Comparative Histochemical Study of Rat Liver in Bile-Duct Ligation and in Alpha-Naphthyl Isothiocyanate (ANIT) Intoxication

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ALTHOUGH MUCH WORK has been done describing acute extrahepatic bile-duct obstruction in contrast to acute intrahepatic cholestasis,[1-8] the similarities between both types of cholestasis have been stressed more than the differences.

The histochemical approach to this problem is of particular interest, since by relatively simple techniques a clear picture can be obtained of the morphology of some submicroscopic cellular elements (e.g., canaliculi) together with an, until now, insufficiently understood indication of the functional disturbances of the liver cell.[2,4]

Alpha naphthyl isothiocyanate (ANIT) has been described as a suitable model for the study of acute intrahepatic cholestasis,[9] although some authors[10,11] have indicated that besides the hepatocellular damage, at a certain period of intoxication an extralobular mechanical obstruction also occurs. These findings reveal the necessity of a critical reevaluation of the histologic, histochemical, and biochemical data observed in ANIT-induced cholestasis, in comparison with the early phase of experimental bile-duct ligation.

Materials and Methods

A total of 66 male albino rats (MOL strain) weighing 250–350 gm. were used. During the experiment all animals had free access to laboratory diet and tap water.

Group I: Standards

Five completely normal animals were sacrificed and used as standards for histochemical and biochemical analyses.

Group II: Bile-Duct Ligation

In 13 experimental animals a median laparotomy was performed under constant-flow ether anesthesia. The bile duct was exposed by blunt dissection, doubly ligated

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halfway between the duodenum and the hilus of the liver, and sectioned between the ligatures. The operation was performed under clean but not aseptic conditions.

In 3 of the 12 control animals in this group, a laparotomy was performed and the bile duct dissected. Five animals underwent a simple laparotomy, and 4 animals received an ether anesthesia of a duration comparable to the time necessary for the complete operation.

Group III: ANIT Intoxication

The 24 experimental animals received a daily dose of ANIT (45 mg./kg. body weight) dissolved in 0.1 ml. of olive oil. The preparation and administration of the drug has been described elsewhere.[11]

The 16 control animals received the same amount of oil, without ANIT.

The animals were sacrificed by exsanguination in groups at 12, 24, and 36 hr., and 2, 4, and 6 days after the start of the experiment. The blood of all the animals was analyzed for bilirubin using a micromethod derived from the technique of Jendrassik and Grof,[12] and for alkaline phosphatase using a commercially available micromethod (Noury, Nourypharma, Deventer, Holland) by Bessey, Lowry, and Brock.[13]

After exsanguination, the liver was quickly removed from 10 experimental and all sham-operated animals in Group II and from the rats in Groups I and III, and processed as described in a previous paper.[11]

Results

Group I: Standards

Histochemistry. The histochemical characteristics of the rat liver found in this experiment did not differ from the picture described elsewhere.[11,14–16]

Biochemistry. No statistically significant differences could be found between the normal and the control groups. The means of the pooled data were 0.35 mg./100 ml. for total bilirubin (SD = 0.15), and 6.53 Bessey-Lowry units for alkaline phosphatase (SD = 1.60).

Group II: Bile-Duct Ligation

Experimental Animals

Macroscopic Findings. From 12 hr. on, a striking dilatation of the ligated duct was observed, together with external and internal signs of jaundice. The liver showed an increased consistency with an accentuated lobular pattern.

Microscopic Findings. The tissue was stained with hemalum and eosin. The moderate inflammatory infiltration of the portal fields seen at the twelfth hour, changed to strong infiltration during the 24- to 36-hr. period, and thereafter decreased gradually, showing a fibroblastic and histiocytic predominance. The interlobular bile ducts were slightly dilated during the first 24 hr. The epithelium was always found intact although mitotic figures were frequently seen in the later phase.

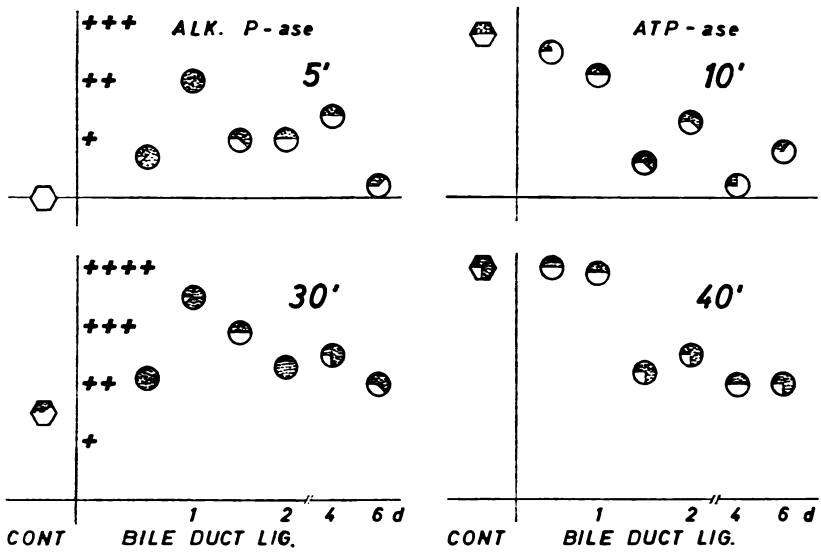
Bile duct hyperplasia had started already at the twenty-fourth hour and progressed until Day 6 when numerous small, newly-formed ducts were found in the periphery of the portal fields and proliferating in the hepatic parenchyma.

The parenchymal necrosis which appeared at the twelfth hour was most pronounced at 24–36 hr., regressed thereafter and was gradually organized by granulation tissue. Most of the necrotic foci were found in the periportal and midzonal areas; only a few were observed in the centrilobular zones.

At 48 hr. after the ligation of the common duct, the liver cells frequently showed anisokaryosis, mitotic figures, and hyperchromatic nuclei, while some cells appeared binucleated. In the same period hyperplasia of the Kupffer cells began.

Enzyme histochemistry. Canalicular adenosine triphosphatase (ATPase) activity was in the control range during the first 24 hr. After this period enzyme activity gradually decreased toward the very low values seen at the end of the experiment (Text-fig. 1).

The changes in canalicular structure were best observed in free-floating sections. Twelve hours after the bile duct ligation most of the periportal canaliculi were dilated. Centrilobular canaliculi were straight, and only a few of them were slightly dilated. After 24 hr., conspicuous dilatation was observed in the peripheral part of the lobuli, and the converging



TEXT-FIG. 1. Enzyme activity in AlkPase and ATPase staining. Figures indicate incubation times. Activity is expressed from 1+ to 4+. Spreading from periportal toward centrilobular zones is presented by dark part of circles.

points showed saccular formations. Numerous tiny paracanalicular dots were seen and most of the centrilobular canaliculi were dilated, but no tortuosity could be seen (Fig. 1 and 2). After 36 hr. the previously dilated canaliculi collapsed partially: dilated parts alternated with narrowed segments (Fig. 3 and 4). Pericanalicular dots were still numerous. Around larger portal fields canaliculi showed a fading of the enzyme staining. At 48 hr. the canaliculi were still more collapsed and showed an irregular tortuosity and conspicuous side-branching. This pattern did not change much until the end of the experiment (Fig. 5).

The activity of alkaline phosphatase (AlkPase) in normal livers (Fig. 6) is confined to a narrow periportal rim. After 12 hr. a slight increase was noticed which reached a maximum at 36 hr., when the whole lobule showed enzymatic activity. In the subsequent period the enzyme activity decreased to levels only slightly higher than in the control livers (Text-fig. 1). The distribution of positivity was irregular and patchy (Fig. 7), not at all corresponding to the pattern described by Rappaport.

During the 24–36-hr. period the activity of ATPase (Fig. 2) and AlkPase (Fig. 8) could be seen in all liver cell membranes. In addition, a correlation was observed between the decrease in enzyme activity and the morphologic changes of the canaliculi. This was particularly clear in sections incubated during short incubation times.

At the twelfth hour, after the ligation of the common bile duct, the acid phosphatase (AcPase) positive granules showed a displacement in a broader pericanalicular zone. From Day 2 on the granules were scattered in a wide zone between the nucleus and the canalicular membrane. After Day 2, and especially in the later phase of the experiment, a conspicuous proliferation of the Kupffer cells occurred: numerous large Kupffer cells showed increased staining for AcPase.

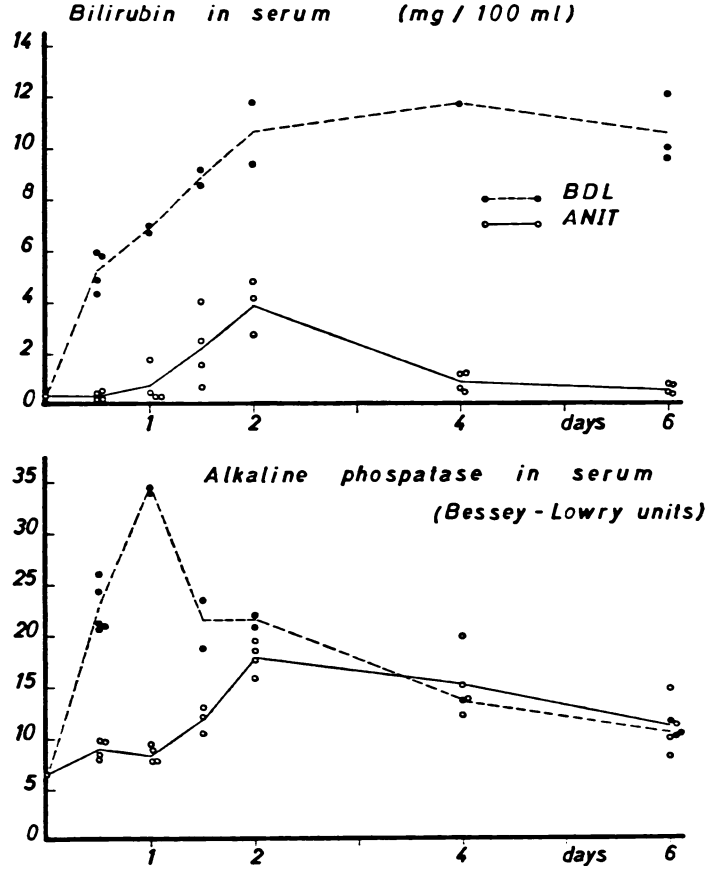
In the preparations stained for 5′-nucleotidase a decrease in canalicular enzyme activity was noticed after 36 hr., which progressed until, in the later phase of the experiment, no canalicular positivity could be seen. No significant changes in the sinusoidal activity could be observed throughout the experiment.

Biochemistry. From the very first hours after the operation a sharp increase was noticed in the serum bilirubin and AlkPase levels. The bilirubin concentration remained at the same high level throughout the experiment. In contrast, the AlkPase levels decreased sharply in the 36-hr. period and in later phases returned rather slowly to values slightly above the control range (Text-fig. 2).

The control animals did not show any significant histologic, histochemical, or biochemical changes, in comparison to the Group I animals.

Group III: ANIT Intoxication

The histologic and histochemical findings were reported in detail elsewhere.[11] As a summary we can mention that for the first 24 hr. only tortuosity and increased side-branching of the canaliculi were noticed in the free-floating sections stained for ATPase. The 36–48-hr. period was characterized by important inflammation of the portal fields and necrosis of the bile-duct epithelium. At this time some paraportal necrotic foci appeared. The histochemical stainings (ATPase and AlkPase) revealed a striking dilatation of the periportal canaliculi. These lesions showed a typical Rappaport distribution (AlkPase). In the following phase of the experiment (4–6 days), a substantial decrease of all canalicular phosphatases, together with a picture of collapse and increasing irregularity of the



TEXT-FIG. 2. Serum bilirubin (mg./100 ml.) and AlkPase (Bessey-Lowry units) concentrations. Interrupted line indicates data in bile-duct ligation; unbroken line, ANIT intoxication. For control values arithmetic mean is presented. For experimental values, all data are plotted. Curve is drawn through arithmetic means of every group.

canaliculi, was noticed. In this period a bile duct proliferation occurred.

Biochemistry. The serum bilirubin levels did not show any significant changes during the first 24 hr. At 36–48 hr. a sharp increase of the serum bilirubin concentration was noticed. At 4 and 6 days a gradual decline of the levels to nearly normal values was observed. The serum alkaline phosphatase levels were slightly increased during the first 24 hr. and evaluated later in a parallel relationship with the bilirubin concentrations.

Discussion

In the results described above, it is clear that two different phases can be distinguished in the liver changes following bile duct ligation.

The early phase (12–24 hr.) is characterized by a conspicuous dilatation of the canaliculi together with a strong increase in AlkPase activity,[4,7,17–19] while the ATPase activity remains about normal. At the same time the serum bilirubin and AlkPase levels are markedly elevated.[20,21]

The most important findings in the second phase (from 36 hr. on) are a decrease in the histochemically demonstrable canalicular phosphatases.[19] A scattering of the AcPase-positive granules is noticed. Especially important is the partial collapse of the previously dilated canaliculi and their increasing morphologic irregularity. At the same period the AlkPase levels in the serum drop to much lower values, while the bilirubin levels remain about as high.[20,21]

These findings point toward a preponderance of mechanical effect in the early period, while the second phase is dominated by a progressively increasing hepatocellular alteration. This alteration can probably be explained as a partial decompensation of the biliary pole of the liver cell, induced by the presence of toxic secretion products under an increased pressure.

The importance of the toxicity of the bile components and in particular of the bile acids, in comparison with the role played by the hyperpressure, has been debated extensively in the literature.[22–24] The present opinion formulated by Heimann[24] indicates that both the pressure and the toxicity are responsible for the liver cell damage: the mechanical factor produces bile stasis and anoxia, which enable the chemical toxic compounds to penetrate and damage the liver cell. Although the cited studies try to explain the pathogenesis of the bile infarcts, it is conceivable that a less pronounced cellular damage is caused by an analogous mechanism.

The interpretation of the second phase as a partial failure of the liver cell was suggested by the finding of partially and irregularly collapsed canaliculi (staining for ATPase) coinciding with a drop in the histochemically demonstrable canalicular phosphatases and the serum AlkPase

levels. Birns and Masek [19] observed the same evolution of the histochemical characteristics but attributed them to the opening of direct shunts between the canaliculi and the sinusoidal spaces. Whether these shunts really exist has been the object of a long controversy between different authors. A summary of this debate can be found in the works of Steiner,[25] Rouiller,[26] and Aterman.[27] However, the studies of Hampton,[28] Biava,[29] and Ehrenbrand and Waldeck [30] seem to exclude definitively the existence of a direct passage between the bile canaliculi and the sinusoids.

The fact that the gradual disappearance of the canalicular phosphatases starts in the periportal area might reflect a more severe impact of the hyperpressure of the toxic components in this part of the lobule, since these canaliculi also showed earlier and more important dilatation.

The work of Polin *et al.*,[31] Keiding,[32] and Sebesta, Bradshaw, and Prockop [33] made it clear that the elevated AlkPase levels in bile duct obstruction have their origin in the liver itself. Bauer [20] stressed the importance of the rate and degree of elevation and decline of the alkaline phosphatase levels in relation to the severity of the hepatic injury. Thus the decrease in serum AlkPase levels might be the reflection of a decrease in the functional capacities of the liver cell. A similar hypothesis was formulated by Bengmark, Edlund, and Olson [34] to explain the drop in serum transaminases between 2 and 4 days after bile-duct ligation. It is noteworthy that in their experiments the drop in the serum glutamic pyruvic transaminase levels was preceded by a decrease in the liver homogenate activity of this enzyme.

The ATPase-positive pericanalicular dots observed in bile duct ligation do not correspond to lysosomes.[7] Presumably they represent resorption vacuoles from the bile canaliculus.[7,35] The canalicular side-branching observed in the later phases of bile-duct ligation [7,19,37] and in ANIT intoxication [7,11] probably corresponds to the intracellular invagination of the canaliculi observed by Hampton [35] and Steiner, Phillips, and Baglio.[36]

The positivity for ATPase (Fig. 2) and AlkPase (Fig. 8) of all liver cell membranes in the early phase of bile-duct obstruction was also noticed by Goldfischer,[4] but could not be detected by Rondez and Rüttner in histochemical investigations [7] or by Wills (ATPase) [38] in electron microscopic studies. However, in this laboratory a distinct positivity for AlkPase was found with the electron microscope in all liver cell membranes.[39] The discussion of these findings together with a general discussion of the histochemical findings in ANIT intoxication and in particular of the changes in the staining for ATPase are reported in a previous paper from this laboratory.[11]

The occurrence of a transient (36–48 hr.) and acute increase in the

levels of both serum bilirubin and AlkPase are in agreement with the hypothesis of the superposition of an acute extralobular mechanical obstruction at this period of the intoxication.[10,11]

In the obstructive phase of ANIT-induced cholestasis (36–48 hr.) the distribution of the lesions shown in the sections stained for AlkPase takes a typical Rappaport pattern,[40] while in bile-duct ligation, the lesions are more homogeneously distributed throughout the lobule (Fig. 7). These findings suggest a different pathogenesis: whereas in ANIT intoxication the lesions are centered upon a particularly damaged portal field, suggesting an obstruction in this portal tract, in bile-duct ligation the more diffuse distribution is due to the *total* bile-duct obstruction with a diffuse effect throughout the liver tissue.

Regarding the importance of the differential diagnosis between the intrahepatic and the extrahepatic obstruction,[1–8,41,42] the differences in the canalicular morphology between bile duct obstruction and the early phase of ANIT-induced cholestasis can be stressed with interesting results. While in extrahepatic obstruction a striking dilatation of regular canaliculi starts from the periportal area, rapidly extending toward the central vein, in ANIT intoxication the earliest changes are a typical side-branching and tortuosity of the centrilobular canaliculi extending towards the periportal area. This is visible throughout the experiment. At the obstructive phase (36–48 hr.) a dilatation of the previously altered periportal canaliculi occurs, leaving a centrilobular area where the specific ANIT-induced changes can still be noticed. At this phase no confusion between the pure extrahepatic obstruction and the ANIT-induced obstruction is possible. Another distinctive sign is the appearance of the tiny ATPase-positive dots in bile duct ligation, which were not frequently seen during the obstructive phase in ANIT intoxication. Probably the resorption mechanisms in the ANIT-induced cholestasis are much less active due to the previous changes in the cellular membrane.

However, in the later phases (4–6 days), at the period of decompensation and intoxication of the liver cells in bile-duct ligation, the canalicular structure resembles that of the canaliculi in ANIT intoxication. Nevertheless in bile-duct ligation (6 days) the patchy distribution of the activity in the partly dilated and tortuous canaliculi surrounded by numerous tiny paracanalicular dots (ATPase) can readily be distinguished from the more homogeneous distribution in the lobule of the narrow, tortuous, and twisted side-branching canaliculi in ANIT intoxication where paracanalicular dots are seldom seen. Analogous observations were made by Rondez and Rüttner.[7]

As a conclusion we can describe the extralobular obstruction as a pri-

mary dilatation of the periportal canaliculi with the appearance of numerous ATPase-positive paracanalicular dots, whereas the intralobular (cellular) cholestasis is characterized by an increased tortuosity and side-branching resulting in an irregular and interrupted canalicular picture. Analogous conclusions are found in the publications of Steiner[3] in electron microscopic studies, and in Holzner[2] and Holzner, Stefenelli, and Wewalka[5] in histochemical investigations of human liver biopsies. At a certain phase in ANIT intoxication an extralobular obstruction occurs superimposed on the primary intralobular cholestasis.

The description of cholestasis as extralobular and intralobular (cellular) instead of extra- and intrahepatic is useful in the histochemical study of ANIT intoxication. The extralobular obstruction can be localized at any level of the biliary tree, resulting in more-or-less widely distributed damage to the liver cells.

Summary

In 1 group of male rats the bile duct was ligated. Another group of rats received a daily dose of ANIT. The histologic and histochemical changes of the liver were compared with the changes in serum bilirubin and alkaline phosphatase levels.

This study shows that in bile duct obstruction, after an initial phase when mechanical effects dominate, a period occurs in which the failure of the liver cells prevails.

A differential diagnosis with ANIT-induced cholestasis can be established on histochemical grounds in the early phases of both types of pathology of the liver. The origin of the differences between these experimental models is discussed.

The concept of intralobular and extralobular cholestasis reveals its usefulness in enzyme histochemical investigations.

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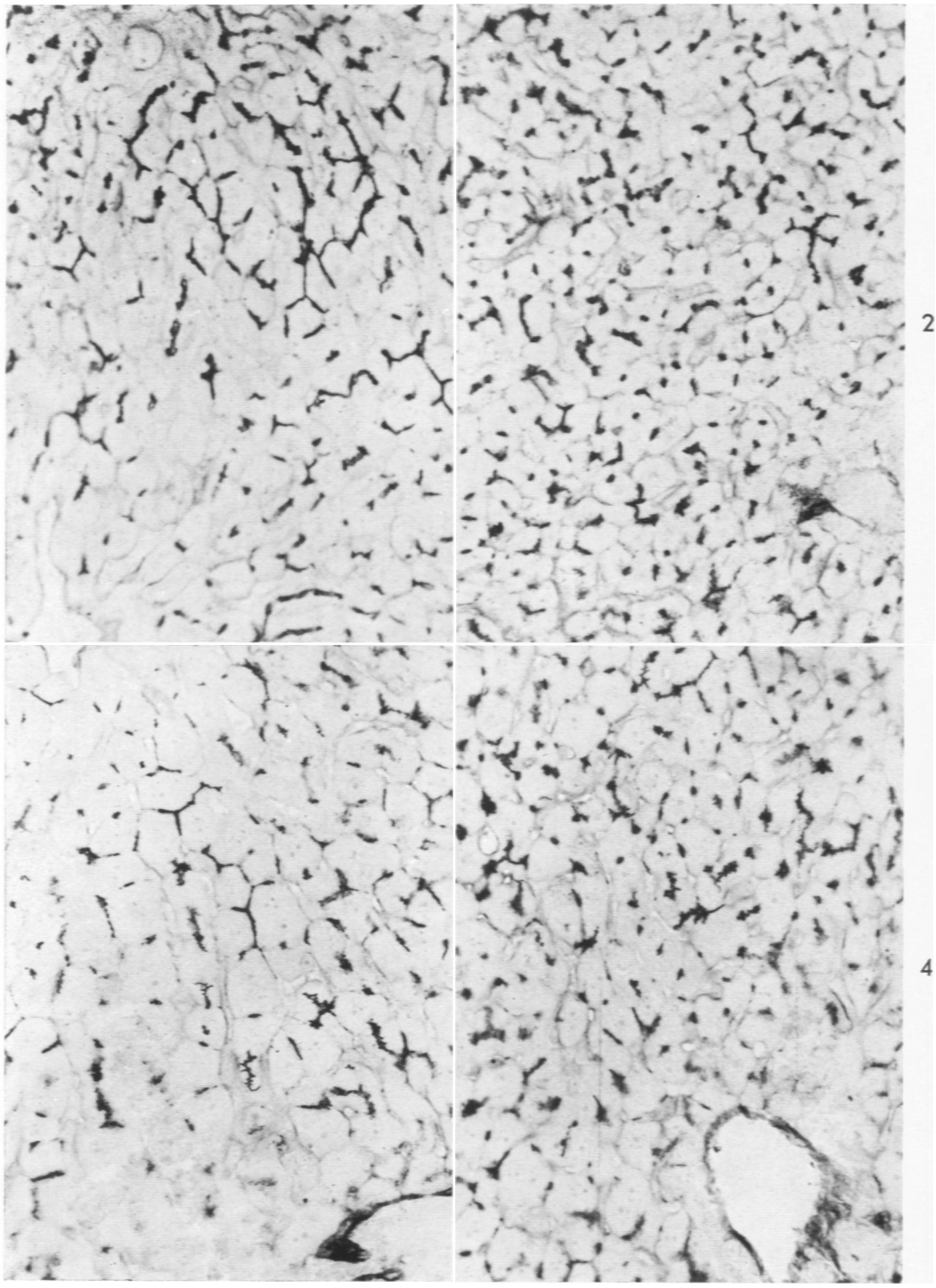
Legends for Figures

Fig. 1. Bile duct ligation (BDL) for 24 hr. Stained for ATPase; free-floating section incubated for 40 min. Conspicuous canalicular dilatation. No centrilobular tortuosity of canaliculi. × 550.

Fig. 2. BDL for 24 hr. Stained for ATPase; free-floating sections incubated for 40 min. Positive granules can be found around dilated canaliculi. All cell membranes show enzyme activity. × 550.

Fig. 3. BDL for 36 hr. Stained for ATPase; free-floating section incubated for 40 min. Partial canalicular collapse with irregular staining intensity. No centrilobular tortuosity of canaliculi. × 550.

Fig. 4. BDL for 36 hr. Stained for ATPase; free-floating section incubated for 40 min. Partial canalicular collapse with irregular staining intensity. No centrilobular tortuosity of canaliculi. × 550.



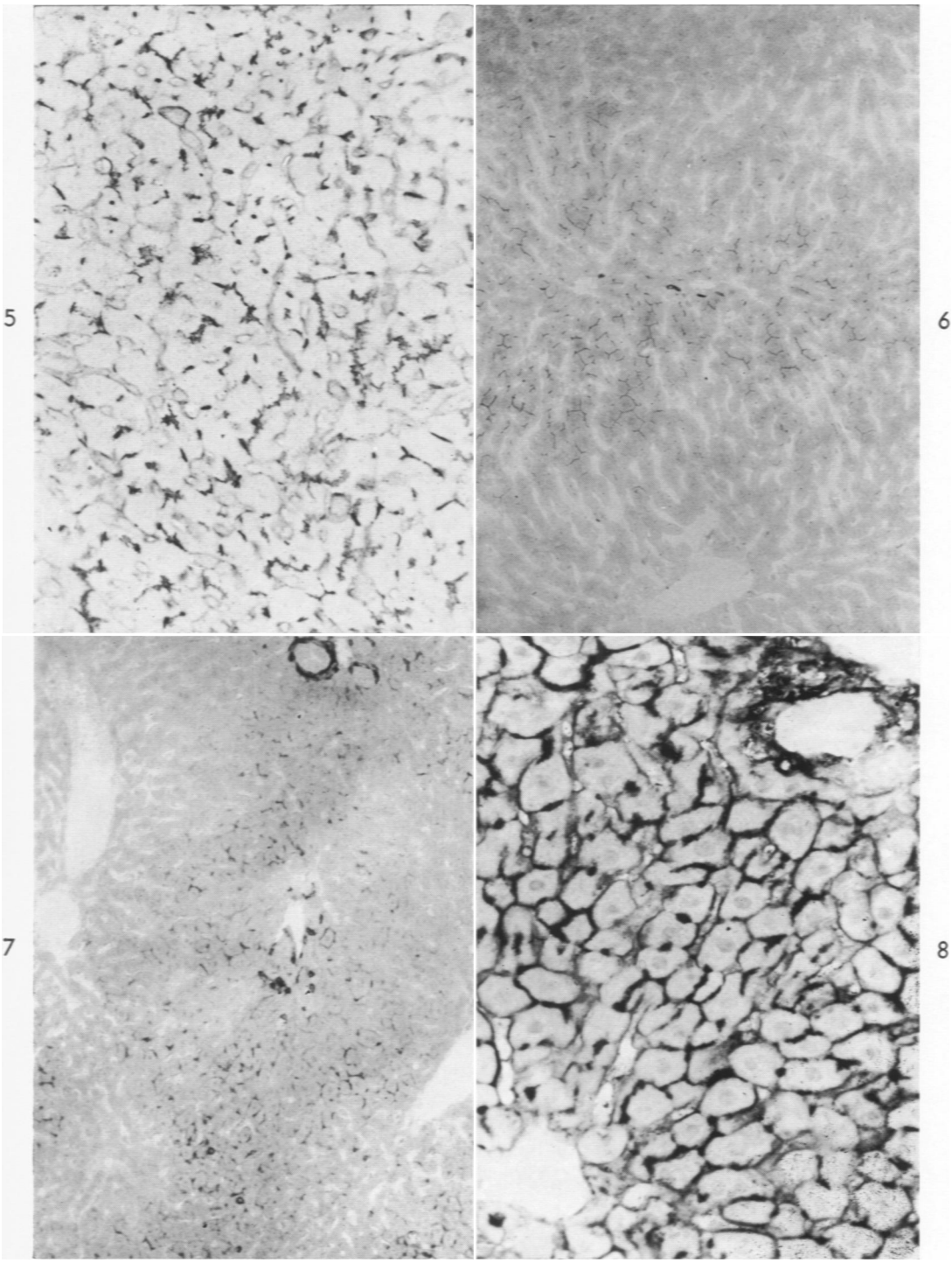


Fig. 5. BDL for 4 days. Stained for ATPase; free-floating section incubated for 40 min. Irregular dilatation and tortuosity of canaliculi with slight staining of all liver cell membranes. × 550. **Fig. 6.** Control rat liver. Stained for AlkPase; free-floating section incubated for 30 min. Low enzyme activity confined to narrow periportal area. × 220. **Fig. 7.** BDL for 4 days. Stained for AlkPase; free-floating section incubated for 30 min. Slightly increased enzyme activity with patchy distribution. Some canaliculi are still dilated. × 220. **Fig. 8.** BDL for 2 days. Stained for AlkPase; free-floating section incubated for 30 min. High enzyme activity throughout lobule. Canaliculi are conspicuously dilated. Positive staining of all liver cell membranes. × 550.