J. Membrane Biol. 77, 63–76 (1984)

The Journal of
Membrane Biology
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Requirement of Cl^- and Na^+ for the Ouabain-Resistant Control of Cell Volume in Slices of Rat Liver

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Summary. The ability of liver cells to control their volume in the presence of ouabain has been studied in tissue slices that were recovering at 38°C from a period of swelling at 1°C. Morphological observations were made in conjunction with measurements of the net movements of water and ions. Extrusion of water in the presence of ouabain (2 mM) was accompanied by a net loss of Na^+ and Cl^- and by the formation of characteristic, rounded vesicles in the peri-canalicular regions of the hepatocytes; bile canaliculi were patent. When incubation was carried out in a medium in which either NO_3^- or SO_4^{2-} replaced Cl^-, ouabain-resistant water extrusion was prevented and the cytoplasmic vesicles normally found with ouabain were almost totally absent. When these slices were subsequently transferred to Cl^- medium with oubain, extrusion of intracellular water was initiated and cytoplasmic vesicles reappeared. Replacement of medium Na^+ by Li^+ mimicked the effects of ouabain on water and ion movements and ultrastructure. In addition, the ouabain-resistant extrusion of water and Cl^- was reduced and there was some diminution in the number of vesicles induced by ouabain. Furosemide (2 mM) had little effect on water movement or ultrastructure in the absence of ouabain, but it slowed the net water loss and substantially reduced the formation of cytoplasmic vesicles in the presence of ouabain. The results show a close relationship between ouabain-resistant water extrusion and the formation of the cytoplasmic vesicles that are characteristic of treatment with ouabain. They further suggest that a cotransport of Na^+ and Cl^- forms an important part of the mechanism underlying ouabain-resistant water extrusion and, specifically, that this cotransport may take place across the membranes of the cytoplasmic vesicles.

Key Words cell volume · cell water · volume regulation; ouabain-resistant · chloride movement, liver cells · sodium movement, liver cells · furosemide, effects on volume regulation · vesicles, in liver cytoplasm

Introduction

The osmotic activity of intracellular macromolecules induces a continual entry of water into cells incubated *in vitro* in an isosmotic, saline medium so that swelling will occur unless metabolic processes intervene to regulate cellular volume (Opie, 1949; Robinson, 1950; Macknight & Leaf, 1977). In liver cells, the volume regulation may be studied by following the net extrusion of water from liver slices during recovery from a period of swelling. The extrusion is partly associated with the ouabain-sensitive transport of Na^+ and K^+, as originally suggested by Leaf (1956), and partly with an ouabain-resistant mechanism (Macknight, Pilgrim & Robinson, 1974; Russo, Galeotti & van Rossum, 1976; Russo, van Rossum & Galeotti, 1977), and it is the latter with which our work has been concerned.

The water extruded in the presence of ouabain is accompanied by Na^+ and Cl^- and is associated with the formation of many rounded, membrane-limited vesicles which are characteristically distributed between the nucleus and bile canaliculi (Russo et al., 1977; van Rossum & Russo, 1981). Combined studies of water extrusion and cellular morphology under a number of incubation conditions, and the responses to a variety of inhibitors (e.g. cyanide, oligomycin, cytochalasin B, Ni^{2+}), led us to postulate that the ouabain-resistant mechanism involved the accumulation of water in cytoplasmic vesicles followed by its expulsion by exocytosis into the bile canaliculi (van Rossum & Russo, 1981). Garfield and Daniel (1974) have also postulated a role for vesicles in the control of cell volume as a result of experiments on myometrial cells.

Our previous work gave no direct indication of the mechanism underlying the postulated passage of water into cytoplasmic vesicles, although we suggested that it would probably follow a movement of Na^+ or Cl^-. In the work described below, we show that Cl^- is required both for the formation of vesicles and for extrusion of water from liver cells in the presence of ouabain and that a cotransport of Na^+ and Cl^- may be involved. Some of these results

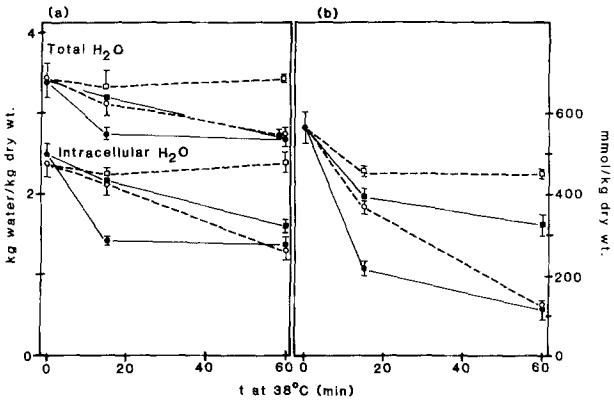


Fig. 1. Effects of ouabain and chloride-free, NO_3^- medium on the time-course of changes of (*a*) water and (*b*) intracellular Na^+ contents of liver slices during recovery at 38°C from a 90-min period of swelling at 1°C. The values for "0 min at 38°C" are those of slices taken for analysis after 90 min at 1°C. Intracellular water calculated as the difference between the total tissue water and the water estimated to contain inulin at the same concentration as the medium. Intracellular Na^+ was calculated by assuming that the inulin-containing water had the same Na^+ concentration as the medium. Points in the graph represent the mean ± standard error of the mean of 4 to 6 observations. Other experimental details as in Materials and Methods. —●— Cl^- medium; --O--NO_3^- medium; —■— Cl^- medium with 2 mM ouabain; --□--NO_3^- medium with 2 mM ouabain

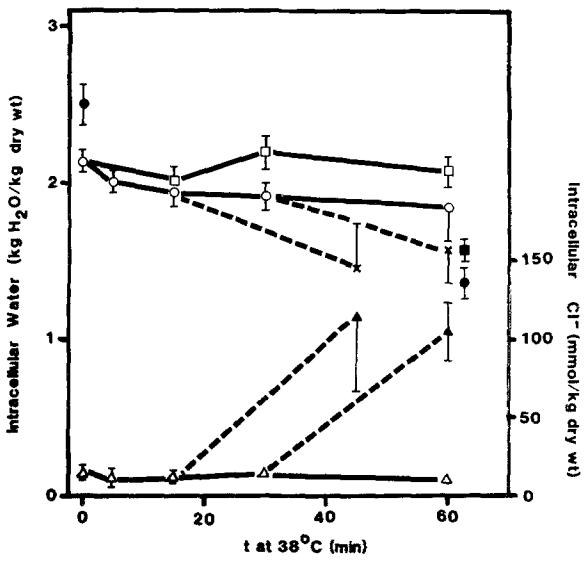


Fig. 2. Changes of intracellular water and Cl^- contents during incubation at 38°C in SO_4^{2-} medium with and without ouabain, and the effect of restoring Cl^- in the absence of ouabain. Slices were incubated for 90 min at 1°C (0 min at 38°C) followed by incubation at 38°C for the times indicated. *Intracellular water:* ○ incubated in SO_4^{2-} medium throughout (n = 10, except at 60 min when n = 4); × incubated in SO_4^{2-} medium and transferred to Cl^- medium for final 30 min (n = 6); □ incubated in SO_4^{2-} medium with 2 mM ouabain throughout (n = 8, except at 30 min when n = 3); ● incubated throughout in Cl^- medium; ■ incubated throughout in Cl^- Ringer's containing 2 mM ouabain. Both sets of samples in Cl^- Ringer's throughout are the same as those illustrated in Fig. 1; the points at 0 and 60 min only are illustrated to permit a comparison with the results in SO_4^{2-} medium. *Intracellular Cl^-:* △ incubated in SO_4^{2-} medium throughout; ▲ Incubated in SO_4^{2-} medium and transferred to Cl^- medium for final 30 min. For other experimental details, *see* Fig. 1 and Materials and Methods

have been published in a preliminary form (van Rossum, Russo & Ernst, 1983).

Materials and Methods

The experimental techniques and analytical methods broadly followed those described previously (Russo et al., 1976, 1977; van Rossum & Russo, 1981). Briefly, slices (0.2 to 0.3 mm thick) of liver from adult rats (200 to 400 g) were cut free-hand with a razor blade and allowed to swell during pre-incubation at 1°C for 90 min. They were then induced to extrude water, and to bring about net transport of ions, by subsequent incubation at 38°C in oxygenated medium. At intervals, samples of the slices were collected for analysis and for morphological examination. The control medium contained (in mM): 143 Na^+, 5 K^+, 1.0 Mg^{2+}, 1.2 Ca^{2+}, 157 Cl^-, 1.0 SO_4^{2-}, 2.0 phosphate, 10 Tris (hydroxymethyl) amino methane and 0.5% (wt/vol) inulin; the pH was 7.4 and the medium was gassed with O_2. The chloride-free media contained either NO_3^- or SO_4^{2-} as replacement for Cl^- (referred to as "NO_3^- medium" and "SO_4^{2-} medium," respectively); in the latter case, SO_4^{2-} was added to replace the anionic equivalents of Cl^- and the molar difference was made up with mannitol. The sodium-free medium contained Li^+ instead of Na^+ ("Li^+ medium").

Slices were divided between control and experimental media from the onset of pre-incubation at 1°C and were transferred to fresh portions of the same medium three times during the first 30 min at 1°C; this greatly lowered the endogenous Na^+ and Cl^- contents of slices incubated in the sodium-free and chloride-free solutions, as will be seen in Results. After 30 min, the slices were distributed in lots of approximately 100 mg wet wt into Erlenmeyer flasks (25 ml capacity) containing 3.0 ml of the appropriate medium. Where required, the inhibitors ouabain and furosemide were added to the medium at this stage so that they were allowed 60 min at 1°C to penetrate the tissue. Duplicate or triplicate flasks were prepared for each treatment, in each experiment. The flasks were gassed with O_2 and stoppered prior to transfer to a shaking water bath for incubation at 38°C. Slices were collected for study at times indicated in Results. For morphological study, one slice was carefully removed from each flask with a pointed wooden stick and put straight into 2% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer with $CaCl_2$ for fixation at 4°C; further treatment was as described by Russo et al., (1976). The remaining tissue was collected by filtration and blotted (Russo et al., 1976); half of the slices from each flask were used for assay of dry wt, water and ions while the others were assayed for inulin. When O_2 consumption was to be measured, the incubation at 38°C was carried out in the Warburg manometric apparatus. Readings of O_2 consumption were taken at 10-min intervals after a preliminary equilibration period of 10 min. Standard manometric techniques were used (Umbreit, Burris & Stauffer, 1949).

Values in the text and Tables are expressed as mean ± standard error of the mean (number of observations). Statistical significance of differences was examined by "Student's" t-test.

Table 1. Effects of ouabain on the intracellular water and ions of liver slices incubated in anion-substituted media[a]

Incubation	Principal anion	Ouabain (mM)	Water (kg/kg dry wt)	Cl^-	Na^+	K^+	(*n*)
				(mmol/kg dry wt)			
(*a*)							
90 min at 1°C	Cl^-	0	2.60 ± 0.09	319 ± 29	387 ± 22	94 ± 2	(10)
	NO_3^-	0	2.53 ± 0.09	19 ± 5	430 ± 23	88 ± 3	(10)
90 min at 1°C followed by	Cl^-	0	−1.08 ± 0.11	−136 ± 24	−230 ± 26	+98 ± 7	(15)
60 min at 38°C	NO_3^-	0	−0.84 ± 0.10	+21 ± 10	−220 ± 24	+117 ± 13	(15)
	Cl^-	2	−0.57 ± 0.11	−29 ± 24	−64 ± 18	−28 ± 3	(15)
	NO_3^-	2	−0.09 ± 0.13[b]	−9 ± 8	−52 ± 23	−4 ± 3	(14)
(*b*)							
90 min at 1°C	Cl^-	0	2.58 ± 0.08	352 ± 26	370 ± 32	83 ± 5	(12)
	SO_4^{2-}	0	2.26 ± 0.10[b]	37 ± 9	314 ± 20	71 ± 4	(12)
90 min at 1°C followed by	Cl^-	0	−0.84 ± 0.10	−121 ± 19	−176 ± 28	+69 ± 8	(18)
60 min at 38°C	SO_4^{2-}	0	−0.35 ± 0.10[b]	−18 ± 7	−127 ± 18	+72 ± 5	(18)
	Cl^-	2	−0.47 ± 0.11	−90 ± 31	−73 ± 21	−22 ± 5	(18)
	SO_4^{2-}	2	−0.11 ± 0.08[b,c]	+9 ± 14	−36 ± 17	+4 ± 4	(18)

[a] Incubation techniques are as described in Materials and Methods. The values given at 1°C represent the intracellular contents determined after 90 min in the media indicated. Values for incubation at 38°C represent the net increase (positive values) or decrease (negative values) occurring from the contents shown at 1°C. Intracellular water values were calculated from the total tissue water content and the fraction of the water that was penetrated by inulin. Intracellular ions were calculated similarly, assuming that the inulin-containing water contained ions at the concentration found in the medium.

[b] Significantly different from value on line above, $P < 0.01$.

[c] Significantly different from value in SO_4^{2-} without ouabain, $P < 0.05$.

Results

Substitution of Cl^- in Medium

The total and intracellular water contents of liver slices which had swollen during pre-incubation at 1°C underwent a net decrease upon subsequent incubation at 38°C in the control, Cl^- Ringer's solution. The loss of water was already maximal after 15 min (Fig. 1*a*) and was accompanied by a net extrusion of intracellular Na^+ (Fig. 1*b*). When the incubation was conducted in a chloride-free medium in which NO_3^- replaced Cl^- (NO_3^- medium), the net extrusion of water and Na^+ was slower, but reached the same final level as the controls after 60 min at 38°C (Fig. 1). Water loss in the Cl^- medium containing ouabain was also slower initially than in controls but, again, the same total water loss was attained by 60 min; however, the total Na^+ extrusion was reduced by about 50% in the presence of ouabain. By contrast, there was no significant loss of water from slices incubated in the NO_3^- medium in the presence of ouabain and the Na^+ extrusion was much reduced (Fig. 1). Similar results were obtained in the SO_4^{2-} medium where, however, the loss of water in the absence of ouabain was even slower than in NO_3^- medium (Fig. 2). No loss of water occurred at any incubation time when ouabain was present in the SO_4^{2-} medium.

An incubation time of 60 min at 38°C was chosen for a comparison of the physiological and morphological effects of omitting Cl^- from the incubation medium; both NO_3^- (Table 1*a*) and SO_4^{2-} (Table 1*b*) media were used. Replacement of Cl^- by NO_3^- had no significant effect on the composition of the slices at the end of the swelling phase of the experiments, except greatly to reduce the Cl^- content of the tissue. The net alterations of water and Na^+ content upon subsequent incubation at 38°C were similar to those seen after 60 min in Fig. 1 except that, in this larger series of experiments, ouabain caused a partial (45%) inhibition of water loss in the Cl^- medium; i.e. a loss of 0.57 kg water/kg dry wt in the presence of ouabain compared to 1.08 in its absence. Such quantitative variations in the effect of ouabain on water extrusion as those seen here between Fig. 1 and Table 1 are not unusual (*see* Discussion); for the present work, directed principally to study of the ouabain-resistant volume control, the important point is that a large, statistically significant extrusion of intracellular water still occurred in the presence of ouabain in the control, Cl^- medium whereas ouabain totally abolished the extrusion in the NO_3^- medium (Table 1*a*). The uptake of K^+ was unimpaired in the NO_3^- medium without ouabain but was totally inhibited when ouabain was present.

Seen under the light microscope, cells which

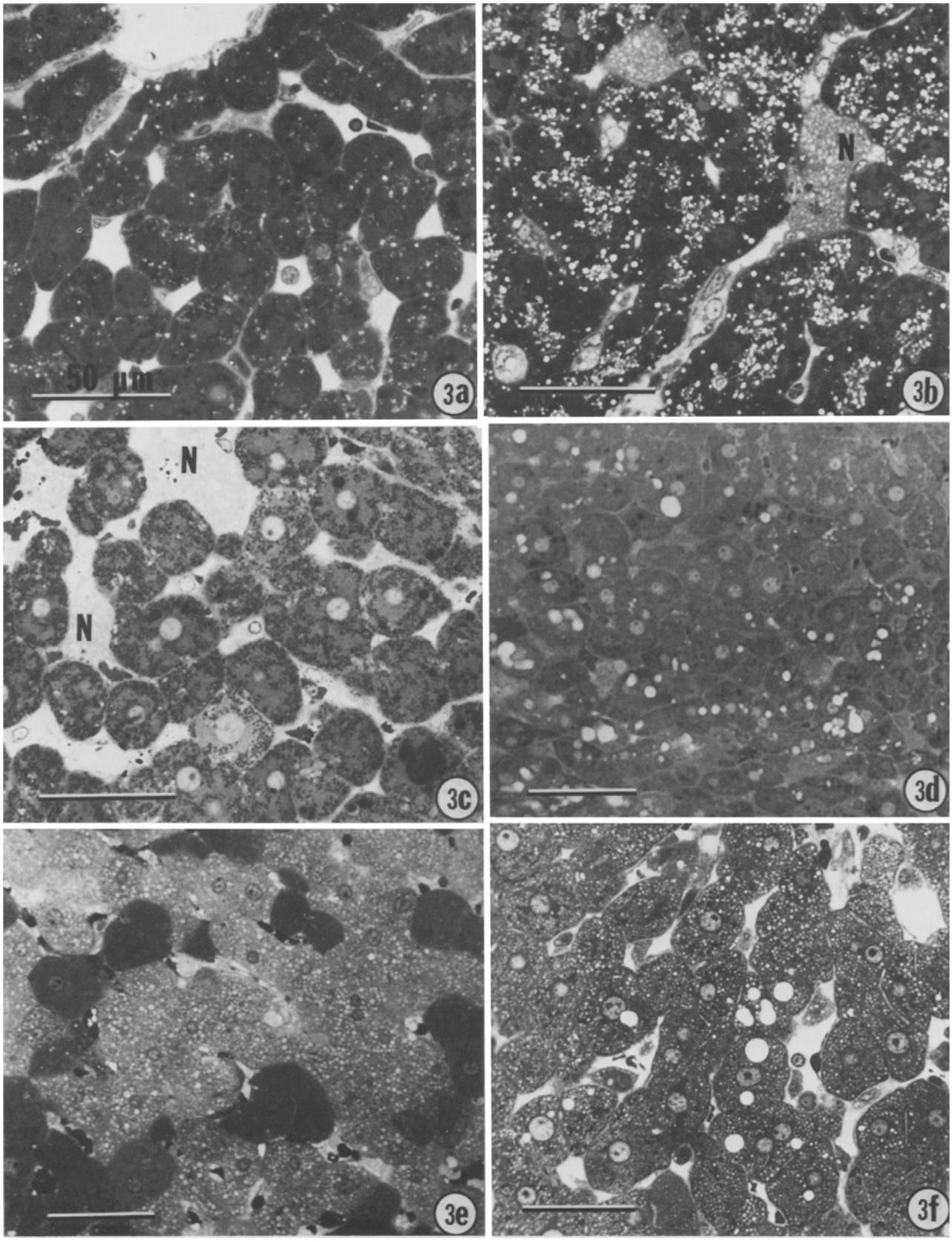
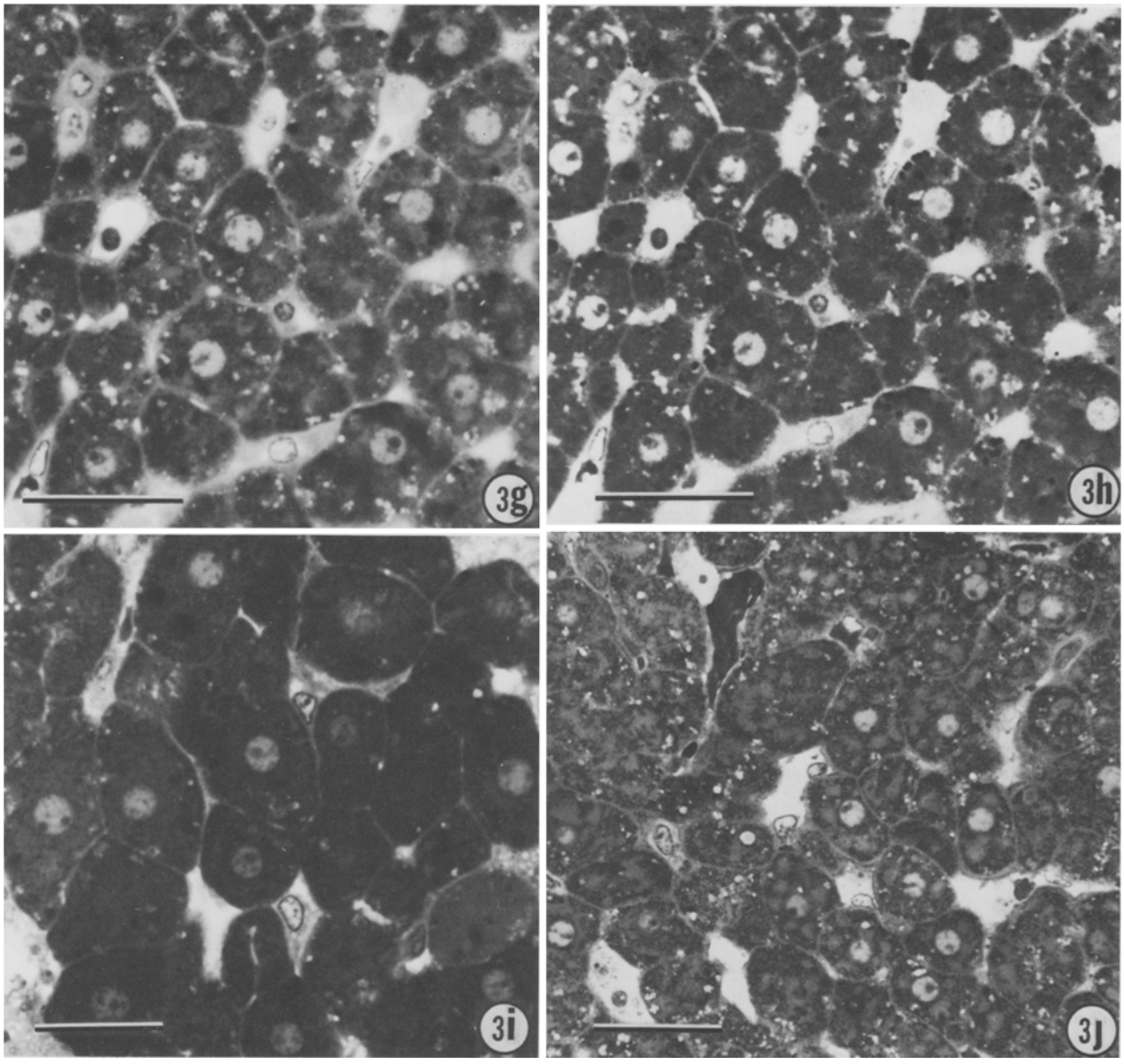


Fig. 3. Optical micrographs of liver slices subjected to pre-incubation for 90 min at 1°C followed by incubation for 60 min at 38°C (unless otherwise stated) in various conditions. This Figure demonstrates in particular the varying numbers and size of cytoplasmic vesicles postulated to be related to water extrusion, as well as the general appearance of cellular and extracellular swelling and recovery. *N* necrotic cell. The bar in each plate represents 50 μm. Magnification 400× in each case. *a*) Control slice. A few vesicles are to be seen in the immediate vicinity of open bile canaliculi (arrows). Note rather wide extracellular spaces and clear delimitation of



cellular boundaries. *b*) Incubation with ouabain (2 mM). Compared to plate *a*, there is a great increase in the number, and also size, of cytoplasmic vesicles. Most vesicles surround bile canaliculi, which are wide open. Extracellular spaces are similar to plate *a*. *c*) Incubation in NO_3^- medium containing ouabain (2 mM). Compared to both plates *a* and *b*, cytoplasmic vesicles are almost completely absent; most canaliculi are closed and the rest are smaller in size. There is a general appearance of some swelling as shown by the clear cytoplasm; mitochondria tend to be in the condensed form. Extracellular spaces are similar to *a* and *b*. Scattered necrotic cells are present. A very similar picture was presented by slices incubated for only 15 min at 38°C under these conditions so that this plate may act as a control for plate *d*. *d*) Slice incubated in NO_3^- medium with ouabain for 15 min at 38°C followed by transfer to Cl^- medium with ouabain for a further 30 min at 38°C. Compare to plate *c*. Small and large vesicles are present in many cells, although not in all. Canaliculi between cells with vesicles are now wide open. There is a partial recovery from the cell swelling noted in *c*; all mitochondria are in the orthodox form. *e*) Incubated in SO_4^{2-} medium containing ouabain (2 mM). No cytoplasmic vesicles are to be seen. The general picture is of a more marked cell swelling than in plate *c* with high amplitude swelling of the mitochondria. Extracellular spaces are reduced compared to plates *a* and *b*. A similar picture is seen after 15 min at 38°C under these conditions so that this plate may act as a control for plate *f*. *f*) Slice incubated in SO_4^{2-} medium with ouabain for 15 min at 38°C followed by transfer to Cl^- medium with ouabain for a further 30 min at 38°C. Compare to plate *e*. Several cells now contain vesicles. Cell swelling is much less than in plate *e* and many mitochondria have reverted to the condensed form. Extracellular spaces are larger. *g*) Incubation in Li^+ medium. The cells contain a number of vesicles quite similar to those seen in the presence of Na^+ and ouabain (plate *b*). Other features also similar to plate *b*. *h*) Incubation in Li^+ medium containing ouabain (2 mM). Canaliculi are wide open. Vesicles are similar to those in plate *g*. *i*) Incubation with furosemide (2 mM). These conditions are characterized by some swelling of the cells and an absence of vesicles, when compared to plate *a*. The canaliculi tend to be closed. The mitochondria are mainly in the condensed form. Extracellular spaces are slightly swollen compared to plate *a*. *j*) Incubation with furosemide (2 mM) and ouabain (2 mM). Some vesicles are present but many fewer than with ouabain alone (plate *b*). The canaliculi are sometimes closed and when open they tend to be smaller than in plate *b*. Mitochondria are mainly in the condensed form

had been incubated for 60 min at 38°C in the control, Cl^- medium, showed a good general structure and contained a few, rather small vesicles in the cytoplasm (Fig. 3*a*). The number and average size of cytoplasmic vesicles was greatly increased in the presence of ouabain (Fig. 3*b*), a result noted earlier (Russo et al., 1977). Details of the vesicles as seen in the electron microscope are illustrated in Fig. 5*a*. In the absence of ouabain, slices in the NO_3^- medium were generally similar in appearance to those in Cl^- medium; mitochondria were condensed in the former case, but the normal uptake of K^+ noted above suggests that energy metabolism was not markedly affected. By contrast, marked differences of appearance were noted between slices in Cl^- and NO_3^- media when ouabain was present (Fig. 3*c*, 5*b*). In this case the cytoplasmic ground substance was much less electron dense, a finding apparently consistent with the retention of intracellular water seen in Table 1*a*. Most marked, however, was the virtually total absence of large cytoplasmic vesicles and the much less widely open canalicular lumina (compare Fig. 3*b* with 3*c* and Fig. 5*a* with 5*b*). A semi-quantitative indication of the numbers of the characteristic vesicles observed is given in Table 2.

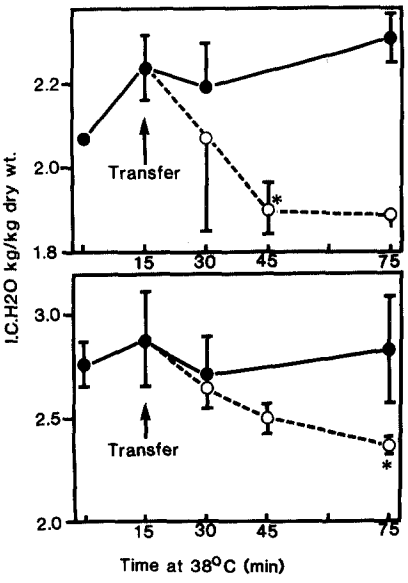


Fig. 4. Effect on intracellular water content of restoring Cl^- to slices incubated with ouabain in SO_4^{2-} medium (upper panel) or NO_3^- medium (lower panel). Slices were incubated for 90 min at 1°C plus 15 min at 38°C in the appropriate chloride-free medium containing 2 mM ouabain. At 15 min, slices were transferred to other flasks containing fresh amounts of pre-oxygenated, chloride-containing (○) or chloride-free (●) medium with ouabain. Incubation was then continued for the times indicated. Other experimental details as in Fig. 1 and Materials and Methods. *Significantly different from the value in chloride-free medium at the time of transfer (i.e. 15 min), $P < 0.05$

Use of SO_4^{2-} to replace Cl^- resulted in reduced swelling at 1°C, as indicated by significantly smaller cellular water contents after pre-incubation (Table 1*b*). This presumably arose from a poor penetration of the divalent anion and/or mannitol through the cellular membranes. The net extrusion of intracellular water which took place upon warming to 38°C in the SO_4^{2-} medium was rather smaller than in Cl^- medium but the accumulation of K^+ was unimpaired in SO_4^{2-} medium. Thus, from a functional aspect the slices appeared to be in a reasonably satisfactory state in the SO_4^{2-} medium. It is therefore significant that an ouabain-resistant extrusion of intracellular water was totally absent from the slices in the SO_4^{2-} medium (Table 1*b*).

Despite their somewhat lower water content, slices preincubated at 1°C in SO_4^{2-} medium had a very similar structural appearance to those in Cl^- medium (*not shown*). After subsequent incubation at 38°C for 60 min, they retained a general appearance of some swelling in the optical microscope, which presumably reflected the somewhat greater retention of cell water seen in Table 1*b*. This was also shown by the homogeneously light cytoplasmic ground substance throughout the entire slice in the electron microscope (Fig. 5*d*). The mitochondria also showed swelling, with light matrices and no dense granules, but they were still well organized with intact cristae. These morphological changes

Table 2. Semi-quantitative estimates of the number of cytoplasmic vesicles in slices of liver[a]

Incubation conditions	Ouabain present (mM)	
	0	2
1. Control medium (Na^+, Cl^-)	+	++++
2. NO_3^- medium	−	−
3. NO_3^- medium for 15 min followed by control (Cl^-) medium for 30 min	*N*	++
4. SO_4^{2-} medium	−	−
5. SO_4^{2-} medium for 15 min followed by control (Cl^-) medium for 30 min	*N*	+
6. Control medium, 2 mM furosemide	−	±
7. Li^+ medium	+++	+++

[a] Incubation was for 90 min at 1°C followed by 60 min at 38°C, unless otherwise indicated. The numbers of vesicles per cell were counted on electron micrographs at a magnification of approx. 3,000×. For each estimate, 20 entire cell images were counted on each of 10 electron micrographs. The symbols indicate approximately the number of vesicles in proportion to those seen in slices incubated with ouabain in the control medium, these last being represented as ++++. Other symbols are: − no vesicles seen: ± most cells contained no vesicles, but some contained 1 or 2 per cell: *N* not examined.

were of a type that suggested that the cellular swelling persisting at 38°C should be reversible and its reversibility was directly demonstrated in later experiments (*see below*).

The presence of 2 mM ouabain in the SO_4^{2-} medium induced little change in the general appearance of the slices from that described above without ouabain (Fig. 3*e*, 5*e*). It is particularly noteworthy that very few of the vesicles normally associated in great numbers with the presence of ouabain were to be seen (Fig. 5*e*, Table 2). There was some degree of cellular disorganization in a maximum of 20% of the cells in the SO_4^{2-} medium with ouabain; but this quantity of damaged tissue is too small to account for the total inhibition of ouabain-resistant water extrusion that was observed.

Restoration of Cl^- to the Medium

The above experiments showed that incubation in the NO_3^- or SO_4^{2-} media prevented both the ouabain-resistant extrusion of water and the formation of the vesicles characteristically associated with the presence of ouabain in the Cl^- medium, providing evidence both that the vesicles are an important aspect of the water extruding mechanism and that Cl^- is important for their formation. In order to confirm these associations, slices were incubated for 90 min at 1°C *plus* 15 or 30 min at 38°C in chloride-free medium before being transferred to Cl^- medium for a further 30 min incubation at 38°C.

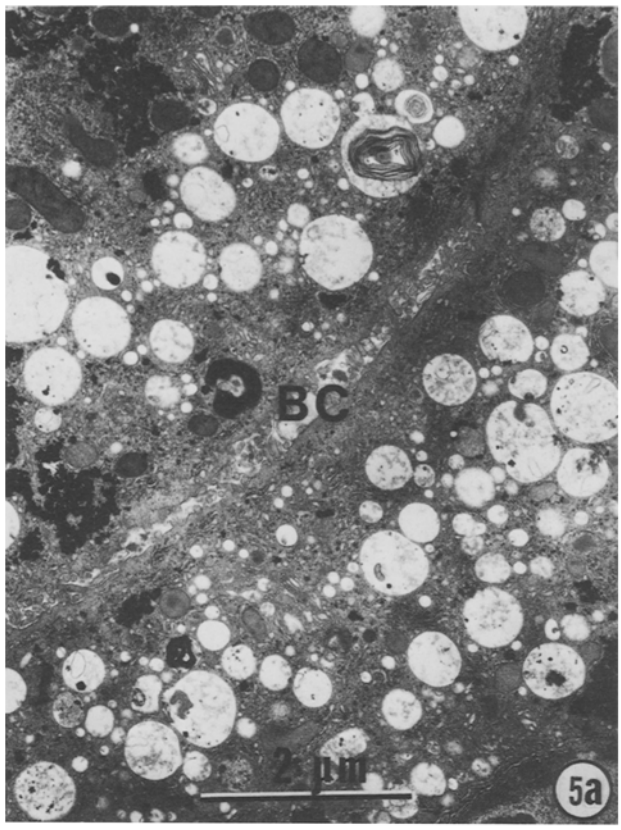
As a control, such experiments were performed in the absence of ouabain. Only the SO_4^{2-} medium was used in this case, and the results are included in Fig. 2. Transfer of the slices to Cl^- medium after the first 15 or 30 min at 38°C resulted in a substantial increase in the rate of water extrusion over the ensuing 30 min, this despite a net entry of Cl^- into the cells which might have been expected to be accompanied by water. Accumulation of K^+ took place at the same rate before and after transfer to the Cl^- medium (*not illustrated*). This transfer also resulted in a good reversal of the morphological swelling described above, and it is important that no cytoplasmic vesicles appeared (*not shown*).

Similar experiments were next carried out in the presence of ouabain, using both NO_3^- and SO_4^{2-} media (Fig. 4). In each case, a significant net extrusion of intracellular water was observed after transfer to the Cl^- medium, while slices remaining in the appropriate chloride-free medium showed no water loss.

Morphological study of the slices incubated for 15 min in NO_3^- medium at 38°C in the presence of ouabain, gave a very similar picture to that described above 60 min (Fig. 3*c*, 5*b*) with, in particular, closed canaliculi and an almost complete absence of cytoplasmic vesicles. Upon transfer to Cl^- medium with ouabain, vesicles appeared in the region of the canaliculi which were morphologically indistinguishable from the vesicles produced upon direct incubation in the Cl^- medium with ouabain (Fig. 3*d* and 5*c*), although they were somewhat fewer in number (Table 2). The canaliculi between cells with the vesicles appeared to be in the process of re-opening for some were quite widely open (Fig. 5*c*) while others were still closed or occupied by microvilli. The mitochondrial structure reverted from the condensed form noted in NO_3^- medium (*see* legend to Fig. 5*b*) to the orthodox configuration (Fig. 5*c*).

The morphological recovery of slices transferred from SO_4^{2-} to Cl^- medium in the presence of ouabain was rather less good than in its absence, both the cytosol and, to a lesser extent, the mitochondria showing some morphological evidence of continued swelling (*compare* Fig. 3*e,f*, Fig. 5*e,f*). However, several vesicles of the type normally associated with ouabain treatment appeared after

Fig. 5. (*next page*). Electron micrographs of lateral and peri-canalicular regions of liver cells after pre-incubation for 90 min at 1°C followed by incubation at 38°C for 60 min (unless otherwise stated) under various conditions. The bar in each plate represents 2 μm. *BC* bile canaliculi. *a*) Incubation with ouabain (2 mM). Note the open bile canaliculus and a large number of vesicles of varying sizes around it. *b*) Incubation in NO_3^- Ringer's containing ouabain (2 mM). Note absence of vesicles in the cytoplasm and almost complete closure of bile canaliculi. Mitochondria are mainly in the condensed form. *c*) Incubation in NO_3^- medium with ouabain for 15 min followed by transfer to Cl^- medium with ouabain for a further 30 min at 38°C. Many vesicles are seen around an open canaliculus. The general form and arrangement of these vesicles is similar to that in plate *a*, although their numbers are fewer. *d*) Incubation in SO_4^{2-} medium. Open bile canaliculi are seen but no vesicles are present in the cytoplasm. The mitochondria, which should be contrasted to those in plates *b* and *c*, tend to be swollen and are without dense granules. *e*) Incubation in SO_4^{2-} medium with ouabain (2 mM). The general picture is similar to that of plate *d*. The canaliculi illustrated are open, but many others were closed. No vesicles are to be seen. Mitochondria are swollen. *f*) Incubation in SO_4^{2-} medium with ouabain for 15 min at 38°C followed by transfer to Cl^- Ringer's with ouabain for a further 30 min. The bile canaliculus is open and vesicles of various sizes, similar to those of plates *a* and *c*, are present. Some swelling of the mitochondria is apparent. *g*) Incubation in Li^+ Ringer's. Vesicles very similar to those in the presence of ouabain (plate *a*) are numerous in the vicinity of the bile canaliculus; the latter is open. A similar picture is seen when ouabain is present in the Li medium. *h*) Incubation with furosemide (2 mM) and ouabain (2 mM). As in plate *b*, the bile canaliculus is occluded by a few microvilli; vesicles of the type usually seen with ouabain are very few in number compared to plate *a*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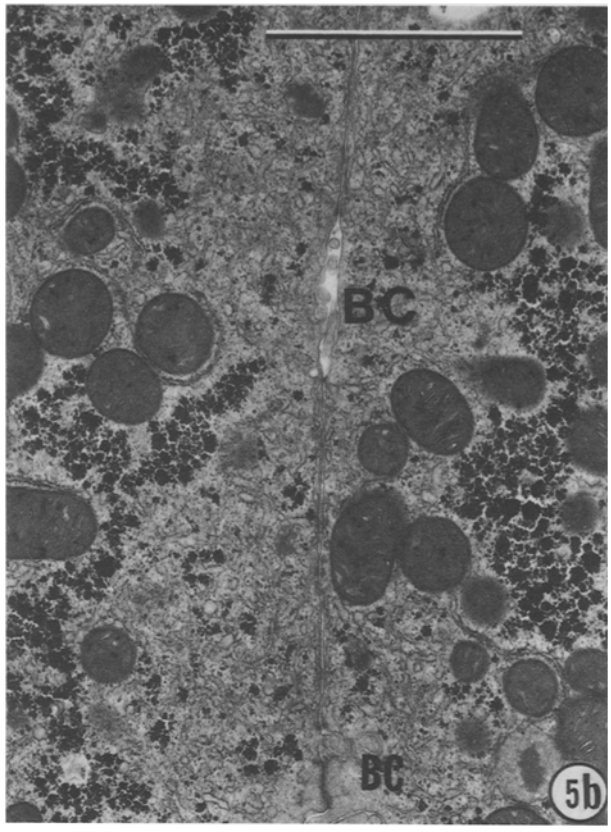
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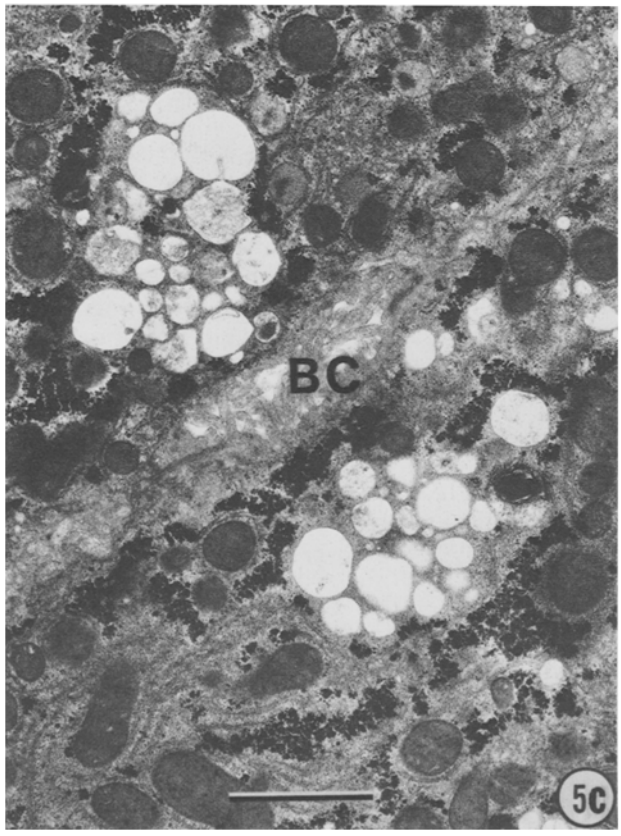
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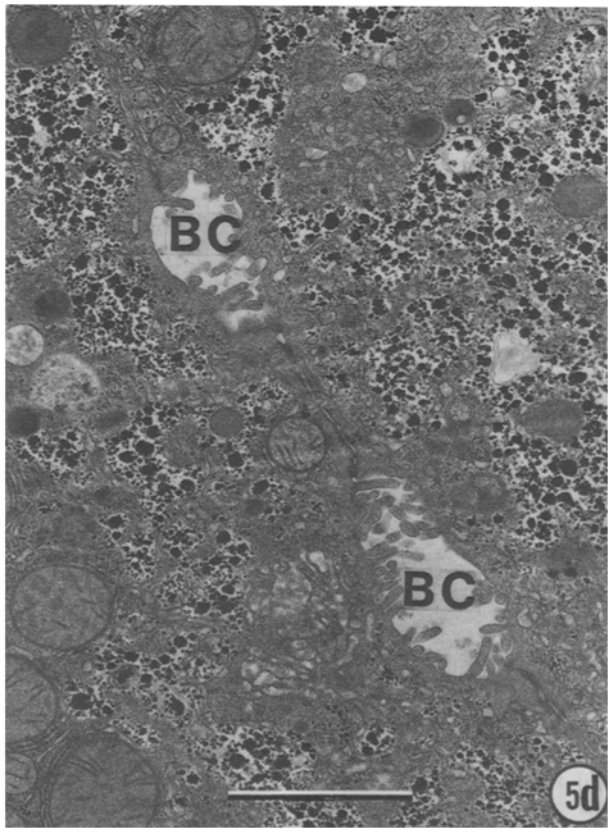
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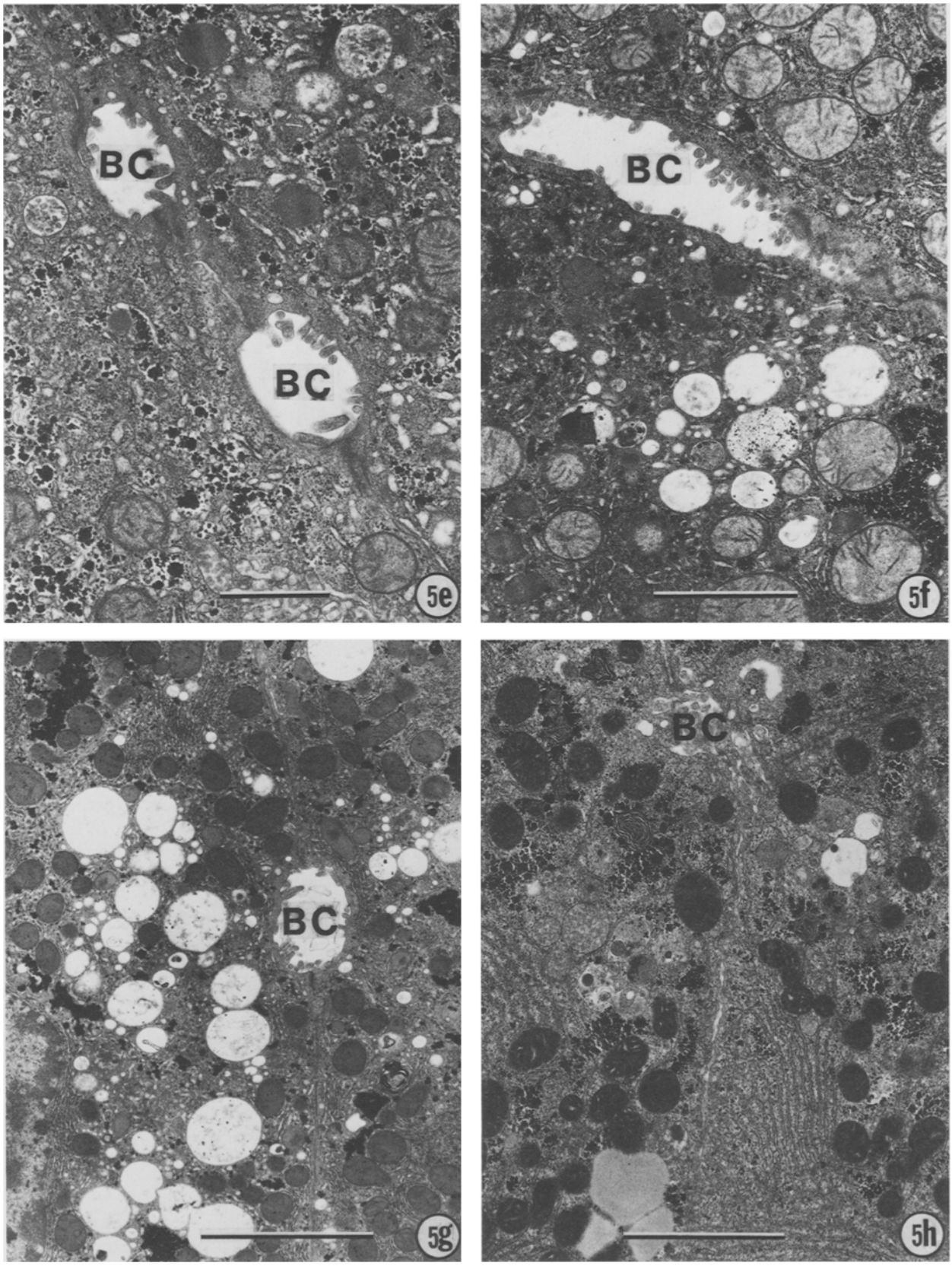
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Table 3. Effects of ouabain on the intracellular water and ions of liver slices incubated in media with Na^+ or Li^+ as principal cation[a]

Incubation	Principal cation	Ouabain (mM)	Water (kg/kg dry wt)	Na^+	Li^+	K^+	Cl^-	(*n*)
				(mmol/kg dry wt)				
90 min at 1°C	Na^+	0	2.46 ± 0.04	356 ± 16	—	70 ± 3	317 ± 12	(28)
	Li^+	0	2.26 ± 0.04	60 ± 11	331 ± 12	76 ± 4	278 ± 10	(28)[b]
90 min at 1°C followed by 60 min at 38°C	Na^+	0	−0.86 ± 0.04	−225 ± 10	—	+119 ± 4	−103 ± 10	(44)
	Li^+	0	−0.29 ± 0.04[e]	−17 ± 6	+16 ± 16	−24 ± 2	+1 ± 8	(58)
	Na^+	2	−0.47 ± 0.06[e]	−84 ± 14	—	−13 ± 2	−63 ± 9	(44)
	Li^+	2	−0.22 ± 0.04[e]	−15 ± 7	−3 ± 15	−26 ± 2	−1 ± 9	(58)[c,d]

[a] Incubation techniques as described in Materials and Methods. Other details as in legend to Table 1.
[b] For Li^+, $n = 10$.
[c] For Cl^-, $n = 52$.
[d] For Li^+, $n = 30$.
[e] Significantly different from line above, $P < 0.01$.

transfer to Cl^- medium (Fig. 3*f* and Fig. 5*f*). It is again apparent that Cl^- was required for the formation of these vesicles and that their appearance was associated with the onset of a net extrusion of water.

Substitution of Na^+ in the Medium

The water and ionic content of slices preincubated at 1°C was little affected by the replacement of Na^+ by Li^+ as the main cation of the medium, except that the Na^+ of the tissue was largely replaced by Li^+ (Table 3). Upon subsequent incubation at 38°C, slices in the Li^+ medium extruded much less water than controls in Na^+ medium. They were unable to accumulate K^+, suggesting that the reduced water extrusion was at least partly due to inhibition of the ouabain-sensitive component of volume control. The ouabain-like consequences of incubation in the Li^+ medium extended to the occurrence of many cytoplasmic vesicles which were in all respects similar to those induced by ouabain (Fig. 5*g*) although they appeared to be present in somewhat smaller numbers (Table 2 and *compare* Fig. 3*g* with 3*b*). On occasion these vesicles were very large. Canaliculi were wide open, as is normally the case with slices incubated in the presence of ouabain in the Na^+ medium.

However, it is clear that factors other than a ouabain-like inhibition of Na^+ and K^+ transport also came into play in the absence of Na^+ for the quantity of water extruded in the Li^+ medium was significantly less than that permitted by ouabain in the Na^+ medium. Moreover, the quantity of water extruded in the Li^+ medium was little affected by the presence of ouabain (i.e. 0.29 kg water/kg dry wt without, and 0.22 kg/kg with ouabain, Table 3). Thus, it can be concluded that the absence of Na^+ inhibited not only all the ouabain-sensitive water extrusion but up to 50% of the ouabain-resistant extrusion as well. This was accompanied by a complete inhibition of the net extrusion of Cl^- suggesting that the movement of this ion is important for that part of the ouabain-resistant water extrusion that is inhibited by the absence of Na^+ (Table 3). As seen in Fig. 3*h* and 5*g*, many cytoplasmic vesicles were present in slices in the Li^+ medium with ouabain, but the number of vesicles was again somewhat less than in the Na^+ medium with ouabain (Table 2). While these results in the absence of Na^+ give a more complex picture than those obtained in the absence of Cl^-, they nevertheless confirm that a reduction in the ouabain-resistant extrusion of water was associated with an inhibition of Cl^- movements and with some reduction in the numbers of cytoplasmic vesicles.

The mechanism underlying the small extrusion of water which persists in the Li^+ medium with ouabain (amounting to about 25% of the control extrusion, Table 3) has not been specifically studied, but it can be seen from Table 3 that it is associated with a small net loss of K^+ and of residual tissue Na^+. There was no net loss of the two ions, Li^+ and Cl^-, which predominated in the tissue under these conditions. The persistence of many cytoplasmic vesicles suggests that they may also be active in the extrusion of water that continues in the Li^+ medium.

Inhibitors of Cl^- Movements

Since both Cl^- and Na^+ appeared to be required for the ouabain-resistant extrusion of water and for the formation of cytoplasmic vesicles, we examined the effects of furosemide. This agent is known to inhibit

Table 4. Effect of furosemide on the intracellular water and ions and on the rate of O_2 consumption of liver slices incubated with and without ouabain[a]

Incubation	Furosemide (mM)	Ouabain (mM)	Water (kg/kg dry wt)	K^+ (mmol/kg dry wt)	QO_2 (μl/mg dry wt · hr)	(*n*)
90 min at 1°C	0 and 5[b]	0 and 2[b]	2.52 ± 0.10	79 ± 4	—	(14)
90 min at 1°C followed	0	0	−0.90 ± 0.10	+144 ± 11	7.8 ± 0.3	(14)
by 60 min at 38°C	2	0	−1.06 ± 0.09	+97 ± 9	6.5 ± 0.2	(14)
	5	0	−1.18 ± 0.11	+69 ± 20	5.5 ± 0.6	(14)
	0	2	−0.78 ± 0.15	−25 ± 4	5.7 ± 0.4	(13)
	2	2	−0.51 ± 0.10	−31 ± 3	5.8 ± 0.2	(13)
	5	2	−0.54 ± 0.09	−29 ± 3	4.1 ± 0.3	(14)

[a] Details as in Materials and Methods and in footnote "*a*" to Table 1.
[b] In each experiment samples were taken after pre-incubation at 1°C with no inhibitors and with 2 mM ouabain plus 5 mM furosemide. There was no significant difference between these two groups with respect to any of the values studied and the results have therefore been pooled.

the coupled movements of Na^+ and Cl^- in several tissues (e.g. Frizzell, Field & Schultz, 1979). However, furosemide also inhibits mitochondrial energy metabolism at certain concentrations (Manuel & Weiner, 1976; Cunarro & Weiner, 1978; van Rossum, Ernst & Russo, 1981) and we observed that the O_2 uptake of liver slices was inhibited by 16 and 30% at 2 and 5 mM furosemide, respectively, in the absence of ouabain, while K^+ accumulation was reduced to similar extents (Table 4). Ouabain reduced O_2 consumption by about 30%, probably due to the "pacemaker" effect of the utilization of ATP for the transport of Na^+ and K^+ (Whittam, 1962; Elshove & van Rossum, 1963); no further reduction was caused by furosemide at 2 mM, although the ouabain-insensitive respiration was reduced by 5 mM. This last observation suggests that the small effect of 2 mM furosemide on respiration was not due to a direct inhibition of electron transfer and, furthermore, we have shown that this concentration has no effect on the ATP content of renal cortical slices (van Rossum et al., 1981). We therefore chose 2 mM as an appropriate concentration to study the effects of the diuretic on volume regulation.

In the absence of ouabain, furosemide caused no reduction of the extrusion of intracellular water (Table 4) although there was some morphological indication of a slight swelling (Fig. 3*i*). In the presence of ouabain there appeared to be a tendency for furosemide to reduce the extrusion of water after 60 min at 38°C in these experiments, but the variation between slices was large (Table 4). However, a study of the time-course of water extrusion in the presence of ouabain showed that 2 mM furosemide clearly reduced the rate at which water was lost between 15 and 30 min and at the latter time there were significant differences between the net extrusion of water, Na^+ and Cl^- with and without diuretic ($P < 0.01$ in each case; Fig. 6). During the ensuing 30 min, there was a continued, slow loss of water from the cells with furosemide so that this agent was apparently only partially effective as an inhibitor of the ouabain-resistant water loss. Morphological examination of the slices after 60 min at 38°C with ouabain *plus* furosemide showed that some 70% of the slice area had an appearance in the optical microscope that was generally similar to that of the slices in the chloride-free, NO_3^- medium (Fig. 3*j*, 5*h*; *cf.* Fig. 3*c* and 5*b*). Furosemide induced a substantial reduction in the number of cytoplasmic vesicles compared to the situation with ouabain alone (Table 2) and also induced closure of the bile canaliculi (Fig. 5*h*). A small proportion of the area of slices treated with ouabain *plus* furosemide showed a degree of morphological damage after 60 min at 38°C and this may have contributed to the continued loss of apparently intracellular (i.e. inulin-free) water at this time, to the extent that the membranes of the damaged cells may have become permeable to inulin. Such an effect clearly cannot account for the inhibition of water loss noted at 30 min.

Certain types of Cl^- movement are affected by di-isothiocyano-diethyl stilbene (DIDS), which inhibits Cl^-/HCO_3^- exchanges in red blood cells (Knauff, Fuhrmann, Rothstein & Rothstein, 1977), and by tributyl tin, which acts as a Cl^- ionophore (Selwyn, Dawson, Stockdale & Gains, 1970; Wieth & Tosteson, 1979). DIDS was examined at concentrations of 0.2 and 1.0 mM in the presence and absence of 2 mM ouabain, but had no effect on the net movements of intracellular water and ions. Tributyl tin (0.1, 0.2 and 1.0 mM) was likewise without effect.

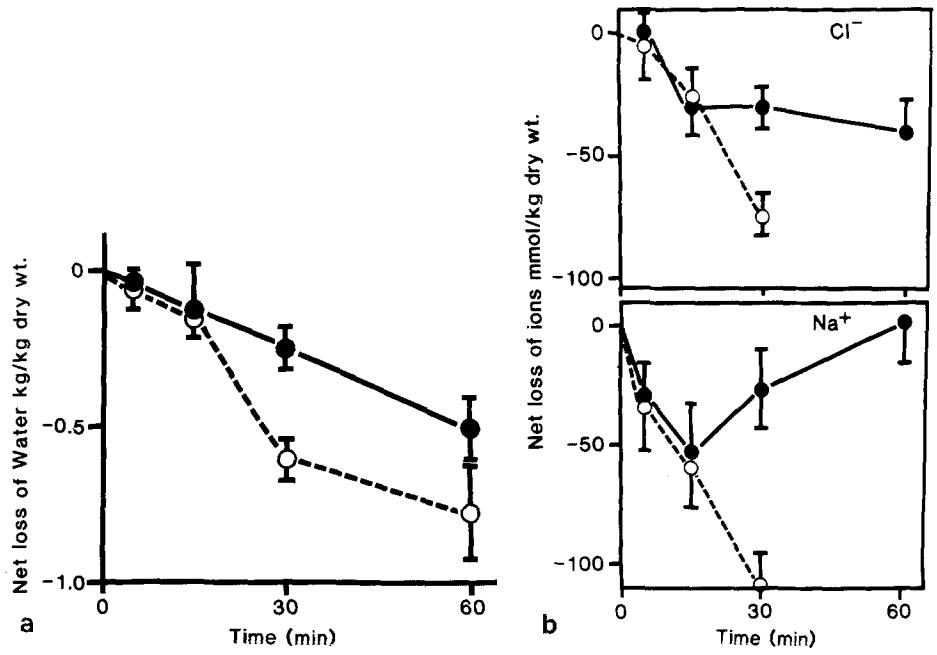

Fig. 6. Effect of furosemide on the ouabain-resistant extrusion of (*a*) intracellular water and (*b*) intracellular Na^+ and Cl^- from liver slices. Incubation was in Cl^- medium containing either 2 mM ouabain (○) or 2 mM ouabain *plus* 2 mM furosemide (●). The points represent the content after 90 min at 1°C *minus* content after further incubation at 38°C for the times indicated; $n = 12$

Discussion

Our results confirm that water extrusion from previously swollen liver slices comprises ouabain-sensitive and ouabain-resistant portions. The former is presumably a consequence of the transport of Na^+ and K^+ as originally postulated by Leaf (1956), and our present work has attempted to elucidate the mechanism underlying the ouabain-resistant extrusion of water.

The proportion of the total water extrusion from liver slices which is resistant to ouabain has been found to be somewhat variable (Russo et al., 1977; van Rossum & Russo, 1981). In the present work, it ranged from 100% (Fig. 1) through 87% (Table 4) to 55% (Tables 1 and 3) of the extrusion noted in the absence of ouabain, when measured after 60 min at 38°C. We have shown previously that, in the presence of ouabain, liver slices recovering from swelling contain large numbers of cytoplasmic vesicles, particularly in the regions between the nucleus and canaliculi (Russo et al., 1977). The formation of these vesicles appears to be related to an inhibition of the Na^+- and K^+-dependent adenosine triphosphatase, rather than to the presence of ouabain itself, since identical vesicles have been observed in the absence of ouabain when incubation was conducted in a potassium-free (van Rossum & Russo, 1981) or sodium-free medium (this paper); in each of these cases an inhibition of the adenosine triphosphatase was indicated by an absence of K^+ accumulation and a reduction of Na^+ extrusion.

Since the binding of ouabain to the Na- and K-dependent adenosine triphosphatase can be inhibited by low temperatures, it is possible that a very early phase of the water loss in the presence of ouabain took place by way of the ouabain-sensitive mechanism while the cells were warming from 1° to 38°C, before inhibition of the Na-K-transport system was complete. However, our results show that the bulk of the water extrusion took place *after* the first 15 min at 38°C, when K^+ accumulation was completely inhibited, and that there was no indication of a loss of volume regulating ability even after 60 min (Fig. 1; *see also* van Rossum & Russo, 1981). Further, the ouabain-resistant water extrusion taking place in Fig. 4 occurred, upon addition of Cl^-, after the slices had been incubated for 15 min at 38°C in the presence of ouabain. Thus, at least the major part of the initial water extrusion, as well as the maintenance of low intracellular water contents after longer times, appears to be due to a ouabain-resistant system.

We have proposed that the vesicles formed in the presence of ouabain are the vehicle for the ouabain-resistant extrusion of water, the final step of which is an exocytotic expulsion into the canaliculi (Russo et al., 1977). In the present work we have obtained further evidence for a close association between the occurrence of the vesicles and the extrusion of water in the presence of ouabain; there was also an association with the state of the canaliculi, although this was less consistent. Thus, conditions which totally inhibited water extrusion, such as incubation in chloride-free media containing oua-

bain (with either NO_3^- or SO_4^{2-} as principal anion) also resulted in the almost complete absence of the vesicles and often to closure, or obstruction by microvilli, of the canaliculi, while subsequent restoration of Cl^- to the tissue (still in the presence of ouabain) induced water extrusion, vesicle formation and opening of canaliculi. Conditions in which the ouabain-resistant extrusion of water was less completely inhibited (i.e. absence of Na^+ or presence of furosemide) resulted in an intermediate reduction in the number of vesicles detected. Thus, under these conditions there was a direct relationship between the presence of vesicles and the occurrence of water extrusion in the presence of ouabain. Yet there is another set of conditions in which inhibition of the ouabain-resistant water extrusion is associated with a large increase in the number of vesicles and with their wider distribution, namely upon treatment with cytochalasin B or, to a less marked extent, Ni^{2+} (van Rossum & Russo, 1981). These contrasting situations can be readily explained if the conditions studied in the present work (i.e. Cl^- or Na^+-free media, presence of furosemide) act by preventing the passage of water from the swollen cytosol into the vesicles, so inhibiting vesicle formation, while cytochalasin B and Ni^{2+} act by preventing exocytosis of the water-laden vesicles into the canaliculi without preventing their formation.

The precise origin of the vesicles appearing in the presence of ouabain is still under investigation, but work done to date shows that they arise as a large number of small vesicles, visible after 5 min incubation at 38°C in the presence of ouabain, which increase in size during the course of incubation (van Rossum & Russo, 1981). Since their contents are largely electron-transparent, the increase in size apparently arises largely from an increased water content. It seems likely that such an entry of water into a membrane-bound vesicle would follow a flux of solute. The almost complete dependence of vesicle formation on the presence of Cl^- indicates an important role for this anion in such a process while the less marked effects of Na^+ and furosemide suggest that a cotransport of Na^+ and Cl^- may also occur. This last conclusion is less clearly supported by our results but cannot be excluded by them. For example, Cl^- extrusion from the slices was blocked when medium Na^+ was replaced by Li^+. That furosemide, a common inhibitor of many Na^+ and Cl^- cotransport processes (Frizzell et al., 1979) did not inhibit the water extrusion totally may be because it failed to attain a high enough concentration inside the cells; the use of higher concentrations than 2 mM was precluded by the likely complicating effects of its inhibition of energy metabolism.

While the major findings of our ion-substitution experiments, as outlined above, appear to be fairly clear-cut, there are some aspects of the experiments which remain less obviously explicable. One of these is the persistence of a net loss of water (about 25% of the total loss) from slices incubated with ouabain in the Li^+ medium. This is associated with small net losses of Na^+ and K^+, but not of Cl^- so that it is not clear how electrical neutrality is maintained. This system is in some ways reminiscent of the water movements accompanying cotransport of K^+ and Na^+ seen in a number of cell types (e.g. Geck et al., 1980; Bakker-Grunwald, 1981). However, the latter is associated with a net movement of Cl^-, in contrast to our results (*see* Table 3), and is sensitive to furosemide whereas in our experiments (Table 4) the loss of K^+ was not prevented by 2 or 5 mM furosemide. A further problem in explaining this same loss of water is that it occurred in the Na^+-free medium, with ouabain, where net extrusion of Cl^- was absent, yet did not take place in the Cl^--free medium with ouabain (Table 1). No explanation for this can yet be offered.

Another unexpected finding was that the extrusion of water in the absence of ouabain was reduced when Cl^- in the medium was replaced by SO_4^{2-} (Table 2*b*). This may arise because the mechanism of water extrusion postulated by Leaf (1956), i.e. that dependent on the coupled, active transport of K^+ and Na^+, requires the outward movement of an anion. While SO_4^{2-} may be able to support swelling at 1°C by passing through the plasma membranes, it may not be able to pass as rapidly through the reformed membrane at 38°C with the result that the rate of water extrusion would be reduced below that seen with Cl^- or NO_3^-.

The formation of vesicles in the presence of ouabain is not only found in liver tissue. An early observation was that of Garfield and Daniel (1977) who noted two types of vesicles in myometrial tissue recovering from swelling. One type was of small vesicles closely associated with the plasma membranes while the other was of larger, cytoplasmic vesicles apparently similar to those we have noted in the liver. Garfield and Daniel suggested that the former type might be involved in the expulsion of water from the cells by exocytosis. We have observed the larger vesicles, similar to those in the liver, in slice preparations of Morris hepatoma 3924A (Russo et al., 1976), of renal cortex (van Rossum et al., 1983) and of avian salt gland (van Rossum & Russo, *unpublished observations*) during incubation in the presence of ouabain. It seems possible that a similar vesicular mechanism may contribute to the regulation of volume in a number of different types of cells.

We thank Mrs. O.O. Holowecky for skillful technical assistance. This work was supported in part by grant no. R806616 from the U.S. Environmental Protection Agency and in part by grant no. AM27559 from the National Institutes of Health.

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Received 7 April 1983; revised 6 June 1983