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HETEROGENEITY OF RABBIT HEPATOCYTES FOR BILE SECRETION AFTER ACINAR ZONE 3 DAMAGE INDUCED BY BROMOBENZENE

EFFECT OF BILIRUBIN AND BILE SALT INFUSIONS

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Abstract—Anaesthetized rabbits were used to study the effect of bromobenzene-induced hepatic damage to the acinar zone 3 on bile flow, bile salt, sodium secretion as well as bilirubin transport in basal conditions or with infusion of sodium glycodeoxycholate. The bromobenzene-pretreated animals exhibited in basal conditions a lower bile flow (44%) than that of the controls, with a smaller decrease in bile salt output (27%) and sodium output (29%), whereas no modification in endogenous bilirubin excretion was observed. The bile salt independent fraction of secretion (BSIF) was reduced significantly after the toxic lesion both in terms of absolute and relative values. The hepatocytes of the periportal zone were capable of excreting the totality of bilirubin presented to the liver, regardless of the extent of bile flow or the input of bile salts. The infusion of bilirubin at 1.0 μmole/kg/min led to a fall in bile flow which was attributed to the interference of the pigment with the BSIF. The maximal bilirubin excretion was significantly smaller in bromobenzene-pretreated animals than in the controls, which could be due to the incapacity of the intoxicated rabbits to recruit quiescent hepatocytes. When glycodeoxycholate was administered under conditions of maximal bilirubin transport, bile flow increased as did bile salt secretion in both controls and animals with damaged livers. However, clear differences persisted between the two, which could be attributed not only to the volume fraction of necrosis but also to an interference by bilirubin with the hepatic handling of bile salts. Maximal bilirubin excretion increased in a similar way in both groups after glycodeoxycholate administration. It is proposed that glycodeoxycholate infusion facilitates the hepatic depletion of bilirubin, probably by stimulating transport processes.

The microcirculatory unit of the liver or hepatic acinus is divided into different zones according to the direction of the blood supply. The periportal hepatocytes which are the first to be exposed to the incoming blood constitute the acinar zone 1 while the hepatocytes situated closer to the central vein make up the acinar zone 3 [1]. These zones seem to be heterogeneous regarding their contribution to different hepatic functions, not only due to their localization with respect to the arrival of blood but also, apparently, to intrinsic cellular differences [2]. The relative contribution to the transport of different exogenous compounds and constituents of bile has been studied after selective acinar lesions of acinar zones 1 and 3 [3–8]. Among the toxic substances used to induce such lesions is bromobenzene [4, 5], a compound which is metabolized in the liver and leads to the formation of an epoxide capable of causing selective necrosis in the acinar zone 3 by altering sulfhydryl groups and other nucleophilic sites of hepatic proteins [9, 10].

In the present study, bromobenzene was used to observe the heterogeneity of the hepatocytes regarding the transport of bile salts, sodium and bilirubin, this latter both at physiological levels and when its maximal excretion in bile is reached. An attempt is also made to study the influence of selective acinar zone 3 damage on the stimulating effect in the excretion of bile pigments exerted by bile salts [11, 12]. Among other possibilities, this effect has been attributed to an increase in the number of cells participating in the transport of such anions [11].

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MATERIALS AND METHODS

Animals and drug treatment. Male and female albino New Zealand rabbits weighing between 2.5 and 3.5 kg were used. Bromobenzene (Aldrich Chemical Co., Milwaukee, WI) dissolved in corn oil (4.0 mmole/kg) was injected intraperitoneally. Control rabbits received corn oil (2 ml/kg, i.p.).

Experimental procedure. Food but not water was withheld for 24 hr before experiments. These were carried out 48 hr after the administration of bromobenzene or corn oil [4]. The animals were anaesthetized through a lateral ear vein with sodium pentobarbitone (Nembutal®, Abbott Laboratories; 40 mg/kg). After inserting a tracheal cannula, a femoral vein was catheterized for infusions. The liver was exposed through a midline incision and the cystic duct ligated. The common bile duct was cannulated with polyethylene tubing (Portex Poly 52, i.d. 1.0 mm). Body temperature was maintained in the range 38–39° by supplemental heating. Bile was collected in preweighed tubes under ice and protected from light to avoid bilirubin decomposition. Bilirubin

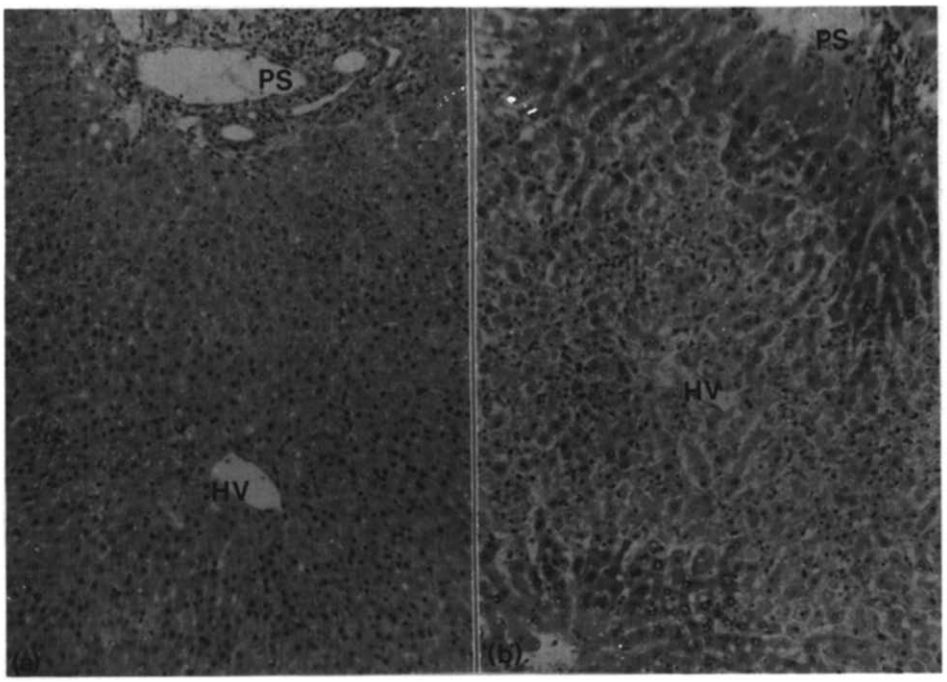

Fig. 1. Histological sections of the liver (H&E × 100). (a) Control liver. (b) 48 hr after bromobenzene pretreatment. PS = portal space. HV = hepatic venule.

analyses were performed within 48 hr of collection.

After an equilibration period of thirty minutes, bile was collected for 3 hr in 15 min samples. Four groups of experiments, involving five animals each, were carried out: CI, CII, BzI and BzII. Group CI received no infusion. Group CII, after 1 hr of basal collection of bile, was infused with bilirubin at 1.0 μmole/kg/min for two hours. This infusion rate was chosen to give maximal hepatic excretion of the pigment [13]. During the second hour of bilirubin infusion, an infusion of sodium glycodeoxycholate was added at 1.6 μmole/kg/min. The other two groups, BzI and BzII, were bromobenzene-pretreated animals with the same protocol as the controls CI and CII, respectively.

Preparation of solutions. Bilirubin (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.1 N NaOH and the pH adjusted to 8.0 with 1.2 N ClH. The solution was prepared immediately prior to experiments and protected from light during preparation. Sodium glycodeoxycholate (Sigma) was dissolved in saline (0.9% NaCl w/v) and the pH adjusted to 7.4 with 0.1 N buffer phosphate; bovine albumin was then added to a final concentration of 4% (w/v) to prevent haemolysis.

Analytical methods. The bile acid concentration in bile was determined by an enzymatic technique [14] using 3α hydroxysteroid dehydrogenase (grade III, Sigma Chemical Co, St. Louis, MO). Biliary phospholipids were also estimated enzymatically [15]. Total bilirubin in bile was measured by the diazo coupling method of Michaelsson [16] using dyphylline activation. Sodium in bile was estimated by flame photometry.

Histological assessment of damage. The livers were removed, weighed and fixed in Heidenhain's SUSA fluid. Paraffin sections were stained by hematoxilin and eosin. Morphological damage induced by bromobenzene was quantitated by the method of Chalkey [17].

Statistical analysis. The means ($\bar{x} \pm$ S.E.M.) were calculated and the significances were assessed using Student's *t*-test. Linear regression analyses were performed using the least squares method.

RESULTS

Histological changes in bromobenzene-treated livers

The two photomicrographs shown in Fig. 1 correspond to the livers of a control animal and another rabbit treated with bromobenzene. The volume fraction of necrosis was 29 ± 6% ($n = 10$), hepatic damage being mainly observed in acinar zone 3 where swollen ballooned cells could be clearly observed, although in some sections the extent of the lesions was wider (Fig. 1).

Treatment with bromobenzene and biliary secretion (groups CI and BzI)

The bile flow registered over the three hours of the experiments was significantly lower in bromobenzene-pretreated animals (BzI) than in the controls (CI) (Fig. 2). In both experimental groups it was possible to detect a progressive decrease in flow which occurred more rapidly and markedly in the BzI group and was not statistically significant until the third hour of assays.

The BzI rabbits exhibited a bile salt output which was significantly lower than that of the CI group throughout the experiments. The secretion pattern in time was very similar, both qualitatively and quantitatively, in the two groups (Fig. 2). The con-

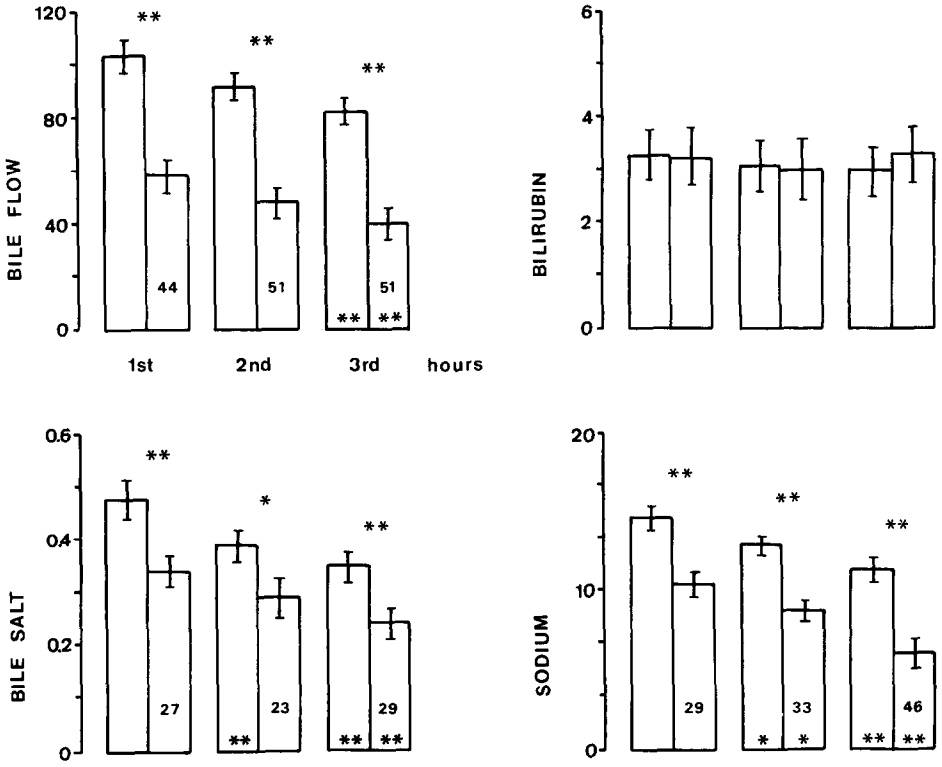

Fig. 2. Variations in bile flow (μl/kg/min) and bilirubin (nmole/kg/min), bile salt (μmole/kg/min) or sodium (μeq/kg/min) output in groups CI (first bar of each pair) and BzI (second bar). Values are means ± S.E.M. of five animals. *P < 0.01; **P < 0.001. Significantly different from group CI values (over bars) or from the first hour of the same experimental group (inside bars). Numbers inside bars indicate percentage difference from CI values.

centration of bile salts was greater in the BzI group than in the controls, a difference which was enhanced as time progressed, since whereas in the BzI group it underwent very little change during the 3 hr of assays (6.2 ± 0.7, 6.4 ± 0.6 and 6.0 ± 0.8 mmole/l), in the CI group it decreased slightly (4.8 ± 0.4, 4.2 ± 0.6 and 4.1 ± 0.5 mmole/l).

Biliary phospholipid secretion during the first hour was less in the BzI group (44 ± 2 nmole/kg/min) than in the CI group (49 ± 5 nmole/kg/min), though the differences were not significant. These changes were very similar to those observed for the bile salts, with a decrease of 34% in the BzI animals and a decrease of 29% in the CI group after the three hours of experiments. The phospholipid concentration in the BzI group was 58% higher, compared with the CI animals during the first hour and 75% higher in the third hour.

Similar to what was observed for the bile salts, the sodium output was less in the BzI animals than in the CI group, in the three periods studied. However, sodium excretion decreased more in the BzI group (41%) than in the CI group (22%) (Fig. 2). Similarly, the sodium concentration was greater in the bile of the BzI animals than in the CI group. In addition, the sodium concentration in the CI group remained unaltered (141 ± 10, 145 ± 9 and 143 ± 10 meq/l), while in the BzI group it decreased significantly (178 ± 12, 173 ± 10 and 152 ± 11 meq/l; $P < 0.005$ from the first to the third hour).

Bilirubin excretion was very similar in both experimental groups, showing small variations within each group of animals (Fig. 2). The concentration of the pigment was significantly higher ($P < 0.001$) in the BzI group than in the CI animals. This became more evident as time progressed: while in the case of the CI group it was only slightly modified (33 ± 3, 35 ± 3 and 36 ± 4 μmole/l), it clearly rose in the BzI animals (58 ± 4, 61 ± 4 and 87 ± 9 μmole/l).

Figure 4 shows the individual variation in the output of bile salts in relation to flow in animals from the BzI and CI groups. As may be seen, there were no significant differences in the slopes of the regression lines obtained, whereas in each case the *y*-intercepts were lower in the BzI animals than in the controls. The equation of the mean regression line of each group (Fig. 4) emphasises this aspect even more. If we accept that the value of the *y*-intercept expresses the bile salt independent fraction (BSIF) of flow, it is clearly smaller in the BzI animals. Even if the BSIF is expressed as a percentage of the mean flow of each group, this difference remains.

Bilirubin infusion (groups CII and BzII)

The infusion of bilirubin to control animals (CII) and to the bromobenzene pretreated group (BzII) at doses which afford maximal excretion of pigments, enhanced the fall in bile flow observed in the groups which were not infused (CI and BzI) (Fig. 3). The

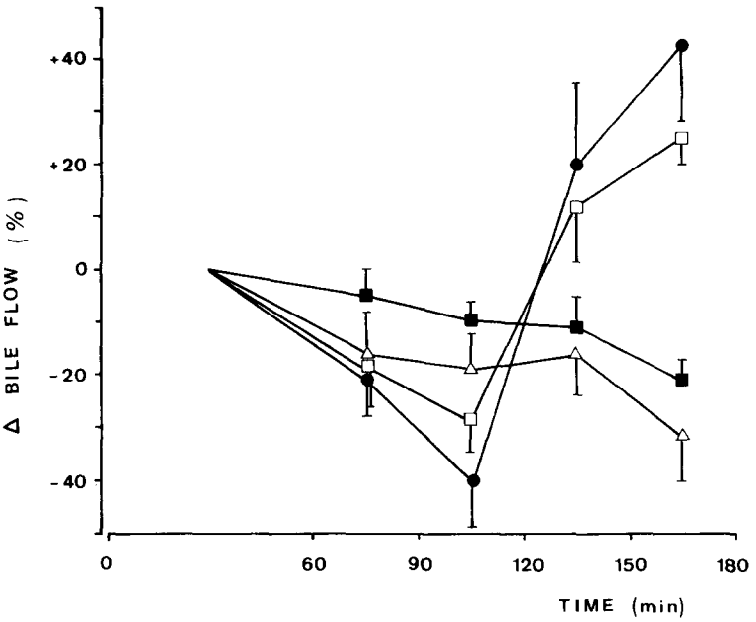

Fig. 3. Percentage changes in bile flow from the first experimental hour. Group CI (■). Group BzI (△). Group CII (□). Group BzII (●). Values are means ± S.E.M. of five animals.

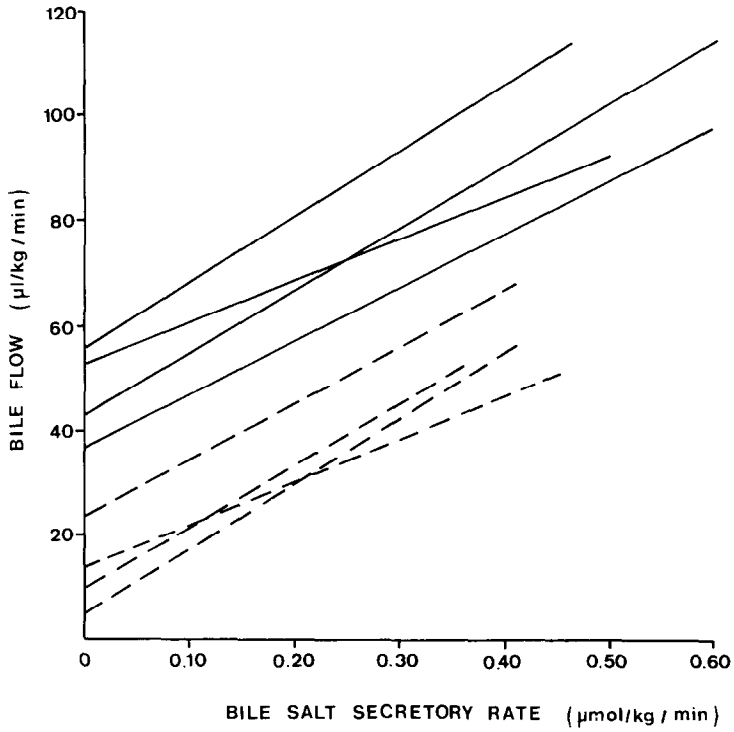

Fig. 4. Relationship between bile flow and bile salt secretory rate in group CI (——) and group BzI (– – – –). Group CI: $y = 107x + 47$; $r = 0.7845$; $n = 48$; BSIF = 47 μl/kg/min; percentage value of BSIF = 49%. Group BzI: $y = 110x + 13$; $r = 0.7427$; $n = 48$; BSIF = 13 μl/kg/min; percentage value of BSIF = 22%.

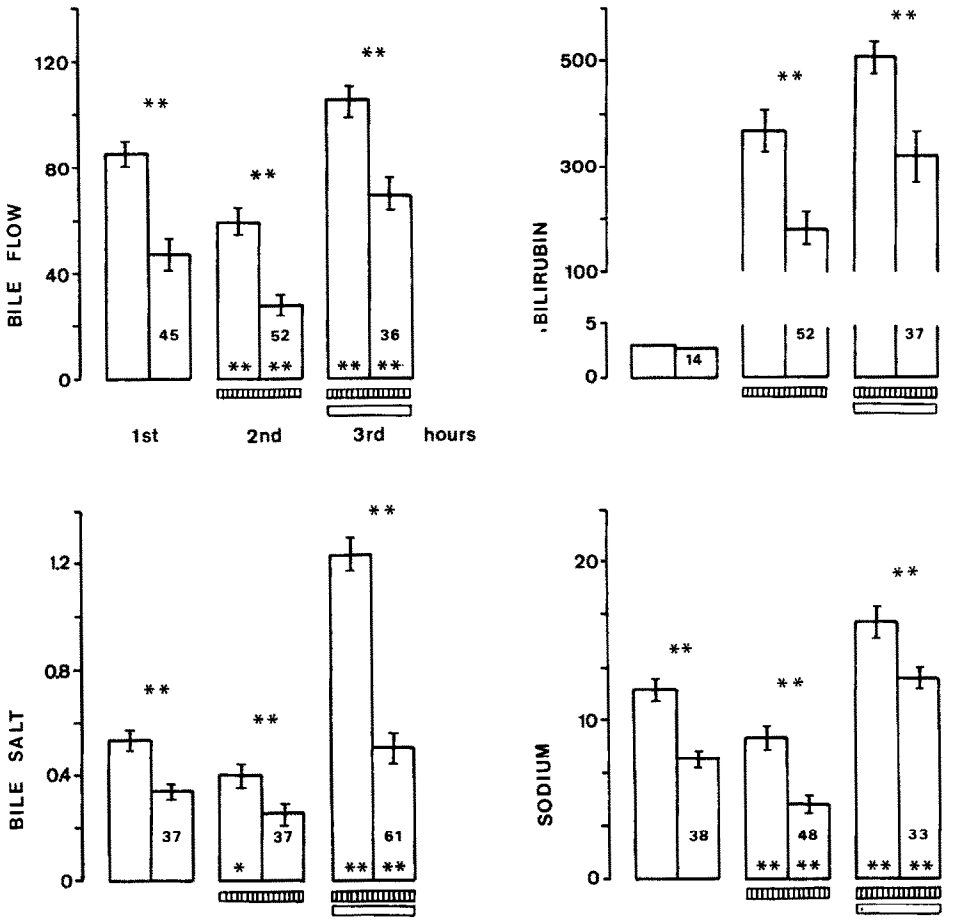

Figure 5. Variations in bile flow (μl/kg/min) and bilirubin (nmole/kg/min), bile salt (μmole/kg/min) or sodium (μeq/kg/min) output in groups CII (first bar of each pair) and BzII (second bar). Values are means ± S.E.M. of five animals and during the 2nd and 3rd hours correspond to the last 30 min of infusion. ▦ : bilirubin infusion at 1.0 μmole/kg/min. ▭ : glycodeoxycholate infusion at 1.6 μmole/kg/min *P < 0.01; **P < 0.001. Significantly different from group CII values (over bars) or from the first hour of the same experimental group (inside bars). Numbers inside bars indicate percentage difference from CII values.

difference in flow between both groups (CII and BzII) was also present in these experiments and in terms of percentage was identical (52%) (Fig. 5) to that observed between groups CI and BzI (51%) (Fig. 2).

Bile salt output decreased throughout this period in a similar way in both groups, thus maintaining a higher secretion rate in the CII animals than in the BzII group, which had already been observed during the first experimental hour (Fig. 5). Infusion of the pigment was seen to modify the pattern of bile salt output of groups CI and BzI only slightly (Fig. 2). The bile salt concentration was greater during the infusion of bilirubin in the BzII animals (8.4 ± 0.4 mmole/l) than in the CII rabbits (6.5 ± 0.7 mmole/l) ($P < 0.01$). The values were similar to those of the preinfusion hour (6.3 ± 0.6 mmole/l) in group CII, though they increased in the BzII animals (7.0 ± 0.6 mmole/l prior to infusion).

Sodium output remained greater in the CII animals than in the BzII group during bilirubin infusion (Fig. 5). In absolute terms, the difference was identical to that described for CI and BzI (Fig. 2). Within each group, infusion of the pigments enhanced the decrease in output of sodium (Fig. 5), as already described for groups CI and BzI. The sodium concentration was not altered significantly by infusion, though it did show a tendency to increase in the CII animals (160 ± 6 vs 141 ± 9 meq/l prior to infusion). Similar to groups CI and BzI, sodium concentration was greater in BzII than in CII animals.

Bilirubin excretion during infusion of the pigment alone was higher in the CII group than in the BzII animals (Fig. 5, 2nd period). However, the concentration of the pigment was very similar in the CII animals (6360 ± 720 μmole/l) and in the BzII animals during this period (6259 ± 623 μmole/l). Figure 6 shows the percentage of infused bilirubin excreted in bile. This value increased progressively and significant differences were maintained in favour of the controls during the infusion period.

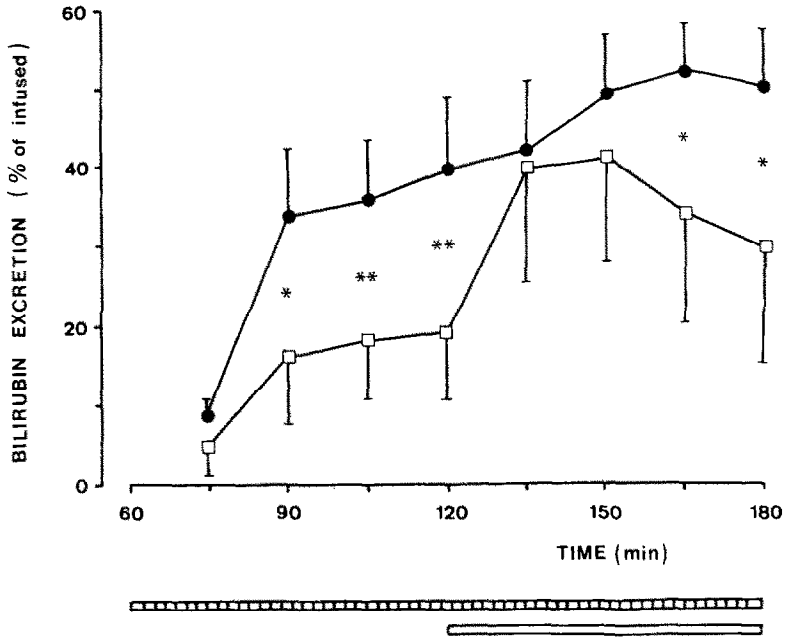

Fig. 6. Changes in bile bilirubin excretion (as % of infused) from the beginning of its infusion in group CII (●) and group BzII (□). Values are means ± S.E.M. of five animals. ▦: bilirubin infusion at 1.0 μmole/kg/min. ▭: glycodeoxycholate infusion at 1.6 μmole/kg/min. *$P < 0.01$; **$P < 0.001$.

Infusion of bilirubin plus glycodeoxycholate (groups CII and BzII)

When glycodeoxycholate was infused together with bilirubin, bile flow increased in a very similar fashion in absolute terms in both experimental groups, exceeding to a significant extent the basal bile flow values corresponding to the first hour of the assays (Fig. 5). Percentage wise, the increase was even more important in the BzII group than in the CII animals.

Bile salt output increased more in absolute and percentual terms in the controls (CII) (218%) than in the bromobenzene-pretreated group (100%). This meant that the difference of 37% between both groups during the previous period (bilirubin alone) in favour of the CII group was increased in this period to 60% (Fig. 5). The bile salt concentration increased markedly in the CII group (11.4 ± 1.0 mmole/l) and in contrast decreased in the BzII animals (7.3 ± 0.6 mmole/l).

The changes in sodium output were similar to those described for bile salts, since there were increases in both groups (Fig. 5). However, the increase was greater in absolute and percentual terms in the BzII rabbits, attenuating the differences between both groups (Fig. 5). The concentration of the cation was not modified in the CII animals (150 ± 6 vs 152 ± 10 meq/l with bilirubin alone) and increased in the BzII group (161 ± 7 vs 184 ± 9 meq/l; $P < 0.01$).

Bilirubin excretion increased in both groups after glycodeoxycholate infusion (Fig. 5). Although it continued to be greater in the CII animals than in the BzII group, as was the case in the previous period (bilirubin alone) (Fig. 5), the concentration of the pigment in the CII group (4714 ± 400 μmole/l) was very similar to the BzII animals (4641 ± 720 μmole/l), as happened in the previous period. Infusion of the bile salt improved bilirubin recovery considerably (Fig. 6) and initially the significant differences between both groups disappeared. However, later on biliary output in BzII group decreased such that significant differences could be established again between both groups (Fig. 6).

DISCUSSION

The present study was carried out in order to investigate the contribution of the hepatocytes from different acinar zones to biliary secretion and bilirubin transport in the rabbit using selective acinar lesions of zone 3 by bromobenzene. Light microscopy of the liver sections showed that in our bromobenzene-pretreated animals hepatic lesions were mainly observed in acinar zone 3. It has been put forward that such morphological lesions do not present an exact functional correspondence and that, in order to elucidate this, scanning electron microscopy or enzyme histochemical techniques would be necessary [6]. However, Gumucio *et al.* [5] have demonstrated for rats that following bromobenzene-induced lesions the gluconeogenic capacity is maintained practically intact.

Differences in biliary secretion in control and bromobenzene-pretreated animals

The bile flow in bromobenzene-pretreated animals compared with controls was less during the first

experimental hour (Fig. 2). This result is similar to those found by other authors for the rat [4, 5]. Though previous studies indicated that in the rabbit bromobenzene-induced lesions, at doses of 0.5–1.5 ml/kg would be smaller than those appearing in the rat [9], the volume fraction of necrosis found in our animals was very similar to that described for the rat at a similar dose of the toxic substance [4, 5]. In principle, the reduced bile flow after bromobenzene-pretreatment could be attributed to a loss of hepatic functional mass. It is known, however, that due to the special organization of the liver acinus [1], the blood in acinar zone 1 is richer in different solutes than that of acinar zone 3. In recent years evidence has accumulated to suggest heterogeneous transport of bile salts. In this sense, in experiments carried out with rats it has been observed that the hepatocytes of acinar zone 3 only contribute at physiological levels by 13–18% to the bile salt clearance [4, 5]. The autoradiography of bile acid derivatives [18] or, more recently, normal perfusions of taurocholate [19], also show that the acinar zone 1 is predominantly involved in the transport of bile acids. In our experiments, the reduced choleresis was accompanied by a reduced output but a greater concentration of bile salts in bile, again in agreement with previous data in the rat [4, 5]. However, the fact that the decrease in flow was greater than that found in the output of bile salts (Fig. 2) seems to support the notion that the acinar zone 1 hepatocytes are mainly involved in bile salt transport at physiological doses and that the decrease in flow was due to the loss of the bile salt independent fraction (BSIF) by the hepatocytes of acinar zone 3. The value of the *y*-intercept of the regression lines relating bile salt output and bile flow (Fig. 4) supports this interpretation, taking into account that the BSIF was smaller in bromobenzene-pretreated animals, both in absolute and percentual terms.

In the lesioned rabbits, sodium and bile salt output were lower than those of the controls by 29 and 27%, respectively, and bile flow was 44% lower (Fig. 2). This means that lesion of acinar zone 3 affected the flow more than it affected the output of the electrolytes which, osmotically, are the main agents responsible for flow; indeed both the sodium concentration and that of the bile salts were greater in the bile of lesioned rabbits than in that of the controls. The lower sodium and bile salt output in the pretreated animals could be ascribed to the loss of hepatocytes, while the greater concentration of both suggests that in these animals the osmotic drag of water was not completed. This last hypothesis is based on the shorter duration of the bile run along the canaliculus, owing to reduced length, and on the disappearance of the canalicular zone of smallest diameter [21, 22] in which, from the physico-chemical point of view, the processes which permit osmotic equilibrium to be reached are more efficient. Both explanations are coherent with the notion that the contribution by the hepatocytes of acinar zone 3 to bile secretion predominantly takes place through the BSIF [4].

Bilirubin excretion was identical in control and bromobenzene-pretreated animals during the first hour (Fig. 2). This allows us to assume that either the hepatocytes of acinar zone 3 do not participate at all in basal excretion of the pigment, or that after intoxication, adaptation of the survivors takes place, until normal values of excretion are reached. In bromobenzene-pretreated rats high glucuronyl transferase activity has been observed in activated microsomes, together with a decrease after lesion of acinar zone 1 by allyl alcohol [22]; this seems to support our first assumption. However, these bibliographical data will not allow us to put forward a final interpretation, since one is dealing with an enzyme which at physiological loads of bilirubin does not play a rate-limiting role in the hepatobiliary transport processes [23].

In untreated animals (CI), a decrease was detected in bile flow throughout the three hours of experiments (Fig. 2). Similar decreases have been reported for conscious or anaesthetized rabbits [13, 24] and in both cases have been attributed to the interruption of the enterohepatic circulation of bile salts. The bile production of bromobenzene-pretreated animals (BzI) showed a similar pattern (Fig. 2), which was also attributable to the same phenomenon. Bile salt output fell at a similar rate in both experimental groups (Fig. 2), an aspect which has already been described for this species [13, 24].

Effect of bilirubin infusion in control and bromobenzene-pretreated animals

The fall in bile flow in animals lesioned with bromobenzene (BzII) or in untreated animals (CII) during bilirubin infusion was greater than that observed in the corresponding groups without infusion (BzI or CI) (Fig. 3). Similar decreases have already been reported for this species during the infusion of identical doses of bilirubin and have been attributed to the partial inhibition of the BSIF [13]. Our results seem to support such an interpretation since, while bile salt output decreased in a similar way with or without infusion of the pigment in animals treated or untreated with bromobenzene, sodium output fell much more in the groups receiving bilirubin infusion.

As we have reported previously [13], i.v. administration of bilirubin at the dose employed permits maximal excretion of the pigment to be reached. However, the value of the latter was significantly less after bromobenzene-induced lesions. It has been observed for bile salts that when high doses are provided, zone 3 cells can be recruited to participate in the removal of bile salts from blood [4, 19]. It is therefore possible to postulate a similar recruitment for bilirubin excretion when the pigment is infused at high doses [25]. It is evident that following lesions of acinar zone 3, such a phenomenon would not occur, or would at least be limited, thereby explaining the lower maximal bilirubin excretion in these rabbits. Klaassen and Plaa [3] have reported decreases in the maximal excretion of dibromosulphthalein (DBSP) in the rat with carbon tetrachloride-induced lesions in acinar zone 3. However, in another study carried out using lower doses of the toxic product, similar effects have not been observed [26]. Groothuis *et al.* [7] using saturating doses of dibromosulphthalein did not find any decrease in its excretion to bile after carbon-tetrachloride-induced

lesions, but in contrast found a marked decrease after administration of N-hydroxy-2-acetyl-aminofluorene, which damages acinar zone 1.

Effect of the infusion of bilirubin plus glycodeoxycholate

The i.v. administration of glycodeoxycholate has a choleretic effect in anaesthetized rabbits [27, 28], even if the infusion of this bile salt is superimposed over that of bilirubin [12]. A similar positive effect appeared both in controls (CII) and bromobenzene-pretreated animals (BzII) (Fig. 5). A quantitative difference in flow was maintained in favour of control animals, in spite of the fact that proportionally the effect of glycodeoxycholate was greater in the lesioned rabbits (Fig. 3).

Bile salt output increased more in the untreated group and bile salt recovery was also greater in this group than in the bromobenzene-pretreated animals. We feel that, apart from possible functional lesions in periportal zones, the high excretion of bilirubin could limit, in the lesioned animals, the hepatic handling of bile salts by the remaining hepatocytes.

On infusing glycodeoxycholate, the excretion of bilirubin improved in both groups. This stimulatory effect of bile salts has been described for several organic anions such as bromosulphthalein [25, 29], DBSP [30] or bilirubin [11, 12] and has been ascribed to different mechanisms. The increased sequestration into mixed micelles is a hypothesis which we feel should be rejected for the action of glycodeoxycholate on the excretion of bilirubin in the rabbit, since bile salts with different capacity to form micelles increase the excretion of the pigment to a similar extent [12]. Another mechanism would be an increase in the number of carriers participating in the transport of bilirubin, due to the high concentration of bile salts reached in acinar zone 3 [11]. We believe that in our experiments such an explanation lacks validity since if we consider the percentage of excretion of infused bilirubin, the effect is more pronounced in bromobenzene-pretreated animals than in controls (Fig. 6) during the first quarter hour of the combined infusion, until a point where the values in both groups are practically equal. Our results seem to indicate that bile salts activate specific mechanisms involved in the transfer of bilirubin to bile. The effect could be accounted for if we accept that glycodeoxycholate infusion facilitates the exit of the pigment stored in the liver, a store which would be greater after bromobenzene-induced damage, and that on depleting the reserves, the positive effect of bile salts is dissipated (Fig. 6). Into this explanation could be integrated others which have been put forward to account for the stimulating action of bile salts, such as the formation of vesicles [20].

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