Scanning Microscopy

Volume 6 | Number 4 Article 25

10-20-1992

Effect of Chronic Treatment with Diuretics on Mouse Liver: A Morphological and Microanalytical Investigation

Ann-Christin Mork
University of Uppsala

Anne von Euler
University of Uppsala

Godfried M. Roomans
University of Uppsala

Scanning Microscopy, Vol. 6, No. 4, 1992 (Pages 1147-1153) 0891-7035/92$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

EFFECT OF CHRONIC TREATMENT WITH DIURETICS ON MOUSE LIVER: A MORPHOLOGICAL AND MICROANALYTICAL INVESTIGATION

Ann-Christin Mörk*, Anne von Euler and Godfried M. Roomans

Medical Ultrastructure Research Group, Department of Human Anatomy, University of Uppsala, Box 571, S-75123 Uppsala, Sweden

(Received for publication March 24, 1992, and in revised form October 20, 1992)

Abstract

In an attempt to produce an animal model for the disease cystic fibrosis (CF), mice were treated chronically with the diuretics amiloride and furosemide, in order to cause chronic inhibition of transepithelial ion transport. Experiments were carried out on adult mice (2 months treatment); in addition, pregnant mice were treated with diuretics, and tissue from offspring 2 and 7 days *post partum* was investigated. Since biliary cirrhosis is a common occurrence in CF, hepatocytes in the treated mice were investigated by X-ray microanalysis and by light and electron microscopy.

Treatment with amiloride caused a significant decrease in cellular Na concentration in adult animals and in *in utero* treated mice 2 days after birth. The decrease in Na was parallelled by a decrease in Cl, but K levels were not affected. Furosemide caused a slight increase of cellular Na concentrations, especially in animals aged 7 days. In the adult animals, both amiloride and furosemide caused a significant decrease of the cellular Na and Cl levels. No signs of cirrhosis could be observed. Inconsistent changes in the accumulation of lipid droplets in hepatocytes of adult animals treated with amiloride were observed by electron microscopy. It can be concluded that chronic treatment with diuretics, even though it causes some, possibly pathological, changes of the liver, is only of very limited value for generating an animal model to study liver disease in CF.

Key Words: Liver, diuretics, ion transport, bile flow, cystic fibrosis, animal model, X-ray microanalysis, transmission electron microscopy, light microscopy.

* Address for correspondence:
Ann-Christin Mörk,
Department of Human Anatomy,
University of Uppsala,
Box 571, S-75123 Uppsala,
Sweden

Phone No.: +46 (18) 174292

Introduction

Cystic fibrosis (CF) is a genetic disease due to a mutation in the gene coding for a chloride channel present in the apical membrane of epithelial cells (Riordan *et al.*, 1989). The main clinical symptoms are chronic lung disease and pancreatic insufficiency. The lung disease is due to the inhibition of transepithelial chloride and water transport, which results in the production of water-deficient (and therefore viscous) mucus (Boucher *et al.*, 1984). In the pancreas, the genetic error causes inhibition of chloride, bicarbonate, and water transport, resulting in obstruction of the pancreatic ducts by secreted enzymes; ultimately, this leads to fibrotic changes in the pancreas (Park and Grand, 1981). Although liver symptoms in CF are considered less serious than lung disease and pancreatic malfunction, the occurrence of biliary cirrhosis in especially older CF patients is common (di Sant'Agnese and Hubbard, 1984). It has been suggested that biliary cirrhosis is caused by obstruction of the bile ducts by a similar mechanism as that present in the pancreas.

Bile flow is driven by the formation of osmotic gradients between the blood, intercellular space and the hepatocytes on one hand, and the lumen of the bile canaliculus on the other hand (reviewed by Sellinger and Boyer, 1990). To a large extent, bile flow rate is dependent on the secretion of bile acids into the bile duct. However, there is an additional component of the bile flow connected with inorganic ions. According to a current model, a chloride channel is present in the canalicular (apical) membrane of the hepatocyte. The chloride in the bile duct lumen is then exchanged for bicarbonate, which in this way enters the bile fluid in a way similar to that by which bicarbonate ions are secreted by the pancreas. Some ion transport mechanisms in the basolateral membrane have been described: an amiloride-sensitive Na^+-H^+ exchange, a Na^+-bicarbonate cotransport, and a Na^+-K^+-ATPase. Whether a furosemide-sensitive cotransporter is present in the basolateral membrane, as it is in that of the hepatocyte, is not known.

Table 1. Effects of amiloride and furosemide treatment on elemental composition of mouse hepatocytes.

	Na	Cl	K
adult animals			
control	104±10	88±9	351±19
amiloride	31± 6***	63±8*	410±21
furosemide	75± 8**	60±8**	441±22*
treatment in utero, animals aged 2 days			
control	69± 8	72±4	391±12
amiloride	47± 5**	81±3	440±11
furosemide	83± 4	71±3	437±16
treatment in utero, animals aged 7 days			
control	53± 5	56±5	373±15
amiloride	64± 5	55±2	347±10
furosemide	96± 5**	83±4**	355±12

Data in mmol/kg dry weight, mean and standard error, n (number of cells analyzed) = 12-19 (three animals per group); significance of differences by analysis of variance is indicated by: * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$).

Previously we have investigated mice and rats chronically treated with diuretics such as furosemide and amiloride as possible animal models for cystic fibrosis (Sagström *et al.*, 1990; Mörk *et al.*, 1991; von Euler *et al.*, 1992). In cystic fibrosis, transepithelial ion and water transport is inhibited because of a defect in the apical chloride channel. In the proposed animal models, transepithelial ion and water transport is inhibited at the basolateral membrane; although the location of the defect is different, the final result should be similar to that in the human disease. In previous investigations on salivary glands and pancreas some changes were noted that indicated that transepithelial ion and water transport had been affected, although not to an extent similar to that observed in the human disease (Sagström *et al.*, 1990; von Euler *et al.*, 1992).

In the present paper, effects of chronic treatment with diuretics on liver were investigated by morphological and microanalytical techniques.

Materials and Methods

Female NMRI (Naval Medical Research Institute) mice were used in the experiments. For experiments with chronic treatment of adult mice with amiloride, 5 week-old mice were given food mixed with amiloride to give a daily dose of 0.5 mg per animal, or with furosemide to give a daily dose of 4 mg per animal (Mörk *et al.*, 1991) for two months. The amiloride and furosemide doses were calculated from the average food intake of the animals. The control group received standard chow, and was kept for two months under identical housing conditions.

In the *in utero* experiments, mice were mated and pregnancy was assessed from the formation of a vaginal plug. The animals received food mixed with amiloride (0.5 mg/day/animal) or furosemide (4 mg/day/animal) (von Euler *et al.*, 1992). A control group of animals was mated and kept for the same period under identical conditions, but received standard chow.

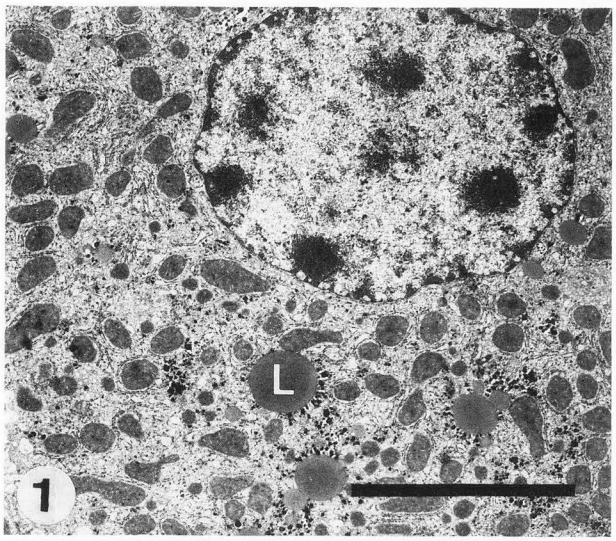
The adult animals were deprived of food 16 hours prior to sacrifice, but had access to water *ad libitum*. The newborn animals that had been treated *in utero* with amiloride or furosemide were sacrificed on day 2 or day 7, respectively, after birth. During the period after birth, they were kept together with the mother, and amiloride- or furosemide-treated food was provided continuously.

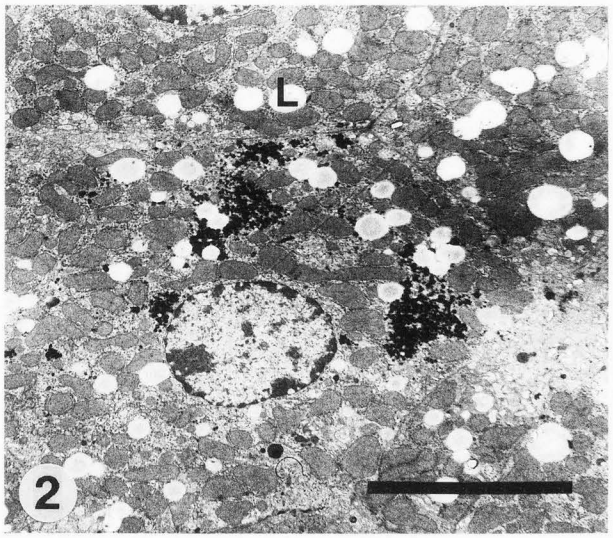
The liver of the animals was removed after heavy sodium pentobarbital anesthesia, to minimize the period of anoxia. Small pieces (about 1 mm^3) were excised from the tissue and rapidly frozen in liquid nitrogen. Thick (16 μm) cryosections were cut on a conventional cryostat at -30°C and mounted on a carbon specimen holder as described earlier (McMillan and Roomans 1990). Specimens were viewed in the scanning mode in a Philips 525 scanning electron microscope (SEM) and X-ray microanalysis was carried out at an accelerating voltage of 20 kV with a LINK AN 10000 energy-dispersive X-ray microanalysis system. Quantitative analysis was carried out by use of the ratio of characteristic intensity and the continuum intensity in the same energy region. The specimen spectra were compared to standard spectra obtained with the help of standards, consisting of a 20% gelatin/5% glycerol matrix to which salts in known concentrations had been added (Roomans, 1988).

For morphological studies, tissue was fixed in buffered glutaraldehyde and embedded for light microscopy in HistoResin (Reichert-Jung, Heidelberg, FRG). Sections were stained with the Verhoeff-van Giemson stain, Jones's periodic acid methenamine silver (PAMS) stain, and Sudan black. Tissue was also postfixed with osmium tetroxide and prepared for transmission electron microscopy, which was carried out in a JEOL 100B or Philips 301 transmission electron microscope at 60 kV.

Results and Discussion

Treatment with amiloride caused a significant decrease in cellular Na concentration in adult animals





Figures 1 and 2. Transmission electron micrographs of the liver of an adult mouse. **Figure 1:** Control; intracellular lipid droplets indicated by L. **Figure 2.** Treated for 2 months with amiloride. Note increased number of intracellular lipid droplets (L). Bar = 5 μm.

---

and in *in utero* treated mouse 2 days after birth; at 7 days after birth no significant difference could be detected (Table 1). The decrease in Na was parallelled by a decrease in Cl; K levels increased slightly. Furosemide had no consistent significant effect on the elemental content of hepatocytes after treatment *in utero* but decreased Na and Cl levels in the hepatocytes of adult mice (Table 1).

Light microscopy failed to show gross signs of cirrhosis after amiloride treatment both in the adult and the newborn animals. In the newborn animals, no increase of connective tissue was noted in the PAMS-stained sections and no increase in fat tissue was noted in the Sudan-black stained tissue.

Electron microscopy showed, however, increased accumulation of lipid droplets in the hepatocytes of amiloride-treated animals as compared to the controls (Figs. 1 and 2). In the hepatocytes of animals aged 2 days very little glycogen or fat is present (Figs. 3-5), irrespective of treatment. "Dark" (electron-dense) cells, in which rough endoplasmic reticulum dominates, and "light" (electron-translucent) cells in which smooth endoplasmic reticulum dominates (Figs. 4 and 5) can be distinguished. At age 2 and 7 days the presence of the hematopoietic cells is still clearly visible (Fig. 5). No difference between control and diuretic-treated animals was noted in this respect. This indicates that treatment with diuretics does not interfere in such a way with general metabolism that the development of the hematopoietic system is affected.

In hepatocytes of control- and furosemide-treated animals aged 7 days numerous fat droplets could be observed (Figs. 6 and 7), but in the amiloride-treated animals there were only few lipid droplets (Fig. 8). Since the amiloride-treated offspring was much smaller than the offspring from the control group and the furosemide-treated group, it is likely that the decreased amount of fat in the hepatocytes after amiloride treatment is due to a general metabolic effect. Bile canaliculi were patent after chronic amiloride treatment (Fig. 8, inset).

The role of the transport mechanisms for inorganic ions in the generation of bile flow has not yet been clarified, and it is unclear which solutes are responsible for the bile flow in the absence of bile acids (the so-called bile-acid independent flow, BAIF). Both the Na^+-K^+-ATPase and the K^+ channel in the sinusoidal membrane are linked to bile secretion, since most bile acids enter the hepatocyte by a Na^+ cotransport mechanism driven by the Na^+ gradient. The driving force for their excretion over the canalicular membrane into the bile is the membrane potential difference (Weinman *et al.*, 1989). Experimental inhibition of BAIF has been shown to result in cholestasis, and animal models for this condition have been developed, based mainly on interference with the Na^+-K^+-ATPase (Keeffe *et al.*, 1980; Layden and Boyer, 1976; Avner *et al.*, 1981). The theoretical basis for attempting to develop an animal model for CF based on an inhibition of transcellular ion and water flow by diuretics is, however, less solid than in, e.g., the pancreas, although it has been suggested that transport of inorganic ions in hepatocytes is similar to that in pancreatic acinar cells (Sellinger and Boyer, 1990). Indeed, several mechanisms are present in both cells. Excretion of chloride and bicarbonate ions by an electrogenic Cl^-

channel in combination with a Cl^--bicarbonate exchange mechanism occurs both in hepatocytes and in pancreatic acinar cells (Kuijpers and De Pont, 1987). However, neither in liver nor in pancreas is it quite certain what ion transport mechanisms are present in the sinusoidal or basolateral membrane, respectively. This much is clear, that in the pancreas transepithelial ion transport can be inhibited both by amiloride and by furosemide. Both drugs cause a decrease in the intracellular chloride concentration, which is the driving force for fluid transport. In the sinusoidal membrane of the liver cell (adjoining the space of Disse) an amiloride-sensitive Na^+-H^+ exchange mechanism is present. This mechanism appears to be important primarily for the regulation of hepatocyte pH (Renner *et al.*, 1989, Anwer *et al.*, 1989), but whether it affects fluid transport is unclear. It is conceivable that inhibition of this mechanism, by decreasing the intracellular sodium concentration, increases the Na^+ gradient over the sinusoidal membrane and by that the driving force for the Na^+-bile acid cotransport. On the other hand, amiloride, along with a number of other compounds, was found to be a competetive inhibitor of taurocholate uptake across the sinusoidal membrane (Zimmerli *et al.*, 1987). It has been shown that in an animal model for cholestasis (rats treated with ethinyl estradiol), Na^+-H^+ exchange activity is decreased, but that study could not answer the question whether inhibition of that pump was the cause of the cholestasis, or only an expression of a generalized membrane damage. The present study indicates that inhibition of the Na^+-H^+ exchange mechanism as such does not cause cholestasis.

In the pancreas, morphological effects of treatment with amiloride and furosemide were more severe in the *in utero* treatment than when adult animals were treated. This was not the case for the liver. In the newborn rat, BAIF is relatively small, but increases during development till it reaches the normal adult level (Ballatori and Clarkson, 1982). If this is also the case in the mouse, treatment with diuretics *in utero* may be less effective than in the adult animal.

Bicarbonate enters the hepatocyte by a stilbene-sensitive Na^+-bicarbonate cotransport mechanism that is not affected by amiloride (Fitz *et al.*, 1989). How chloride ions enter the hepatocyte is unclear. Chloride uptake in pancreatic acinar cells proceeds via a furosemide-sensitive mechanism of which the nature still is in dispute. The results of this study indicate that chloride uptake into hepatocytes in very young animals is not sensitive to furosemide, but our results are consistent with the presence of a furosemide-sensitive Na^+-Cl^- cotransport mechanism in the sinusoidal membrane in adult animals.

Morphological effects following cholestasis are well-defined (Carpino *et al.*, 1981), but none of these was found in our study. The adult amiloride-treated animals presented with mild steatosis, which, in the human, can develop into cirrhosis. However, the effect of amiloride treatment on lipid accumulation is not consistent, since e.g., in amiloride-treated animals aged 7 days, lipid accumulation was less than in the control group.

In summary, it can be concluded that chronic treatment with diuretics is not very suitable to generate an ideal animal model to study liver disease in cystic fibrosis.

Fig. 3. Hepatocytes in a control animal aged 2 days. Very little glycogen and lipid droplets present.

Fig. 4. Hepatocytes in an animal treated *in utero* with furosemide, aged 2 days. "Dark" cells containing predominantly rough endoplasmic reticulum.

Fig. 5. Hepatocytes in an animal treated *in utero* with amiloride, aged 2 days. "Dark" cells containing predominantly rough endoplasmic reticulum, "light" cells containing predominantly smooth endoplasmic reticulum. Hematopoietic cells are visible (H).

Fig. 6. Hepatocytes of a control animal aged 7 days. A large number of intracytoplasmic lipid droplets (L) is present.

Fig. 7. Hepatocytes and hematopoietic cells in an animal treated *in utero* (and after birth) with furosemide, aged 7 days. In the hepatocytes, lipid droplets (L) are present.

Fig. 8. Hepatocytes in an animal treated *in utero* (and after birth) with amiloride, aged 7 days. Only few lipid droplets are present in this tissue. *Inset*: Patent bile canaliculus.

Bars = 5 μm.

Acknowledgements

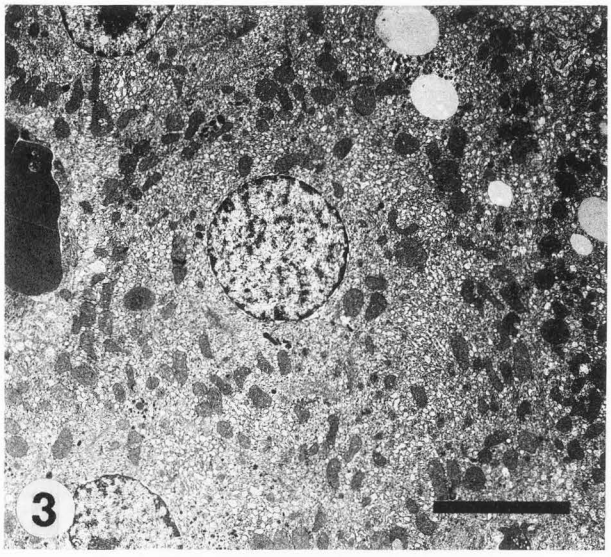
The skillful technical assistance of Anders Ahlander is gratefully acknowledged. This study was supported by the Swedish Medical Research Council (grant 07125), the Swedish Association for Cystic Fibrosis, the Swedish Association against Heart and Chest Diseases and the Swedish Association for Medical Research.

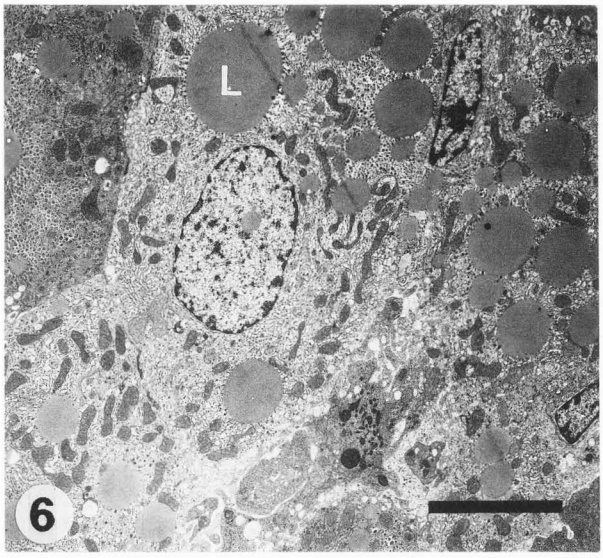
References

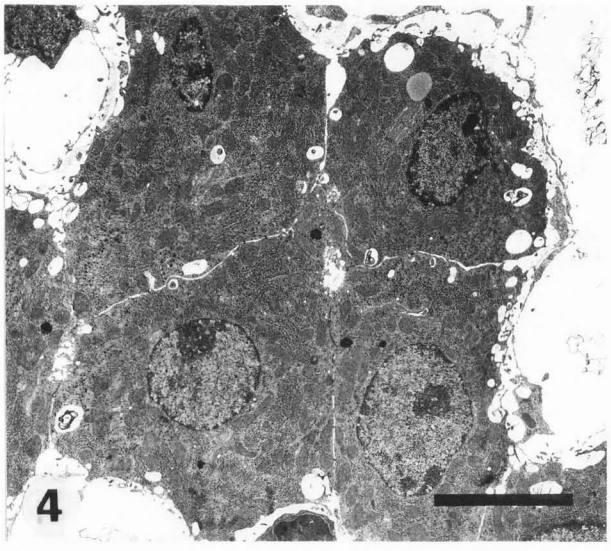
Anwer MS, Hondalus MK, Atkinson JM (1989) Ursodeoxycholate-induced changes in hepatic Na^+-H^+ exchange and biliary HCO_3^- secretion. Am J Physiol **257**, G371-G379.

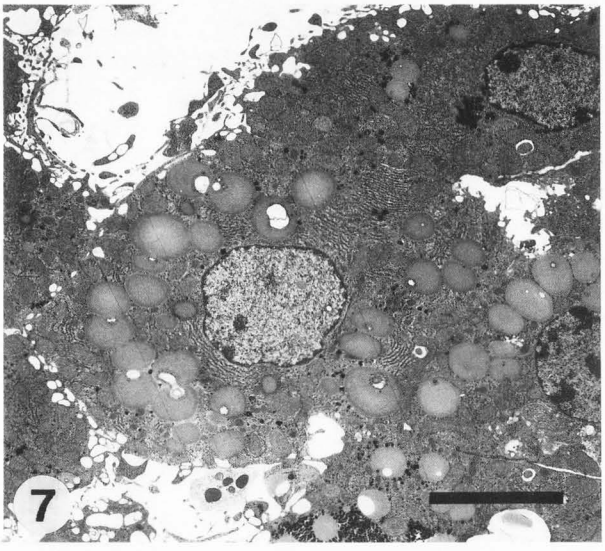
Avner DL, Randall GL, Berenson MM (1981) Protoporphyrin-induced cholestasis in the isolated in situ perfused rat liver. J Clin Invest **67**, 385-394.

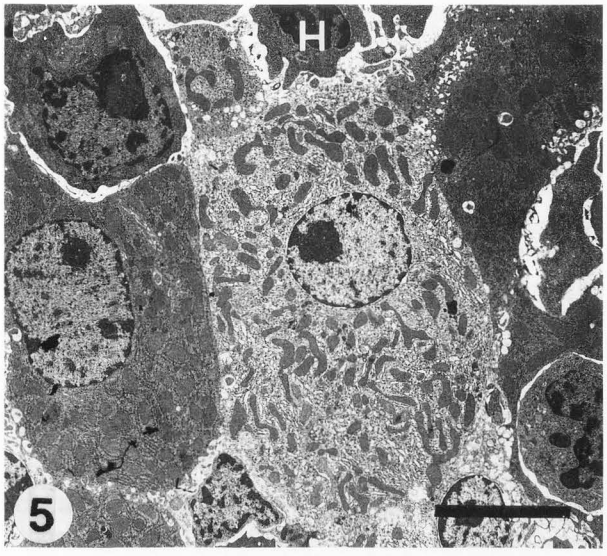
Ballatori N, Clarkson TW (1982) Developmental changes in the biliary excretion of methyl mercury and glutathione. Science **216**, 61-63.

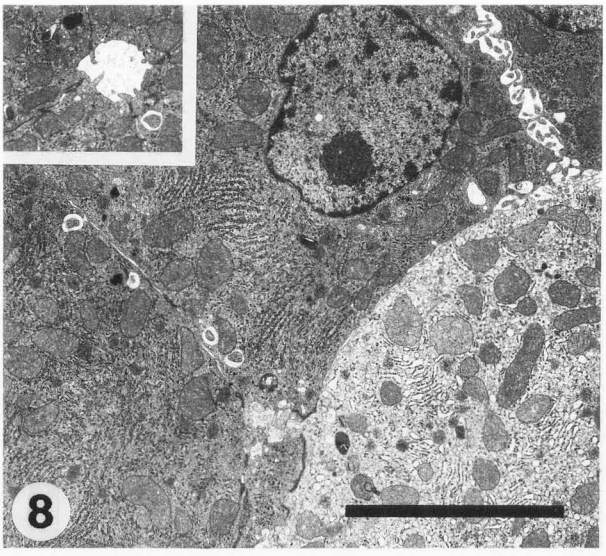

3


L
6


4


7


H
5


8

Boucher RC, Knowles MR, Cotton C, Stutts MJ, Yankaskas JR, Gatzy JT (1984) On a unified theory of cystic fibrosis lung disease. In: *Cystic Fibrosis: Horizons* (D Lawson, ed), Wiley, Chichester, pp 167-177.

Carpino F, Gaudio E, Marinozzi G, Melis M, Motta PM (1981) A scanning and transmission electron microscopic study of experimental extrahepatic cholestasis in the rat. J Submicrosc Cytol **13**, 581-598.

di Sant'Agnese PA, Hubbard VS (1984) The hepatobiliary system. In: *Cystic Fibrosis* (Taussig LM, ed) Thieme-Stratton, New York, pp 296-322.

Fitz JG, Persico M, Scharschmidt BF (1989) Electrophysiological evidence for Na^+-coupled bicarbonate transport in cultured rat hepatocytes. Am J Physiol **256**, G491-G500.

Keeffe EB, Blankenship NM, Scharschmidt BF (1980) Alterations of rat liver plasma membrane fluidity and ATPase activity by chlorpromazine hydrochloride and its metabolites. Gastroenterol **79**, 222-231.

Kuijpers GAJ, De Pont JJHHM (1987) Role of proton and bicarbonate transport in pancreatic cell function. Annu Rev Physiol **49**, 87-103.

Layden TJ, Boyer IL (1976) The effect of thyroid hormone on bile-salt independent flow and Na/K-ATPase activity in liver plasma membranes enriched in bile canaliculi. J Clin Invest **57**, 1009-1018.

McMillan EB, Roomans GM (1990) Techniques for X-ray microanalysis of intestinal epithelium using bulk specimens. Biomed Res **1**, 1-10.

Mörk AC, von Euler A, Roomans GM (1991) Effects of chronic amiloride treatment on epithelial cells determined by X-ray microanalysis. Biomed Res **2**, 182-190.

Park RW, Grand RJ (1981) Gastrointestinal manifestations of CF: a review. Gastroenterol **81**, 1143-1161.

Renner EL, Lake JR, Persico M, Scharschmidt BF (1989) Na^+-H^+-exchange activity in rat hepatocytes: role in regulation of intracellular pH. Am J Physiol **256**, G44-G52.

Riordan JR, Rommens JM, Kerem BS, Alon N, Rozmahel R, Grzelczak Z, Zielinski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS, Tsui LC (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science **245**, 1066-1073.

Roomans GM (1988) Quantitative X-ray microanalysis of biological specimens. J Electron Microsc Techn **9**, 19-44.

Sagström S, McMillan EB, Marijianowski M, Mulders H, Roomans GM (1990) Changes in rat and mouse salivary glands and pancreas after chronic treatment with diuretics: a potential animal model for cystic fibrosis. Scanning Microsc **4**, 161-170.

Sellinger M, Boyer JL (1990) Physiology of bile secretion and cholestasis. In: *Progress in Liver Diseases* (Popper H, Schaffner F, eds) WB Saunders, Philadelphia, vol 9, pp 237-259.

von Euler A, Mörk AC, Roomans GM (1992) Effects of chronic and *in utero* treatment with diuretics on mouse exocrine cells studied by X-ray microanalysis. J Submicrosc Cytol Pathol **24**, 225-230.

Weinman SA, Graf J, Boyer JL (1989) Voltage-driven taurocholate-dependent secretion in isolated hepatocyte couplets. Am J Physiol **256**, G826-G832.

Zimmerli B, Valantinas J, Meier PJ (1987) Multispecificity of Na^+ dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane (b1LPM) vesicles. Hepatology **7**, 41 (abstr).

Discussion with Reviewers

P.M. Motta: Comparing Figures 1 and 2, it is noted that in treated liver cells, besides an increase of lipid droplets, mitochondria and glycogen masses are also apparently increased. Further, the lipids in Fig. 2 have a different electron density compared to those in Fig. 1. In addition, they are closely assembled with mitochondria and glycogen masses. If these aspects have been commonly observed in this study, they might reflect rather significant alterations of the hepatocyte's structural and functional machinery. Further, looking collectively to Figs. 3 to 8 and making a general morphological evaluation, it is noted that dense liver cells appear somewhat more abundant in treated animals. The possibility cannot be ruled out that these structural changes reflect a different metabolism of the cells or may even be an expression of a cellular functional deficiency. Please comment.

I. Zs.-Nagy: It would be interesting to include data on the body weights of the embryos since the dose applied is around the 50% inhibition of DNA synthesis (Koch and Leffert, 1979).

Authors: It appears evident that chronic treatment with amiloride, and to a lesser extent furosemide, induces significant structural and functional changes both in the animal as a whole and in the liver. As an example, the weight of the amiloride-treated newborn animals was half of that of the control group. Since we were specifically interested in cystic fibrosis-related changes, we have not made a quantitative evaluation of the material with regard to the changes in lipid droplets, glycogen masses, and mitochondria. We have no doubt, however, that such an evaluation would show significant changes.

A. Warley: The results remind me of work carried out by a collegue of mine who showed that, in rabbit, tissues such as heart, brain, and liver were able to adapt to

K depletion. Do you think that in your system the liver is adapting to the presence of the drug?

Authors: In humans, resistance against diuretics is a well-known phenomenon (Beermann, 1982; Sjöström *et al.*, 1988). This is one possible mechanism by which the effects of chronic treatment with diuretics could be minimized. But we agree that it is possible that the cells may adapt to a different intracellular ionic environment.

Additional References

Beermann B (1982) Kinetics and dynamics of furosemide and slow-acting furosemide. Clin Pharmacol Ther **32**, 584-591.

Koch KS, Leffert HL (1979) Increased sodium ion influx is necessary to initiate rat hepatocyte proliferation. Cell **18**, 153-163.

Sjöström PA, Beermann BA, Odlind BG (1988) Delayed tolerance to furosemide diuresis. Influence of angiotensin converting enzyme inhibition by Lisinopril. Scand J Urol Nephrol **22**, 317-325.