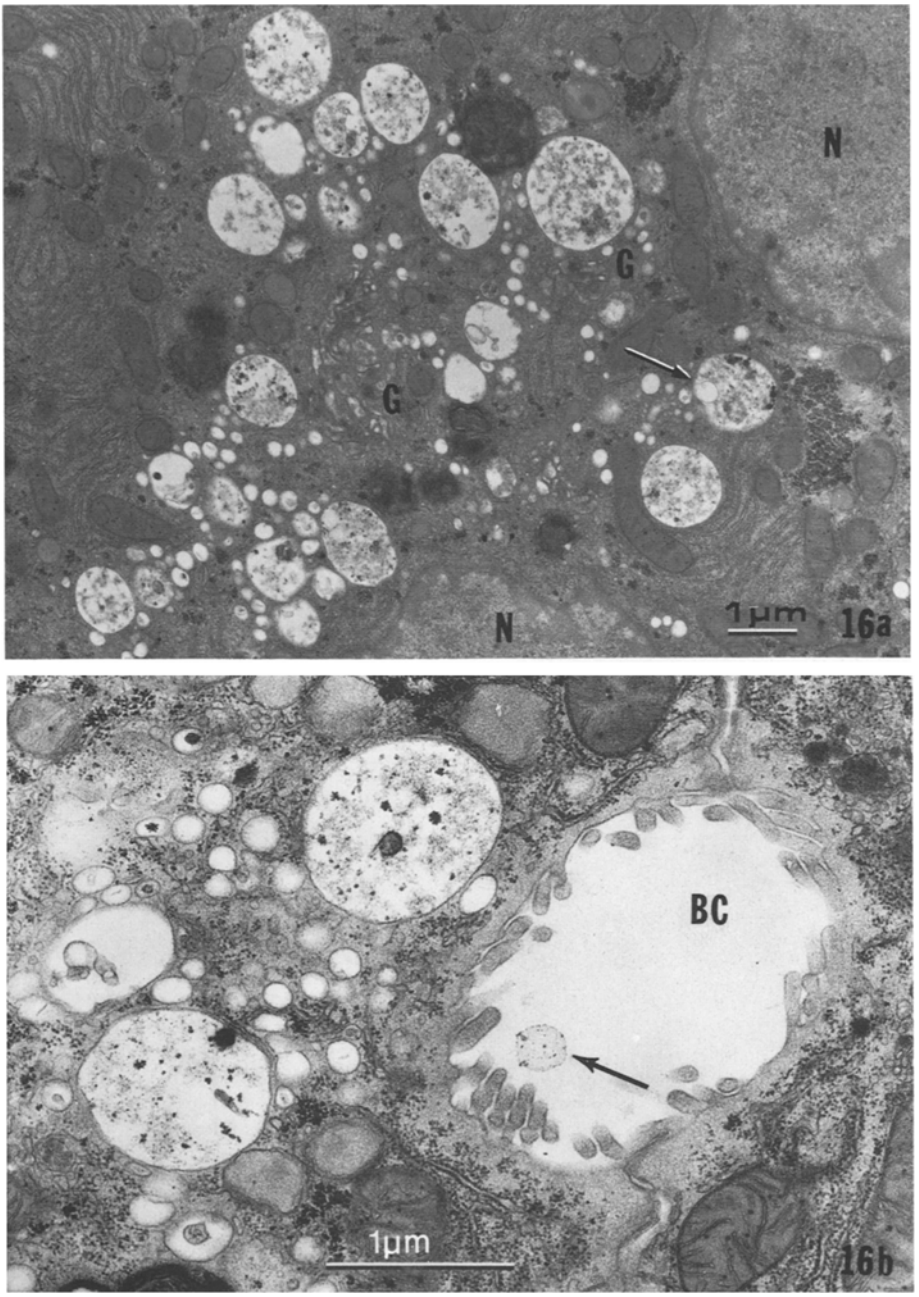

Fig. 16. Slice incubated for 70 min at 38 °C in the presence of ouabain (1 mM). (*a*) Detail of the Golgi region. The typical finding of this experimental condition is that of numerous vesicles containing thorium dioxide. Often, smaller vesicles from the Golgi maturing-face appear to be coalescing with one of the larger vesicles (arrow). The mitochondria are almost identical to those of control, incubated slices; compared to oligomycin-treated slices (Fig. 11), they are rich in dense granules. Golgi apparatus, *G*; nucleus, *N* ; ×11,000. (*b*) Detail of the peri-canalicular region. The close relationship of both small and large vesicles to the widely dilated lumen of the canaliculus can be seen. The lumen contains a rounded formation (arrow) of the same density as the contents of the large vesicles.

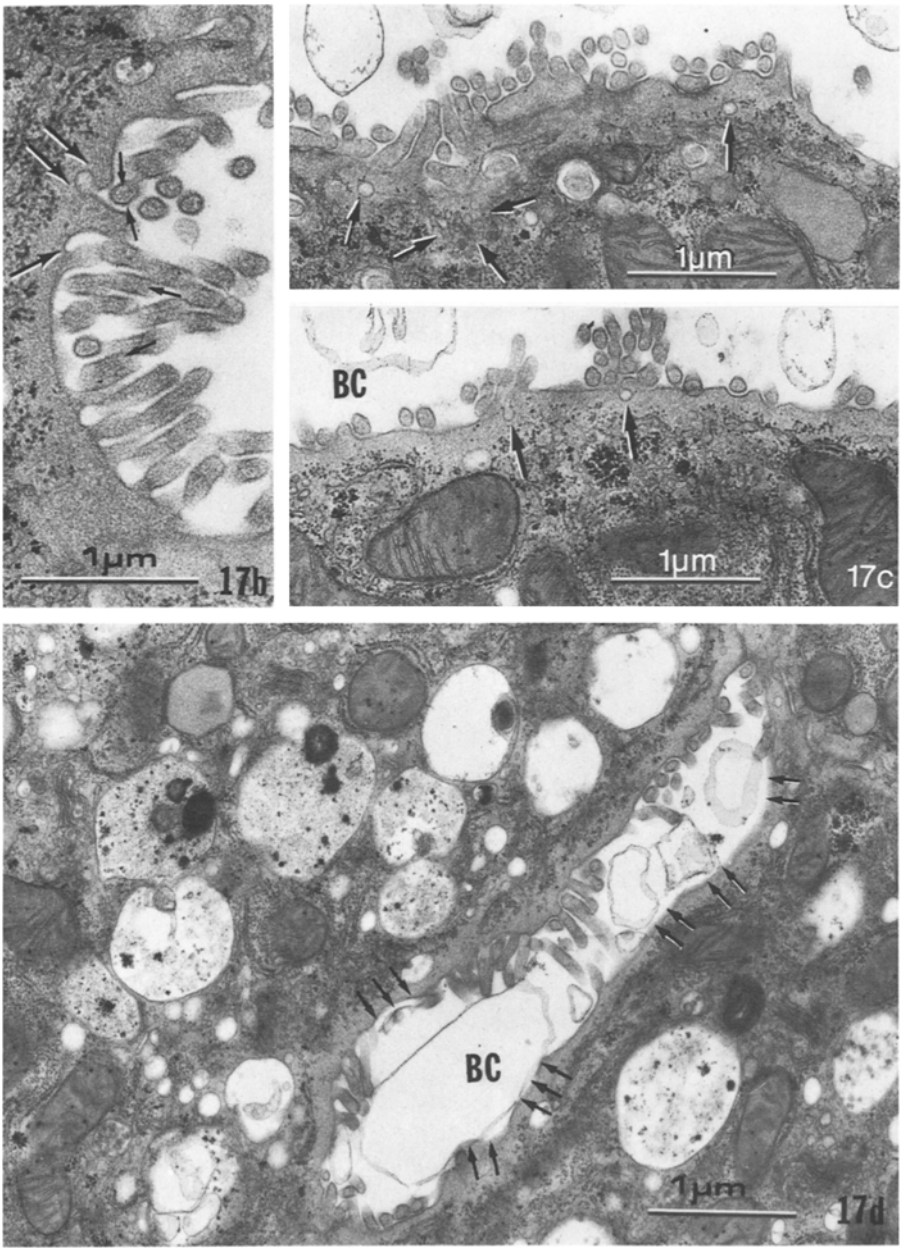

Fig. 17. Slices incubated for 70 min at 38 °C in the presence of ouabain (1 mM); further details of the peri-canalicular regions. (*a*) The presence of small vesicles (arrows) in the immediate vicinity of the microvilli of a canaliculus which has been cut longitudinally. ×25,000. (*b*) Part of transverse section of a canaliculus, in which small vesicles can be seen opening into the lumen (large arrows). Microtubules are evident in those microvilli which have been cut transversely (small arrows) and microfilaments in those sectioned longitudinally. ×30,000. (*c*) This again illustrates the opening of small vesicles (arrows)

electron density as the contents of the large vesicles, including occasional particles of thorium dioxide (Figs. 16*b*, and 17*d*); (iii) this electron-dense material was often situated in regions of the canaliculi that were free of microvilli, and these zones may therefore represent the point of exocytosis of the large vesicles (Fig. 17*c*, *d*).

In view of the different relative effects of ouabain and oligomycin on the transport of water and ions, their combined effects were studied. There was no significant additivity of their effects on respiration or adenine nucleotides, the inhibition being equal to the greater effect caused by either agent alone (Table 3). Similarly, the effect of the combination on the intracellular water and ion movements was similar to that of ouabain alone, and the increase of inulin-containing water induced by oligomycin alone was also seen in the additional presence of ouabain (Table 2). Clearly, there remains an extrusion of cell water, Na^+ and Cl^- which is insensitive to both oligomycin and ouabain, but which, in view of our discussion of the effects of cyanide, is dependent upon respiratory energy.

Discussion

Control of Cell Volume and Ultrastructure

We have shown that the tissue swelling and alterations of ionic composition that occur under conditions of reduced metabolic activity are accompanied by a marked disorganization of tissue and organellar structure, particularly with respect to disruption of intercellular connections,

into the lumen of the canaliculus (which has been sectioned longitudinally). Further, the lumen contains formations of the same density as the contents of the large vesicles; frequently, such formations are adjacent to regions of the canaliculus that are free of microvilli. ×25,000. (*d*) Longitudinal section of a bile canaliculus, summarizing the characteristics of the peri-canalicular region as they pertain in particular to the large cytoplasmic vesicles. That is, the very close association of the large vesicles with the canaliculus, the presence in the canaliculus of formations containing material similar to the vesicular contents, and the occurrence of indentations of the canalicular membrane which are free of microvilli and situated close to these formations (arrows). The indentations of the canalicular membrane may represent the points at which large vesicles have just opened into the canaliculus, releasing their contents. Furthermore, in unstained sections both the formations in the lumen of the canaliculus and the contents of the vesicles in the cytoplasm are seen to contain extremely electron-dense particles which, in this section, are represented by fine bodies varying in diameter from approximately 60 to 130 Å, and which we conclude to be particles of thorium dioxide. ×25,000

surface structures and endoplasmic reticulum. Restoration of the liver slices to metabolically favorable conditions leads to a largely respiration-dependent recovery of cell volume and composition which is accompanied by a remarkable restoration of cellular organization to a condition often nearly indistinguishable from that of the fresh liver. These findings appear to testify to a substantial degree of physiological competence in this slice preparation. The alterations in cell size (e.g., Figs. 1, 4 and 7) support the conclusions from studies of extracellular markers that both the cellular and extracellular compartments gain water during cold-induced swelling [5, 20], but that the recovery of volume at 38 °C is largely confined to the intracellular water. The extracellular water spaces remained somewhat expanded after recovery, probably due to inelasticity of the stroma.

The transition from 1 °C to the incubation at 38 °C brought about many simultaneous changes in the tissue, but several of these can be excluded as causes of the ultrastructural recovery. The increase in temperature *per se* is excluded by the failure of the structures to recover at 38 °C in the presence of cyanide. The cell K^+ content is excluded since recovery of structure (like that of cell water) was well advanced during the lag period which preceded reaccumulation of K^+ [5, 14, 29]. Also, a high level of ATP is not by itself sufficient, since the ATP content was as great, or greater, in the swollen slices at 1 °C as in control or oligomycin-treated slices at 38 °C. We conclude that the recovery of ultrastructure requires active metabolism as well as a moderate level of ATP, and is temporally closely related to the extrusion of water and restoration of cell volume.

The ability of the rat-liver cell to control its volume appears, like that of the kidney-cortex cell [15, 34], to consist of two components, one of which is dependent on the presence of K^+ in the medium and is sensitive to ouabain, while the other is not dependent on these factors. Each component appears to bring about a net extrusion of a roughly isotonic solution of NaCl. Both mechanisms are energy-dependent, as indicated by the total inhibition of volume control by cyanide and Amytal [32]. It is of interest that the volume and structure of reticuloendothelial cells appeared to follow closely the changes of parenchymal cells in the electronmicroscopic studies. The ouabain-sensitive mechanism appears to be a manifestation of the system for the coupled transport of Na^+ and K^+ as envisaged by Leaf [16] and, as such, presumably occurs at all surfaces of the plasma membrane. The molecular mechanism underlying the ouabain-insensitive water extrusion remains to be ex-

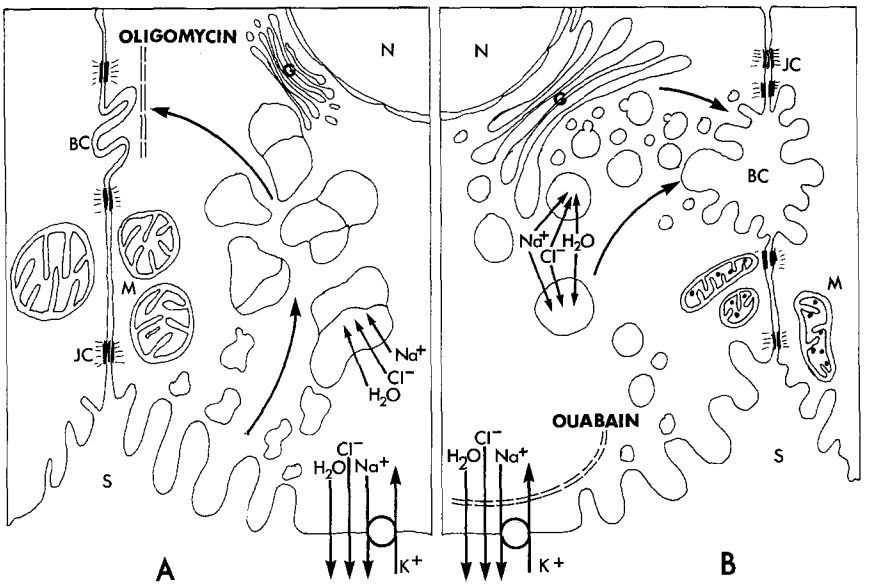

Fig. 18. Postulated schemes for the ouabain-sensitive and -insensitive components of the system for controlling cell volume in liver, and the consequent effects of (*A*) oligomycin and (*B*) ouabain on the intracellular vesicles. Nuclei, *N*; junctional complexes, *JC*; bile canaliculi, *BC*; Golgi apparatus, *G*; sinusoids, *S*; mitochondria, *M*. For further description, *see* text

plored, but the marked differences in size and structure of the intracellular vesicles under various incubation conditions leads us to suggest a role for them in this extrusion. In control slices, where both water-transporting mechanisms are potentially active, only small vesicles in the Golgi region were seen. Inhibition by ouabain of the water extrusion coupled to Na^+ and K^+ transport led to the appearance of many, moderately large vesicles between the nucleus and the canaliculi. The almost universal occurrence of thorium particles in their contents, their occasional appearance near the sinusoids, and the stimulation of microvillar formation by ouabain all suggest an origin of these vesicles by pinocytosis at the sinusoidal surface of the cells, with subsequent passage to the canaliculi (Fig. 18). We suggest that during this passage their volume is increased by secretion into them of Na^+, Cl^- and water by an ouabain-insensitive mechanism, and that they then fuse with the Golgi vesicles prior to extruding their contents by exocytosis into the canalicular lumen. Our electronmicrographs clearly show the fusion of small vesicles with the canalicular membranes, and the presence in the canaliculi of material (including Thorotrast) similar to the contents of the large vesicles. More-

over, there are further indications of the exocytosis of large vesicles into the canaliculi, such as the microvilli-free indentations of the canalicular surface (e.g., Fig. 17*c*, *d*). However, we have not yet found a clear example of the fusion of a large vesicle with this surface, probably because the tension of the fluid in the canaliculi results in a very rapid stretching of the vesicular membrane after its coalescence with the canalicular boundary. The vesicles are more evident in the presence of ouabain because of the greater load placed on this system by inhibition of the water extrusion coupled to K^+ and Na^+ transport. We envisage the latter system as resulting in an extrusion of water through "pores" in the plasma membrane at, or close to, the sites of the "carriers" for Na^+ and K^+ (Fig. 18).

The larger, multiple vesicles seen in the presence of oligomycin appear to have a similar origin to those seen with ouabain. Their increased size and the greater dilution of the thorium particles they contain suggest that fluid is secreted into them from the cytosol, but that they are unable to release their contents into the canaliculi, possibly as a result of the reduced ATP levels caused by oligomycin. The inactive appearance of the Golgi apparatus and the closure of the canalicular lumina in the presence of oligomycin are in accord with this suggestion. Thus, the ouabain-insensitive water extrusion proceding by way of the vesicles is blocked, inulin remains within the confines of the cell (in the vesicles), but the extrusion of inulin-free water (from the cytosol) continues at control levels because of a compensating increase in the activity of the water transport coupled to K^+ and Na^+ transport. However, the vesicular transport system here proposed cannot be the only ouabain-insensitive system in the liver cells, since there remains a substantial excretion of water even when ouabain and oligomycin are present simultaneously. Further studies to clarify the proposed ouabain-insensitive vesicular mechanisms of volume control are in progress, including time-course studies in the presence of ouabain and the use of more satisfactory electron-dense markers (e.g., Ferritin and horseradish peroxidase).

Mitochondrial Conformation and Metabolic State

The various incubation conditions used in these experiments placed the cells in different metabolic states, as a result of alterations in the activity of energy-conserving and energy-utilizing pathways, and the resulting changes in mitochondrial conformation may be compared to those

observed in isolated mitochondria under apparently analogous metabolic states [9, 10]. In samples of fresh liver, the mitochondria were in the "orthodox" configuration, and upon incubation at 1 °C they underwent changes of two types: some came to show the condensed pattern which is also seen in cold-stored, isolated mitochondria [9], while others became swollen. Both types of change were often seen in the same cell (*cf.* ref. 8) suggesting that they represent two stages of a single process.

The initiation of state 4 respiration (as defined in ref. 3) in isolated mitochondria results in their restoration to the orthodox conformation, this change being prevented by inhibitors of respiration and oxidative phosphorylation [10]. A very similar transformation (also prevented by cyanide) occurred upon re-initiation of respiration and metabolic activity in control liver slices at 38 °C. However, the respiration of these slices is unlikely to be truly in state 4, since the cells carry out a number of energy-requiring processes (including the transport of Na^+ and K^+) which ensure the regeneration of some ADP. Some evidence of the condensed configuration associated with state 3 respiration [9, 10] was therefore anticipated but was not found. The explanation of this apparent contradiction may be that the orthodox-to-condensed transition only takes place in an advanced form of state 3 which can only be attained in isolated mitochondria. State 4 should be more closely approached [27, 28, 33] in the presence of ouabain (*cf.* the increased ATP, Table 3), and the mitochondria then also undergo the transition to the orthodox configuration. Another difference between the isolated mitochondria and those *in situ* was that the condensed-to-orthodox transition accompanying restoration of respiration to the slices was not prevented by oligomycin. However, this difference is probably explicable by the substantial activity of respiration and ATP synthesis (apparently from substrate-level phosphorylation) which persists in the slices [31]. Another marked finding in the slice mitochondria was the direct relation between the number of dense granules observed and the tissue content of ATP, especially when comparing oligomycin-treated, control and ouabain-treated slices. However, the significance of these bodies is not clear at present.

In conclusion, while the mitochondrial configurations observed in isolated mitochondria can also be observed in slices of liver and other tissues [25], transformations between them are less clearly attributable to metabolic states of the cell. The reason is probably that the conditions of integrated metabolism within the functioning, intact cells prevent more than an approximation to the clear-cut metabolic states (especially states 3 and 4) obtainable in isolated mitochondria.

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