Solvent isotope effect on bile formation in the rat

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^2H_2O affects many membrane transport processes by solvent and kinetic isotope effects. Since bile formation is a process of osmotic filtration where such effects could be important, we investigated the effects of ^2H_2O on bile formation in the *in situ* perfused rat liver. Dose finding experiments showed that at high concentrations, ^2H_2O increased vascular resistance and induced cholestasis; at 60% ^2H_2O however, a clear dissociation between the vascular and biliary effects was observed. Therefore, further experiments were carried out at this concentration. The main finding was a reduction in bile salt-independent bile flow from 0.99 ± 0.04 to 0.66 ± 0.04 $\mu l \cdot min^{-1} \cdot g^{-1}$ ($P < 0.001$). This was associated with a 40% reduction in biliary bicarbonate concentration ($P < 0.001$). Choleretic response to neither taurocholate nor ursodeoxycholate was altered by ^2H_2O; in particular, there was a similar stimulation of bicarbonate secretion by ursodeoxycholate in the presence of 60% ^2H_2O. To further elucidate this phenomenon, the effect of ^2H_2O on three proteins potentially involved in biliary bicarbonate secretion was studied *in vitro*. ^2H_2O slightly inhibited cytosolic carboanhydrase and leukocyte Na^+/H^+-exchange, these effects reached statistical significance at 100% ^2H_2O only, however. In contrast, Cl^-/HCO_3^--exchange in canalicular membrane vesicles was already inhibited by 50% ($P < 0.001$) at 60% ^2H_2O. Finally, there was a slight reduction in biliary glutathione secretion while that of the disulphide was not affected. Our results are compatible with an inhibition of canalicular Cl^-/HCO_3^--exchange by ^2H_2O. Whether this is due to altered hydration of the exchanger and/or of the transported bicarbonate remains to be determined.

INTRODUCTION

The formation of canalicular bile is a process of osmotic filtration driven by different ion transporters in the basolateral and apical membrane of the hepatocyte [1]. Two main processes are responsible for the canalicular formation of bile: bile salt secretion, leading to the so-called bile salt-dependent fraction of canalicular bile, and transport of different organic and inorganic anions, in particular bicarbonate [2] and glutathione and its disulphide [3] that contribute significantly to the elaboration of the so-called bile salt-independent fraction of bile. It is thought that a bicarbonate/chloride exchanger in the apical membrane is prominently involved in formation of bile salt-independent bile [4]. The separation into bile salt-dependent and -independent fractions is somewhat artificial however, since bile acids alter ion transport [5], in particular hypercholeretic bile acids such as ursodeoxycholate induce hypercholeresis, by formation of a bicarbonate-rich bile [6].

Water, which follows these actively secreted ions, leading to equilibration of the osmotic gradient created, is thought to enter the canaliculus para- and trans-cellularly [1]. Heavy water (^2H_2O) has been widely used to probe membrane transport processes, however, no use of its isotope effects have yet been made in studies of biliary physiology. Replacement of H_2O by ^2H_2O has two main effects, a solvent isotope effect and a kinetic isotope effect. The solvent effects are due to the greater viscosity, the higher melting-point and the greater heat capacity of ^2H_2O [7,8], which are due to the higher degree of structural order of ^2H_2O compared with H_2O. However, many important physical characteristics such as the dipole moment, dielectric constant, hydrogen bond length and molecular dimensions are quite similar [7,8]. The kinetic isotope effects are due to an exchange of H^+ for $^2H^+$ [8]. Thus, in plant cells, substitution of hydrogen by deuterium leads to membrane depolarization [9] and inhibits plasma membrane H^+-ATPase [10]. In animal cells, deuterium oxide slows the sodium pump by decreasing the apparent affinity of Na^+/K^+-ATPase for sodium but increasing it for potassium [11] apparently by stabilizing the E2 conformation [12]. It influences the conductance and the half-life of different ion channels [12–14] and can interfere with receptor function [15,16].

Since many of these processes, including Na^+/K^+-ATPase and ion channels are involved in bile formation, solvent effects were studied by substitution of H_2O by ^2H_2O in the *in situ* perfused rat liver.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats were obtained from the Deutsche Versuchstierfarm Hartmutt-Voss (Tuttlingen, Germany). Animals were housed in temperature- and humidity-controlled animal quarters on a 12 h light/dark cycle. They were allowed free access to pelleted rat diet and water. At the time of study, body and liver weight averaged 218 ± 15 and 10.3 ± 2.0 g respectively.

Deuterium oxide with a purity of 99.7% was obtained from the Paul Scherrer Institute (Würenlingen, Switzerland). ^{36}Cl was obtained from Amersham International (Amersham, Buckinghamshire, U.K.) as a 0.18 M HCl solution (sp.A. 145 μCi/mmol). Sodium taurocholate was obtained from Calbiochem (Läufelfingen, Switzerland) and ursodeoxycholic acid from Sigma Chemical Co. (Saint Louis, MO, U.S.A.). Fatty acid-free

Abbreviations used: ANOVA, analysis of variance; MIBA, methyl isobutyl amiloride; pH_i, intracellular pH; BCECF/AM, biscarboxyethyl carboxyfluoresceinacetoxymethyl ester.

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albumin and carbonic anhydrase from bovine erythrocytes were obtained from Boehringer (Mannheim, Germany). Biscarboxyethyl carboxyfluoresceinacetoxymethyl ester (BCECF/AM) and tissue culture (TC) medium 199 were obtained from Gibco BRL (Berne, Switzerland), methyl isobutyl amiloride (MIBA) was from Research Biochemicals Inc. (Natick, MA, U.S.A.) and nigericin and monensin from Serva Feinbiochemika GmbH (Heidelberg, Germany). All other reagents were of analytical grade from different commercial sources.

Methods

Rat liver perfusions

Perfusions were carried out *in situ* with a recirculating system using a pressure head, as previously described [17], with Krebs–Ringer bicarbonate buffer containing 2% (w/v) BSA and 0.1% (w/v) dextrose. All experiments were carried out after an equilibration period of 20 min. In a first set of experiments, dose–response curves with respect to haemodynamic characteristics and bile flow in perfusion media containing 100, 80, 60, 50 and 0% ^2H_2O were established. Because a clear dissociation between biliary and vascular effects was observed at 60% ^2H_2O, all further experiments were carried out at this concentration of ^2H_2O and compared with perfusions in a control medium of 100% H_2O.

Viability of the preparation was assessed by monitoring flow, pressure and release of ALT and K^+ into the perfusate. All experiments, except the dose–response studies, met the following criteria: portal pressure, < 11 cm H_2O; increase in perfusate K^+, < 0.2 mmol/l; and increase in perfusate ALT activity, < 20 i.u./l.

Extraction and biliary excretion of taurocholate were determined after intraportal injection of 0.5 μCi of [^{14}C]taurocholate by collecting hepatic venous outflow for 2 min and bile for 50 min following the injection. The choleretic effects of taurocholate were determined by using graded infusions (0.5–4 μmol/min per kg body weight) of sodium taurocholate into the portal vein. The effects of ursodeoxycholate were studied at one infusion rate only (8 $\mu mol \cdot min^{-1} \cdot kg^{-1}$). To determine the effect of ^2H_2O on the excretion of glutathione and its disulphide into bile, perfusions were carried out as described above: bile was collected into tubes containing 100 μl of 4% sulphosalicylic acid after an equilibration period of 30 min, then perfusate was switched to 60% ^2H_2O and another bile sample obtained after a further equilibration period of 30 min ($n = 4$); in control experiments ($n = 4$) perfusion was continued with normal perfusion medium.

Taurocholate uptake by isolated hepatocytes

Hepatocytes were isolated by collagenase perfusion [18]; only suspensions with a Trypan Blue exclusion of > 95% were employed. Hepatocytes were preincubated for 20 min at 37 °C in Krebs–Ringer bicarbonate buffer (pH/^{2}H 7.4), made up either with H_2O or 60% ^2H_2O. [^{3}H]Taurocholate uptake was determined at taurocholate concentrations ranging from 6.25 to 100 μmol/l by filtration on Whatman GF/D glass fibre filters as previously described [18]. At each concentration, five time-points at 12 s intervals were obtained. In agreement with previous studies uptake was linear over this time interval [18].

Carbonic anhydrase activity

Rat liver cytosol was prepared by differential centrifugation: livers were minced and homogenized in 4 vols. of ice-cold 5 mmol/l Tris/HCl buffer (pH 7.4) containing 0.25 mol/l sucrose and 1 mmol/l $MgCl_2$. The homogenate was centrifuged for 10 min at 10000 *g* and the resulting supernatant was centrifuged for 1 h at 105000 *g*. Carbonic anhydrase activity was determined in the supernatant according to Garcia-Marin et al. [19] in an assay buffer of 0.02 mol/l Tris/HCl made up in 0–100% ^2H_2O. The method had been validated by using dialysed and freeze-dried carbonic anhydrase from bovine erythrocytes.

Na^+/H^+-antiporter activity

Leukocytes from human blood, obtained from volunteers after an overnight fast, were isolated by dextran sedimentation [20]; after lysing remaining erythrocytes by hypo-osmotic shock, leukocytes were loaded with the pH-sensitive dye BCECF/AM by incubation in 10 μmol/l for 30 min at 37 °C. Then, the cells were washed twice in TC 199 medium and left at room temperature for 30 min. All measurements were made in duplicate in 0, 60 and 100% ^2H_2O media. Measurement of intracellular pH (pH_i) was performed in a buffer containing NaCl (140 mmol/l), KCl (5 mmol/l), $MgSO_4$ (0.8 mmol/l), $CaCl_2$ (1.8 mmol/l), glucose (5 mmol/l), Hepes (15 mmol/l) and fatty acid-free BSA (1 g/l) at pH 7.4 and 37 °C. Fluorescence was measured on a Perkin-Elmer luminescence spectrophotometer (LS 50) at excitation and emission wavelengths of 500 and 530 nm respectively. Excitation at 439 nm was used to correct the 500 nm readings for dye concentration, leakage and bleaching. To convert the fluorescence ratio 500:439 into pH units, leukocytes were suspended in KCl buffer (140 mmol/l) containing $CaCl_2$, $MgSO_4$, glucose and Hepes at the concentrations indicated above; intracellular and extracellular pH were equalized by addition of nigericin (2 μmol/l) and monensin (5 μmol/l) [20]. Buffer pH was changed by adding aliquots of Tris (1 mmol/l); pH was directly measured in the cuvette by a conventional pH electrode. Calibration curves were obtained from each cell preparation in each buffer (H_2O, 60% and 100% ^2H_2O). There was no difference in the calibration curves between the three media. Buffering power was determined by the alkaline shift in pH_i on the addition of 8 mmol/l ammonium sulphate.

Na^+/H^+-antiport activity was studied in leukocytes clamped to a pH_i of 6.5 in the KCl/monensin/nigericin buffer described above; thereafter, the cells were washed once in an identical buffer without the ionophores, but containing fatty acid-free BSA to remove any membrane-bound ionophore. On re-introduction of external Na^+, pH_i increased rapidly. About 95% of this alkalization could be blocked by MIBA (10 μmol/l). The rate of alkalization, dpH_i/dt, after adding external Na^+ was calculated from an linear regression analysis over the first 15 s after addition of external Na^+. The activity of the antiport was defined as the product of the MIBA-sensitive rate of change of pH_i on introduction of external Na^+ and the buffering power.

Na^+/H^+-antiport activity in hepatocytes, isolated by collagenase perfusion as described and referenced above, was performed essentially in the same fashion. The details and validation of the determination have recently been published [21].

Cl^-/HCO_3^--exchange activity

Canalicular liver plasma membrane vesicles were prepared and characterized as previously described [4]; vesicles were stored at −70 °C. Frozen vesicle suspensions were quickly thawed in a water-bath at 37 °C and revesiculated by ten passes through a 25-gauge needle; the suspension was washed in a buffer (pH 7.7) containing sucrose (150 mmol/l), tetramethylammonium glucon-

ate (100 mmol/l), calcium gluconate (0.2 mmol/l), magnesium gluconate (5 mmol/l), Tris and Hepes (each 35 mmol/l) and choline bicarbonate (50 mmol/l) by centrifugation for 1 h at 150000 *g*. The pellet was resuspended in the same buffer and diluted to the desired protein concentration (5 mg/ml). All buffers were made with H_2O, 60% or 100% ^2H_2O. The vesicles were revesiculated again by passing ten times through a 25-gauge needle. A 10 μl portion of this membrane vesicle suspension was incubated at 25 °C in 90 μl of incubation medium. To generate an outwardly directed HCO_3^- gradient, the incubation medium consisted of the gluconate salts at the concentrations indicated above, sucrose (184 mmol/l), Tris (30 mmol/l), Hepes (14 mmol/l) and Mes (90 mmol/l) set at pH 7.4. An outwardly directed pH gradient was generated by lowering the pH of the incubation buffer to 6.0. Chloride was added to all incubation media as a neutral TMA–$^{36}Cl^-$ solution at a final concentration of 5 mmol/l. The reaction mixture was gassed with 95% N_2 and 5% CO_2. After the indicated time intervals, $^{36}Cl^-$ uptake was stopped by the addition of 3.5 ml of ice-cold stop solution (204 mmol/l sucrose, 150 mmol/l potassium gluconate, 0.2 mmol/l calcium gluconate, 10 mmol/l Hepes/Tris, pH 7.5). Membrane vesicle-associated ^{36}Cl radioactivity was separated from free $^{36}Cl^-$ radioactivity by rapid filtration (1 ml/s) through a 0.65 μm cellulose nitrate filter (Sartorius, Göttingen, Germany) which had been presoaked in cold, de-ionized water. Then, the filters were washed twice with 3.5 ml of ice-cold stop solution. Non-specific binding to the filter and the membrane vesicles was determined in each experiment by addition of ice-cold stop solution to 10 μl of membrane suspension and 90 μl of incubation buffer kept at 0 °C. This filter blank was subtracted from all determinations.

Analytical techniques

The radioactivity of ^{36}Cl was measured by liquid scintillation counting using Filter Count (Packard, Groningen, The Netherlands) as scintillator, with quench correction carried out by an external standard. ^3H- and ^{14}C-radioactivity were similarly determined using Lumagel (Lumac/3M B. B., The Netherlands). Bile flow was determined gravimetrically, assuming a density of 1 g/ml. Bile salts in bile were determined photometrically by the 3-hydroxysteroid dehydrogenase method [22]. Sodium and potassium concentrations were determined by flame photometry (Model 143, Instrumentation Laboratory Inc., Lexington, MA, U.S.A.); biliary chloride and pCO_2 were determined by potentiometry (Chlor-O-Counter; Marius, Utrecht, The Netherlands) and microgasometry (Natelson model 650, Scientific Industries Inc., Queens Village, NY, U.S.A.) respectively. Biliary bicarbonate concentration was calculated by the Henderson–Hasselbach equation.

Alanine aminotransferase activity was assayed photometrically on an autoanalyser (Cobas Bio, Hoffmann-LaRoche, Basel, Switzerland) and protein concentrations according to Lowry et al. [23] using BSA as a standard. Total glutathione was determined enzymically according to Tietze [24]; glutathione disulphide was determined according to Griffith [25]. Reduced glutathione was calculated as the difference between the two. Owing to the low concentrations, only total glutathione was determined in perfusate. Glutathione release into perfusate was calculated as the product of flow rate and the difference between glutathione concentration in perfusate in- and out-flow.

Statistics

All data are expressed as mean ± 1 standard deviation (S.D.). Means of two groups were compared by Student's *t* test. Multiple means were compared by analysis of variance (ANOVA) followed by the Newman–Keuls test [26]. Linear regression analysis was performed by the method of least squares [26]; regression lines were compared by ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

The effect of increasing concentrations of ^2H_2O in perfused rat liver is shown in Figure 1. Beginning at 50% ^2H_2O, a decrease in bile flow could be observed, while portal resistance only began to increase at 80% ^2H_2O. This was due to both an increase in portal pressure and a decrease in perfusate flow. A clear dissociation between the biliary and vascular effects could be seen at 60% ^2H_2O, therefore, all further perfusion experiments were carried out at 60% ^2H_2O. The characteristics of perfusions under these experimental conditions are reported in Table 1; neither haemodynamics nor ALT and potassium release were affected, while bile flow was decreased by 60% ($P < 0.0001$).

The handling of taurocholate by the perfused rat liver and in isolated hepatocytes is reported in Table 2. In the perfused liver, both extraction and biliary excretion of a tracer dose of [^{14}C]taurocholate were slightly decreased in ^2H_2O medium; however, when excretion was expressed as a percentage of the

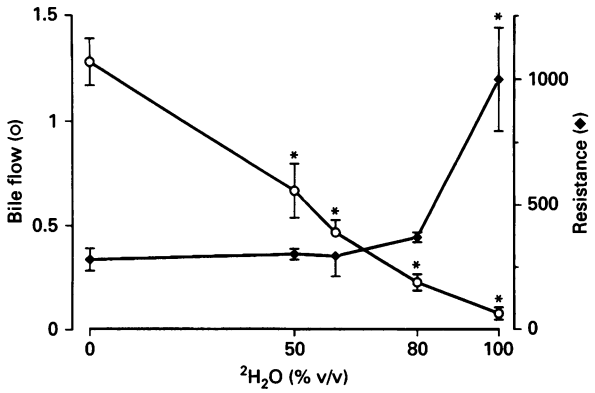

Figure 1 Effect of different ^2H_2O concentrations in the perfusate on bile flow (μl/min per g of liver) and vascular resistance (dyn·cm^{-5}·s^{-1}) in the *in situ* perfused rat liver

At 0% ^2H_2O ($n = 9$), at 50, 80 and 100% ($n = 3$) and at 60% ($n = 5$). * Indicates significant differences compared with 0% ^2H_2O by ANOVA. Vascular effects were seen at the highest concentration studied only, while there was cholestasis starting at the lowest concentration tested, namely 50% ^2H_2O. Therefore, all further experiments were carried out at 60% ^2H_2O.

Table 1 Characteristics of perfused rat liver perfused in normal (H_2O) or ^2H_2O (60% v/v) medium

Results are given as mean ± 1 S.D. Differences between the two groups were calculated by Student's *t* test. * $P < 0.0001$.

	H_2O ($n = 4$)	^2H_2O ($n = 5$)
Perfusion pressure (cm H_2O)	8.5 ± 0.5	9.5 ± 1.3
Perfusate flow (ml/min)	33.1 ± 7.4	32.8 ± 4.8
ALT release (i.u./l)	< 5	< 3
K^+ release (mmol/l)	< 0.2	< 0.2
Bile flow (μl·min^{-1}·g^{-1})	1.2 ± 0.2	0.5 ± 0.1*

Table 2 Handling of [^{14}C]taurocholate by the perfused rat liver and isolated hepatocytes

Results given as mean ± 1 S.D. Differences between the two groups were calculated by Student's *t* test. * $P < 0.05$.

	H_2O	^2H_2O
Perfused liver	($n = 4$)	($n = 5$)
Extraction (% of dose)	93 ± 2	82 ± 8*
Excretion (% of dose)	82 ± 3	73 ± 14
Excretion (% of uptake)	89 ± 3	88 ± 8
Isolated hepatocytes	($n = 6$)	($n = 6$)
$V_{max.}$ (nmol/min^{-1} per 10^6 cells)	2.5 ± 0.2	2.8 ± 0.6
K_m (μmol/l)	44 ± 10	52 ± 1

Table 3 Effect of ursodeoxycholate (UDCA: 8 μmol · min^{-1} · kg^{-1}) on bile flow and composition in livers perfused with H_2O and 60% ^2H_2O

Results are given as mean ± 1 S.D. ($n = 4$ in each group). Bile flow is given as μl/min per gram of liver and bile acid and electrolyte concentrations in mmol/l. Differences were evaluated by two-way ANOVA followed by the Newman–Keuls test. * $P < 0.05$ compared with control experiment in H_2O; ** $P < 0.05$ compared with basal condition in same medium.

	H_2O Basal	H_2O UDCA	^2H_2O Basal	^2H_2O UDCA
Bile flow	1.0 ± 0.1	2.0 ± 0.3**	0.4 ± 0.1*	1.4 ± 0.2**
[Bile acids]	3 ± 1.2	29 ± 7**	8 ± 2	30 ± 1**
[Na^+]	143 ± 5	156 ± 8	141 ± 8	151 ± 7
[K^+]	5.7 ± 0.3	6.4 ± 0.2**	5.8 ± 0.5	6.4 ± 0.4**
[Cl^-]	114 ± 4	95 ± 2**	99 ± 2*	87 ± 6*,**
[HCO_3^-]	20 ± 1	30 ± 2**	12 ± 1*	23 ± 0*,**

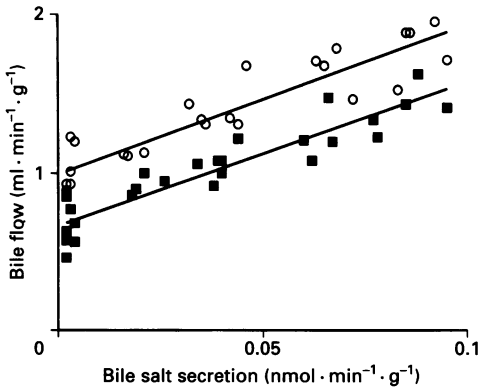

Figure 2 Relationship between bile flow (ml · min^{-1} · g^{-1}) and bile salt secretion (nmol · min^{-1} · g^{-1}) in rat livers perfused with medium made up in 100% H_2O (○) or 60% ^2H_2O (■)

Each point represents the mean of a triplicate determination; $n = 7$ perfusion experiments. Bile flow was linearly related to bile salt excretion in both media. The equations describing the relationship were $y = 0.99 + 9.50x$ ($r = 0.920$; $P < 0.001$) and $y = 0.66 + 9.19x$ ($r = 0.927$; $P < 0.001$) in H_2O and in 60% ^2H_2O respectively. The slopes were not significantly different while the intercept was shifted downwards ($P < 0.001$) in ^2H_2O medium, suggesting a decrease in the bile salt-independent fraction of bile.

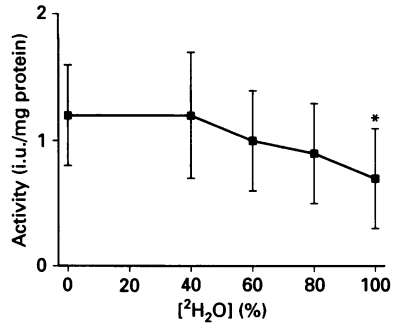

Figure 3 Effect of ^2H_2O on carbonic anhydrase activity (expressed in i.u./mg protein) in rat liver cytosol

A dose-dependent inhibition was observed ($P < 0.02$ by ANOVA); however, at 60% ^2H_2O, the concentration used in the perfused organ, this inhibition amounted to only 16% and was not statistically significant (Newman–Keuls test). The only statistically significant inhibition was observed at 100% ^2H_2O (* $P < 0.02$ by Newman–Keuls test). Results are given as mean ± 1 S.D.

dose taken up, there was no difference between the two media. Because of this reduction in extraction, taurocholate uptake was also studied in isolated hepatocytes. ^2H_2O did not affect hepatocyte viability as judged by Trypan Blue exclusion. Taurocholate uptake was saturable in both media and neither apparent maximal uptake velocity ($V_{max.}$) nor apparent affinity (K_m) were affected by 60% ^2H_2O (Table 2).

Infusing increasing amounts (0.5–4 μmol · min^{-1} · kg^{-1}) of taurocholate induced an increase in bile flow in both media (Figure 2). Bile flow was linearly related to bile salt excretion under both experimental conditions. The slope of the regression line, an expression of the choleretic activity of taurocholate, was not affected by ^2H_2O (9.50 ± 0.83 versus 9.19 ± 0.76 μl/μmol in H_2O and ^2H_2O respectively). The downward parallel shift of the regression line describing the ^2H_2O experiments suggested a decrease in the bile salt-independent fraction of bile by 60% ^2H_2O; indeed, the intercept was significantly reduced in ^2H_2O-perfused livers (0.99 ± 0.04 versus 0.66 ± 0.04 μl · min^{-1} · g^{-1}; $P < 0.001$).

The effect of ursodeoxycholate infusion (8 μmol · min^{-1} · kg^{-1}) on bile flow and biliary electrolytes is reported in Table 3. Again, bile flow was significantly reduced under basal conditions in the presence of 60% ^2H_2O; this was associated with a marked (40%) decrease in biliary bicarbonate and a lesser (15%) decrease in biliary chloride concentration. Ursodeoxycholate infusion induced a marked choleresis in both media; this appeared even more pronounced in ^2H_2O when compared with the corresponding basal bile flow. The choleretic property of ursodeoxycholate (calculated as the increment in bile flow divided by the increment in bile acid excretion) averaged 18 ± 5 and 23 ± 4 μl/μmol in H_2O and ^2H_2O respectively (results not shown). The corresponding figures for the HCO_3 stimulatory activity were 0.73 ± 0.21 and 0.64 ± 0.08 μmol/μmol.

To further investigate the reduced biliary bicarbonate concentration induced by perfusion with ^2H_2O, potential steps of bicarbonate secretion, namely the activity of carbonic anhydrase, the Na^+/H^+-antiporter and the Cl^-/HCO_3^--exchanger were investigated.

^2H_2O inhibited rat liver cytosol carbonic anhydrase in a dose-dependent fashion (Figure 3). However, at 100%, an inhibition of only 42% was observed. At 60% ^2H_2O, the concentration used in the perfusion experiments, inhibition was only 16% and not statistically significant.

The resting pH_i of leukocytes was slightly higher in cells

Table 4 Effect of ^2H_2O on Na^+/H^+-antiporter characteristics in human leukocytes

Results are given as mean ± 1 S.D. Na^+/H^+-exchange and H^+-efflux are given as the MIBA-sensitive part; for further details see text. Differences between the groups were calculated by ANOVA, followed by the Newman–Keuls test. * $P < 0.05$ compared with 0% ^2H_2O.

	^2H_2O (% v/v)		
	0	60	100
pH_i	7.56 ± 0.06	7.53 ± 0.07	7.78 ± 0.02
Buffering power (mmol/l per pH unit)	37.2 ± 7.7	46.4 ± 6.3	43.5 ± 8.3
Na^+/H^+-exchange (pH/min)	0.67 ± 0.06	0.58 ± 0.08	0.38 ± 0.15*
H^+-efflux (mmol · min^{-1} · l^{-1})	25.2 ± 7.7	27.2 ± 6.9	17.2 ± 8.8

Table 5 Effect of ^2H_2O on Na^+/H^+-antiporter characteristics in rat hepatocytes

The initial (first 30 s) rate of pH_i recovery from an acid load of 20 mmol/l NH_4Cl (dpH_i/dt) observed in Na^+-containing medium, in Na^+-free medium and in the presence of 10 μmol/l methyl isobutyl amiloride (MIBA). Na^+/H^+ exchange is calculated as the MIBA-sensitive and Na^+-dependent part. pH_i represents the starting pH_i achieved by acid loading. Results are given as mean ± 1 S.D. Differences between the groups were calculated by ANOVA; none of the effects were statistically significant.

	^2H_2O (% v/v)		
	0	60	100
pH_i	6.78 ± 0.31	6.79 ± 0.20	6.74 ± 0.16
dpH_i/dt Na^+ (pH/min)	0.16 ± 0.09	0.15 ± 0.06	0.14 ± 0.07
dpH_i/dt Na^+-free (pH/min)	0.04 ± 0.08	0.06 ± 0.03	0.05 ± 0.05
dpH_i/dt MIBA (pH/min)	0.07 ± 0.08	0.08 ± 0.02	0.07 ± 0.04
Na^+/H^+-exchange (pH/min)	0.12 ± 0.04	0.08 ± 0.04	0.08 ± 0.03

Table 6 Effect of ^2H_2O on $^{36}Cl^-$ uptake, expressed in nmol/mg, by canalicular liver plasma-membrane vesicles in the absence and presence of a pH gradient

Results are given as mean ± 1 S.D.; differences were evaluated by ANOVA followed by the Newman–Keuls test. * $P < 0.05$ compared with 0% H_2O.

	^2H_2O (% v/v)		
	0	60	100
No pH gradient 1 min	4.15 ± 1.36	2.51 ± 0.63*	2.43 ± 0.63*
pH gradient 1 min	11.59 ± 2.98	6.67 ± 2.04*	5.55 ± 1.20*
No pH gradient 3 h	8.67 ± 5.31	5.86 ± 2.57	6.26 ± 2.87
pH gradient 3 h	13.32 ± 9.07	9.12 ± 5.56	6.90 ± 3.32

resuspended in 100% ^2H_2O medium compared with H_2O medium (Table 4); this difference, however, was not statistically significant. The buffering power of the resting cells was similar in the three groups. When the activity of the Na^+/H^+-antiport was studied by acid loading (pH_i 6.5), no effects at 60% ^2H_2O were observed. At 100% ^2H_2O, the MIBA-sensitive exchange was significantly reduced. When proton fluxes were calculated (buffering power × initial MIBA-sensitive rate of alkalization), however, there was no difference between the three experimental conditions (Table 4). Similar experiments, performed in isolated hepatocytes, showed no effect of ^2H_2O on Na^+/H^+-exchange activity or on dpH_i/dt at any of the conditions studied (Table 5).

The effects of ^2H_2O on HCO_3^-/Cl^--exchange in canalicular plasma membrane vesicles are reported in Table 6. Initial Cl^- uptake in the presence of a pH gradient was reduced by 40% at 60% ^2H_2O; there was no further inhibition at 100% ^2H_2O. In the absence of a pH gradient this reduction was also present. The activity of the Cl^-/HCO_3^--exchanger (the difference between Cl^- uptake in the presence and absence of a pH gradient) is shown in Figure 4. Again, there was already marked inhibition (about 50%) in the presence of 60% ^2H_2O, suggesting that ^2H_2O interferes with HCO_3^-/Cl^- exchange. This was not due to an unspecific membrane effect of ^2H_2O, since under equilibrium conditions at 180 min no difference between the six experimental conditions could be observed by two-way (ANOVA) (Table 6).

Finally, the effect of perfusion with 60% ^2H_2O on biliary excretion of glutathione and its disulphide was investigated

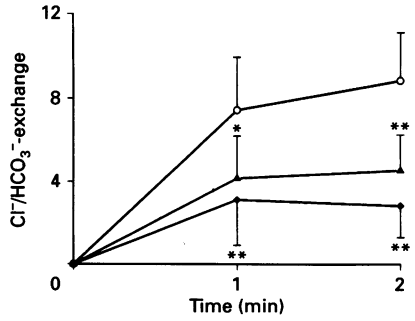

Figure 4 Chloride/bicarbonate exchange (nmol $^{36}Cl^-$ per mg of protein) in canalicular liver plasma membrane vesicles in H_2O (○), 60% ^2H_2O (▲) and 100% ^2H_2O (◆)

Results are given as mean ± 1 S.D. ($n = 4$ preparations) each assay performed in triplicate. ^2H_2O at both concentrations significantly (* $P < 0.03$; ** $P < 0.005$) inhibited chloride/bicarbonate exchange. Differences were evaluated by two-way ANOVA followed by the Newman–Keuls test to localize significant differences.

(Table 7). Perfusion with ^2H_2O induced a slight reduction in biliary concentration of glutathione but not of the disulphide; a similar reduction was seen in biliary glutathione excretion but this failed to reach statistical significance (Table 7). Glutathione release into perfusate averaged 119 ± 33 and 76 ± 40 nmol/min in

Table 7 Effect of ^2H_2O on biliary concentration (mmol/l) and excretion (nmol/min per gram of liver) of glutathione (GSH) and its disulphide (GSSG) in the *in situ* perfused rat liver; both are given in mmol/l

Under basal conditions, normal perfusate was used; then (experimental), perfusate was switched to 60% ^2H_2O or continued with normal perfusate. Results are given as mean ± 1 S.D.; differences were evaluated by ANOVA followed by the Newman–Keuls test. * Significant effect by ANOVA ($P < 0.02$); the effect of ^2H_2O perfusion reached borderline significance only ($P < 0.10$).

	GSH		GSSG	
	H_2O	^2H_2O	H_2O	^2H_2O
Concentration				
Basal	1.91 ± 1.01	1.40 ± 0.39	1.45 ± 0.19	1.55 ± 0.37
Experimental	1.73 ± 0.29	0.93 ± 0.34*	1.71 ± 0.24	1.37 ± 0.27
Excretion				
Basal	1.14 ± 1.00	1.01 ± 0.52	1.07 ± 0.49	1.01 ± 0.32
Experimental	1.20 ± 0.40	0.57 ± 0.13	1.11 ± 0.42	0.87 ± 0.25

the two groups under basal conditions; after switching perfusate, this remained unchanged at 95 ± 22 in the H_2O perfused livers but decreased to 12 ± 12 nmol/min in the presence of 60% ^2H_2O ($P < 0.05$).

DISCUSSION

The present study demonstrates that ^2H_2O induces cholestasis by inhibiting the bile salt-independent fraction of canalicular bile. This is achieved mainly by inhibiting biliary bicarbonate secretion. Analysis of different ion pumps potentially involved in the elaboration of bicarbonate secretion demonstrated that ^2H_2O selectively interferes with the canalicular Cl^-/HCO_3^--exchanger; in contrast, hepatic cytosolic carbonic anhydrase and the Na^+/H^+-exchange in leukocytes or isolated hepatocytes were not affected by ^2H_2O. The inhibition of bicarbonate secretion appeared to be specific for hepatocytes, since the presumably ductular hypercholeresis [27] induced by ursodeoxycholate was not affected. In addition, it is conceivable that a slight reduction in biliary glutathione, but not glutathione disulphide, secretion contributed to the observed phenomenon.

Dose–response studies demonstrated that ^2H_2O inhibited bile formation before vascular phenomena occurred (Figure 1). The mechanism of the latter remains unclear but could be related to interactions of ^2H_2O with the cytoskeleton of endothelial cells, since at least in leukocytes ^2H_2O at high concentrations leads to reversible contraction of actin [28]; a decrease of hepatic perfusion induces cholestasis in the perfused liver [29]. This mechanism is unlikely to have contributed to the decrease in bile flow in the present study since at the concentration selected no vascular effects were apparent.

At high concentrations, ^2H_2O depletes ATP content of the heart, presumably as a consequence of increased energy requirement [30]. Although we have not measured ATP content this is unlikely to have contributed to the observed phenomena, since bile salt uptake, which is a process dependent upon the ATP-mediated sodium gradient [31,32], was not affected by our experimental conditions (Table 2). Similarly, the inhibition of Na^+/K^+-ATPase observed at 100% ^2H_2O [11] appears not to occur at the present concentration since taurocholate uptake remained unaffected.

The main effect of ^2H_2O in the present experiments was on bile salt-independent bicarbonate secretion, a major component of the bile salt-independent fraction of bile [2]. We therefore investigated several potential steps in bicarbonate delivery to the canaliculus.

Activity of cytosolic carbonic anhydrase was modestly decreased at 100% ^2H_2O only (Figure 3); this has been initially described by Pocker and Bjorquist [33] who ascribed it to an alteration of intramolecular proton transfer. It appears unlikely that inhibition of carbonic anhydrase activity is responsible for the decrease in basal bicarbonate secretion since at 60% the effect was not statistically significant. However, it has to be pointed out that we studied the cytosolic form of the enzyme. A membrane-bound form has been postulated to exist in liver [19] and it is conceivable that the membrane-bound enzyme reacts differently than the cytosolic form. Thus, cytosolic and membrane-bound carbonic anhydrase differ in their sensitivity to inhibition by sulphonamide derivatives [34] and the membrane-bound form is able to form ion channels when incorporated into lipid bilayers [35].

The main role of the Na^+/H^+-antiporter, localized at the basolateral membrane of the hepatocyte [36], is regulation of pH_i [37,38]; it may also be involved in volume regulation [39]. A potential role for this antiporter in the hypercholeresis induced by ursodeoxycholate has been invoked [40], but this phenomenon is thought to reflect secondary activation since ursodeoxycholate does not directly influence its activity [41]. Activation via phosphorylation results from an increased affinity for H^+ at a cytoplasmic site [42]. Only at 100% ^2H_2O was there a slight inhibition of activity in leukocytes but not in hepatocytes, suggesting that this ion transporter is not involved in cholestasis induced by ^2H_2O. Since the set-point of the antiporter is rectified by phosphorylation [42] and the observed pH_i was not affected by ^2H_2O (Tables 4 and 5), changes in phosphorylation can be ruled out in the present experiments.

Canalicular Cl^-/HCO_3^- exchange is thought to play a role in bile acid-independent bile formation [43,44]; indeed, a Cl^-/HCO_3^- exchanger has been described in canalicular plasma-membrane fraction [4]. Aside from its putative role in bile formation, this exchanger is also involved in pH regulation [45]. In contrast to carbonic anhydrase and Na^+/H^+-exchange activity, ^2H_2O already inhibited Cl^-/HCO_3^--exchange at 60%, the concentration used in the whole organ studies (Table 6, Figure 4); at this concentration, the initial uptake rate of chloride was reduced by almost 50%.

The mechanism of ^2H_2O-induced inhibition of Cl^-/HCO_3^--exchange remains unclear. It appears unlikely that this is due to alterations of the lipid membrane since detailed analysis of model membranes failed to detect any alteration of the dielectric structure [46]. Another possibility is increased solvent viscosity, since according to the Nernst–Planck equation for electrodiffusion, ion flux is directly proportional to the mobility, which in turn is inversely proportional to the viscosity of the solution. This should also affect other membrane transport processes such as taurocholate uptake or Na^+/H^+-exchange, which were not observed in the present study however (Tables 2, 4 and 5). Deuteration of hydrogen-bonding sites can affect the conformation of proteins [13], although such changes are usually very small and cannot be detected by physical techniques they may have large functional effects by altering the probability of the open state of an ion channel [13,47]. Since the Na^+/H^+- and the Cl^-/HCO_3^--exchangers share large hydrophobic and hydrophilic regions [42,48] it appears unlikely, but not impossible, that altered hydration of a crucial region is responsible for the inhibition of Cl^-/HCO_3^--exchange. The most likely explanation for the inhibition of Cl^-/HCO_3^--exchange by deuterium oxide is altered hydration of the bicarbonate molecule itself. This contention is supported by the marked inhibition of the highly

selective gap junction aqueous channel by ^2H_2O [49,50]. This would also explain the differential effects of ^2H_2O on the Na^+/H^+-antiporter and the Cl^-/HCO_3^--exchanger since HCO_3^- is larger than the other involved ions and has a larger hydration shell.

Our data are not compatible with altered permeability of ^2H_2O through biomembranes. Reports on the diffusion coefficient of ^2H_2O through biological membranes are controversial [8,47]. The present finding of unaltered osmotic response to taurocholate and ursodeoxycholate suggest that in the liver, diffusion of ^2H_2O, at least in the reduced concentration used, is not affected. The finding that ursodeoxycholate-induced bicarbonate secretion is not affected, in contrast to basal bicarbonate secretion, lends further support to the cholehepatic shunt hypothesis [27].

Alternative effects of ^2H_2O, in particular, on cytoskeletal elements such as microtubules [51] or microfilaments [28] could also contribute to cholestasis induced by ^2H_2O, since both microtubules and microfilaments are involved in certain aspects of bile formation. However, this would not explain the selective decrease in HCO_3^--excretion observed in the present study since neither microtubule nor microfilament inhibitors selectively affect the bile salt-independent HCO_3^--excretion [1]. Anions other than bile acids and bicarbonate account for about 20% of the bile salt-independent fraction of bile [52]; recently, Ballatori and Truong [3] have found this to be related in part to the excretion of glutathione, glutathione disulphide and its degradation products. We found a reduction in biliary glutathione but not glutathione disulphide concentration in livers perfused with 60% ^2H_2O (Table 7). This seems to be mainly related to a decreased rate of synthesis of glutathione in view of the fact that release of total glutathione into the perfusate was also reduced. However, other mechanisms cannot be excluded at present. Whatever the mechanism, it appears unlikely that it contributes markedly to the cholestasis observed in the present experiments since the magnitude of glutathione-related bile flow is 20% at best [52].

In conclusion, ^2H_2O is cholestatic in the perfused liver. It inhibits the bile salt-independent fraction of bile flow by reducing the basal, but not the ursodeoxycholate-induced, bicarbonate secretion. This is mainly due to inhibition of the canalicular Cl^-/HCO_3^--exchanger; whether this is due to conformational changes of the exchanger and/or the increased hydration shell of HCO_3^- ions remains to be determined.

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