

Response of Chemically Induced Hepatocytelike Cells in Hamster Pancreas to Methyl Clofenapate, A Peroxisome Proliferator

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ABSTRACT Administration of *N*-nitrosobis(2-oxopropyl)amine during peak DNA synthesis of regenerating pancreas in hamsters has been shown to induce hepatocytelike cells in pancreas. We now present evidence to demonstrate that such cells respond to methyl clofenapate, a peroxisome proliferator. The response includes a marked proliferation of peroxisomes and enhanced activity of peroxisomal enzymes enoyl-CoA hydratase (8.5- to 13-fold), [^{14}C]-palmitoyl-CoA oxidation (2.8- to 3.9-fold), catalase (1.6 to 3.4-fold), and carnitine acetyltransferase (>2,000-fold). Cytochemical localization of catalase by the alkaline 3,3'-diaminobenzidine procedure and immunofluorescence localization of heat-labile enoyl-CoA hydratase showed that these peroxisome-associated enzymes are localized strictly in pancreatic hepatocytelike cells, while adjacent acinar, duct, and islet cells appeared consistently negative. Morphometric analyses of hepatocytelike cells showed a significant increase in the numerical density and an eightfold increase in the volume density of peroxisomes in methyl clofenapate treated animals. These results demonstrate that the hepatocytelike cells are responsible for the observed peroxisomal enzyme activity in pancreas of hamsters and suggest that the derepressed peroxisome specific genes in these cells respond to a peroxisome proliferator as do parenchymal cells in hamster liver.

A single dose of the pancreatic carcinogen *N*-nitrosobis(2-oxopropyl)amine (NBOP) administered to regenerating pancreas of hamster during peak DNA synthesis leads to the appearance of cells strikingly similar to hepatocytes (16, 21). These cells show many of the morphological and cytochemical features characteristic of normal differentiated hepatocytes. Immunofluorescence staining with appropriate specific antibodies indicated the presence of albumin in the cytoplasm of these cells and the absence of α -amylase and carboxypeptidase-two marker proteins characteristic of pancreatic acinar cells. Although these cells closely resemble the hepatocytes, their definitive identification and origin remain to be determined. Since exposure of rodents to any of a group of structurally dissimilar compounds which lower plasma triglyceride levels is predictably associated with a marked proliferation of peroxisomes in hepatic parenchymal cells (2, 18-21, 28), and to a lesser extent in proximal tubular epithelium of kidney (4), it appeared particularly relevant to assess the response of these

hepatocytelike cells in pancreas to a peroxisome proliferator as a way of further characterizing their nature. This study documents the response of hepatocytelike cells to methyl clofenapate (methyl-2[4-(*p*-chlorophenyl)phenoxy]2-methyl propionate), a potent peroxisome proliferator and inducer of augmented synthesis of peroxisome-associated enzymes (19).

MATERIALS AND METHODS

Initiation of Pancreatic Regeneration and Induction of Peroxisomes

The experimental procedure for initiation of pancreatic regeneration is described in a previous communication (25). Briefly, male Syrian golden hamsters maintained on a methionine deficient semisynthetic diet were injected i.p. with DL-methionine (500 mg/kg body weight [b. wt.]) in saline daily for 8 d. On the ninth day they were returned to a full amino acid diet and given a single i.p. injection of methionine 800 mg/kg b. wt. Rapid regeneration of pancreas is observed with peak DNA synthesis at 60 h and maximum mitotic activity at 72 h. During the period of peak DNA synthesis, 50 animals were injected s.c. with

30 mg/kg b. wt. of NBOP (24). 6 mo later, 30 animals were fed Purina Rat Chow containing 0.2% methyl clofenapate obtained from Dr. Neal Handly (California Institute of Technology) for 3 wk (Group 4), at which time they were killed for morphological and biochemical studies. The remaining 20 animals served as NBOP controls (Group 3). A group of 15 hamsters were used as normal untreated controls (Group 1), and another group of 15 normal hamsters were fed methyl clofenapate for 3 wk (Group 2).

Enzyme Assays

Pancreas (dissected free of fat and surrounding lymph nodes), and portions of liver from experimental and control groups were homogenized in ice cold 0.25 M sucrose using a Potter Elvehjem homogenizer with a teflon pestle. These homogenates were used for measuring enzyme activities: KCN insensitive palmitoyl-CoA oxidizing activity was assayed by the method of Lazarow (7); the heat-labile enoyl-CoA hydratase activity was measured by the method outlined by Osumi and Hashimoto (15). Catalase and carnitine acetyltransferase activities were assayed by the spectrophotometric method as described previously (10). A small portion of pancreas from all experimental animals that were used for the enzyme assays was fixed for light microscopy to confirm the presence of hepatocytelike cells.

Immunofluorescence Procedure for Enoyl-CoA Hydratase Demonstration

Portions of the pancreas were fixed in cold 96% ethyl alcohol for 24 h and cold xylene for an additional 24–40 h, then processed by the procedure of Sainte-Marie (26). 2 μ m thick paraffin sections were stained for enoyl-CoA hydratase with antibodies raised in rabbits against purified enzyme from rat liver as described by Reddy et al. (22).

Electron Microscopy

Pancreas was minced into small pieces, fixed in cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 4 h and rinsed overnight in 0.1 M cacodylate buffer, pH 7.4, containing 0.2 M sucrose. This tissue was incubated at 37°C for 1 h in the alkaline 3-3'-diaminobenzidine (DAB) reaction medium of Novikoff and Goldfischer (13), for the localization peroxidatic activity of catalase. Controls consisted of incubations in which 0.02 M 3-amino-1,2,4-triazole (K & K Laboratories Inc., Plainview, NY) was added to the medium (9). After incubation the tissue was washed in cacodylate buffer and examined by light microscopy. Dark brown stained islands of tissue representing clusters of hepatocytelike cells, were dissected from adjacent nonstained pancreatic tissue, postfixed in 2% OsO₄ buffered to pH 7.4 with 0.1 M *S*-collidine and processed for electron microscopy.

Pancreas, not incubated in DAB medium, was also processed for electron microscopy. Unstained 1 μ m thick sections were examined in a Zeiss Ultraphot III microscope. Thin sections were examined with a Hitachi-HU 12 electron microscope.

Morphometric Analyses

Morphometric analysis of peroxisomes and mitochondria in hepatocytelike cells in pancreas from three animals treated with clofenapate and three NBOP control animals was carried out by the method described by Weibel (30). Three blocks were randomly selected from each animal and morphometric measurements were made on 30 electron micrographs from each group (10 from each animal). Micrographs were taken at \times 4,000 and magnified 2.5 times at printing. Points of intersection overlying cytoplasm, mitochondria, and peroxisomes were counted using a 5-mm spaced lattice grid. The volume density of mitochondria and peroxisomes was determined in relation to cytoplasmic volume.

RESULTS

Peroxisomal Enzyme Induction

The activities of peroxisomal enzymes in the liver and pancreas of various experimental and control groups are shown in Table I. Catalase activity, a marker enzyme for peroxisomes (1) was increased twofold in pancreas after the induction of hepatocytelike cells by NBOP and 3.4-fold following their exposure to methyl clofenapate. The activity of carnitine acetyltransferase, which was not detectable in the pancreatic homogenates of NBOP-treated or normal control animals, increased to a level of 2,178 nmol/min/g tissue in the NBOP plus methyl clofenapate treated group. Levels of enoyl-CoA hydratase activity and palmitoyl-CoA oxidation were increased 8.5- to 13-fold and 2.8- to 3.9-fold, respectively ($P < 0.01$), in the pancreas of NBOP plus methyl clofenapate-treated animals in which hepatocytelike cells were induced as compared to the pancreas of animals treated with methyl clofenapate alone and normal controls in which such cells were not present. The presence of islands of induced hepatocytelike cells in the pancreas of animals showing elevated activities of the various enzymes and conversely their absence in pancreata with low enzyme activities were confirmed by light microscopy. As expected, the activities of peroxisomal catalase, fatty acid β -

TABLE I

Effect of Methyl Clofenapate on Peroxisomal Enzyme Activities in Hamster Liver and Pancreas Containing Hepatocytelike Cells

Treatment groups*	Peroxisomal enzyme activities			
	Catalase U/g tissue	Carnitine acetyl transferase nmol/min/g tissue	Enoyl-CoA Hydratase μ mol/min/g tissue	1-C ¹⁴ -palmitoyl CoA oxidation μ mol/min/g tissue
Control				
Pancreas	237 \pm 7.4‡	§	12 \pm 2.5‡	0.065 \pm 0.01‡
Liver	9,620 \pm 473	1,238 \pm 429	426 \pm 34.0	0.767 \pm 0.088
Control + methyl clofenapate				
Pancreas	226 \pm 29.0	§	15.75 \pm 5.8	0.058 \pm 0.01
Liver	25,247 \pm 1,357	13,473 \pm 3,821	3,224 \pm 817	3.87 \pm 2.33
NBOP				
Pancreas¶	492 \pm 125	§	8 \pm 2.0	0.047 \pm 0.02
Liver	9,519 \pm 2,066	1,486 \pm 742	200 \pm 119	0.942 \pm 0.20
NBOP + methyl clofenapate				
Pancreas¶	814 \pm 153	2,178 \pm 767	103 \pm 25	0.182 \pm 0.09
Liver	17,330 \pm 1,669	7,801 \pm 525	3,395 \pm 1,503	6.574 \pm 0.28

* Animals in Groups 2 and 4 were fed with 0.2% methyl clofenapate in diet for 3 wk before being killed. Age of the control animals correspond to experimental groups. Preparation of tissue extracts for measuring enzyme activity is described in experimental procedure.

‡ Each value represents a mean \pm SD of three to four experiments. Two to three pancreata were pooled for each determination.

§ Barely measurable activity.

|| Significantly increased compared with corresponding controls ($P < 0.01$).

¶ Hepatocytelike cell clusters were induced in the pancreas of these hamsters by injecting with NBOP (30 mg/kg b. wt. subcutaneously) at "S" phase during ethionine-induced pancreatic regeneration; the animals were sacrificed 6–8 mo later.

oxidation, and carnitine acetyltransferase were also increased in liver after treatment with methyl clofenapate (23).

Immunofluorescence Localization of Heat-labile Peroxisomal Enoyl-CoA Hydratase in Hepatocytelike Cells in Pancreas

In NBOP control animals hepatocytelike cells, an indirect immunofluorescence reaction using antibody to enoyl-CoA hydratase revealed a weak granular cytoplasmic localization of the enzyme (Fig. 1*A*). The pancreas of methyl clofenapate fed animals on the other hand contained islands of cells with bright and diffuse cytoplasmic staining (Fig. 1*B*). Ductal, acinar, and islet cells of adjacent pancreas and pancreas devoid of hepatocytelike cells were negative.

Light and Electron Microscope Observations

Histologic examination of hematoxylin-eosin stained sections of pancreas from animals treated with NBOP during regeneration showed multiple islands of hepatocytelike cells (Fig. 2) as described previously (24). Hepatocytelike cells from both NBOP and NBOP + methyl clofenapate treated animals stained positively for the peroxidatic activity of catalase by the alkaline DAB method (Fig. 3). Aminotriazole controls were consistently negative. Examination of semi-thin sections of such cells after methyl clofenapate treatment showed increased numbers of intensely stained peroxisomes in the cytoplasm (Fig. 4). Electron micrographs of pancreatic hepatocytelike cells (Fig. 5) from animals that were not fed methyl clofenapate

showed morphological features characteristic of normal hamster liver cells, including small numbers of peroxisomes. Peroxisomes in these cells contained subcrystalloid nucleoids (see *inset* to Fig. 5) that were straight, curved, or angulated identically to those found in normal hamster liver hepatocytes (27). Both pancreatic hepatocytelike cells (Fig. 6) and liver hepatocytes from NBOP plus methyl clofenapate-treated animals showed increased numbers of peroxisomes. Many of these organelles proliferated under the influence of methyl clofenapate and lacked nucleoids, but they all stained positively for catalase indicating their peroxisomal nature (Fig. 7). The cytochemical reaction in these organelles was abolished when the catalase inhibitor 3-amino-1,2,4-triazole was added to the incubation medium. Hepatocytelike cells in the pancreas of NBOP controls (Group 3) contained substantially fewer peroxisomes. The usually sparse complement of peroxisomes normally present in pancreatic acinar cells remained unchanged following methyl clofenapate treatment.

Morphometric Analysis

The results of mitochondrial and peroxisomal measurements in hepatocytelike cells, in hamsters not treated with methyl clofenapate, are in general agreement with measurements in liver parenchymal cells from rats (8, 30). The qualitative difference in peroxisome content in hepatocytelike cells in control and methyl clofenapate-treated hamsters reflected significant quantitative differences. The percentage of cytoplasm occupied by peroxisomes in hepatocytelike cells from methyl clofenapate-treated animals showed an eightfold increase as compared

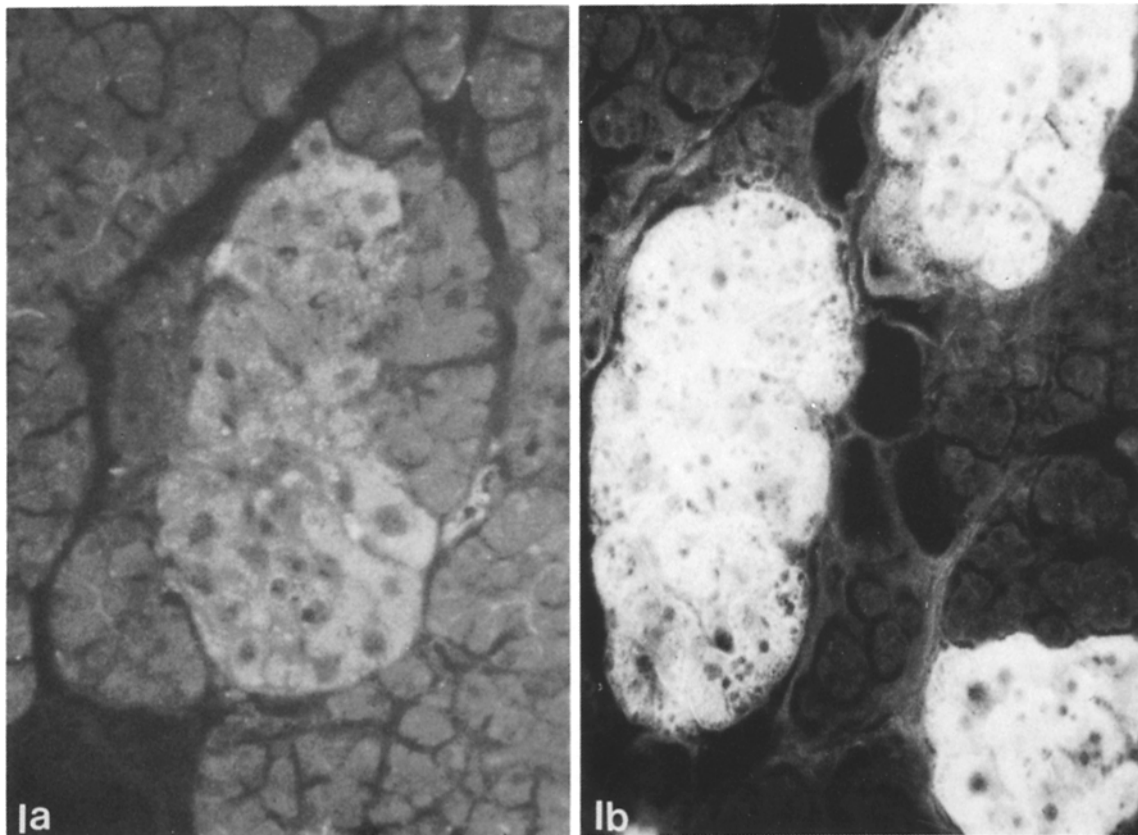


FIGURE 1 Immunofluorescent staining of heat-labile peroxisomal enoyl-CoA hydratase in hepatocytelike cells in pancreas after NBOP treatment with and without feeding of methyl clofenapate (0.2% for 3 wk). (a) An island of hepatocytelike cells in pancreas of NBOP-treated control animals showing a granular cytoplasmic localization of the enzyme characterized by weak fluorescence. (b) Intense fluorescence of islands of similar cells following methyl clofenapate treatment. $\times 350$.

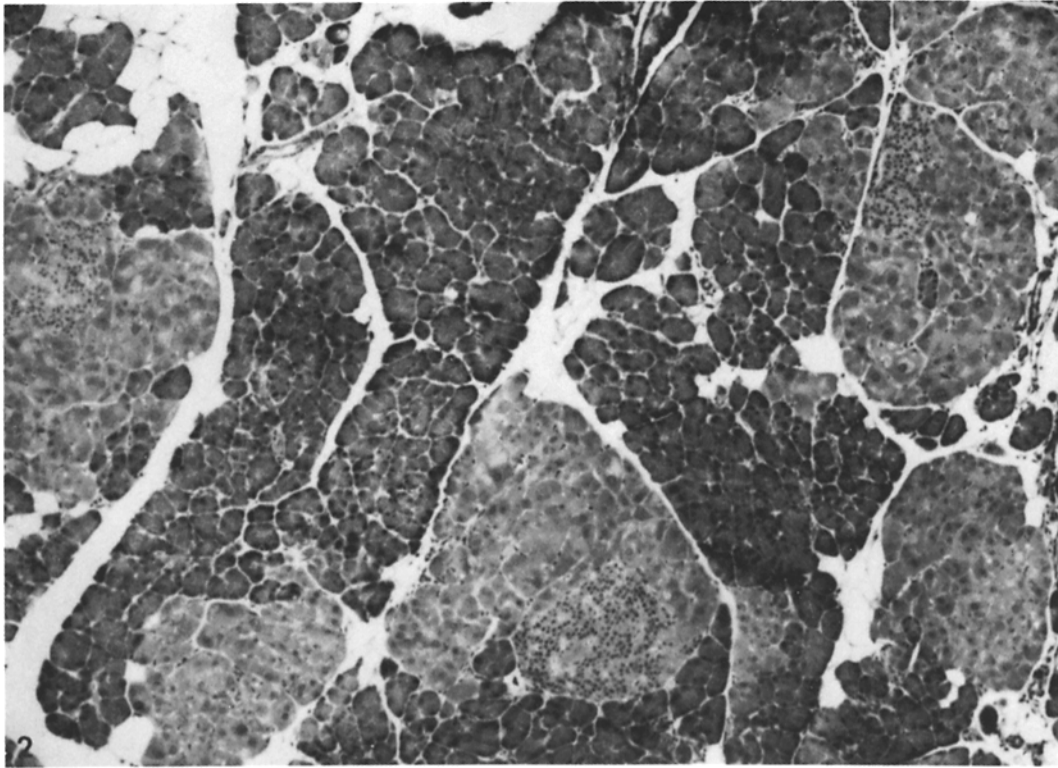
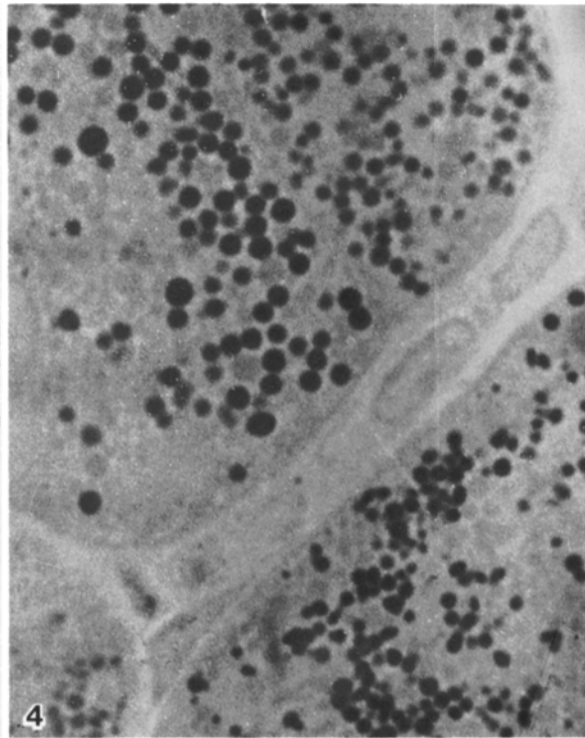
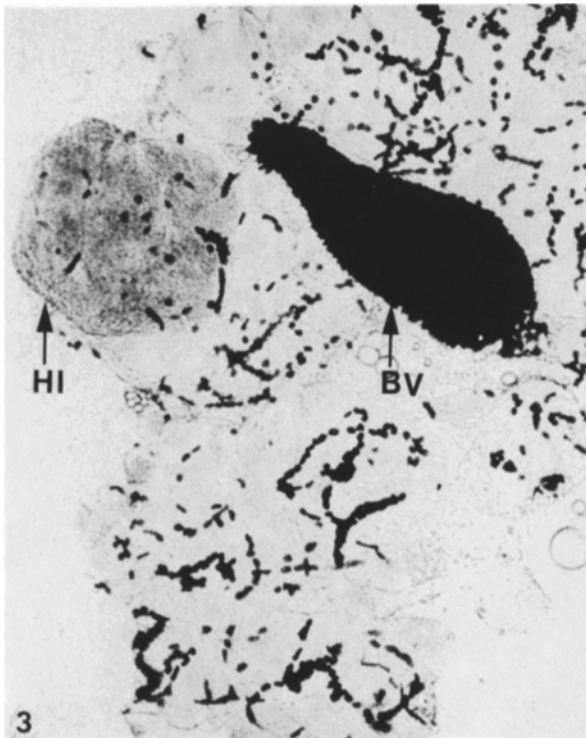


FIGURE 2 Low power photomicrograph illustrating several foci of hepatocytelike cells in hamster pancreas induced by a single dose of NBOP administered during regeneration. $\times 80$.

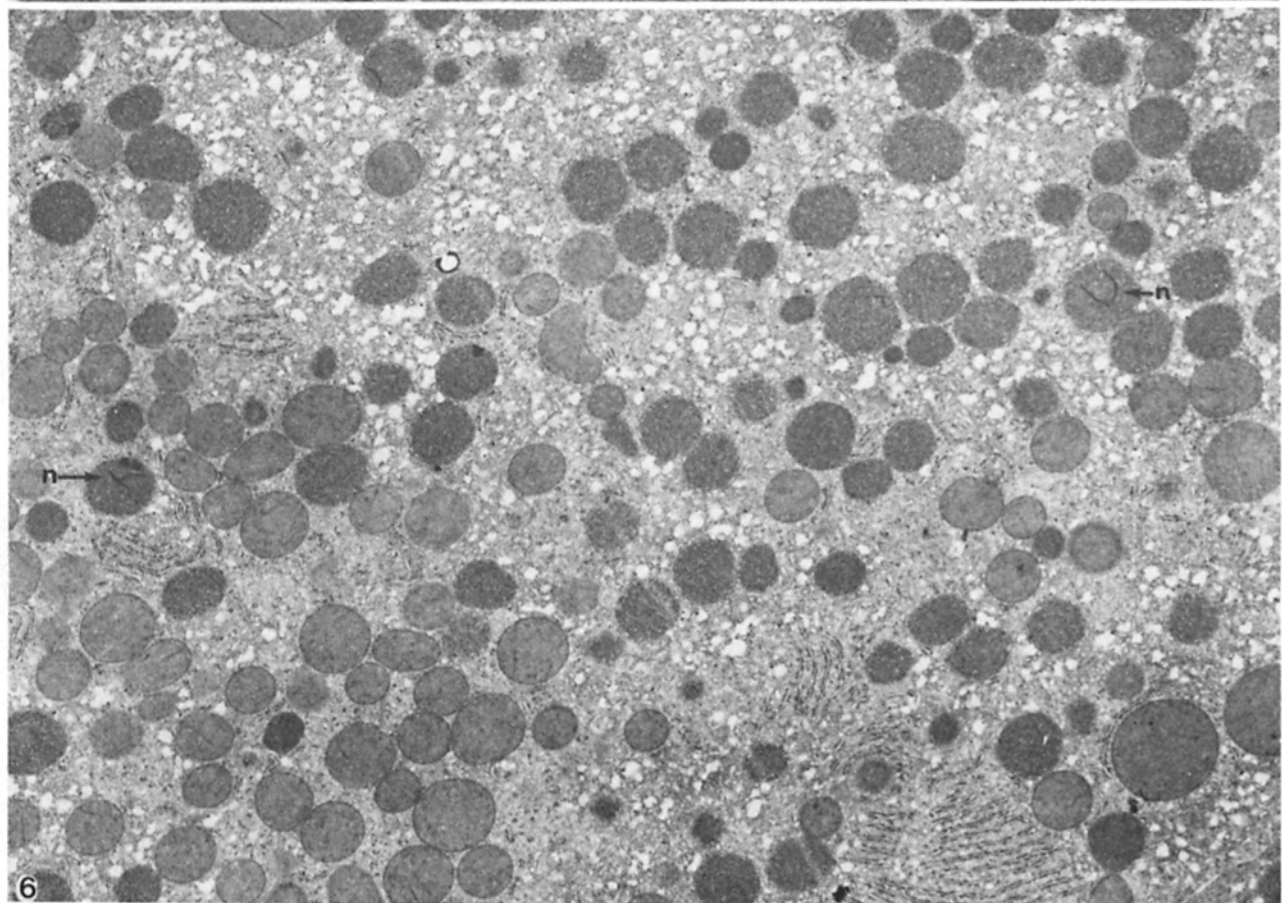
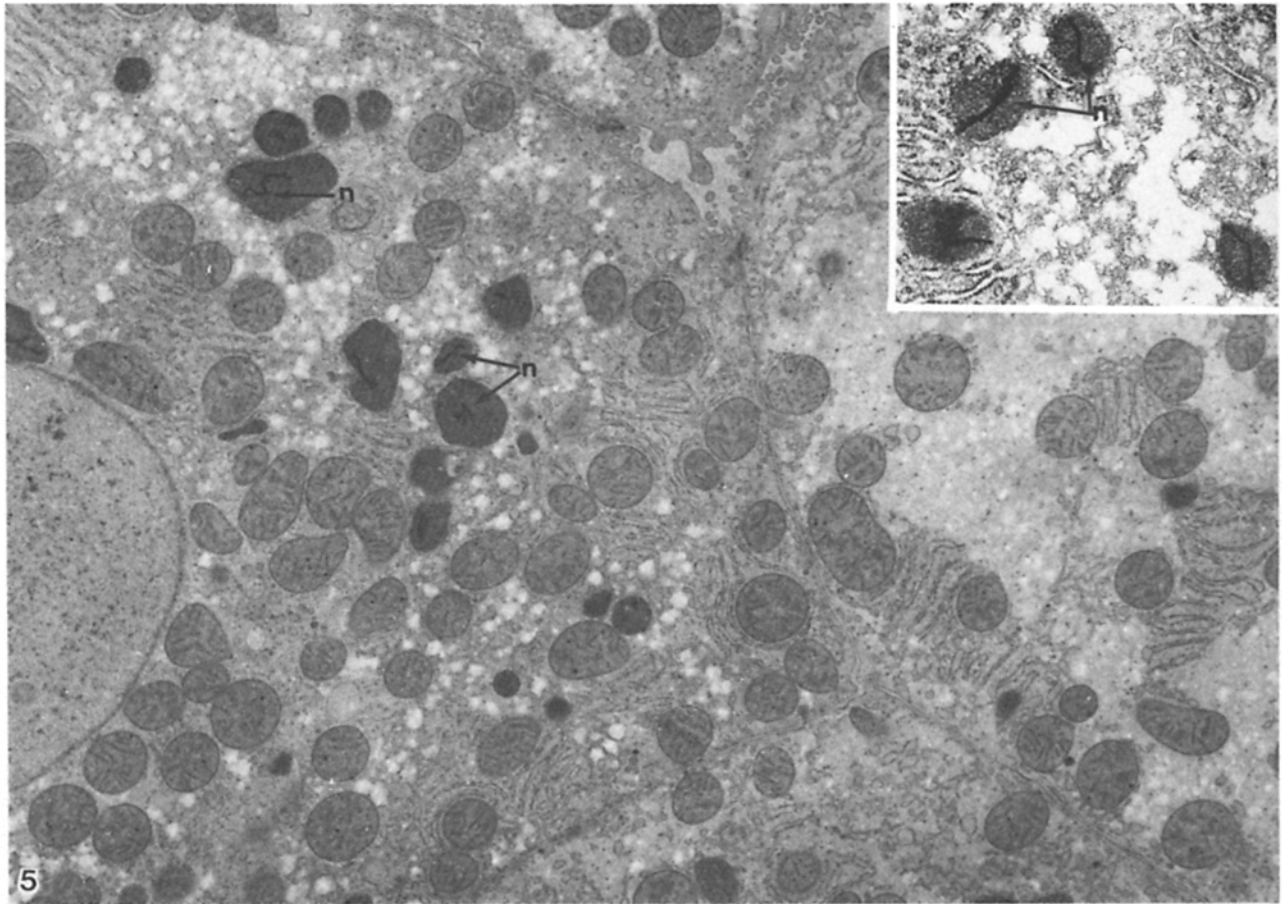


FIGURES 3 and 4 Fig. 3: A fragment of pancreatic tissue from a methyl clofenapate-treated animal incubated in alkaline DAB medium. An island of hepatocytelike cells (*HI*) is darkly stained. A large blood vessel (*BV*) and adjacent smaller capillaries are intensely stained due to blood catalase. $\times 90$. Fig. 4: Phase-contrast micrograph of $1\ \mu\text{m}$ thick section of plastic embedded tissue reveals a portion of a hepatocytelike cell from the pancreas of hamster fed methyl clofenapate. Tissue was incubated in alkaline DAB medium before embedding. Reaction product is localized in peroxisomes. $\times 2,600$.

to cells from untreated controls (Table II). The numerical density of peroxisomes was also increased in these cells. The volume density of mitochondria in hepatocytelike cells remained essentially unchanged in methyl clofenapate-treated hamsters compared to untreated controls.

DISCUSSION

Certain hypolipidemic drugs and some industrial plasticizers are capable of inducing marked peroxisomal proliferation which appears to be limited to parenchymal cells of liver and



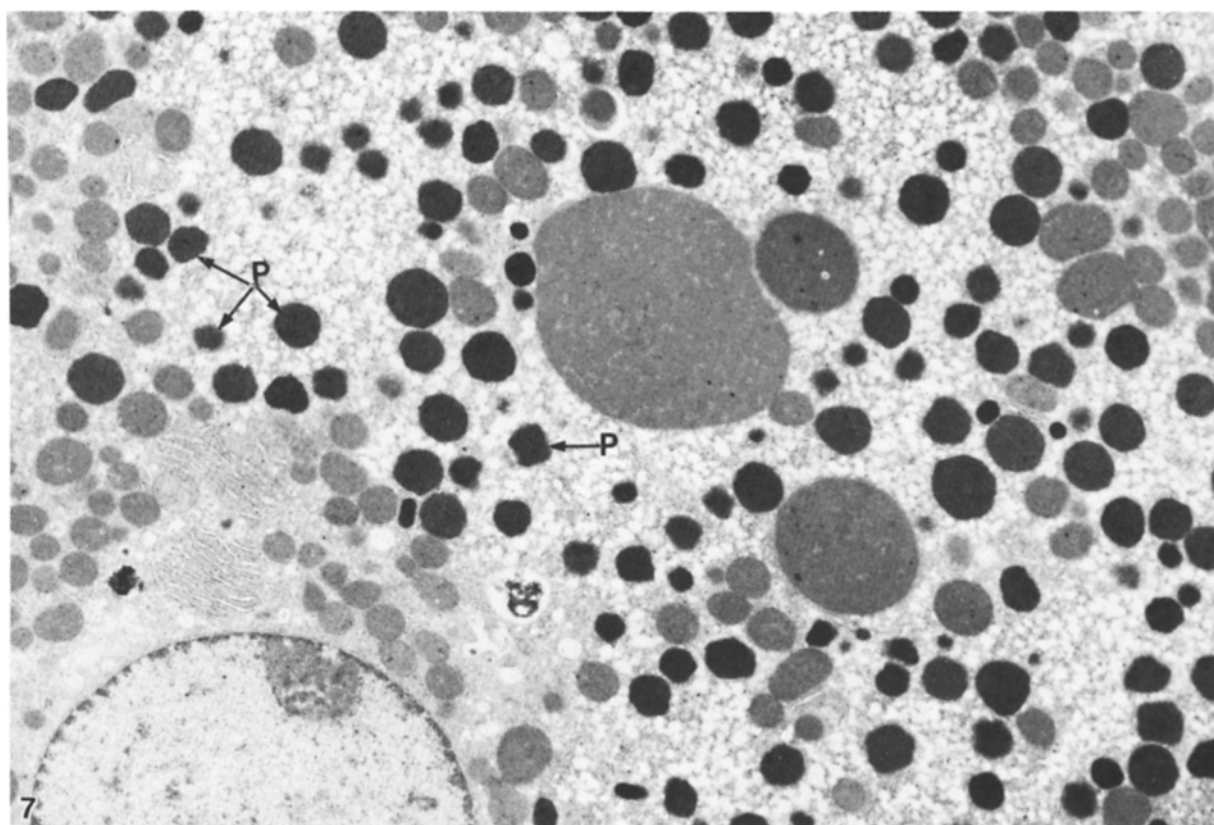


FIGURE 7 Electron micrograph of a pancreatic hepatocytelike cell induced in regenerating hamster pancreas by NBOP and followed by feeding of methyl clofenapate and incubated in the alkaline DAB medium. Electron-dense reaction product indicative of the presence of catalase is localized strictly to peroxisomes (P). $\times 10,600$.

kidney tubular epithelial cells (2, 12, 20, 21, 28, 29). Such proliferation is invariably accompanied by significant increases in the activity of peroxisome-associated enzymes such as catalase, carnitine acetyltransferase, and the enzymes responsible for β -oxidation of fatty acids (4-6, 10, 18, 20). Hepatocytelike cells in pancreas appear to respond to methyl clofenapate in a fashion similar to hepatocytes in liver. In both cells this compound led to increased numbers of peroxisomes and high levels of peroxisome-associated enzymes. In the case of the enoyl-CoA hydratase assay, the identity of enzyme as the peroxisomal one was confirmed by its inhibition by heat (15). Further, Reddy et al. (22) have shown that an 80,000-dalton protein induced in cells during peroxisomal proliferation is immunologically identical to the heat labile enoyl-CoA hydratase. This finding validates the immunochemical localization of enoyl-CoA hydratase to peroxisomes. Morphometric analysis not only further documented that methyl clofenapate induced peroxisome proliferation in hepatocytelike cells, but also established that the extent of proliferation was quite similar (eight-fold increase) as compared to an 8.4-fold increase observed by Moody and Reddy (11) in rat hepatocytes treated with the hypolipidemic drug SaH 42-348.

TABLE II
Relative Volume of Mitochondria and Peroxisomes in
Hepatocytelike Cells in Hamster Pancreas *

Treatment	% Cytoplasmic volume	
	Mitochondria	Peroxisomes
Control	19.77 \pm 1.74	1.78 \pm 0.21
Methyl clofenapate \ddagger	22.72 \pm 2.27	14.22 \pm 2.38 \S

* Each group consisted of three hamsters; 10 micrographs ($\times 10,000$) of hepatocytelike cells from each animal were subjected to morphometric measurement.

\ddagger Methyl clofenapate (0.2% in diet) was fed for 3 wk.

\S Significantly increased over control group $P < 0.05$.

Peroxisomes are not exclusive cell markers for hepatocytes (3, 14). However, the presence in hepatocytelike cells of four of the enzymes associated with peroxisomes namely, catalase, carnitine acetyltransferase, enoyl-CoA hydratase, and palmitoyl-CoA oxidizing system, the striking morphologic similarity of peroxisomes in these cells to those in liver hepatocytes, and their almost identical quantitative response to a peroxisome proliferator as compared to normal liver cells, strongly support

FIGURES 5 and 6 Fig. 5: Electron micrograph showing portions of hepatocytelike cells induced by NBOP in the regenerating pancreas of hamster and not treated with methyl clofenapate. Note the close resemblance of cytoplasmic architecture of these cells to hepatocytes. The peroxisomes contain subcrystalloid nucleoids (n) characteristic of hamster hepatocytes. Inset demonstrates the irregular nature of the peroxisome nucleoids (n) in these hepatocytelike cells. $\times 8,600$. Inset, 19,500. Fig. 6: Electron micrograph of a pancreatic hepatocytelike cell induced in regenerating hamster pancreas by NBOP and followed by the feeding of methyl clofenapate. The cytoplasm contains increased numbers of peroxisomes. In many of these peroxisomes the nucleoids are absent or out of the plane of section or obscured by an increased electron density of the organelle matrix. A nucleoid (n) is discernible in a few peroxisome. $\times 9,600$.

their identity as hepatocytes. Further, these results suggest that the repressed peroxisome specific genes in pancreatic cells are expressed with full fidelity upon derepression during the induced differentiation of these cells to hepatocytelike cells. The mechanism by which a remarkable proliferation of peroxisomes is induced in parenchymal cells of liver (2, 20, 28), proximal tubular epithelium of kidney (4) and now in these hepatocytelike cells in the hamster pancreas by structurally diverse chemicals remains to be elucidated.

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