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A Morphologic and Histochemical Study of Biliary Atresia in Lamprey Liver*

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Summary. A morphologic and histochemical study was carried out on the liver of larval and adult lampreys at the optical and electron microscopic level.

In the larva the liver is composed of blind ending single cell thick tubules of hepatocytes. The tubular lumina provided with microvilli are morphologically comparable with the canalicular lumens of the higher species of animals. The cytoplasm of the hepatocytes contains numerous inclusions with heterogeneous appearance and crystalline material. The biliary system is composed of numerous bile ductules and ducts.

In the adult lamprey, the biliary system has disappeared. The hepatocytes loose their tubular arrangement and the characteristic differentiation of their biliary pole. In contrast to previous reports in the literature the presence of bile pigment in the adult lamprey liver could not be demonstrated with any histochemical technique.

Key words: Liver — *Lampetra* — Physiological biliary atresia — Electron microscopy — Histochemistry.

Introduction

During the metamorphosis of a larval to an adult lamprey profound changes in physiologic and morphologic characteristics occur: the entire bile-transport apparatus completely disappears during the period of sexual maturation. This atresia creates a cholestatic condition in the adult lamprey liver which usually has been described to be accompanied with bile pigment accumulation in *Lampetra fluviatilis* L. and *Lampetra zanadreai* Vladykov (Bertolini, 1965). In this investigation we have studied the liver of the larval and adult lamprey at the optical and electron microscopical level. An attempt was made to identify the pigment inclusions in the hepatocytes with histochemical techniques, while changes on the biliary pole of the hepatocyte were studied with the help of enzyme histochemical techniques.

Materials and Methods

The liver of 14 ammocoetes larva (*Lampetra planeri* Block) living in the same biotope and with the same external morphological characteristics (average weight 4.0 g, average length 14.0 cm) was studied. Two adult lamprey's of the same biotope and each of identical external morphology were also included in this study.

Two specimens of liver tissue were taken for optical microscopy. One fragment was immediately frozen in isopentane cooled by liquid nitrogen. Cryostat sections were cut at 7μ.

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They were incubated for 10 minutes in Gomori's medium for alkaline phosphatase (Gomori, 1939; Takamatsu, 1939), for acid phosphatase (40 minutes) Gomori, 1941) and in Wachstein-Meisel's medium for ATPase (40 minutes) and 5'nucleotidase (30 minutes) (Wachstein, Meisel, 1957). Control sections were incubated in substrate free incubation media. Further cryostat sections were stained for total bilirubin with Fouchet's reagent (Hall, 1960), and with a diazonium salt (Raia, 1965); for direct reacting bilirubin with ethylanthranylate reagent (Desmet, Bullens, De Groote, Heirwegh, 1968) and with 2,4 dichloraniline (Raia, 1965) and for lipids with Oil Red 0 (Lillie, 1944). A second specimen was fixed in Bouin's fixative and embedded in paraffin. Sections cut at 4 μ were stained with hemalum and eosin, with PAS and after amylase digestion (Takeuchi, 1958), with Perls, Schmorl's, and Van Gieson's methods and with reticulin stain (Pearse, 1960).

A third specimen of the same liver was used for electron microscopy. Thin slices (1/1/10 mm) were fixed by immersion for 2 hours in cold (4°C) 6.00 g/100 ml glutaraldehyde, plus 3.04 g/100 ml formaldehyde in cacodylate buffer, pH 7.2 (Töro, Joo, 1966) followed by buffer rinse overnight.

For morphological study portions of each specimen were postfixed in 1% OsO_4 in phosphate buffer pH 7.2 for 1 hour at 4°C (Millonig, 1961), dehydrated in graded alcohols and embedded in Epon (Luft, 1961).

For cytochemical study, 40 μ thick sections were prepared with the TC_2 Smith and Farquhar tissue sectioner, and incubated for the following:

Alkaline phosphatase activity with beta-glycerophosphate (2 mg/ml) as substrate in a 0.05 M Tris HCl buffer, pH 8.2 for 30 minutes at room temperature. Lead citrate was used as capture reagent (Saito, Ogawa, 1968).

ATPase activity with adenosinetriphosphate (0.5 mg/ml) as substrate in a 0.05 M Tris-maleate buffer, pH 7.2 for 45 minutes at 37°C (Wachstein, Meisel, 1957).

5'nucleotidase with adenosine-5' -monophosphate (0.5 mg/ml) as substrate in a 0.05 M Tris-maleate buffer, pH 7.2 for 30 minutes at 37°C (Wachstein, Meisel, 1957).

Control sections were incubated in each incubation medium without substrate. After incubation the sections were rinsed in cacodylate buffer, postfixed for 1 hour in 1% OsO_4 in phosphate buffer, pH 7.2 (Millonig, 1961) and embedded in Epon (Luft, 1961).

Thin sections were stained with uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963) and examined in a Zeiss EM 9A electron microscope.

Observations

Optical Microscopy

1. Larval Forms. Morphologically the structure of the liver corresponds to a ramified tubular gland composed of blind ending tubules; there are no portal tracts. The cytoplasm of the hepatocytes contains pigment inclusions which are mostly situated near the apical pole of the cell. These inclusions are doubly refractile, slightly PAS positive after amylase digestion, strongly Schmorl positive, partly positive with Oil Red O stained sections and negative with Fouchet's reagent and Perls method. The single layered liver tubules are surrounded by fibres which show positive reactions with PAS, reticulin and Van Gieson stains. The sinusoids between the liver tubules are bordered by endothelial cells.

The biliary system is composed of numerous small bile ductules, distributed between the hepatocytic tubules and larger bile ducts which fuse together in the central axis of the liver. The bile ductules are lined by cuboidal epithelial cells resting on a basement membrane; the larger ducts are bordered by cylindrical epithelial cells, also surrounded by a basement membrane. The latter structures are embedded in loose connective tissue.

After incubation for alkaline phosphatase, ATPase and 5'-nucleotidase the luminal side of the hepatocytes shows a homogeneous positive reaction; a few

positive reacting bodies can be seen in the apical cytoplasm. These enzyme histochemical staining reactions show that the tubular lumina, homogeneously distributed over the whole liver, are plump and rectilinear with side branchings (Fig. 3). After incubation for acid phosphatase no specific reaction can be demonstrated.

2. Adult Lamprey. The bile ducts and ductules have disappeared. The hepatocytes loose their tubular arrangement and form multicellular muralia surrounded by fibre like structures, which appear positive with PAS, Van Gieson and reticulin stains. Some parenchymal areas show foci of eosinophilic necrosis. In the cytoplasm of the hepatocytes the same pigment inclusions are found as in the larval forms. Moreover Perls positive structures can now be demonstrated in the hepatocytes while the sinusoidal fluid also shows a positive reaction. After incubation for ATPase, alkaline phosphatase, 5′-nucleotidase and acid phosphatase no enzyme activity could be detected.

Electron Microscopy

1. Larval Lamprey. The liver parenchyma is composed of tubules formed by 4 to 6 hepatocytes surrounded by collagen fibres and reticulin fibres (Fig. 1).

The biliary lumina of the tubules are separated from the intercellular space by junctional complexes. The biliary pole of the hepatocytes shows long, fine and closely packed microvilli and a broad zone of ectoplasm. The mitochondria are long or oval, they contain few but long cristae. Numerous free ribosomes are scattered through the cytoplasm, amongst them some polysomes are found. The R.E.R. forms numerous parallel lamellae; the S.E.R. appears in the form of small vesicles.

The Golgi apparatus is composed of long and slender cisternae. The hepatocytes also contain more or less scattered glycogen particles of the alpha form (Fig. 2). The numerous cytoplasmic inclusions have a heterogeneous appearance and can roughly be divided in two types (Fig. 1). One type is round or ovoid, is surrounded by a distinct membrane and has a dark matrix; such inclusions may contain myelin figures and granular or lamellar material; the other type appears irregular and even angular in form, and is strongly osmiophilic; these latter inclusions are not clearly delineated by a membrane and contain crystalline material embedded in an electron dense matrix.

The cells lining the bile ducts are provided with short microvilli and contain only a few of the cytoplasmic inclusions described in the hepatocytes. They are characterized by a basement membrane, by deep and numerous interdigitations and wide intercellular spaces (Fig. 5).

After incubation for alkaline phosphatase, ATPase and 5′-nucleotidase a positive reaction is noted on the luminal side of the microvilli of the hepatocytes (Fig. 4).

2. Adult Lamprey. The picture of the adult is characterized by a disorderly arrangement of hepatocytes, endothelial cells, collagen fibres and amorphous granular material (Fig. 5).

Electron microscopic characteristics of the biliary pole of the hepatocyte have disappeared: the microvilli on the apical side are no longer present, the lateral and basal cell membranes become indistinct while in other hepatocytic areas intra-

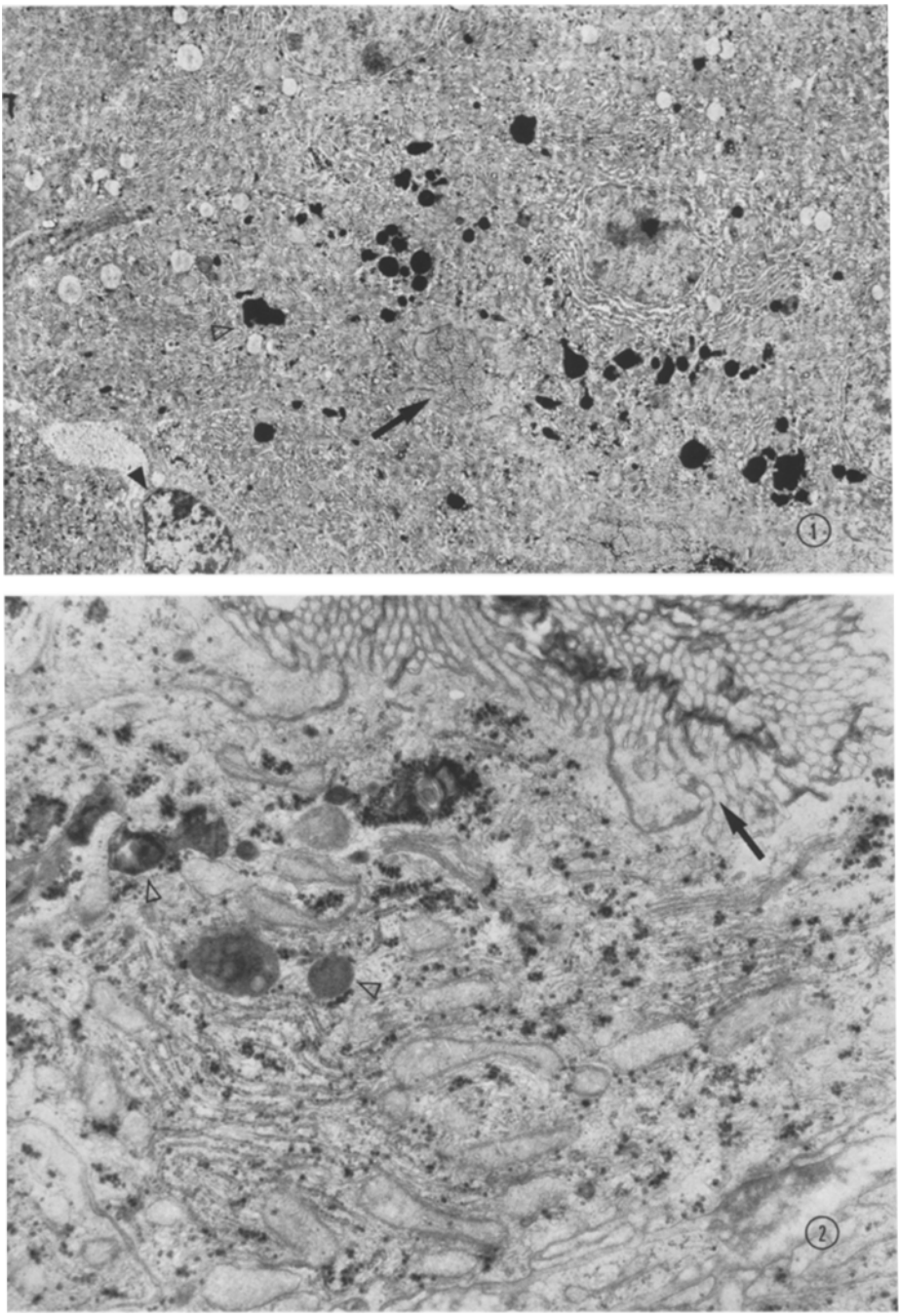
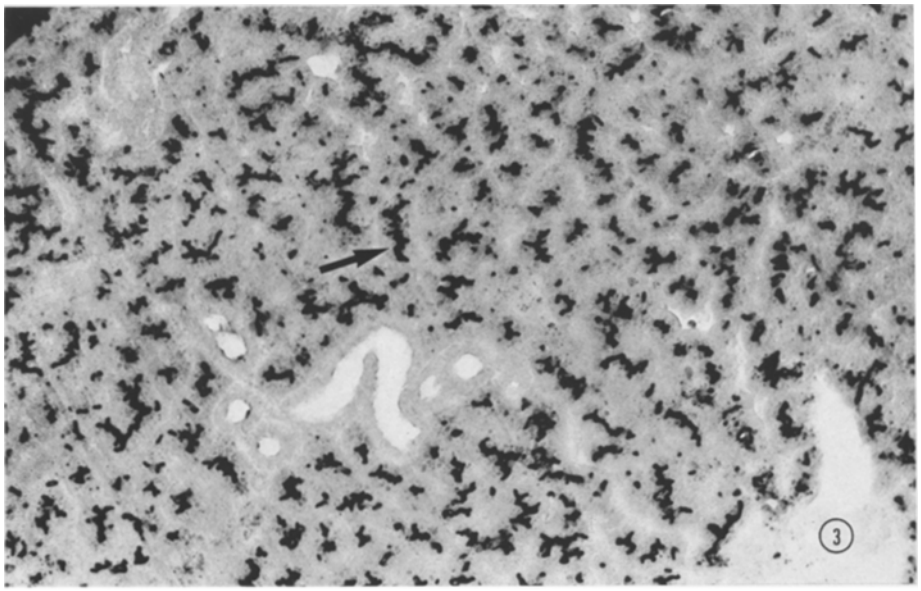


Fig. 1. Larval lamprey liver tissue: thin section prepared for morphologic study, stained with uranyl acetate and lead citrate. A low power micrograph showing the tubular arrangement of the hepatocytes (➔) — with their special cytoplasmic inclusions (▷) — and part of a liver sinusoid (▶). × 5700

Fig. 2. Larval lamprey liver tissue: thin section prepared for morphologic study, stained with uranyl acetate and lead citrate. Numerous long microvilli (➔) and different kinds of cytoplasmic inclusions (▷). × 28500



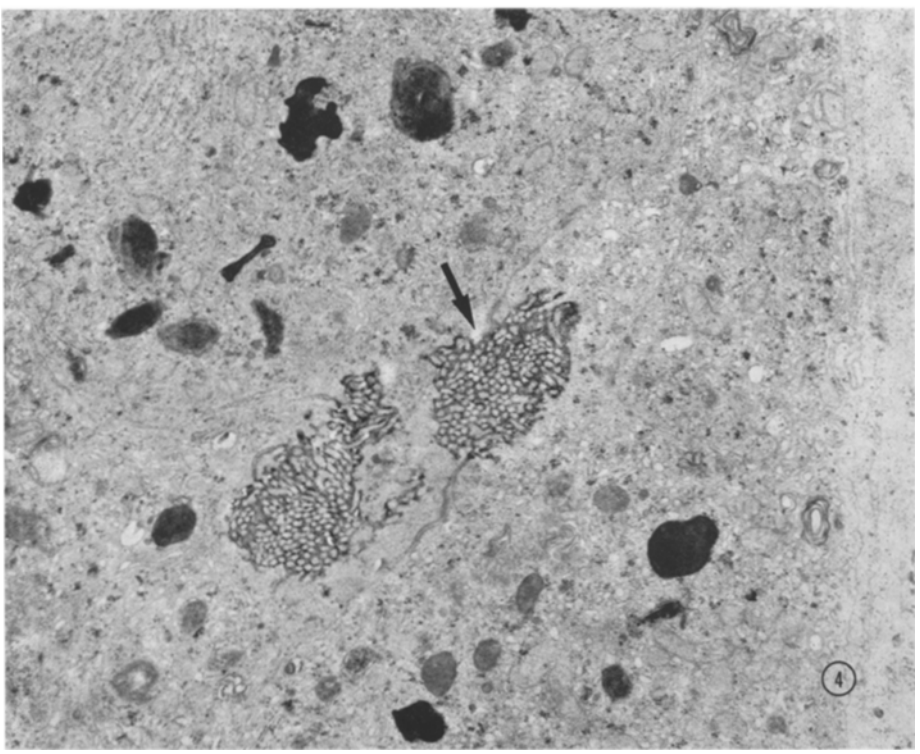
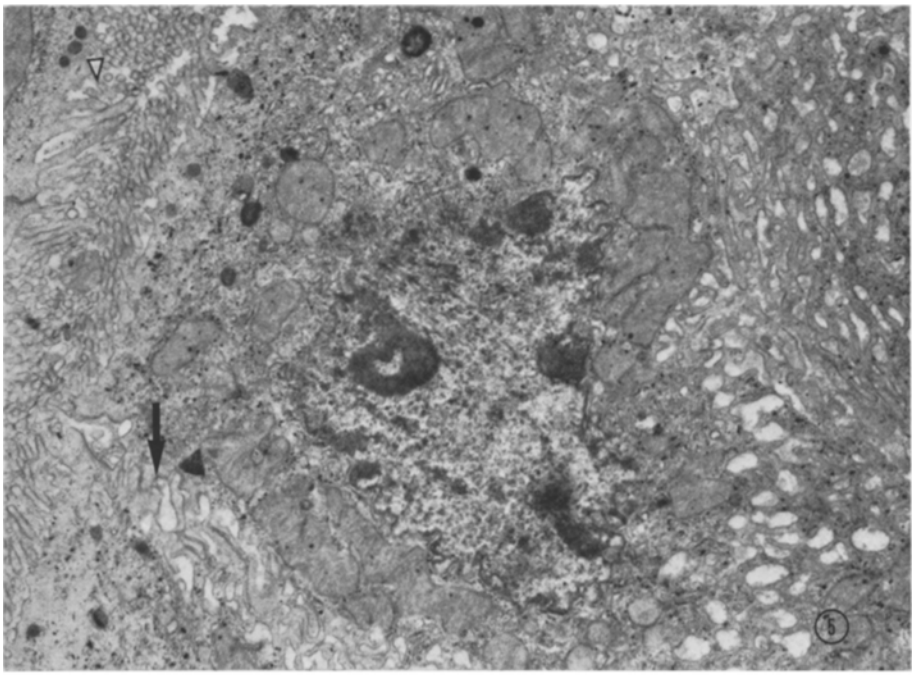


Fig. 3. Larval lamprey liver tissue: fresh frozen section prepared for cytochemical study. Alkaline phosphatase positive tubular lumina (→). × 2000

Fig. 4. Larval lamprey liver tissue: thin section prepared for cytochemical study, stained with uranyl acetate and lead citrate. Alkaline phosphatase positive microvilli of the tubular pole of the hepatocytes (→). × 13500



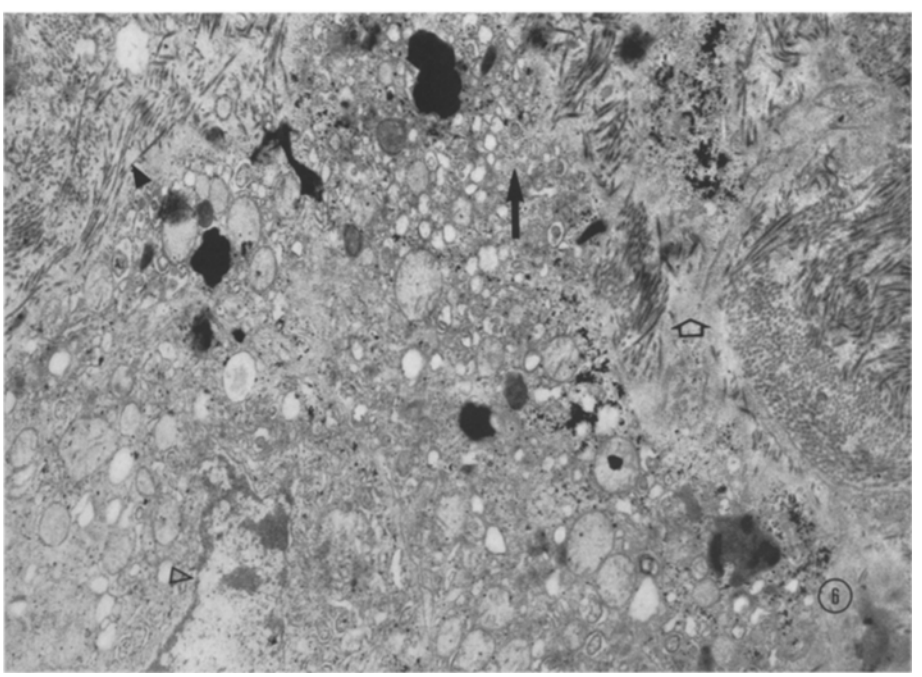


Fig. 5. Larval lamprey liver tissue: thin section prepared for morphologic study, stained with uranyl acetate and lead citrate. Bile duct cells showing numerous deep lateral interditations (➔); short microvilli (▷). × 13500

Fig. 6. Adult lamprey liver tissue: thin section prepared for morphologic study, stained with uranyl acetate and lead citrate. Disorderly arrangement of hepatocytes – with indistinct cell borders (➔) –, sinusoidal cells (▷), collagen fibres (▶) and amorphous material. (◊). × 13500

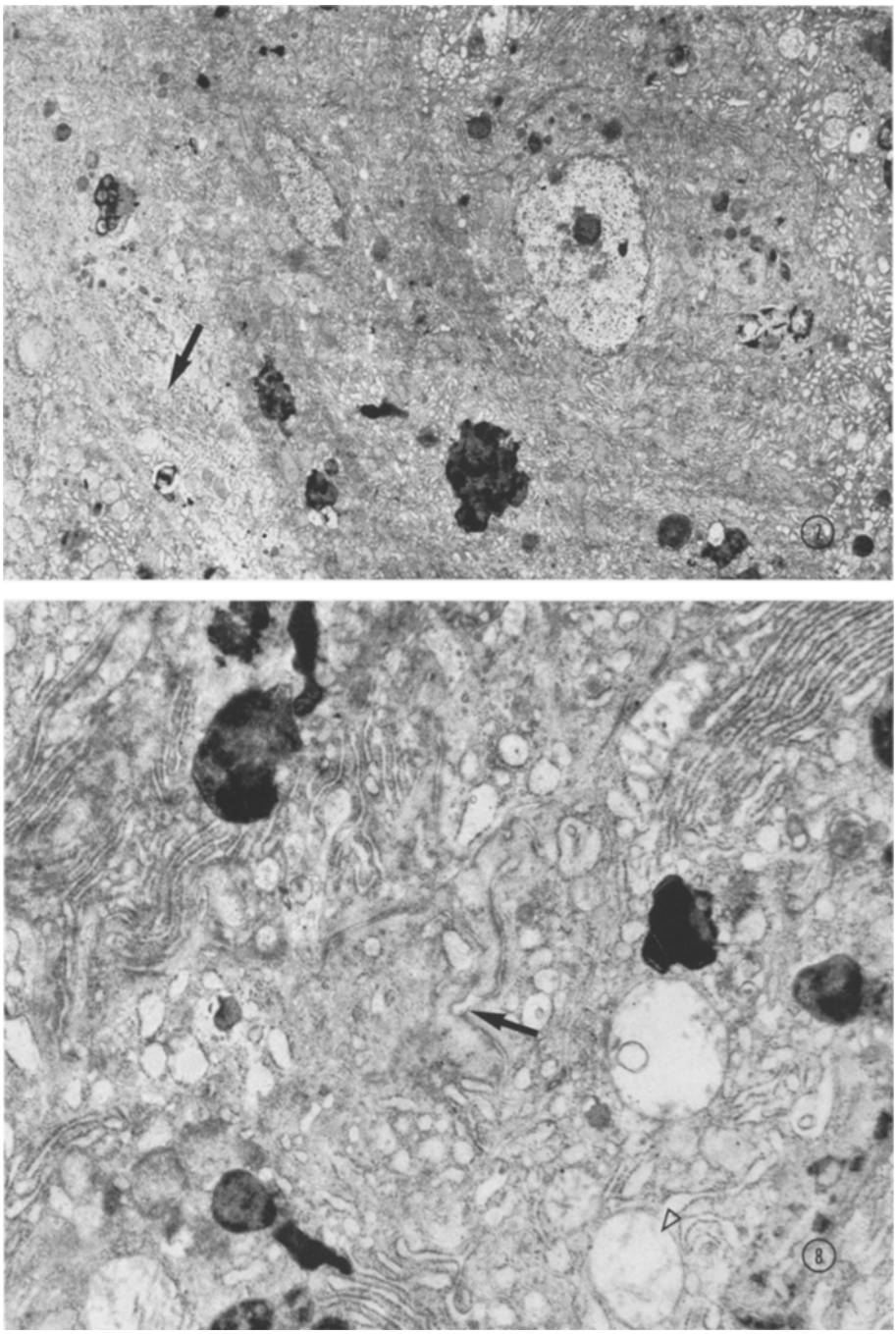
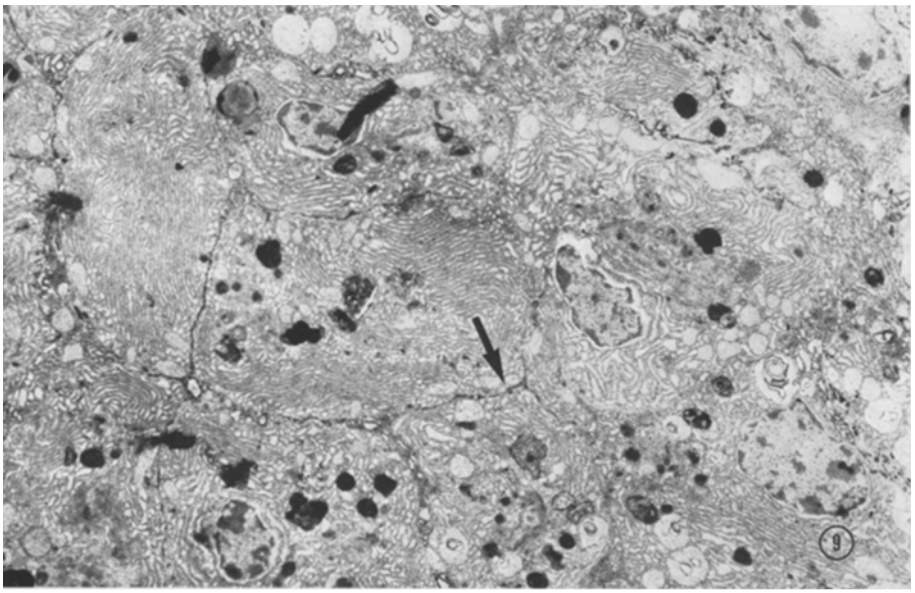


Fig. 7. Adult lamprey liver tissue: thin section prepared for morphologic study, stained with uranyl acetate and lead citrate. A low power micrograph showing the syncytial disorder-like appearance of the liver parenchyma with necrotising hepatocytes (➔). × 5700

Fig. 8. Adult lamprey liver tissue: thin section prepared for morphologic study, stained with uranyl acetate and lead citrate. Canalicular like structure without lumen and without microvilli (➔); mitochondria with vesicular cristae (▷). × 28500



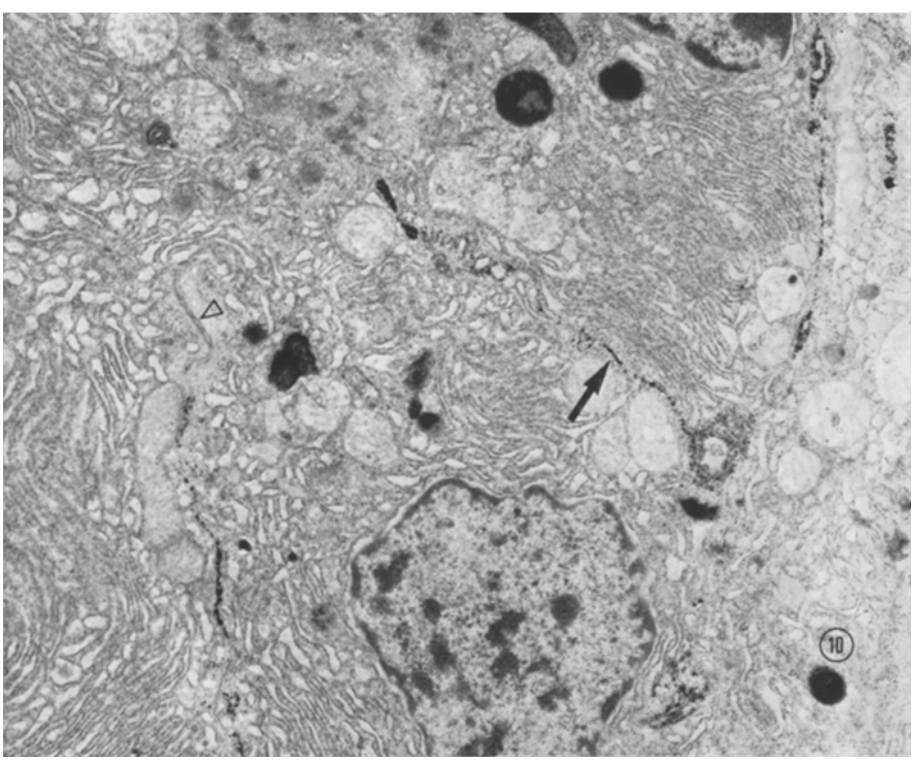


Fig. 9. Adult lamprey liver tissue: thin section prepared for cytochemical study, stained with uranyl acetate and lead citrate. Low power micrograph showing ATPase positive cell membranes (➔). × 5700

Fig. 10. Adult lamprey liver tissue: thin section prepared for cytochemical study, stained with uranyl acetate and lead citrate. Parts of the cell membrane showing a positive ATPase activity (➔); desmosome on one side lying free in the cytoplasm (▷). × 13500

cytoplasmic invaginations of the cell membrane appear (Fig. 7). Remnants of desmosomes can be found free in the cytoplasm, without connection with the cell membrane. The mitochondria are now characterized by an electron lucid matrix and by very short cristae, which often appear tubular to vesicular (Fig. 8). The Golgi apparatus is clearly hypertrophic: the distal ends of cisternae are swollen and contain small droplets of moderate electron density. In some hepatocytes, the R.E.R. is strongly dilated, fragmented and filled with amorphous, granular electron dense material. The S.E.R. remains similar to that of the larval stage.

The cytoplasmic inclusions, although less numerous, have the same appearance as in the larva; in some areas they are larger and have a more heterogeneous content. In contrast to the larval stage, a great number of siderosomes or ferritin containing inclusions surrounded by several membranes are now observed. After incubation for alkaline phosphatase and ATPase the intercellular spaces and the deep intracytoplasmic membrane invaginations show a positive lead phosphate deposition (Fig. 9, 10). A high number of cytoplasmic inclusions also show partly a positive reaction after incubation for alkaline phosphatase and ATPase. No reaction product could be detected after incubation for acid phosphatase.

Discussion

In the literature two hypotheses are made about the structure of the liver of the larval lamprey: according to Elias and Bengelsdorf (1952), the liver is composed of two cell thick hepatocytic muralia; according to Mugnaini and Harboe (1967) the liver is formed of compound blind ending single cell thick tubules of the hepatocytes. Our morphologic and enzyme histochemical findings are in agreement with the latter concept. The tubular lumina can be compared with the canalicular lumina of the higher species of animals. In both, the lumen is separated from the rest of the intercellular space by junctional complexes. The wall is provided with numerous slender microvilli surrounded by an ectoplasmic area.

Also histochemically, this part of the cell membrane is comparable to the mammalian canalicular cell membrane: it shows positive reactions for alkaline phosphatase, adenosine triphosphatase and 5′-nucleotidase (Desmet, 1963; Wachstein, 1963).

Although animals in metamorphosis could not be studied, the picture of the adult liver suggests a disappearance of the bile ducts during metamorphosis and a loss of the single cell layer tubular arrangement.

In light microscopy, the zones of more eosinophilic necrotic cells probably correspond to disappearing bile ducts while the multicellular muralia of hepatocytes have replaced the tubular structures, At the ultrastructural level, the disappearance of distinct cell membranes and the presence of free desmosomes in the hepatocytic cytoplasm imply the formation of syncytia (Okudaira, Shunsaku, Okudaira, Hashimota, Hayakawa, 1968). The numerous cytoplasmic inclusions, found in larval and adult lamprey's are not seen in this amount in the liver of other normal species of animals.

The enzyme histochemical findlings in the adult lamprey fit in with the morphologic reconstruction of the liver, As the microvilli of the hepatocytes disappear, the enzyme activity at the tubular lumen can no longer be demon-

strated either at the light or electron microscopic levels. Intracytoplasmic cell membrane invaginations and interdigitations with distinct enzyme activity appear. These findings are comparable to changes in liver tissue of humans and of other animals in pathological (cholestatic) conditions. It has been demonstrated that a disappearance of hepatocytic microvilli is accompanied by a loss of enzyme activity. This is usually associated with changes in the lateral cell membranes, which develop numerous microvilli and intracytoplasmic invaginations which show positive histochemical reactions for alkaline phosphatase, ATPase and 5′-nucleotidase. Such alterations are most typically observed in conditions of extrahepatic cholestasis (Krstulovic, Van Damme, Desmet, 1968; Orlandi, 1962; Steiner, Jezequel, Phillips, Miyai, Arakawa, 1965; Wills and Epstein, 1966).

In analogy, we interpret our findings as follows: the disappearance of the luminal pole is partly compensated by an enlargement with concomitant histochemical change of the rest of the hepatocellular cell membrane, apparently reflecting a functional change of these membrane areas.

In contrast with previous reports we cannot demonstrate with any histochemical technique – even on fresh frozen sections which are known to best preserve bilirubin (Desmet, Bullens, De Groote, Heirwegh, 1968) –, the presence of bile pigment in the adult lamprey liver. The cytoplasmic inclusions do not correspond to the electron dense amorphous material described in cholestatic liver tissue of other species and presumed to represent bile constituents (Barone, Carozza, Inferrera, 1968; Biava, 1964; Okuda, Tanikawa, 1967; Tanikawa, 1966). This contradiction may arise by the fact that other authors have studied other species or subspecies of lampreys like Bertolini (1964), or that they have based their interpretation on biochemical results like Sterling, Meranze, Windsten, Krieger (1967), or that they have drown their conclusion only on macroscopical and morphologic observations without making histochemical confirmation like Fontaine (1958).

The reconstruction of the liver and the disappearance of tubular lumina in the adult lamprey is accompanied by circulatory changes. In the adult lamprey liver endothelial cells are found in a disorderly way between the syncytial hepatocytes, intermingled with fibres and amorphous electron dense material. This material shows morphologic similarity to the fibrinoid material observed in the human glomerulus in pathological conditions as in intravascular coagulation (Mc Cluskey, Vassali, 1969) and may indicate a disturbed circulation in the remodelled liver.

The finding that no remnants of tubular lumina or only partly atrophied tubular poles of the hepatocytes persist in adult lamprey indicates that the bile duct atresia occurring during metamorphosis from the larval to the adult lamprey is a phenomenon that leads to a complete disappearance of the normal biliary secretory pole of the hepatocyte.

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