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CHANGES IN BILIARY SECRETION AND LACTATE METABOLISM INDUCED BY DIETHYL MALEATE IN RABBITS

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Abstract—Diethyl maleate is a compound which binds with glutathione by means of a glutathione *S*-transferase and is excreted into bile leading to a rapid depletion of hepatic glutathione. In the rabbit, the activity of the enzyme is fairly low and we were thus prompted to study the possible effects of diethyl maleate on biliary secretion and metabolic status in this species. The administration of diethyl maleate induced a transient choleresis followed by cholestasis. The choleresis coursed with increases in the biliary output of sodium and unaccounted anions, whereas those of chloride, bicarbonate and bile acids were unaffected. Our data seem to confirm that choleresis is due to the osmotic activity of diethyl maleate compounds excreted into bile, as has been reported in rats and dogs. The cholestasis observed coursed with falls in the outputs of sodium, chloride and bicarbonate though that of bile acids remained constant. Following diethyl maleate administration, a metabolic acidosis appeared with progressive increases of blood lactate concentration. In bile the concentration of this anion closely followed that of plasma. The cholestasis is attributed to a lowered biliary secretion of bicarbonate probably secondary to the metabolic alteration. The hepatic values of cytoplasmatic and mitochondrial NADH/NAD ratios and of adenine nucleotide concentrations suggest that the increase in blood lactate results rather from a fall in its hepatic utilization that from an increase in its production.

Glutathione is a compound involved in the maintenance of cellular redox states which also plays a fundamental role in protection against the toxicity of several xenobiotics. Cellular glutathione depletion induced by different agents has proved to be a useful tool in detoxification and drug metabolism studies. The most commonly employed compound is diethyl maleate, first used by Boyland and Chasseaud in 1967 [1]. Diethyl maleate is an α,β-unsaturated carbonyl compound which reacts with glutathione in the presence of glutathione transferases, giving rise to derivatives which are then excreted in bile, producing a choleretic effect [2–4]. The compound causes a marked reduction in glutathione levels in the liver and, to a lesser extent, in other tissues in species such as the rat, dog and mouse [2, 3, 5]. Apart from glutathione depletion, other effects have also been reported to take place at cellular level; these include inhibitory effects on the activity of cytochrome P-450 [3], inhibitory or stimulatory effects on mixed function oxidase activity [6] and modifications in microsomal heme oxygenase activity [7]. However, such effects do not seem to alter biliary secretion, and in the rat, once the choleretic period has ended, bile flow returns to normal values [2, 5].

The rabbit is a species characterized by its extremely low glutathione transferase activity against different substrates such as 1-chloro-2,4-dinitrobenzene, ethacrynic acid or sulfobromophthalein [8]. Diethyl maleate conjugating activity is also lower than in most other mammals [1], and it is hence likely that diethyl maleate excretion would be lower compared with other species with greater enzymatic activities and that possible effects at hepatocyte levels would have a different repercussion in the mechanisms of bile secretion.

The present study was designed to assess the effects of intraperitoneal administration of diethyl maleate on bile flow and composition in the rabbit and also on different aspects of the hepatic metabolism in this species.

MATERIALS AND METHODS

Chemicals. Diethyl maleate, 5,5′-dithiobis-nitrobenzoic acid, 3α-hydroxysteroid dehydrogenase, glutathione reductase, glutathione-reduced form, β-nicotinamide adenine dinucleotide, β-nicotinamide adenine dinucleotide phosphate and phosphoenolpyruvate were obtained from Sigma Chemical Co. (St Louis, MO); ^{14}C erythritol from Amersham International (Amersham, U.K.); Aquasol 2 fluid from New England Nuclear (Dreieich, F.R.G.); β-hydroxybutyrate dehydrogenase, L-lactic dehydrogenase and pyruvate kinase from Boehringer (Mannheim, F.R.G.). All other reagents were the highest quality available commercially.

Animals and experimental procedures. Male albino New Zealand rabbits weighing between 1.5 and 2.0 kg were used. They were kept in a room maintained at 24° with a 12 hr:12 hr light–dark cycle. Food but not water was withheld for 18 hr prior to surgery. The animals were anaesthetized with pentobarbital sodium (Nembutal, Abbott Laboratories, Madrid, Spain; 40 mg/kg of body weight) through a lateral ear vein. After tracheotomy, cath-

eters were inserted into the left femoral artery for blood sampling. The catheters contained normal saline with 5 U/ml of heparin. The livers were exposed through a midline incision and the cystic duct ligated. The common bile duct was cannulated with polyethylene tubing (PE 50). A cannula was also inserted into the first part of the duodenum. Rectal temperature was monitored via a thermistor probe and maintained at 38.5–39.0° by heating the operating table.

After an equilibration period of 30 min to allow bile flow to stabilize, bile was collected over 3 hr in 10–15 min samples. A part of the samples (<10%) was kept for analyses and the rest reinfused through the duodenal cannula. After discarding the first 200 μl fraction of blood diluted with the heparinized solution of the catheters 300 μl blood samples were obtained; this fraction was then reinjected to minimize blood loss.

After collecting four baseline 15 min samples of bile, diethyl maleate was administered by intraperitoneal injection at a dose of 3.2 mmol/kg of body weight mixed 1:1 (v:v) in corn oil. Controls received only corn oil.

To measure biliary clearance of erythritol, immediately after cannulation of the common bile duct, the rabbits received an injection of 4 μC of ^{14}C erythritol followed by an infusion of 0.02 μC/min (in 0.15 M NaCl at 0.015 ml/min). In order to allow uniform distribution of erythritol in the body fluids, investigation of biliary clearance started 120 min later.

In a separate series of experiments, hepatic parameters were determined in rabbits given corn oil or diethyl maleate at 50 min, 90 min and 180 min.

Analytical methods. Bile volume was determined gravimetrically without correction for specific gravity. Bile acid concentration in bile was measured by an enzymatic technique using 3α-hydroxysteroid dehydrogenase [9]. Sodium and potassium concentrations were measured by flame photometry (Nak II flame photometer, Meteor, Madrid, Spain). Chloride concentration was determined by titration with a silver electrode (chloridometer model 160, Analytical Control, Italy). pO_2 and pCO_2 in blood and pH and bicarbonate concentration in blood and bile were measured in an automated gas analytic system (model 168, Corning Medical, Medfield, MA). The osmolality of bile and plasma was determined by a vapour pressure osmometer (model 5100 C, Wescor, Logan, UT). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined in plasma using commercial kits (Boehringer, Mannheim, F.R.G.).

To measure plasma lactate, blood samples were collected and immediately centrifuged at 4° for 10 min at 1000 *g* to separate plasma. Liver samples were removed from the animals before sacrifice and immediately freeze-clamped in liquid nitrogen. Liver metabolites and adenine nucleotide concentrations were assayed after cold homogenization (1:5; w:v) in 5% $HClO_4$, 10 min centrifugation at 1000 *g* and immediate neutralization of the supernatant with 20% KOH.

Lactate was determined as described by Hohorst [10], β-hydroxybutyrate according to Williamson and Mellanby [11] and acetoacetate by the method of Mellanby and Williamson [12]. ATP was assayed by the method of Lamprecht and Trautchold [13]. Pyruvate, ADP and AMP were determined as described by Adam [14]. Freeze-clamped liver tissue was used for total glutathione determination in a kinetic assay using NADPH, glutathione reductase and 5,5′-dithiobis nitrobenzoic acid [15].

^{14}C erythritol radioactivity was determined in bile and plasma by liquid scintillation spectrometry (model LS 1800, Beckman, Fullerton, CA). Ten millilitres of liquid scintillation fluid (Aquasol 2) were added to 20 μl of bile or plasma. Correction for quenching was made by the external standard channels ratio method.

Statistical analysis. Data are expressed as means ± SEM. Statistical comparisons were performed using a Student's two-tailed *t*-test.

RESULTS

Diethyl maleate administration induced a marked increase in bile flow (Fig. 1). This increase reached a maximum at 15–30 min post-injection, after which it declined slowly and progressively until values significantly lower than those of the controls were reached at 120 min after the start of the assays. At the end of the experiments, flow was reduced to half that of the controls (Fig. 1).

The modifications in bile flow after administration of diethyl maleate were accompanied by others in the ionic composition of the bile collected. In this sense, chloride (Fig. 2) and bicarbonate (Fig. 4) concentrations declined rapidly and markedly at 10–20 min after injection of diethyl maleate. In contrast to bicarbonate, whose concentrations remained low until the end of the assays, those of chloride started a slow recovery which was completed at 75–90 min after injection. On the other hand, potassium concentrations (Fig. 2) hardly underwent any changes in the first samples after diethyl maleate administration and reached concentrations twice those of the controls in the last samples. Although the concentrations of sodium and bile acid did vary slightly, they did not undergo significant modifications (Fig. 2).

Regarding the secretory rates of the electrolytes assessed, those of sodium and potassium (Fig. 3) increased significantly after injection of diethyl maleate and those of sodium (Fig. 3), chloride (Fig. 3) and bicarbonate (Fig. 4) decreased significantly during the second hour post-injection. The bile acid secretory rate hardly underwent any modifications.

The variations in the "anion gap" calculated from the individual values of concentration or secretory rate of the different ions that were determined are shown in Figure 5. It may be seen that whereas the concentration of undetermined anions increased at 10 min after diethyl maleate injection and remained high until the end of the assay period, its secretory rate rose and returned to basal levels in the last hour of the experimental period.

The evolution of bile osmolalities is shown in Fig. 6. As may be seen, their values fell non-significantly with respect to the controls during the choleretic period and clearly increased during cholestasis.

Bile pH was noticeably stable and the values

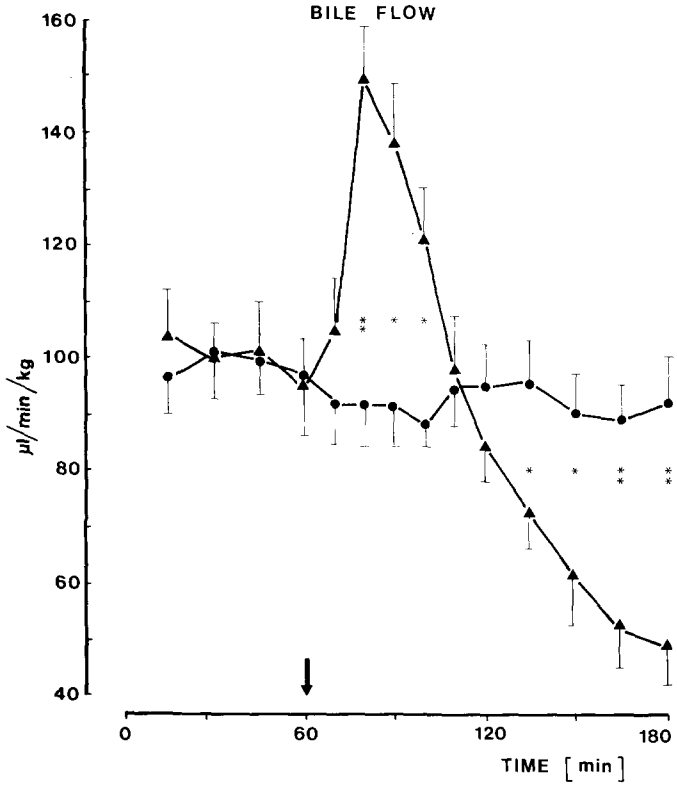

Fig. 1. Bile flow in control (●) and diethyl maleate (▲) treated rabbits. Arrow indicates point at which diethyl maleate was administered. Values are given as means ± SEM for 5–8 rabbits: *P < 0.05; **P < 0.005; significantly different from the controls.

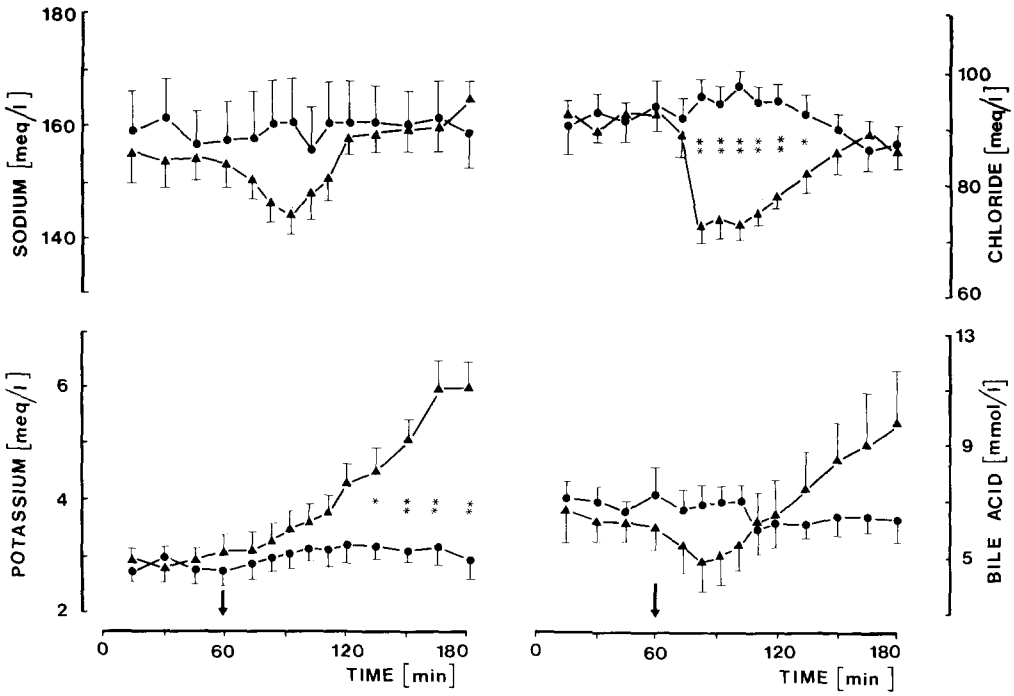

Fig. 2. Sodium, potassium, chloride and bile acid biliary concentrations in control (●) and diethyl maleate (▲) treated rabbits. Arrows indicate point at which diethyl maleate was administered. Values are given as means ± SEM for 5–8 rabbits: *P < 0.05; **P < 0.005; significantly different from the controls.

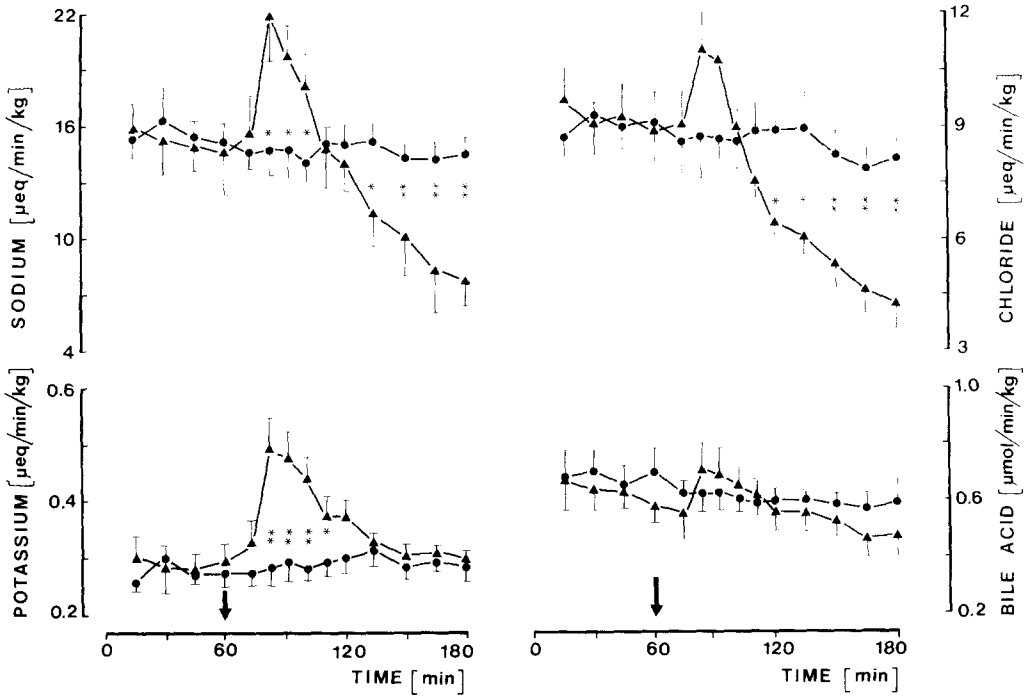

Fig. 3. Biliary secretory rates of sodium, potassium, chloride and bile acid in control (●) and diethyl maleate (▲) treated rabbits. Arrows indicate point at which diethyl maleate was administered. Values are given as means ± SEM for 5–8 rabbits: *$P < 0.05$; **$P < 0.005$; significantly different from the controls.

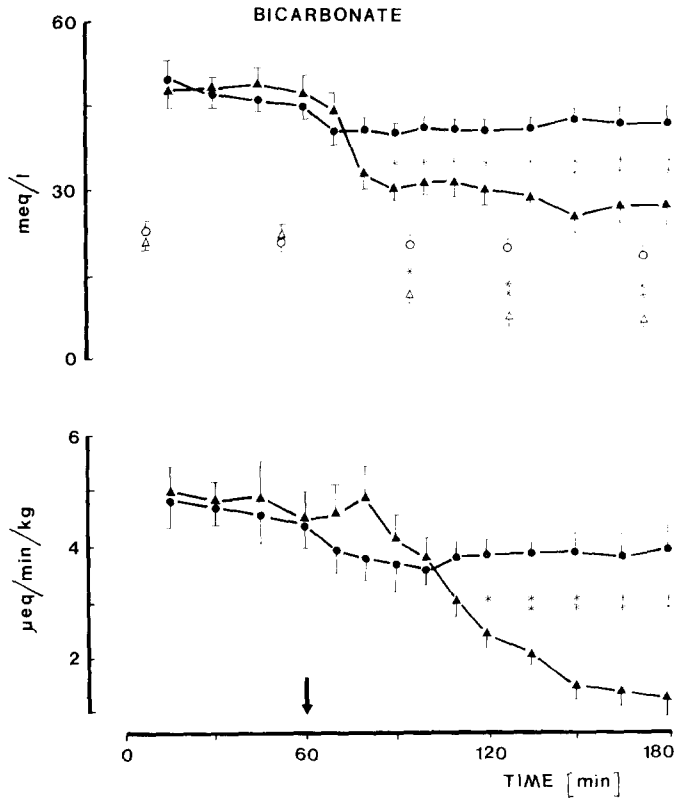

Fig. 4. Bile and blood concentration and biliary secretory rate of bicarbonate in control (●, bile; ○, blood) and diethyl maleate (▲, bile; △, blood) treated rabbits. Arrow indicates point at which diethyl maleate was administered. Values are given as means ± SEM for 5–8 rabbits; *$P < 0.05$; **$P < 0.005$; significantly different from the controls.

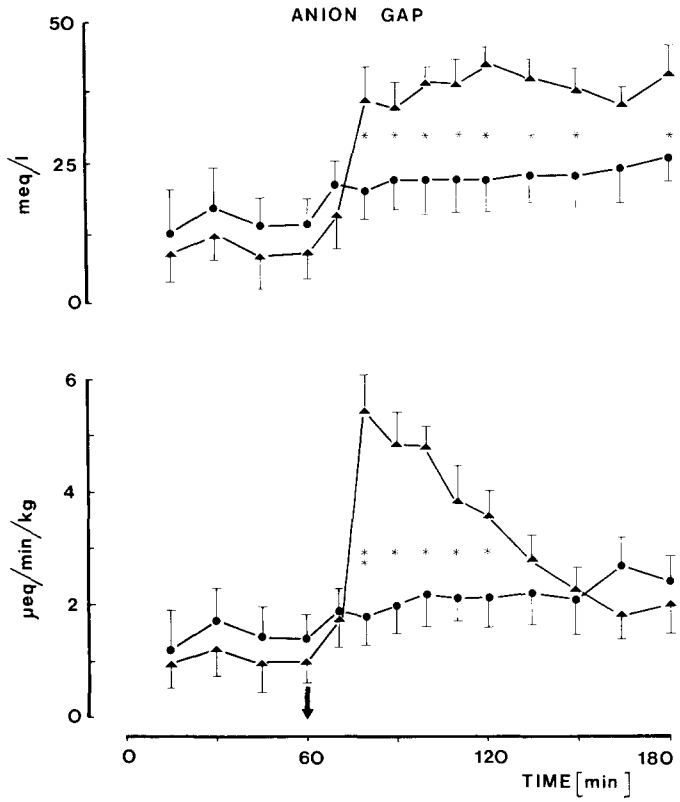

Fig. 5. Anion gap values in control (●) and diethyl maleate (▲)treated rabbits. Arrow indicates point at which diethyl maleate was administered. Values are given as means ± SEM for 5–8 rabbits: *P < 0.05; **P < 0.005; significantly different from the controls.

obtained did not leave a range between 7.71 and 7.79 both for the control assays and for those involving diethyl maleate treatment (data not shown).

Erythritol clearance was determined in three rabbits administered with diethyl maleate and in a further three controls. The bile/plasma ratio for the radioactive compound reached a value of 1.21 ± 0.11 in the controls and was not significantly modified after diethyl maleate injection.

pH, pO_2, pCO_2, osmolality and bicarbonate concentrations were determined in blood. Figure 6 shows the existence of a significant difference between the osmolality of the control and diethyl maleate experiments in the last 15 min of the assay period, similar to what was found for bile. Figure 7 shows the modifications in pH, pO_2 and pCO_2 and Fig. 4 those in blood bicarbonate concentrations. From all these values it may be inferred that after diethyl maleate administration an uncompensated metabolic acidosis is set up with decreases in pCO_2, pH and bicarbonate concentration and increases in pO_2. Respiratory rate (not shown) also increased in these animals though it did not become significantly different from that of the controls, perhaps owing to the great dispersion in these values. Accordingly, respiratory rates of 40–60 insp/min before diethyl maleate or corn oil, rose to values of 80–120 and 50–90 insp/min, respectively.

In view of these findings we were then prompted to determine lactate values in bile and plasma (Fig. 8). In the former fluid of the control animals lactate concentrations were very low and constant, whereas after diethyl maleate administration they rose slowly and progressively, reaching values about 8-fold higher than those of the controls (Fig. 8). The biliary output of lactate increased rapidly and remained high until the end of the assays (Fig. 8). Plasma lactate values exhibited parallel changes to those reported for bile both in the control animals and the diethyl maleate-treated ones; for both cases there was a slight plasma/bile gradient.

Finally, a series of hepatic determinations were performed in an additional group of animals. Glutathione concentrations fell significantly at 30 min after administration of diethyl maleate and remained decreased at 120 min after injection of the substance (Fig. 9). The cytosolic NADH/NAD ratio was measured as the lactate/pyruvate ratio. As may be seen in Fig. 9, this ratio followed an inverse trend to that of glutathione, with significant increases after diethyl maleate-treatment. The β-hydroxybutyrate/acetoacetate ratio (Fig. 9), the concentrations of ATP, ADP and AMP and also the ATP/ADP ratio (data not shown), and adenilate energy charge (Fig. 9), all determined at the same moment as the lactate/pyruvate ratio, did not undergo significant modifications. In these same animals blood samples were taken 1 min before sacrifice to analyze ALT and AST activities. Control values were respectively 69 ± 9 (N = 4) and 48 ± 5 (N = 4) Sigma units/ml, with no significant differences with the group of diethyl maleate-treated animals.

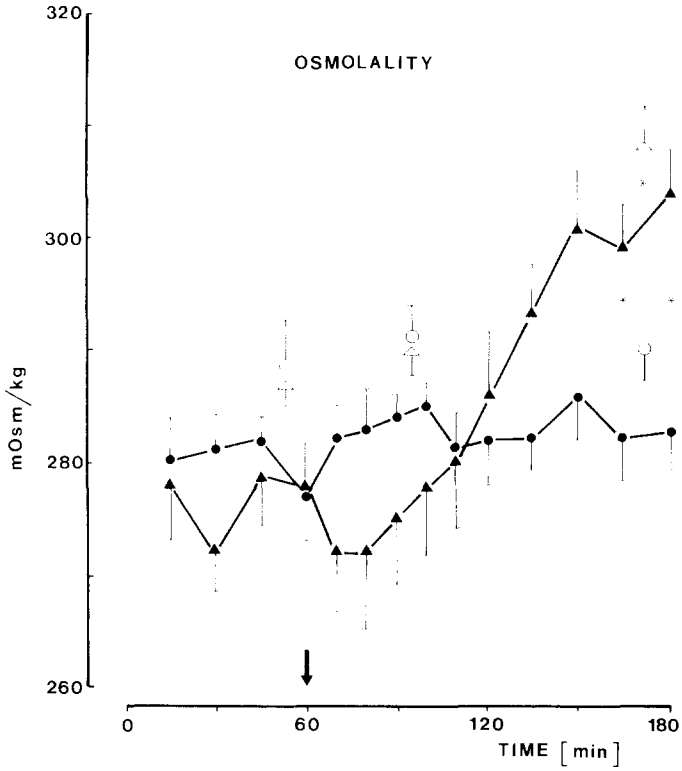

Fig. 6. Bile and plasma osmolality in control (●, bile; ○, plasma) and diethyl maleate (▲, bile; △, plasma) treated rabbits. Arrow indicates point at which diethyl maleate was administered. Values are given as means ± SEM for 5–8 rabbits: *P < 0.05; significantly different from the controls.

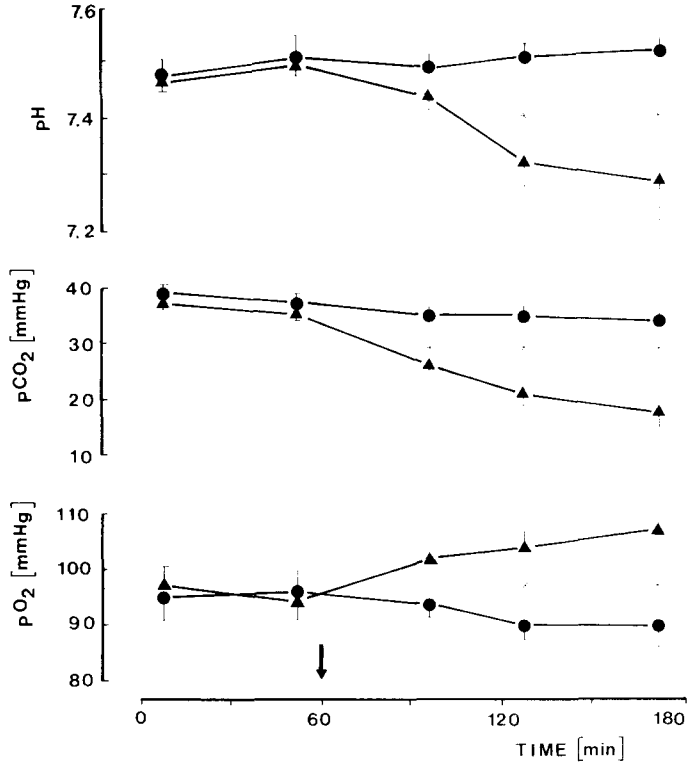

Fig. 7. pH, pCO_2 and pO_2 values in arterial blood of control (●) and diethyl maleate (▲) treated rabbits. Arrow indicates point at which diethyl maleate was administered. Values are means ± SEM for 3–6 rabbits: *P < 0.05; significantly different from the controls.

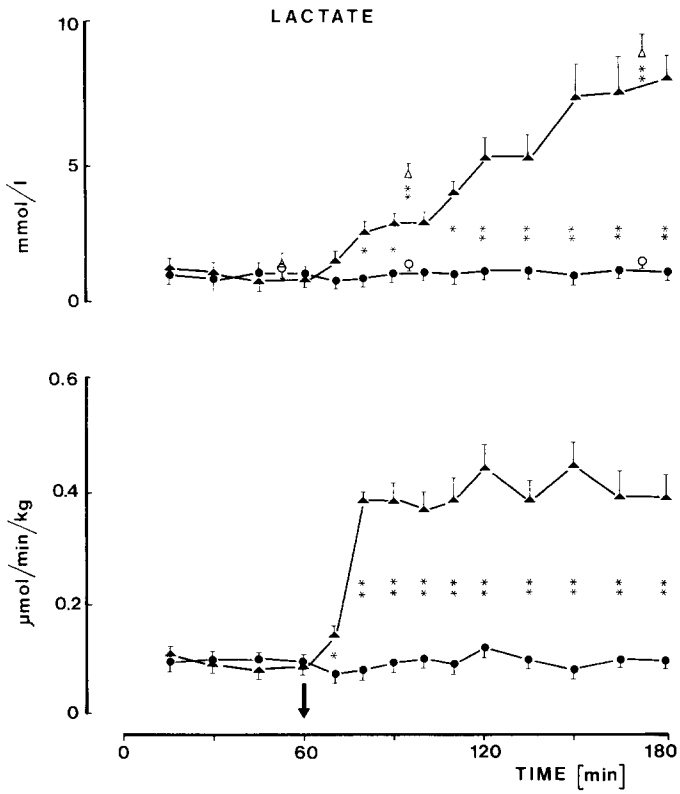

Fig. 8. Bile and plasma concentration and biliary secretory rate of lactate in control (●, bile; ○, plasma) and diethyl maleate (▲, bile; △, plasma) treated rabbits. Arrow indicates point at which diethyl maleate was administered. Values are means ± SEM for 4–7 rabbits: *P < 0.05; **P < 0.005; significantly different from the controls.

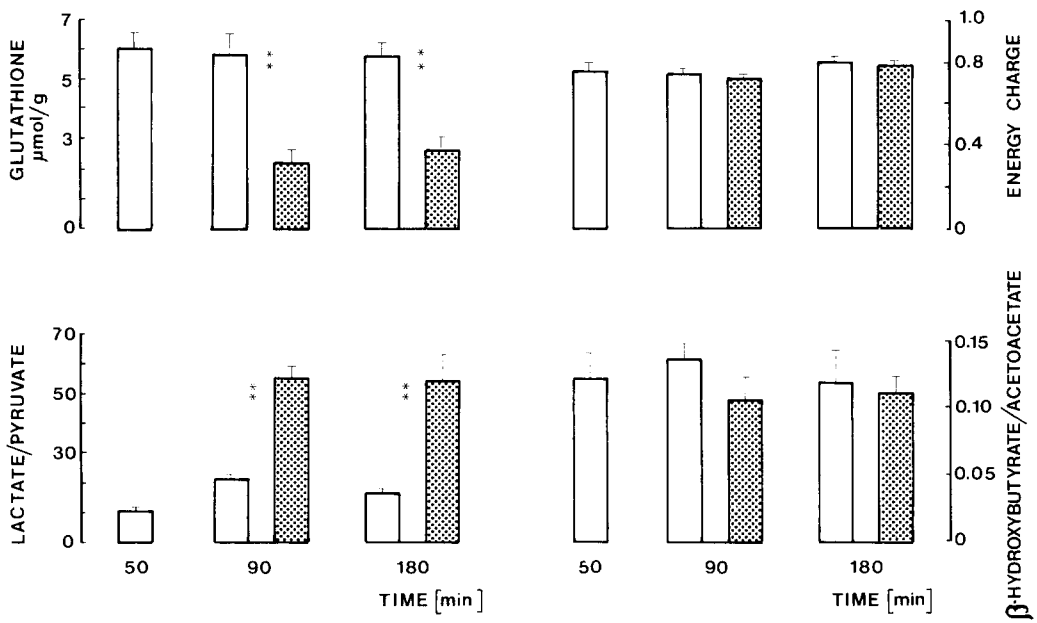

Fig. 9. Liver glutathione concentration, adenylate energy charge and lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios in control (□) and diethyl maleate (▨) treated rabbits. Diethyl maleate was administered at 60 min. Adenylate energy charge = ½[2(ATP)+(ADP)/(AMP)+(ADP)+(ATP)]. Values are means ± SEM for 3–5 rabbits: *P < 0.005; significantly different from the controls.

DISCUSSION

In 1978 Barnhart and Combes [2] showed that, when administered to the dog and the rat, diethyl maleate induces a choleresis which seems to be of canalicular origin and which courses with no changes in bile acid secretion. These authors suggested that the choleresis could be attributed to the osmotic activity of the diethyl maleate compounds excreted into bile and that the conjugation of this substance could account for the depletion of hepatic glutathione. These data were later confirmed in the rat [4, 5] and it was demonstrated that diethyl maleate did not alter hepatocyte morphology in this species except for certain modifications in the Golgi complex [4] that point to the involvment of this organelle in the biliary excretion of osmotically active diethyl maleate conjugates.

In the rabbit, diethyl maleate induces a rapid choleresis with no appreciable changes in the bile acid secretory rate nor in the bile/plasma ratio of ^{14}C-erythritol. These findings confirm those described previously indicating that choleresis is probably of canalicular origin and independent of bile acid secretion. By contrast, our results differ with those reported in the rat [2] in several aspects: in our experiments choleresis is apparently less intense, it does not last as long and is followed by an intense cholestasis which was undetected in the rat [2]. Like Barnhart and Combes [2], we believe that the choleresis must be due to the biliary excretion of the products resulting from the conjugation of diethyl maleate with glutathione and the consequent osmotic drag of water. In the rat it has been established, basing on the measurement of the clearance of labelled aminoacids which form part of glutathione, a correlation between the excretion of conjugates and the measurement of the "anion gap" calculated in bile [2]. In our animals, after diethyl maleate injection, a significant increase was detected in the bile output of one or more undetermined anions. The increase in the cumulative "anion gap" with respect to the controls was 122 μEq/kg; i.e. if the "anion gap" represents the excretion of diethyl maleate, only 3.7% of the dose administered had been excreted, a percentage which is very close to that calculated by us for the previous results in the rat [2]. A comparison of our results concerning bile flow and the "anion gap" for the periods in which both are significantly increased allows us to calculate a choleretic capacity of 15 ml/mEq, a value very similar to that determined for the rat or the dog [2] and within the ranges described for different endogenous and exogenous anions in several species [16]. At 30 min after diethyl maleate administration the hepatic glutathione concentration had fallen by 114 μmol/kg. This value coincides acceptably well with the amount of diethyl maleate conjugates excreted over the same period of time calculated by the cumulative "anion gap", a coincidence that has also been reported in the rat [2].

In the light of such findings it may be inferred that the differences detected by us between the diethyl maleate-induced choleresis in the rat [2, 5] and rabbit do not seem to be due to a different excretion of diethyl maleate, nor to a different choleretic capacity of this compound in one or other species. We believe that the discrepancies are due to the progressive appearance of a cholestatic phenomenon which limits and shortens the choleretic effect, with an origin and development that will be discussed below.

During choleresis sodium and potassium secretory rates increased significantly, whereas those of chloride and bicarbonate did not. However, the concentrations of these two anions were strongly decreased during the same period. The ionic modifications indicated are probably secondary to the excretion of diethyl maleate compounds, which owing to their anionic nature would affect the cations and the anions in different ways. In the case of bicarbonate, bile concentrations did not recover at the end of the choleretic period. We believe that this fact might be related, as was the case of flow, to the above-mentioned cholestatic phenomenon.

At 30 min after diethyl maleate administration the metabolic status of the animals was modified, with a series of changes that suggest the appearance of a metabolic acidosis accompanied by the start of compensatory respiratory mechanisms, such that the former was still not very intense. It is precisely this metabolic phenomenon which we feel is related to the drop in biliary bicarbonate and the cholestatic phenomenon. Hardison and Wood [17] have reported that substituting bicarbonate for some other anion in the perfusion liquid of isolated rat livers induces a 50% decrease in bile flow and an identical decrease in the secretory rates of sodium and chloride, without any effect on the secretion of bile acids. The changes observed in our experiments were similar and when the metabolic acidosis later became manifest and the decreases in blood bicarbonate were more pronounced, we found—as did the authors cited—50% reductions in flow and in the secretory rates of sodium and chloride.

During the 80–120 min period after diethyl maleate administration, with an overt uncompensated metabolic acidosis, a significant increase was observed in bile osmolality which, at least in the last 15 min, was accompanied by a comparable increase in plasma osmolality. This effect could be explained by the rise in lactate and may be an additional component of the cholestasis observed. In this sense, Mathisen and Raeder [18] have found inverse relationships between plasma osmolality and bile flow in piglets. The cholestasis rather appears to be the result of specific alterations, because the secretion of bile acids remains unmodified. This is to say that the processes which depend directly or indirectly on energy consumption are not affected. In this sense, it should be pointed out that neither the hepatic ATP concentrations nor the adenylate energy charge were modified during the cholestatic phenomenon.

Both in the controls and in the diethyl maleate-treated animals it was possible to note a parallel trend in the bile and plasma lactate concentrations, though in all cases there was a slight plasma/bile gradient. This suggests that this anion is equilibrated between both compartments, following concentration gradients. Its passage could take place through the transcellular and or paracellular route.

The fact that the pH range in blood and bile remained between 7.1 and 7.9 allows us to assume that most of lactate was in anionic form. Taking into account the reports of carriers for anionic lactate uptake in the baso-lateral membranes of the hepatocytes [19, 20], and that the paracellular passage is more hindered for anionic forms [21], we are inclined to believe rather in the transcellular route.

At cell level, among other originating causes for the appearance of a lactic metabolic acidosis, a fall in the transport of reduced equivalents from the cytosol to mitochondria, the destruction of the pyruvate dehydrogenase complex, NAD depletion and the inhibition of gluconeogenesis from lactate have been proposed [22–26]. The most debated point is whether a reduced utilization of lactate is sufficient for its implantation or whether an increased production of this substance would be necessary [24, 27]. In our experiments tissue hypoxia does not seem to be important since pO_2 did not decrease and nor did ATP concentrations, at least in liver; according to Blitzer *et al.* [28], this decrease is the first consequence of hypoxia in the rabbit. Moreover, according to these authors [28] the plasma levels of lactate dehydrogenase and AST increase consequent to hepatocellular damage, and in our assays the plasma levels of ALT and AST did not vary.

A plausible hypothesis would be that diethyl maleate might cause the intrahepatocyte pH to descend in a specific way, which would thus lead to the inhibition not only of pyruvate carboxylase but also of the step from lactate to pyruvate due to the accumulation of end products of the reaction (pyruvate and H^+). We detected an increase in the hepatocyte lactate concentration, with no alterations in that of pyruvate. Notwithstanding, pyruvate might be being oxidized in the Krebs cycle, since according to several workers [29] for changes in the intrahepatocyte pH ranging from 6.5 to 8.0 neither mitochondrial oxidation nor ATP output are affected. This latter would explain the constancy of this nucleotide in our experiments. Another hypothesis to account for the metabolic acidosis would be the specific inhibition of some of the key enzymes in gluconeogenesis caused by diethyl maleate administration. Very recently, Saez *et al.* [30] have demonstrated that glutathione depletion by diethyl maleate or the inhibition of its synthesis by buthionine sulfoximine induces in rat hepatocytes an inhibition of phosphoenolpyruvate carboxykinase with no exit of lactate dehydrogenase and no modification in intrahepatocyte ATP deposits. These findings are in keeping with our own and, moreover, both hypotheses might complement each other. In fact, the influence of phosphoenolpyruvate carboxykinase may induce the accumulation of lactate; this would decrease plasma and hepatic deposits of bicarbonate and would lead to the appearance of acidosis, hence reinforcing the inhibition of hepatic lactate metabolism.

Some workers have suggested that the fall in pH could inhibit lactate uptake and might be the cause of the hyperlactataemia [31]. However, Fafournoux *et al.* [19] and Monson *et al.* [20] have demonstrated that decrease in extracellular pH favours hepatic lactate uptake. Although we are unable to evaluate the importance of lactate uptake in the development of the acidosis in question, the latter work cited [20] and the similarities between the plasma and bile concentrations of lactate already mentioned suggest that it cannot be decisive.

Finally, regarding the differences found in the biliary response to diethyl maleate in the rat [2, 5] and the rabbit, the different glutathione *S*-transferase activity [1] is probably not responsible since the excretion of diethyl maleate seems to be similar in both species. It is possible that diethyl maleate also induces an impairment of lactate metabolism in the rat and the inhibitions of gluconeogenesis described in that species [30] point in this direction. Perhaps the phenomenon is not as intense as in the rabbit and it must be considered that, though biliary secretion in the rat is related to bicarbonate secretion, it is probably so to a lesser extent than in the rabbit [32]. Indeed, it has been found that significant drops in blood pH and bicarbonate concentrations, comparable to our own, produce no appreciable changes in bile flow in the rat [33].

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