Immunohistochemical Localization of α1-Antitrypsin in Normal Mouse Liver and Pancreas

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Using horseradish peroxidase and fluorescence immunohistochemistry, α1-antitrypsin (α1AT) was demonstrated in normal mouse hepatocytes and pancreatic islet cells. All hepatocytes were positive; 1-3% stained intensely for α1AT. These were located mainly in the periportal area as well as randomly distributed, both singly and in clusters, throughout the liver lobule. Nonparenchymal liver cells were negative for α1AT. The type of hepatocyte cytoplasmic staining appears to alter during ontogeny, changing from a localized granular to a diffuse pattern. The use of immunohistochemistry to demonstrate α1AT in normal mouse liver allows us to examine the acute phase response at a cellular level. (Am J Pathol 1980, 101:723-736)

α1-ANTITRYPSIN (α1AT) is one of the major proteinase inhibitors present in serum. Although the proposed major function is the inhibition of leukocyte neutral proteases, this molecule also inhibits the active enzyme of the three major biochemical pathways of inflammation: the complement, coagulation, and kinin systems. α1AT has been shown to inhibit trypsin, chymotrypsin, plasmin, and elastase but has also been implicated as acting on thrombin, kallikrein, and Hageman factor. It is a well-known acute phase reactant, increasing two- to threefold during an acute inflammatory reaction in man, though the increase in the rat is not quite as marked.[1]

We have previously demonstrated that α1AT is synthesized in the liver by radiolabeled amino acid incorporation studies in isolated rat liver[2] perfusions, but the exact cellular location of the site of synthesis has not been established, although a number of reports would implicate the hepatocyte as the major source of the serum α1AT.[3-8]

There are various well-documented demonstrations using immunohistochemistry that α1AT is present in the hepatocytes of humans homozygous for the Z gene (Pi ZZ),[4-7,9,10] while there has been a consistent claim that the normal human liver cannot be shown to contain α1AT by immunohistochemical means.[4,6,7,9] There is a single notable exception to this claim with the demonstration by Feldman et al[5] that with appropriate reagents and fixation, α1AT could be demonstrated to be present in normal

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Supported by Grant MA-5956 from the Medical Research Council of Canada.
Accepted for publication July 1, 1980.
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0002-9440/80/1209-0723$01.00

human hepatocytes. There have been other reports that α1AT is present in normal human pancreas [11-13] in platelets and megakaryocytes,[14-16] in mast cells [13,17] and leukocytes,[18] and on the surface of stimulated lymphocytes.[19]

We report here the successful immunohistochemical localization of α1AT in the mouse in the normal hepatocyte and, in addition, demonstrate localization of α1AT in pancreatic islet cells.

Materials and Methods

Animals

CBA/J (Jackson Laboratories, Bar Harbor, Maine) mice (12–16 weeks) were used in all experiments performed.

Tissue Sections

Liver and pancreas sections were cut from buffered-formalin–fixed paraffin embedded blocks.

Tissues were also fixed by the St. Marie [20] and Carnoy [21] methods.

Antigens and Antiserums

Mouse α1-Antitrypsin

Mouse α1-antitrypsin was isolated from a pool of normal mouse plasma by a combination of ion exchange and affinity chromatography and preparative acrylamide gel electrophoresis (unpublished results). The purified protein was found to be homogeneous by analytic gel electrophoresis and had a molecular weight of 53,000 daltons, determined by SDS acrylamide gel electrophoresis. The optimum specific activity of the preparation was 350 μg of trypsin inhibited per milligram of α1AT with N-benzoyl-DL-arginine-*p*-nitroanilide as substrate (theoretical optimum specific activity, 412).

The purified protein was used to immunize sheep to produce an antiserum that reacted specifically with mouse α1-antitrypsin. There was a small amount of antialbumin activity (detected only on crossed immunoelectrophoresis), which was absorbed with highly purified mouse albumin. The resulting antiserum was shown to be monospecific for α1AT by double diffusion and immunoelectrophoresis (Figure 1). The presence of the two forms of α1AT as previously reported by Myerowitz [33] and confirmed in our experiments is marginally visible in the double diffusion shown in Figure 1.

Other Reagents

Rabbit antisheep IgG and sheep antirabbit IgG were raised with purified sheep and rabbit IgG as antigens. Specificity was established by immunoelectrophoresis and double diffusion in agar.

Fluorescence Immunohistochemistry

All procedures were carried out at room temperature. Paraffin sections, 6 μ thick, were dewaxed in xylol and rehydrated. Sections were washed in phosphate-buffered saline (PBS) (0.03 M, pH 7.2) for 20 minutes. The tissue sections were then incubated, for 1 hr, with specific sheep anti-mouse α1AT, diluted in PBS. The sections were subsequently washed 3 times with PBS for 5 minutes each. Sections were incubated, for 45 minutes, with fluorescein- or rhodamine-conjugated rabbit antisheep IgG (Cappel Laboratories Inc., Cochran-

ville, PA), diluted in PBS. This period of fluorescent labeling was followed by 3 PBS washes, 5 minutes each. Sections were coverslipped with freshly prepared 90% glycerol in PBS. Microscopic examination was carried out with a Leitz Dialex microscope fitted with a Ploem-pak multiple filter system and epifluorescence optics.

Controls for mouse α1AT specificity consisted both of normal sheep serum or PBS used at the sheep antimouse α1AT incubation stage, with the remaining procedures as specified above. Other controls included the adsorption of sheep antimouse α1AT with pure mouse α1AT and with purified mouse albumin.

Horseradish Peroxidase Conjugation

Horseradish peroxidase (Grade VI, RZ = 3.2, Sigma, St. Louis, Mo) was conjugated to sheep antirabbit IgG (prepared in our laboratory) as described by Nakane.[22] A conjugate RZ value of 0.43 was obtained from optical densities at 280 nm and 403 nm. Maximum theoretical RZ value is 0.60.

Horseradish Peroxidase Immunohistochemistry

All procedures were carried out at room temperature. Liver and pancreas sections were dewaxed in xylol and taken into ethanol. The sections were then incubated, for 30 minutes, with fresh 0.5% methanolic hydrogen peroxide. The tissue sections were then incubated, for 30 minutes, with 10% ovalbumin (Grade III, Sigma, St. Louis, Mo), in PBS to reduce nonspecific reactions particular to rodent tissues.[23] Tissue sections were subsequently incubated for 30 minutes with sheep antimouse α1AT, diluted 1:150 in PBS. Rabbit antisheep IgG, diluted 1:150 in PBS, was applied to sections for 30 minutes. All incubation steps were interspersed with 3 5-minute washes in PBS (0.03 M, pH 7.2). Subsequently, horseradish-conjugated sheep antirabbit IgG, diluted 1:10 in PBS, was applied to the tissue sections for 30 mintues. (0.5 M Tris HCl, pH 7.6, diluted 1:10 with 0.85% saline). The horseradish peroxidase was developed by incubating, for 8 minutes, with 0.6 mg/ml of 3, 3′-diaminobenzidene tetra-HCl (DAB) (ICN Pharmaceuticals Inc., Plainview, NY), dissolved in Tris–saline buffer (0.01% hydrogen peroxide). The sections were washed in distilled water, counterstained with methyl green or hematoxylin, and dehydrated in ethanol and xylol. The stained sections were mounted in Permount (Fischer Scientific Co., Fairlawn, NJ). Controls for mouse α1AT were specified in the fluorescence labeling technique described above.

Results and Discussion

Using both the fluorescence and horseradish peroxidase (HRP) techniques, α1AT was shown to be present in normal mouse hepatocytes. Although there was a clear overall impression that all hepatocytes were slightly positive for α1AT, there were a number of hepatocytes, ranging from 1% to 3%, that stained intensely for α1AT. These were distributed mainly around the portal triad areas as well as being scattered singly or in small clusters randomly throughout the liver lobule. There was no restriction to a particular size of hepatocytes, and single and multinucleated hepatocytes were positive. There was no staining of Kupffer cells, nor any evidence of staining in the bile duct (Figures 2–5). The specificity controls confirmed the staining to be due to the presence of α1AT as absorption of the antibody with highly purified α1AT abolished the staining, while ab-

sorption with purified mouse albumin, a common contaminant of α1AT preparations, did not alter the intensity or pattern of positivity.

Under high power, the α1AT appeared to be located in diffuse granular deposits throughout the cytoplasm in the adult mouse. This was particularly evident when immunofluorescence was used (Figure 5).

There are fewer of the densely staining large hepatocytes in fetal and neonatal liver (Figures 6 and 7) than in the adult (Figure 2). The cells that are positive show a marked granular pattern of staining (Figure 7) and may reflect the reduced levels of α1AT measured in circulation at this developmental stage (unpublished observations). The different cytoplasmic distribution that alters with ontogeny was a consistent finding in these studies, reflecting a functional change in the hepatocytes during development.

While it is true that we have not ruled out the possibility that the positive-staining hepatocytes represent those cells taking up α1AT from the circulation, studies showing an increase in the number, distribution, and intensity of staining of hepatocytes positive for α1AT subsequent to inflammatory challenge, but prior to increased circulating levels (unpublished observations), would support the proposal that the positive-staining hepatocytes in the normal mouse liver represent those cells actively synthesizing α1AT. These findings are consistent with those showing increased hepatocyte staining for fibrinogen [24] and haptoglobin [25] in the dog and C-reactive protein in the rabbit [26] after inflammatory stimulus.

We have shown with isolated hepatocyte culture studies of normal and transformed cells that the hepatocyte clearly synthesizes a significant amount of α1AT (35 ng/10^7 cells/hr for hepatoma and significantly more for normal hepatocytes). In addition, 72-hour cultures of isolated pancreatic islets give an indication of significant synthesis of α1AT. These results are preliminary, and further studies to establish synthetic rates are in progress. We must also rule out the limited possibility of release from pancreatic cells that have died, though viability studies on the cultures would tend to negate this possibility.

There have been many reports demonstrating globular deposits of α1AT in the hepatocyte of the human carrying the Pi ZZ phenotype (α1AT deficiency).[4,6,7,9,10] Each of the authors was careful to point out that when the particular experimental conditions described were used no α1AT was demonstrable in the normal hepatocyte. However, since the liver has been demonstrated as being the prime synthetic source,[2,3,7] it was expected that by appropriate manipulation of good reagents the hepatocyte could be shown to contain α1AT. This has proved to be the case in the mouse (current results), rat, and rabbit (unpublished results) and may

represent a difference in the activity of the hepatocyte in these species from that of the human. Indeed, the ontogeny of α1AT in the mouse differs markedly from that of the human (unpublished observations), which may reflect just such a different rate of hepatic synthesis.

The initial investigations were carried out with liver fixed in formalin and embedded in paraffin, a technique which leads to slow fixation and sometimes is open to criticism due to destruction of antigenic sites during the paraffin embedding. However, even with this slow fixation, the distribution of single and small clusters of cells positive for α1AT was similar to that described for α1AT in normal human liver,[5] the cells being located mainly in the periportal areas and some being randomly distributed throughout the liver lobule. In terms of the classification of Rappaport,[27] the α1AT-containing hepatocytes in the mouse are located mainly in Zone 1 of the liver acinus, little being found around the terminal hepatic veins.

We investigated other fixatives using the Carnoys and St. Marie methods of fixation. Carnoy's fixative gives rise to very rapid fixation, and the fluorescence obtained confirmed the presence of specific cells staining very brightly for α1AT, but in addition, this fixative indicated that the majority of hepatocytes were capable of specific staining for α1AT. There was, in addition, much interstitial staining. The St. Marie technique gave results similar to the formalin fixation, but the advantage of the slow formalin fixative and the permanence of the paraffin embedding led us to adopt this approach for the majority of our assessments. A similar effect of fixation had been reported by Chan [28] for the localization of human α1AT in the deficiency syndrome.

The distribution of α1AT-positive cells in the liver is very similar to that shown for fibrinogen in the dog [24] and haptoglobin in the human.[25] In addition, the distribution of cells positive for α-fetoprotein in the mouse [29] and human fetal liver [30] showed a similar pattern.

The single description of the immunohistochemical localization of α1AT in normal human liver [5] and the recent report by Palmer et al [7] that the tumor cells of hepatomas induced by oral contraceptives contained α1AT deposits even though the individuals were not α1AT-deficient may point to the fact that the human liver cell, though synthesizing α1AT, exports it rapidly into the circulation. The use of high-affinity reagents and appropriate fixation or an abnormality of transport mechanisms in the human or perhaps a slower transport in the mouse, rat, and rabbit would allow the immunohistochemical demonstration of α1AT in the normal hepatocyte.

In all of the cases demonstrating α1-antitrypsin deposits in hepatocytes, there was never any indication of the Kupffer cells being positive. This

was also a finding in our studies. Fibrinogen and haptoglobin, other acute phase reactants, had been demonstrated to be synthesized in the liver and in some cases were shown to be also present in the Kupffer cells, though the authors pointed out that this was probably due to phagocytosis and degradation as a result of inflammatory reaction, as opposed to synthesis.[24,25]

The lack of staining in the bile duct contrasts somewhat with the finding of α1AT in a number of human cholangiocarcinomas.[3] However, this may again be due to species differences or reflect specificity differences in the reagents.

The recent demonstration of α1AT localized to the islet cells of the pancreas in the human [11-13] were confirmed by our studies in the mouse (Figure 8). However, it is not clear whether the α1AT in the pancreas represents another source of synthesis of α1AT or is absorbed from serum after having been synthesized in the liver, though our preliminary studies with cell cultures would indicate synthetic capacity. Since the tissues were taken and fixed immediately after death, there would have been no opportunity for autolysis to occur, confirming the assumption by Ray et al [12] that the pancreatic α1AT was not an artifact of protease-inhibitor complexing in autopsy material due to autolysis. The role of α1AT in the pancreas may be to protect the islet cells against the various exocrine enzymes in the parenchymal cells of the pancreas.[31] However, one cannot rule out the islet cells as being another source of α1AT for circulation.

The association of human pancreatitis with decreased levels of circulating α1AT [32] may indicate that the pancreas is an important source of circulating α1AT, though the findings of Hood et al [8] that in liver transplantation the recipient converts totally to the phenotype of the donor may indicate that the pancreas does not contribute very much to the circulation. It would be most interesting to examine the pancreas before and after transplantation of the Pi ZZ or Pi _ (total lack of α1AT) recipients to see whether the islets were positive for α1AT. A negative islet converting to a positive islet staining after transplantation would indicate that the islet cells picked up the α1AT from the circulation.

Using both the fluroescence and peroxidase techniques, we have been unable to confirm the findings in the human that the mast cell [13,17] and the polymorphs [18] contain α1AT. We have examined a variety of mast-cell–containing tissues, including gut and thymus, in normal and parasite-infected animals; and neither the structural nor mucosal mast cell could be shown to be positive for α1AT. This finding may reflect a species difference or a difference in the specificity of the reagents used.

The finding by Lipsky et al [19] that human lymphocytes after stimulation

by Concanavalin-A (Con-A) demonstrate the presence of α1AT on their surface is interesting, particularly in the light of the known involvement of proteolytic enzymes in the triggering of cellular transformation. As yet we have not been able to demonstrate the presence of α1AT on the surface of Con-A–stimulated mouse peripheral blood lymphocytes. We have not yet examined the platelet or megakaryocyte for positively in the mouse.

We have demonstrated α1AT to be present in a measurable quantity in a limited number of hepatocytes in the normal mouse liver, and it would appear that all hepatocytes contain a small amount of α1AT. The distribution of the strongly positive cells is in the periportal area of the liver. In addition, α1AT was shown to be present in the islet cells of the pancreas. It would appear that these two tissues represent the major deposits of α1AT in the body.

The localization of α1AT in normal mouse liver tissue by immunohistochemical techniques and the demonstration of an altered distribution of positive cells during an inflammatory response should prove a powerful tool in examining the response of the liver to inflammation and the role of protease inhibitors in the regulation of an inflammatory process.

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Acknowledgment

The editorial assistance of Mrs. J. Hickey is gratefully acknowledged.

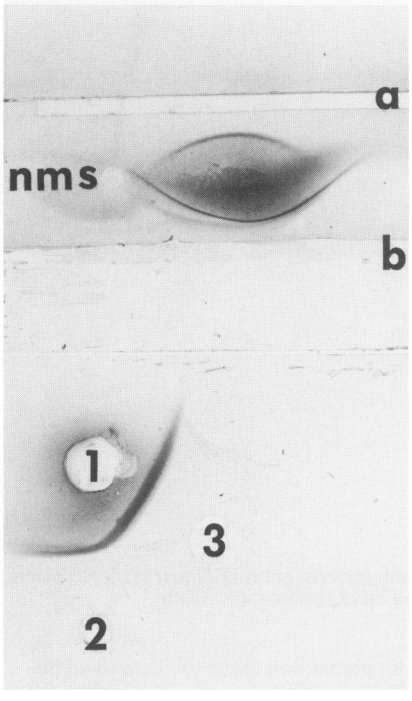
[*Illustrations follow*]

Figure 1—*Upper*, immunoelectrophoresis of normal mouse serum: Trough *a*, Sheep antimouse α1-antitrypsin absorbed with albumin, Trough *b*, Rabbit anti-whole-mouse serum. Single arc of reactivity against mouse serum, which crosses with albumin arc. *Lower*, double diffusion analysis: *1*, Sheep antimouse α1-antitrypsin absorbed with albumin; *2*, Normal mouse serum; *3*, Purified α1-antitrypsin.

Figure 2—Normal adult liver stained with horseradish peroxidase for α1-antitrypsin. (×100)

Figure 3—Normal adult liver negative control for peroxidase stain. (×100)

Figure 4—Normal adult liver stained for α1-antitrypsin (HRP). Evidence of low-level diffuse cytoplasmic staining in most hepatocytes and high-intensity staining in a limited number of parenchymal cells surrounding the portal triad. (×400)



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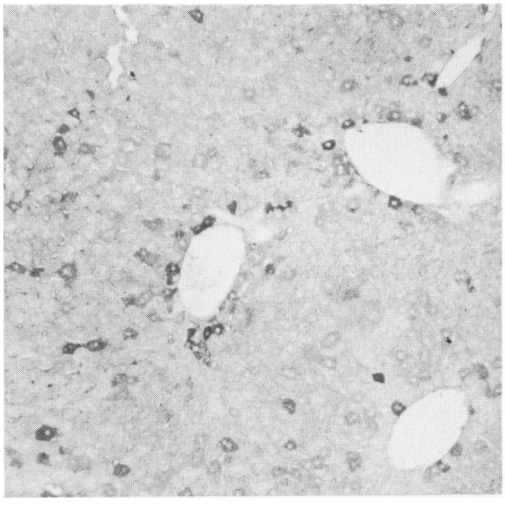

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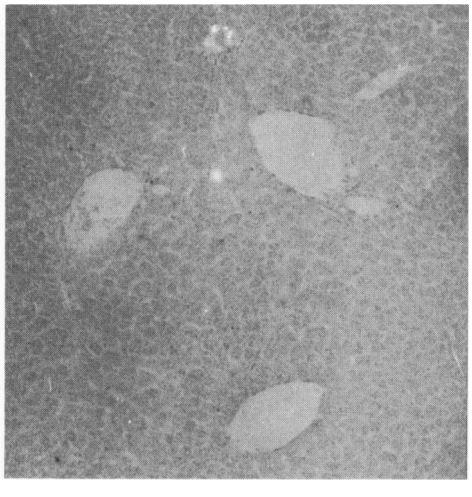

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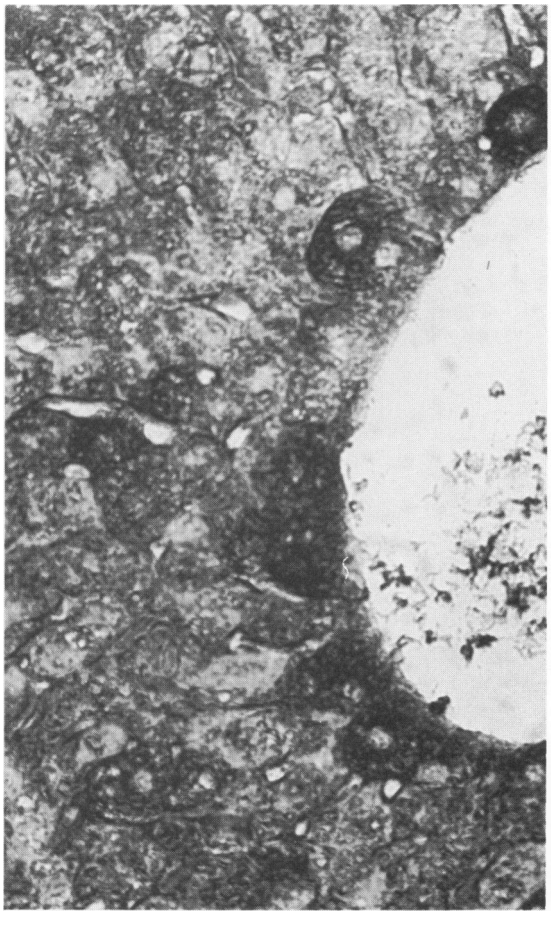

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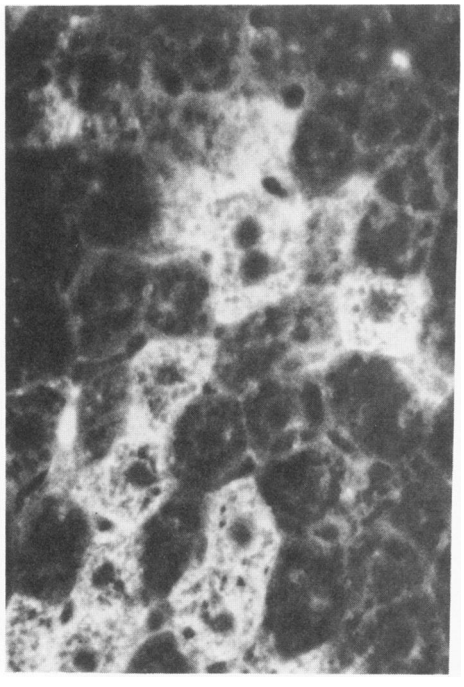
Figure 5—Normal adult liver stained for α1-antitrypsin with fluorescent reagents (FITC). No evidence of Kupffer cell staining. Mono and multinucleated cells positive. (×1000)

Figure 6—Day 15 fetal liver stained for α1-antitrypsin with peroxidase reagents. Dispersed hepatocyte staining with no evidence of erythroid cell staining. (×400)

Figure 7—Day 7 neonatal liver stained for α1-antitrypsin with peroxidase reagents. Similar distribution as adult with decreased numbers and intensity of positive staining cells. (×100)

Figure 8—Normal pancreas stained for α1-antitrypsin with peroxidase reagents. Islet cells strongly positive with no staining seen in the exocrine cells. (×400)



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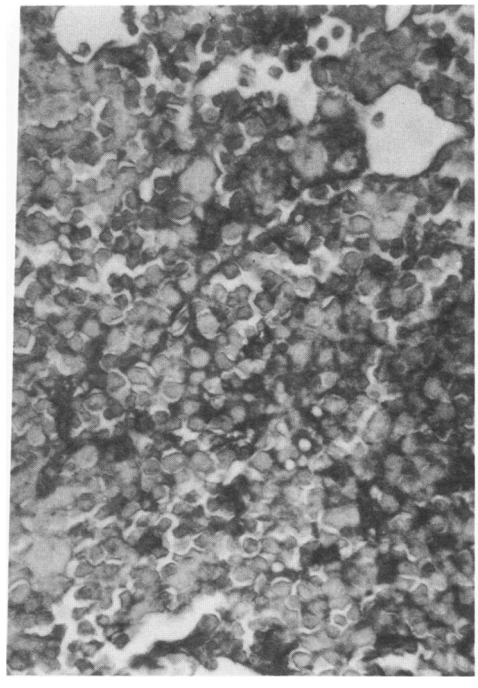

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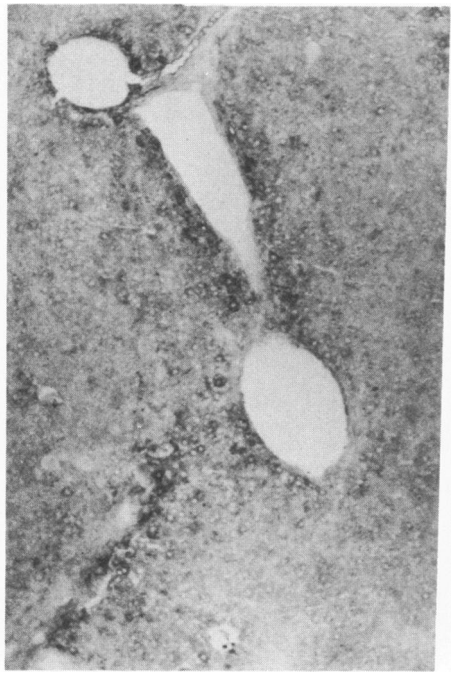

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