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Liver Damage Induced by Intrabiliary Turpentine in Rats

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Abstract—Inflammation of the rat bile duct induced by administration of turpentine into it has been used to study the influence of the impaired duct on liver function. Turpentine was dissolved in olive oil 1 : 1000 and 1 : 500. A 2 h ligation of the bile duct was used to promote a local effect. Contemporary groups of intact, sham-operated, control rats (given 0·9% NaCl by intrabiliary injection) and animals with total chronic obstruction were compared to assess the significance of changes. Serum concentrations of total and conjugated bilirubin, cholesterol and creatinine, activities of *S*-alanine-aminotransferase, *S*-aspartate aminotransferase and alkaline phosphatase, mortality of rats, and also total body weight compared with the weight of the liver, were investigated on days 1, 4, 8, 12, 16, 32 and 64 after surgery and turpentine, or following ligation of the bile duct. An increase in bilirubin and cholesterol, an augmentation of enzymatic activity and the histological changes were indicative of hepatotoxicity or cholestasis. The turpentine concentration – effect, manifested in body-weight change, suggests some specificity of the effect. There were no changes in serum creatinine arterial blood pressure, heart rate or portal blood pressure, when turpentine was administered by the intrabiliary route. These results suggest primary liver damage.

Studies on bile secretion, hepatic extraction and drug metabolism have been made following bile duct obstruction or ligation in man and animals (McLuen & Foust 1961; Hutterer et al 1970; Mackinnon & Simon 1974; Harrison & Gibaldi 1976; Knodell et al 1980; Basseches & DiGregorio 1982; Schacter et al 1983). The mechanisms of altered renal perfusion and salt retention after bile duct ligation have also been examined (Yarger 1976; Allison et al 1978; Hishida et al 1980).

Turpentine, which is known to induce arthritis when administered topically, was used to induce inflammation of the bile duct. Chindavijak et al (1987) showed that there is a decrease in metabolism of both high and low extraction drugs in-vitro in the liver of rats with turpentine-induced inflammation. Changes in intrinsic clearance however, will have more impact on the systemic clearance of low extraction drugs, such as antipyrine, than on those of high extraction. Thus we also compared the effect of bile duct inflammation after turpentine with that when only bile duct ligation was made.

Materials and Methods

Turpentine oil (Chema, Rájec-Jestřabí, CSSR) was dissolved in olive oil 1 : 1000, 1 : 500, w/v. A modified Jendrassik-Grof procedure (1938), see Nezhyba et al (1977), was used to measure total bilirubin (TB). Conjugated bilirubin (CB) was assayed by the method of Jendrassik & Grof (1938) as modified by Garber (1981).

The activities of *S*-alanine-aminotransferase (ALT) and A-aspartate-aminotransferase (AST) were determined according to the recommendations of the Committee on enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1973) by the test-combination "Sera-Pak" fi.AMES on a centrifugal analyser with the modification of Bergmeyer et al (1978). Serum cholesterol was determined by the Biotest Lachema method of Richmond (1973) and Demacker (1983). The activity of alkaline phosphatase (AP) was determined according to Bessey et al (1946) and Morgenstern et al (1965) by means of the Bio-La-Test Lachema on a centrifugal analyser. Serum creatinine was estimated by the method of Fabini & Ertingshausen (1971) as modified by Kammeraat (1978).

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Animals and experimental procedures

Female Wistar rats, bred in Třebeš, maintained on a standard laboratory diet, and initially 230–250 g, were anaesthetized with pentobarbitone (35 mg kg^{-1}. i.p.) and a 2 cm incision made just below the xiphoides process. A double ligature was placed through the mesentery around the common bile duct. The upper knot was below the junction of the lobar ducts, and the lower knot was placed just above the junction of the pancreatic ducts with the common bile duct. A polyethylene cannula was introduced through the duodenal wall into the common bile tract near the confluence of its tributaries (5 mm) and held in position with the upper silk ligature.

Saline or turpentine 0·1 mL/220 g was administered through the cannula which was then removed. A temporary 2 h obstruction of the common bile duct was made (TBDL) to allow the biliary effect of turpentine to develop. This was then removed and the animal allowed to recover. Rectal temperature was maintained between 37 and 37·5°C. The duodenum of all operated rats was topically treated with chloramphenicol.

Animals were divided into 6 groups:

1) Intact rats (Group 1. n = 9) anaesthetized without any operation.

2) Controls (Group 2, n = 32), given sterile 0·9% NaCl (saline, 0·1 mL/220 g) into the bile duct before TBDL.

3) Sham-operated rats (Group 3, n=7) similarly treated except the main duct was neither cannulated nor obstructed. Estimates were obtained from groups 1 and 3 on day 4 as cumulative changes, if any, occurred.
4) To study both the influence of increased turpentine concentration and the severity of the pathological effect following TBDL, we administered 0·1 mL turpentine/220 g 1:1000 (n=35) and 1:500 (n=28) before TBDL.
5) The other groups of turpentine-treated rats were treated before TBDL as follows: turpentine 1:1000 (n=25) and 1:500 (n=17) i.b. These rats were individually caged (15 × 15 cm floor). Intake of water and food, output of urine and changes in body-weight were measured each day for 16 days.
6) A group of 8 animals was used to compare the influence of both temporary (TBDL) and chronic obstruction of the bile duct (BDL), the latter being produced by a double ligation of the common bile duct with a non-resorbable suture. The duct between the ligations was then resected to prevent recanalization (Allison et al 1978). Rats were killed by exsanguination 1, 4, 8, 12, 16, 32 and 64 days after operation and treatment. Blood samples were obtained from the carotid artery and processed. In addition, a visual inspection of the liver and biliary tree was made to verify the development of changes in the common bile duct and liver surface. The presence or absence of bile duct obstruction was confirmed in all experiments by observation of a dilated or non-dilated bile duct.

Arterial blood pressure, heart rate and portal pressure measurements

Polyethylene catheters were inserted into the carotid artery and the jugular vein under anaesthesia (sodium pentobarbitone 35 mg kg^{-1} i.p.). Additional groups of animals were used to ascertain the portal vein pressure via a polyethylene catheter (Williams Cook) inserted into a branch of the ileocolic vein. The catheter was fixed to the vein with a silk ligature and the abdominal incision was sutured. We ensured that the pressure tracing showed a stable plateau with minor respirating variations; blood could be easily aspirated. All catheters were flushed with saline and heparin. Their exteriorized ends were secured. 24 h later the conscious animals were placed in cages maintained at constant temperature. After 60 min to allow the animals to stabilize, arterial blood pressure was recorded on a Statham P 23 pressure transducer connected to a recorder. ECG was obtained through three electrodes placed on the limbs. Portal blood pressure was measured by a manometer and a pressure transducer. The parameters were measured 3 times at 60 min intervals.

Histological methods

Specimens of the common bile duct and liver tissue were fixed in 10% formalin, dehydrated and embedded in paraffin. Sections of the common bile duct were stained with haematoxylin and eosin, Masson's trichrome technique and Gomori's impregnation of reticulin. Liver slices were also treated with Perl's method for haemosiderin and Best's carmine technique for glycogen. Also, frozen sections of liver were stained for neutral lipids with oil red O.

Statistics

Student's *t*-test for unpaired samples was used for the analysis of the differences between groups. All results are expressed as means ± s.e.m.

Results

The effects of non-specific factors influencing biochemical values are presented in Table 1. Surgical preparation of the skin, an abdominal incision, puncture of the bowel wall and administration of chloramphenicol (gp 3) did not affect the bilirubin level but there was an increase in AST activity on day 4. Control (gp 2) rats had a temporary increase in total and conjugated bilirubin. On day 12 their AST activity was more than that of the sham operated rats. Bilirubin levels and AST activity had returned to those of intact rats between 12 and 32 days after surgery (Table 1). There was an increase in

Table 1. Biochemical test values of intact (gp 1), sham (gp 3) and control (gp 2) rats in serum (means ± s.e.m.).

	Days after treatment						
	4 gp1 (n=9)	4 gp3 (n=9)	12 gp3 (n=7)	32 gp3 (n=4)	4 gp2 (n=4)	12 gp2 (n=5)	32 gp2 (n=9)
TB $\mu mol\ L^{-1}$	6·5 ±1·8	9·2 ±2·5	7·0 ±1·6	9·8 ±3·8	*28·0 ±10·0	11·6 ±7·0	6·1 ±2·5
CB					**10·0 ±8·2		
ALT $\mu cat\ L^{-1}$	0·67 ±0·28	0·79 ±0·37	0·73 ±0·29	0·53 ±0·14	1·62 ±0·91	1·50 ±0·90	0·42 ±0·07
AST $\mu cat\ L^{-1}$	1·32 ±0·28	*2·98 ±0·23	1·95 ±0·70	1·94 ±0·69	3·10 ±1·60	*2·96 ±0·38	1·15 ±0·27
AP $\mu cat\ L^{-1}$	0·77 ±0·35	0·73 ±0·21	0·74 ±0·20	0·43 ±0·09	*2·11 ±0·63	1·49 ±1·11	0·57 ±0·19
ChO $mmol\ L^{-1}$	1·63 ±0·38	1·35 ±0·17	1·60 ±0·30	1·48 ±0·05	1·73 ±0·38	1·60 0·29	*2·15 ±0·17

TB = total bilirubin. CB = conjugated bilirubin. ALT = *S*-alanine-aminotransferase. AST = S-aspartate-aminotransferase. AP = alkaline phosphatase. ChO = cholesterol.
* $P<0·05$ gp 3: gp 2 ** $P<0·001$ gp 3: gp 2.

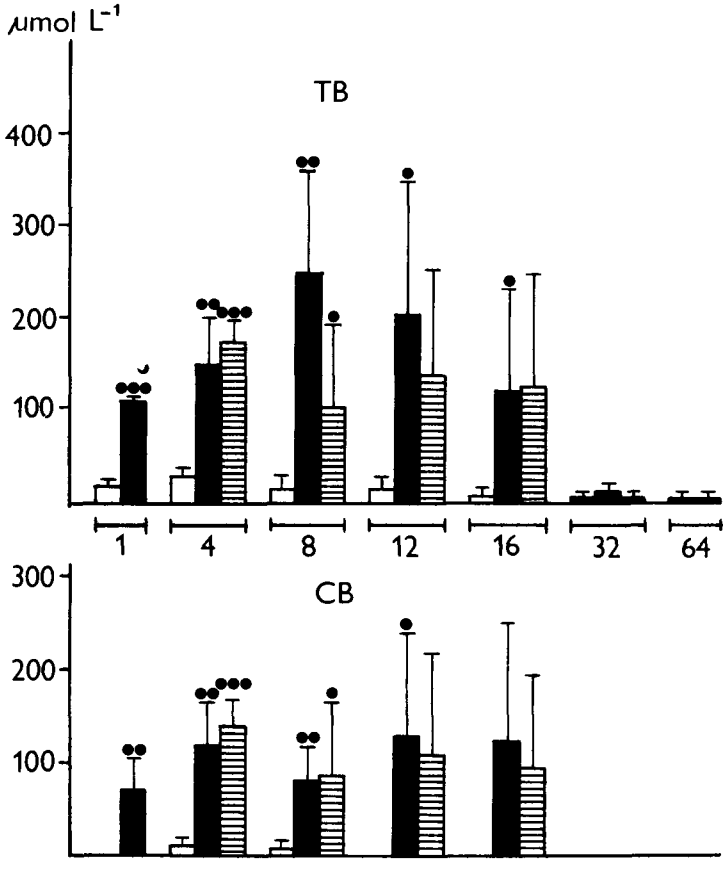

FIG. 1. Changes in TB and CB in group 2 rats (□) and after i.b. turpentine 1:500 (■) and 1:1000 (■). *P* (gp 2 rats vs turpentine-treated rats). · $0{\cdot}01 \leqslant P \leqslant 0{\cdot}05$; · · $0{\cdot}001 \leqslant P < 0{\cdot}01$; · · · $P < 0{\cdot}001$.

serum cholesterol of group 2 rats at 32 and 64 days (Table 1, Fig. 3). An increase in AP activity of the controls had returned to normal values by day 32 (Table 1, Fig. 3).

Turpentine (Fig. 1) caused a significant increase in total and conjugated bilirubin compared with controls without a marked difference between concentrations used. There was a significant increase in AST activity relative to that in group 2 after the 1:500 dose of turpentine (Fig. 2) while there was little difference in ALT activity except immediately after surgery (Fig. 2). By day 32, activity had returned to normal (non-operated) values in all experimental groups. Conversely, serum cholesterol had already increased 24 h after surgery (Fig. 3). Later there was overall a modest decrease in the values but the normal levels of group 1 animals were not reached even after 64 days. In addition there was a delayed increase in AP activity after turpentine (Fig. 3). Serum creatinine concentrations were 74 ± 15 μmol L^{-1} in group 1 animals. In group 2 they were 60 ± 4 μmol L^{-1} (day 1), 71 ± 17 μmol L^{-1} (day 4) falling to 57 ± 6 μmol L^{-1} (day 64). In the rats given turpentine 1:500, serum creatinine concentrations were between 52 ± 3 μmol L^{-1} (day 1) and 54 ± 5 μmol L^{-1} (day 64). No significant differences were seen compared with group 2 rats.

Another consideration in defining this pathological model was body and liver weight (Fig. 4). While the i.b. saline administration reduced the body-weight by only 4%, turpentine 1:1000 i.b. decreased it by 20% during the 16 days after surgery. This corresponded with a decreased intake of food in two thirds of group 2 animals over the same time. The weight loss after T 1:500 was only 8% in 8 days (Fig. 4). Liver tended to increase in weight; there was an increase after both T 1:1000 and 1:500, the former with a maximum on day 12, the latter on day 16. We further explored this difference by considering the liver to body-weight ratio (g/100g). This ratio reached $2{\cdot}90 \pm 0{\cdot}33$ in group 1 rats, $3{\cdot}48 \pm 0{\cdot}22$ in group 3 on day 4 ($P < 0{\cdot}01$ gp 1 vs gp 3) in comparison with $2{\cdot}96 \pm 0{\cdot}31$ on day 12 in the same group without later changes ($2{\cdot}95 \pm 0{\cdot}51$ on day 32). In group 2 there was another increase in the ratio ($4{\cdot}25 \pm 0{\cdot}19$, $P < 0{\cdot}01$ vs gp 3) on day 4 with a gradual fall to a lower value during the following period. I.b. administration of turpentine had a marked effect on the ratio, culminating with a value of $5{\cdot}29 \pm 1{\cdot}30$ ($P < 0{\cdot}01$ vs gp 2) on day 12 for T 1:1000 and with a value of $4{\cdot}64 \pm 1{\cdot}17$ ($P < 0{\cdot}005$ vs gp 2) on day 32 in the case of T 1:500. Although the mean ratio decreased, it remained elevated to the end of the experiment. The rats which died did so during the first 12 days. In group 2 8% died. After i.b. administration of turpentine 1:1000 and 1:500, mortality reached 20 and 17%, respectively.

In addition, we decided to compare the relatively well known and profound alteration induced by surgical ligation of the bile duct (Yam & Roberts 1977; Simko et al 1982; Zambraski & Dunn 1984; Kountouras et al 1985; Van Noorden et al 1987) with our model of supposed reversible

Table 2. Biochemical test values (means ± s.e.m.) of rats given i.b. turpentine 1:500 before 2 h ligation of the bile duct and those of rats with chronic bile duct ligation.

Day	Days of the treatment			
	4		32	
	Turpentine (n=4)	Chronic ligation (n=4)	Turpentine (n=7)	Chronic ligation (n=4)
TB μmol L^{-1}	145·3 ± 66·2	171·0 ± 26·2 NS	9·0 ± 2·1	159·8 ± 54·4 $P<0·001$
CB	114·8 ± 49·4	133·5 ± 23·6 NS	0	124·5 ± 69·1 $P<0·001$
ALT μcat L^{-1}	1·44 ± 0·18	1·89 ± 0·81 NS	0·63 ± 0·26	0·59 ± 0·11 NS
AST μcat L^{-1}	6·80 ± 1·72	3·65 ± 0·10 $P<0·005$*	1·34 ± 0·36	2·21 ± 0·35 $P<0·006$
AP μcat L^{-1}	1·33 ± 0·30	1·38 ± 0·81 NS	1·46 ± 0·58	1·84 ± 0·89 NS
ChO mmol L^{-1}	3·15 ± 0·35	3·90 ± 0·78 NS	2·20 ± 0·45	2·90 ± 0·46 $P<0·04$

n = number of rats.
Day = Time after surgery and turpentine administration.
*Chronic bile duct ligation vs turpentine.

changes. For this purpose similar times were explored and established biochemical tests used. Turpentine (1:500) was administered i.b. The results are shown in Table 2. As expected in rats with chronic ligation of the bile duct, TB and CB levels were high and persisted until day 32. In contrast, TB and CB values after turpentine 1:500 were high on day 4, but low again on day 32. Cholesterol had also returned to low levels in this group by day 32.

There were no changes, on day 4, in arterial blood pressure, portal blood pressure, or in the heart rate in group 3 animals, after T 1:500, or after chronic bile duct ligation (Table 3).

Autopsy findings

Abnormalities were found in the extrahepatic bile ducts of rats given turpentine. At 4–12 days the bile duct had a resistent rigid wall. Later there were large dilated ducts containing bile fluid without stasis.

Chronic ligation of the bile duct was accompanied by biochemical and clinical signs of cholestasis. The ear lobes, tails, sclerae and urine became icteric 24–48 h after duct ligation. With time the liver surface changed and there was

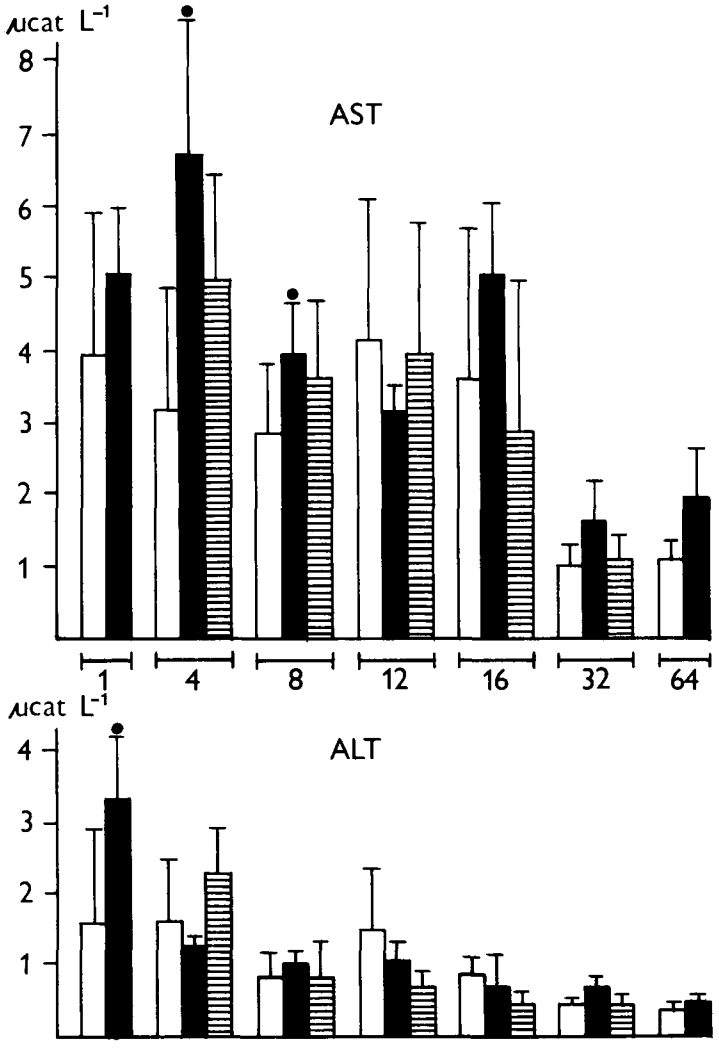

FIG. 2. Changes in serum AST and ALT activities in group 2 rats (□) and after i.b. turpentine 1:500 (■) and 1:1000 (■). P (gp 2 rats vs turpentine treated rats). ● $0·01 \leqslant P \leqslant 0·05$.

Table 3. Some effects on haemodynamics in sham-operated rats, after turpentine 1:500 before 2 h ligation of the bile duct and after chronic bile duct ligation on day 4 of the experiment.

	Portal b.p. mm Hg	Arterial b.p. mm Hg	Heart rate beats min^{-1}	Liver wt g $100\ g^{-1}$
Sham op. (n=3)	6·73±0·82	115±7	414±13	3·35±0·50
Turpentine (n=6)	7·13±1·10	103±11	355±68	*4·22±0·94
Chronic ligation (n=6)	6·87±0·67	108±5	369±66	*4·99±1·21

*$P<0·05$ vs SH

marked icterus, as well as dilation of the bile duct proximal to the obstruction.

Histology

Tissue was obtained at 1, 4, 8, 16, 32 and 64 days after turpentine 1:500. Initially, hepatic parenchyma in portal areas and their vicinity was affected. At 24 h after turpentine administration bile-ducts were dilated with regressive changes of the epithelium. In some animals severe steatosis was found. We found zonal parenchymal necroses with prominent blood extravasation in the more severe lesions. Zonal necroses in various stages of repair were also seen at 8, 16 and 32 days.

From 4 to 64 days bile duct proliferation in and around the portal areas dominated. This change varied between animals. Nevertheless, the number of ductules tended to increase with time. Proliferating ductules invading parenchyma were accompanied by inflammatory cellulization and later by fibroblasts and reticulin fibre accumulation. After 32 and 64 days some animals showed a degree of pseudolobular rearrangement of hepatic parenchyma. Conversely, in other rats, there tended to be a diminution of pathological features. There were deposits of ferric iron pigment in Kupffer cells at the hepatic lobules periphery and in the portal areas, particularly at later times. These obviously resulted from the extravasations seen in the initial stages. Occasionally bile plugs were encountered at 16 and 32 days. Common bile duct pathology was evaluated in the region above the lower ligature. Mucosal erosions had developed at all times studied. These were accompanied by an acute, and later subchronic, inflammatory reaction in the duct wall.

Discussion

There have been many studies, mainly in rats, of the experimental extrahepatic cholestasis induced by common bile duct ligation. These delineated the morphological changes as well as the functional alterations of hepatocytes. Liver changes disappear only slowly after the relief of obstruction (Aronsen 1961) and recovery depends on the duration of obstructive cholestasis. Temporary obstruction of the common bile duct can be effected by recannulation (Yam & Roberts 1977) or by a cannula introduced into the common duct at the beginning of the experiment. The cannula is exteriorized and its end sectioned to permit free flow of bile when required (Accatino et al 1979). Our aim was

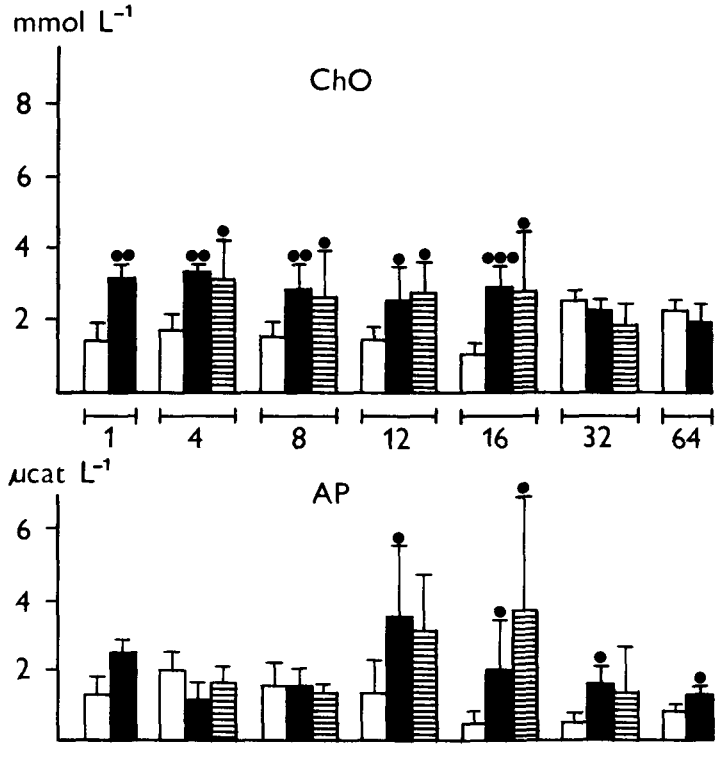

FIG. 3. Changes in serum cholesterol and AP in group 2 rats (□) and after i.b. turpentine 1:500 (■) and 1:1000 (■). P (gp 2 rats vs turpentine treated rats). ● $0·01 \leq P \leq 0·05$; ●● $0·001 \leq P < 0·01$; ●●● $P < 0·001$.

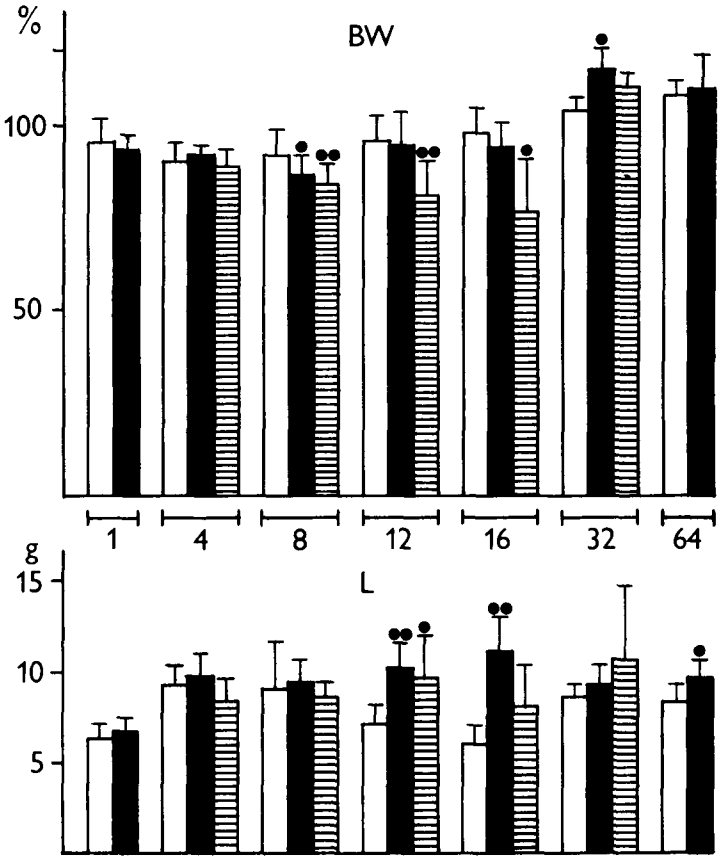

FIG. 4. Changes in body weight (BW) and liver weight (L) in group 2 rats (□) and after i.b. turpentine 1:500 (■) and 1:1000 (■). P(gp 2 rats vs turpentine treated rats. ● $0{\cdot}01 \leqslant P \leqslant 0{\cdot}05$; ●● $0{\cdot}001 \leqslant P < 0{\cdot}01$.

to establish another method of avoiding the second operation while achieving temporary cholestasis, in particular one which might be more analogous to human pathology.

For this purpose we used "chemical inflammation", namely turpentine in olive oil (1:1000 and 1:500). Immediately after turpentine administration into the bile duct, the common duct was ligated for 2 h to standardize turpentine administration. Both total and conjugated bilirubin had increased four days after turpentine administration. Maximum values were reached between day 4 and day 8. The percentage of CB in serum was relatively constant and ranged from 85 to 98% of TB except on day 8 after turpentine 1:500. Thus, the capacity of rats to conjugate bilirubin was not seriously impaired. Bilirubin is considered a better indicator of impaired hepatobiliary excretion than alkaline phosphatase because of its more rapid clearance (Pelligrini et al 1982). Moreover, the finding that the conjugated fraction decreases more rapidly than direct or total bilirubin during relief of cholestasis (Arvan & Shirey 1985) might be useful in diagnosis. The results presented in this report are in agreement with previous data. Furthermore, the bilirubin microsomal conjugation capacity is markedly altered in rats with chronic bile duct ligation (Bengochea et al 1985). The fall of TB and CB, elevated after turpentine i.b. administration, after day 8 occurs more quickly and reaches the values of group 2 and group 1 animals. This could be a sign of the temporary effect of "chemical inflammation" initiating acute hepatotoxicity or cholestasis.

Other criteria of hepatic function are AST, ALT and AP activities and cholesterol level in serum. An increase in these values is also regarded as a measure of hepatotoxicity and cholestasis after bile duct ligation (Allison et al 1978; Franco et al 1979; Hishida et al 1980; Accatino et al 1981; Gliedman et al 1985; Komoda et al 1986; Van Noorden et al 1987).

The fact that hepatic function of rats in groups 3 and 2 returned to normal values indicated that any impairment of the liver, if present, was reversible.

We did not find an increase in serum creatinine concentration in rats after turpentine, in contrast to the results from rats with chronic bile duct ligation (see also Yarger 1976; Hishida et al 1980). According to Yarger, an increase in serum creatinine, which is approximately doubled, is commensurate with the 41% reduction in glomerular filtration in these rats. Thus, it seems that turpentine i.b. does not reduce glomerular filtration and the kidneys are not primarily affected by turpentine i.b. administration.

Biochemical and morphological evidence indicates that the turpentine effect is primarily concentrated in the liver. Also, in the laboratory tests there were no signs of serious haemolysis, or of effects on arterial blood pressure, heart rate, or portal blood pressure. The dynamics of functional changes documented in our report reflect the temporary

hepatocellular toxicity and/or cholestasis induced by bile duct damage, following a 2 h stenosis, where the effect of ligature pressure can be amplified by inflammation changes.

Finally, increasing turpentine concentration affected the total body-weight. In control rats body-weight was slightly reduced immediately after surgery but in the next few days it soon reached or exceeded the preoperative level. There was a difference between the effects of turpentine 1:1000 and turpentine 1:500. There was a greater weight loss and slower recovery of weight with the lower concentration of turpentine. There is no obvious explanation; perhaps a marked ascites developed in rats after turpentine 1:500, as in the rats after chronic bile duct ligation, possibly as a result of a positive sodium balance (similar to that reported by Yarger 1976). The eventual decrease in the liver/body-weight ratio, following the initial gain, could be related to the observed histological changes.

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