Hepatic Foci of Cellular and Enzymatic Alteration and Nodules in Rats Treated With Clofibrate or Diethylnitrosamine Followed by Phenobarbital: Their Rate of Onset and Their Reversibility

Peter Greaves, Edmundo Irisarri, and Alastair M. Monro

ABSTRACT—The histologic appearance and cytochemical characteristics of foci of hepatic cellular alteration, hepatic nodules, and hepatocellular carcinomas occurring in male Sprague-Dawley rats treated with the hypolipidemic agent clofibrate (CAS: 637-07-0), with phenobarbital (CAS: 50-06-6), or with diethylnitrosamine [(DENA) CAS: 55-18-5] followed by phenobarbital were studied after treatment periods from 1 month to 2 years. Rats treated with clofibrate revealed foci of cellular alteration that were more often basophilic and occurred slightly sooner (wk 42) than those in untreated controls (wk 60). Of 36 rats that had received 68 or more weeks of continuous clofibrate, 19 had hepatic nodules. Of the 11 nodules examined cytochemically, none was γ-glutamyltransferase (γ-GT) positive and 2 were positive to glucose-6-phosphate dehydrogenase (G-6-PD) under oxygen. In rats withdrawn from clofibrate for 16-18 weeks after 68-95 weeks of clofibrate, 0 of 14 had nodules. In several of these rats zones of hepatic scarring were observed, suggesting the reversibility of the nodules. Phenobarbital alone had little effect on the incidence of foci of cellular alteration, although the number of γ-GT-positive foci was increased. DENA followed by phenobarbital led to the early appearance of foci of cellular alteration (from wk 4), of nodules (from wk 13), and of hepatocellular carcinomas (from wk 26). γ-GT activity was raised in most of these nodules and carcinomas, while G-6-PD activity was raised in only 3 of 9 nodules but in all 9 carcinomas examined. DENA-phenobarbital given for 13 or 26 weeks followed by withdrawal of phenobarbital for 28 and 26 weeks, respectively, produced an essentially similar pattern of lesions. In view of the growing recognition of the nonspecificity of γ-GT as a marker of carcinogen-initiated foci, the value of G-6-PD (under oxygen) as a marker merits further investigation.—JNCI 1986; 76:475-484.

Following extensive and detailed morphologic, biochemical, and cytochemical studies over a number of years, the sequence of events occurring in the rat liver after administration of powerful genotoxic carcinogens is now well established. It has been shown that there is a fairly rapid development of foci of cellular alteration, which have distinct morphologic and enzymatic characteristics as well as resistance to the effects of iron loading (1-4). After further exposure to carcinogens or promoting environments, nodules become grossly visible; these nodules have been shown to be at least one site of origin for hepatocellular carcinomas (1).

Broadly similar morphologic changes occur in the rat liver following the long-term administration of a wide variety of other chemical agents that are not generally considered as powerful carcinogens or are not mutagenic in short-term tests. Such agents include a number of hepatic enzyme inducers typified by phenobarbital and DDT, as well as hypolipidemic agents, the drug methapyrilene hydrochloride, and a variety of other xenobiotics (5-10). Hepatic nodules may also be produced in rodents by means of other procedures such as portacaval anastomosis (11) or providing choline-deficient diets (12).

Whether all these hepatic nodular lesions are similar is uncertain. While they seem to possess some common features (13), Ward (14) has suggested that certain hepatocarcinogens produce specific morphologic types of liver tumors in the rat. But does the remodeling or redefinition process described by Farber (15) in the resistant hepatocyte model apply to nodules induced by all hepatocarcinogens? Furthermore, although some biochemical differences have been noted between hepatocarcinogens induced in the rat by hypolipidemic agents and by genotoxic carcinogens (16, 17), the biochemistry of foci and nodules generated by nongenotoxic agents has not been well explored.

Some rat strains develop spontaneously with advancing age characteristic foci of cellular alteration, nodules, and frank hepatocellular carcinomas (14, 18-20). Discrimination between initiating and promoting agents in the usual long-term bioassay may then be impossible by classical light microscopy, but histochemical approaches may permit in certain cases partial distinction in terms of the mechanism of evolution of such lesions (20, 21).

The study of such foci is of potentially great importance in the context of safety evaluation. Because the foci often occur within a few weeks and in much greater number than do nodules and tumors, the possibility of characterizing them as early harbingers of the much

ABBREVIATIONS USED: DENA = diethylnitrosamine; G-6-PD = glucose-6-phosphate dehydrogenase; γ-GT = γ-glutamyltransferase; H & E = hematoxylin and eosin; PAS = periodic acid-Schiff.

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5 We thank Mr. P. Mompon for his skilled assistance with the histochemistry and Mme. D. Devauchelle for preparation of the manuscript.
later production of various types of tumor is highly attractive.

The aim of the present study, therefore, was to compare critically the evolution of hepatic foci of cellular alteration, nodules, and hepatocellular carcinomas arising in rats treated with the hypolipidemic agent clofibrate with that found after administration of the carcinogen DENA followed by phenobarbital promotion and with that occurring spontaneously in the same strain of rat. To obtain an indication of the biologic nature of the induced lesions, we also studied groups of rats after periods of withdrawal from treatment with clofibrate and phenobarbital. Inasmuch as focal enzyme changes have been used to identify putative preneoplastic changes in hepatocytes (1), livers were examined cytochemically. We examined not only the classical hepatic enzyme marker for early neoplastic alteration, \( \gamma \)-GT, but also G-6-PD, the activity of which under certain conditions is often elevated in malignant cells (22).

The dose level chosen for clofibrate (400 mg/kg body wt/day) was based on results of a previous in-house study in Sprague-Dawley rats. The dose level was predicted to be well tolerated and to be one that would produce an appropriate crop of hepatic nodules.

**MATERIALS AND METHODS**

Animals and experimental design.—The animals used in this study were male Sprague-Dawley rats [Crl: COBS-CD(SD)BR] from Charles River, St. Aubin-Lès-Elbeuf, France. At the start of the experiment rats were 6 weeks old and had been acclimatized in the laboratory for at least 1 week. They were fed a standard laboratory diet (UAR A04C) and given water ad libitum throughout the experiment. The rats were housed individually under standard conditions as described previously (23).

Rats were killed by carbon dioxide gas, and all animals whether sacrificed or found dead were subjected to a full autopsy.

In experiment I (table 1) 180 rats were divided into 2 groups: Seventy were untreated, and the remaining were given 400 mg clofibrate (CAS: 637-07-0)/kg body weight/day (Siber Hegner Cie, Lyon, France) mixed in the diet. At the intervals indicated in table 1, 3, 4, or 5 untreated controls and treated rats were sacrificed. At week 42 and at subsequent intervals listed in table 1, 3 or 4 treated rats ("reversibility" groups) were withdrawn from treatment with clofibrate and killed 16-18 weeks later.

In experiment II (table 2) rats were divided into 3 groups: Thirty-five remained untreated; 45 were treated with DENA (CAS: 55-18-5; Sigma Chemical Co., St. Louis, MO) given as 1 ip injection, 50 mg/kg body weight each week for 4 weeks, followed by 0.05% phenobarbital (CAS: 50-06-6; Coop Pharmacie Française, Melun, France) in the diet from week 5 onward; a further group of 20 was treated with a similar dose of phenobarbital alone starting at week 5. Groups of 5 controls and 5 treated rats were sacrificed after treatment with DENA (wk 4) and at the subsequent intervals indicated in table 2. In addition, after 13 and 26 weeks of treatment with DENA-phenobarbital, reversibility groups of 4 rats were withdrawn from treatment and sacrificed 28 and 26 weeks later.

Two hepatic nodules found in 2-year-old untreated rats (1 male and 1 female) in concurrent chronic bioassay studies were taken for cytochemical study.

**Histopathologic techniques.**—The livers and any abnormal organs were fixed in 10% formol saline and processed, and 5-µm-thick histologic sections were prepared in a routine fashion. Three standard H & E- and PAS-stained sections of liver, one from each lobe from each animal, as well as any liver lesion observed macroscopically were examined.

Hepatic lesions were classified according to the histologic criteria given in (24) except that the lesion referred to as a "neoplastic nodule" was termed a "hyperplastic nodule," as is usual in this laboratory (25).

**Cytochemical procedure.**—Fragments of liver 0.25 cm³ from the left lobe and fragments from any hepatic lesions seen at autopsy were frozen in hexane at -70°C. The activities of the following enzymes were demonstrated with the use of unfixed, frozen sections measuring approximately 0.25 cm³: \( \gamma \)-GT (26) and G-6-PD [after Chayen et al. (27)] after incubation in an atmosphere of oxygen instead of nitrogen. H & E and PAS stainings were also performed on a section from each block. The number of enzyme-positive foci was recorded, inasmuch as normal hepatocytes contained little or no activities of these enzymes as measured in this experiment. Enzyme activities in 0.25-cm³ sections of nodules and carcinomas were also similarly assessed. Cytochemical studies were not done on rats found dead or moribund.

**RESULTS**

**Survival**

In experiment I, 8 control rats, 16 clofibrate-treated rats, and 3 rats from the reversibility groups died or were sacrificed because of ill health, mostly as a result of spontaneous disease as reported previously in this strain (28). In some clofibrate-treated animals, massive hepatic nodules contributed to death (see below).

In experiment II, no unscheduled deaths in controls were observed, but 5 deaths or early sacrifices occurred in DENA-phenobarbital-treated rats. Hepatocellular carcinoma contributed to death in all 5 cases.

**Body and Organ Weights**

Body weights in clofibrate-treated groups were similar to those in controls until 3 months had elapsed; thereafter weight gain was slightly less, with treated animals weighing about 8% less than controls at 18 months. After cessation of the treatment, the body weight gain reverted to that of the controls. Both absolute and relative liver weights were higher in groups continuously treated with clofibrate than in controls, but they were
similar in control and reversibility groups.

Similarly, body weights in the DENA-phenobarbital-treated groups were less than those in controls toward the end of experiment II, with the difference being about 9% at day 300. Liver weights were higher in both groups tested with phenobarbital compared with those in controls, although the differences were less in the reversibility groups. Large tumors in DENA-phenobarbital-treated rats at the end of the experiment contributed to liver weights of nearly four times those of controls in some cases.

**Histopathologic Findings**

Hepatocytes in zones unaffected by foci and nodules generally only showed minor histologic changes. The livers from controls rats were replete with glycogen and had abundant dense cytoplasm in H & E-stained sections. The hepatocyte cytoplasm in the clofibrate-treated groups was more granular and eosinophilic, and lipofuscin pigment accumulated at later examination periods. Rats treated with phenobarbital with or without DENA showed centrilobular hypertrophy of the hepatocytes. Apart from an occasional single-cell degeneration and minor focal accumulation of chronic inflammatory cells in all groups including controls, there was no evidence of hepatocellular damage.

After cessation of treatment with phenobarbital or clofibrate, loss of the hepatocyte eosinophilic change or hypertrophy occurred, such that these livers resembled those of control rats. Slight fatty change and zones of scarring were sometimes observed in clofibrate-treated reversibility groups (see below).

**Foci of cellular alteration.**—These foci were first observed in H & E-stained sections in control rats at 60 weeks in experiment I and 52 weeks in experiment II and were found at all subsequent examination periods (tables 1, 2). A preponderance of clear cell foci was found (table 3). Generally, 1–5 clear cell foci were found per section; however, occasionally, as many as 100 per section were observed.

In both clofibrate-treated and phenobarbital-alone-treated groups, foci of cellular alteration were observed earlier than in controls—at 42 and 41 weeks, respectively (tables 1, 2). These foci also were mainly clear cell in type, although a slight increase in the proportion of rats with eosinophilic foci in the groups treated continuously with clofibrate was noted (table 3).

In contrast to the above results, DENA-phenobarbital-treated rats developed foci much earlier than controls, the first being observed after 4 weeks of phenobarbital treatment. Although clear cell foci were present in significant numbers, more basophilic and cystic foci (spongiosis hepatis) were observed than in controls. The 2 reversibility groups showed an incidence of foci similar to that in the groups given continuous treatment (table 3).

**Hyperplastic nodules and hepatocellular carcinomas.**—Neither hyperplastic nodules nor hepatocellular carcinomas were found in controls from experiments I and II.

In rats treated continuously with clofibrate, hyperplastic nodules (fig. 1), sometimes characterized by nodular growths of hepatocytes intersected by fibrous tissue bands (not illustrated) compressing but not infiltrating the surrounding liver, were found during week 68 and at most subsequent autopsy periods (table 1). These nodules were composed of uniform but large hepatocytes, with abundant dense cytoplasm and large but uniform round nuclei with prominent nuclear membranes and large dense nucleoli (fig. 1). A total of 19 of 36 rats treated continuously with clofibrate developed hyperplastic nodules. These 19 rats had 1–3 nodules each, with a total of 31 nodules for the group.

In the clofibrate-treated reversibility groups (28 rats), only 1 hyperplastic nodule was found—in a rat that died 11 days after having been treated for 86 weeks. However, in a number of animals from the reversibility

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**Table 1.** Numbers of rats with hepatocellular foci of cellular alteration and nodules, experiment I, clofibrate

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Lesion</th>
<th>Scheduled or not</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>32</th>
<th>42</th>
<th>50</th>
<th>60</th>
<th>68</th>
<th>77</th>
<th>86</th>
<th>95</th>
<th>104</th>
<th>113</th>
<th>Total weeks, 68–113</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>Foci</td>
<td>Scheduled</td>
<td>0/4</td>
<td>0/3</td>
<td>0/3</td>
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<td>0/5</td>
<td>0/5</td>
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<td>3/5</td>
<td>5/5</td>
<td>21/30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unscheduled</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
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<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
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<td>Clofibrate, continuous</td>
<td>Foci</td>
<td>Scheduled</td>
<td>0/4</td>
<td>0/3</td>
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<td>0/5</td>
<td>0/5</td>
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<td>0/5</td>
<td>0/5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Unscheduled</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
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<td>0/1</td>
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<td>Reversibility groups</td>
<td>Foci</td>
<td>Scheduled</td>
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<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/4</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
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<td>0/5</td>
<td>0/5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Unscheduled</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
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<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

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* Animals that were sacrificed when found moribund or that were found dead between the week of the column heading and the subsequent scheduled sacrifice.

* Results are No. of rats with lesion/total No. of rats treated.

* Two other animals had hepatocellular carcinomas.

* These animals were treated with clofibrate-supplemented diet for the period of the column heading and then sacrificed 16–18 wk later.

* Clofibrate-containing diet withdrawn 11 days previous to death.
groups, considerable zones of scarring were evident. These zones were variable in location and were often subcapsular (Fig. 2). The zones were free of nodules, although tinctorial changes were present in some hepatocytes, features similar to those seen in foci of cellular alteration. Larger areas and bands of fibrous scar tissue contained abundant lipofuscin pigment and small islands of entrapped but viable hepatocytes. Necrosis was not seen.

Two hepatocellular carcinomas were found in animals moribund or found dead at or after week 100 in the clofibrate-treated groups. These tumors were well differentiated, infiltrated normal liver tissue, and produced pulmonary metastases in one case.

In rats treated with DENA, hyperplastic nodules and hepatocellular carcinomas appeared early—at 13 and 26 weeks of phenobarbital, respectively—and were seen in both reversibility groups (Table 2). A total of 43 nodules was found in the 21 rats with nodules. No nodules were found in rats treated with phenobarbital alone. The hyperplastic nodules were, by definition, localized growths of hepatocytes compressing but not infiltrating the surrounding liver tissue. However, cell size was more variable and nuclear pleomorphism and mitotic activity were more marked than in clofibrate-treated or untreated rats. Hepatocellular carcinomas were generally very pleomorphic with a high mitotic activity and showed heterogeneous cellular differentiation often in the same neoplasm (Fig. 3). Glandular differentiation was often striking, and infiltration of surrounding liver and blood vessels was widespread. Pulmonary or lymph node metastases were found in 2 cases.

Cytochemical Findings

Foci of cellular alteration.—γ-GT-positive foci were first observed in controls at week 60, in phenobarbital-treated rats at week 26, in clofibrate-treated rats at week

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**Table 2.**—Number of rats with hepatocellular foci, nodules, and carcinomas, experiment II, DENA-phenobarbital

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Lesion</th>
<th>Weeks of treatment with phenobarbital</th>
<th>Total weeks, 13-67</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>Foci</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Nodules</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Carcinomas</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Phenobarbital alone</td>
<td>Foci</td>
<td>0/5</td>
<td>0/5</td>
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<td></td>
<td>Nodules</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Carcinomas</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>DENA-phenobarbital</td>
<td>Foci</td>
<td>0/5</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td>Nodules</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Carcinomas</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Carcinomas</td>
<td>1/4</td>
<td>2/4</td>
</tr>
</tbody>
</table>

a Results are No. of rats with lesion/total No. of rats treated.

b Animals that were sacrificed when found moribund or that were found dead between the week of the column heading and the subsequent scheduled sacrifice.

c Animals were treated for 13 or 26 wk and sacrificed 28 and 26 wk later, respectively.

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**Table 3.**—Summary of main histologic types of foci

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Total No. of rats with foci/total examined</th>
<th>Clear cell foci</th>
<th>Basophilic foci</th>
<th>Eosinophilic foci</th>
<th>Cystic foci</th>
<th>Vacuolated foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated controls</td>
<td>22/70</td>
<td>20</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>0</td>
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<td>Clofibrate</td>
<td>29/74</td>
<td>19</td>
<td>3</td>
<td>17</td>
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<td>0</td>
</tr>
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<td>Clofibrate, reversibility</td>
<td>18/28</td>
<td>15</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Expt II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated controls</td>
<td>5/35</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>2/20</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DENA-phenobarbital</td>
<td>27/36</td>
<td>18</td>
<td>18</td>
<td>8</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>DENA-phenobarbital, turnover</td>
<td>9/9</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

a Including rats that were sacrificed when found moribund or were found dead.

b Total duration, 113 wk.

c Total duration, 71 wk.
52, and in clofibrate-treated reversibility groups at week 86 (table 4). In the controls, 8 of 10 foci were first detected at week 104. In striking contrast to the above groups, all DENA-treated groups developed large numbers of γ-GT-positive foci at and after 13 weeks of phenobarbital treatment.

After incubation under oxygen, a number of foci showed G-6-PD activity, whereas the surrounding liver showed no such activity (fig. 4). These foci were observed in controls and the clofibrate-treated reversibility groups only after 86 weeks or longer; none was observed in rats treated continuously with clofibrate. In rats treated with DENA, foci with this activity were observed as early as week 4, even before the onset of treatment with phenobarbital.

Many enzyme-altered foci were not visible in the corresponding frozen sections stained with H & E. Some, but not all, basophilic foci in all groups showed activity for these enzymes. Furthermore, foci of other types also sometimes showed activity for γ-GT and G-6-PD.

Hyperplastic nodules and hepatocellular carcinomas.—Enzyme activities of these lesions are presented in table 5. γ-GT activity was notably absent from all clofibrate-induced nodules examined, but it was present in 7 of 9 DENA-induced nodules. The activity of G-6-PD when incubated under oxygen was high (fig. 5) in carcinomas (all in the DENA-treated groups) but only occasionally found in nodules of any group.

### DISCUSSION

Although it has been known for some years that clofibrate induces hepatocellular carcinomas in Fischer rats (5, 29), the main finding following administration of clofibrate to Sprague-Dawley rats for up to 2 years in the present study was an approximately 50% incidence of hyperplastic nodules. The carcinomas found in 2 rats treated for more than 95 weeks were probably also treatment related, inasmuch as such tumors rarely occur spontaneously in rats of this strain in our laboratory (5 in the last 250 male rats studied for 2 yr).

This incidence of nodules, however, provided an excellent basis for the study of their potential reversibility. The incidence in rats treated continuously for 68 weeks (the first period at which nodules were found) or longer was 19 of 36, compared with an incidence of 0 of 14 in rats treated for the same periods followed by 16-18 weeks' withdrawal from treatment (table 1, last col.).

The potential reversibility of the 1 nodule seen in an animal found dead in a reversibility group is unknown because the treatment was withdrawn only 11 days before death. Furthermore, striking zones of cellular alteration and scarring consistent with nodule regression were observed in the livers of animals in the clofibrate-treated reversibility groups. These two observations (the zero incidence plus scarring in the reversibility groups) taken together are interpreted as evidence

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Type of lesion</th>
<th>No. examined</th>
<th>γ-GT positive</th>
<th>G-6-PD positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>Hyperplastic nodules</td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>Hyperplastic nodules</td>
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<td>0</td>
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<td>DENA-phenobarbital</td>
<td>Hyperplastic nodules</td>
<td>9</td>
<td>7</td>
<td>3</td>
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<tr>
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<td>Hyperplastic nodules</td>
<td>2</td>
<td>2</td>
<td>0</td>
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<tr>
<td></td>
<td>Hepatocellular carcinomas</td>
<td>3</td>
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</tbody>
</table>

<sup>a</sup> From concurrent studies.
that clofibrate-induced nodules—even large ones—retain the capacity to regress after continuous administration of the stimulus for up to 95 weeks.

Although in many models of carcinogenesis foci of cellular change precede the appearance of nodules and tumors, in the present study clofibrate revealed little such propensity. The first foci were detected slightly sooner (wk 42) than in the controls (wk 60), but by week 77 most treated and control animals showed such foci. The only characteristic difference was that clear cell foci dominated in the controls, whereas clofibrate-treated animals showed similar proportions with clear cell and with eosinophilic foci. Furthermore, in the reversibility groups, although the numbers of rats with foci of cellular change were similar to those in the controls and continuous-treatment groups, the proportion with eosinophilic foci was similar to that in the controls (table 3), which may be an indication that some of these foci are "reversible." A trend toward eosinophilic foci has been reported following administration of another hypolipidemic agent (Wy-14,643) to rats (30).

Other workers have reported higher incidences of foci of cellular change in Fischer and Wistar rats (14, 20, 21), often with basophilic cell types predominating. In our experience the histologic types of foci that occur spontaneously in these Sprague-Dawley rats are quite variable from one study to another, such that little importance can be attached to the pattern of foci found in the present study. Nevertheless, the very presence of these foci in untreated rats is a complicating factor in the interpretation of the results of chronic rodent bioassays (31).

The findings following the DENA-phenobarbital schedule were strikingly different from those following clofibrate administration. Foci of cellular alteration were first seen after 4 weeks of phenobarbital treatment; thereafter, their incidence increased rapidly. The foci were predominantly basophilic and cystic (spongiosis hepatitis); such foci have been particularly associated with carcinogen exposure (32, 33). Subsequently, nodules and hepatocellular carcinomas quickly developed at a time when similar lesions are not prone to occur in untreated rats.

Although remodeling of nodules has been described in rats treated with genotoxic carcinogens (34), there was no evidence in the present study that the DENA-induced lesions were capable of significant regression, for they occurred in similar incidence in both reversibility groups. In addition, these nodules were histologically much more pleomorphic than their counterparts found in the clofibrate-treated groups and those arising spontaneously, although, by definition, they were also sharply demarcated from the surrounding liver. Although earlier studies with another genotoxic hepatocarcinogen, N-2-fluorenylacetamide, indicated that nodules could regress after removal of the stimulus (35), later studies with the same agent indicated that the nodules were persistent and could even grow (32).

Let us consider whether the histochemical data supplement the morphologic findings. The marker γ-GT was detected in foci of controls only at a similar time that the foci of cellular alteration were observed in paraffin wax-processed sections. However, the slightly higher incidence and earlier appearance of positive foci following phenobarbital, the marked effects following DENA-phenobarbital, and the absence of effect with clofibrate (table 4) were useful observations and in keeping with expectations for such agents (17, 31, 36-41). Nodules and carcinomas were only positive for γ-GT in rats treated with DENA-phenobarbital, although the correspondence was not complete in either category.

In view of the growing recognition of the limitations of γ-GT as a marker for putative preneoplastic foci (31, 39, 40, 42, 43), our results for G-6-PD take on a particular interest. While few or no such foci were observed in the control, clofibrate-treated, or phenobarbital-treated groups, they were numerous in the DENA-phenobarbital-treated groups. Furthermore, they appeared even before the phenobarbital treatment commenced. Although some nodules showed G-6-PD activity, all the carcinomas did so. The enzyme is the regulatory enzyme of the pentose-phosphate shunt, which provides ribose for the synthesis of nucleic acids and NADPH for biosynthetic mechanisms and has been, therefore, studied in cancer (22). It has recently been shown that when neotetrazolium chloride is used as an indicator of the activity, oxygen competes with the neotetrazolium and eliminates the reaction in normal cells, whereas a considerable range of human malignant cell types is resistant to this effect and shows activity (22, 44). In view of the paucity of specific markers for new cell populations and lesions in experimental hepatocarcinogenesis (45), the activity of this enzyme in an atmosphere of oxygen warrants further investigation in the rodent hepatic tumor model.

In conclusion, we have demonstrated the heterogeneity of noninfiltrative hepatic nodular lesions in rats by both morphologic and cytochemical criteria. Lesions caused by DENA-phenobarbital were more pleomorphic than those induced by clofibrate and, under the conditions of this study, were irreversible. By contrast, the clofibrate-induced nodules were apparently reversible even after almost 2 years of treatment. Most, but not all, nodules induced by DENA were positive for γ-GT; none induced by clofibrate were. A few of the nodules induced by DENA or by clofibrate were positive for G-6-PD; all the carcinomas were positive. This may be evidence supporting the view of Peraino et al. (39) that while foci of enzymatic change may be indissociable from the administration of hepatocarcinogens, such changes do not lead irrevocably to neoplasia.

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FIGURE 1.—Part of a hyperplastic nodule from a rat treated with clofibrate for 104 wk. Nuclei with prominent nucleoli are in enlarged hepatocytes. Nodule is sharply demarcated from surrounding liver. H & E. × 320

FIGURE 2.—Low-power view of liver from a rat treated with clofibrate for 86 wk and killed 17 wk after cessation of treatment. Large zone of scar tissue contains small groups of hepatocytes but no residual evidence of nodularity. H & E. × 60
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FIGURE 3.—Hepatocellular carcinoma from a rat treated with DENA followed by 13 wk of phenobarbital and then 28 wk free of treatment. Pleomorphic neoplasm showing early glandular differentiation is characteristic of hepatocellular carcinomas in this group. H & E. × 320
FIGURE 4.—Typical foci of G-6-PD activity when the reaction is performed in an atmosphere of oxygen in liver of a rat treated with DENA, followed by 13 wk of phenobarbital. Surrounding liver is characteristically negative for this reaction. Frozen section. X 320

FIGURE 5.—High activity of G-6-PD in hepatocellular carcinoma (same case as in fig. 5), when the reaction is performed in an atmosphere of oxygen. Frozen section. X 320