The Sensitivity and Heterogeneity of Histochemical Markers for Altered Foci Involved in Liver Carcinogenesis

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Subcutaneous injection of iron dextran resulted in a hepatic siderosis within 2 weeks in rats, as previously reported for mice. Hepatic carcinomas as well as neoplastic nodules in rats were entirely or mainly free of stainable iron and, thus, could be readily identified histologically. In addition, early carcinogen-induced altered foci were resistant to iron accumulation. In rats fed 0.02% N-2-fluorenylacetamide (FAA) for 13 weeks, the number of iron-resistant foci identified following iron injection was the same as that observed with dietary iron overload. Histochemical investigation of enzymatic markers that have been used to identify foci in rats revealed that foci characterized by enzymatic reactions of positive gamma-glutamyl transpeptidase and decreased adenosine triphosphatase and glucose-6-phosphatase corresponded to those characterized by resistance to iron accumulation. However, in quantitative analysis of the early carcinogen-induced foci in rats given iron dextran following a diet containing 0.02% 2-FAA for 13 weeks, more lesions were detected by resistance to iron accumulation than by any of these other properties. There was considerable phenotypic heterogeneity among foci for the enzyme markers. It is concluded that resistance to iron accumulation is a more sensitive and reliable marker for early carcinogen-induced altered hepatocellular foci than is any other histochemical property. (Am J Pathol 95:317–328, 1979)

DURING NEOPLASTIC development in rodent liver, a variety of hepatocellular lesions arise that have been described as altered (hyperplastic) foci, neoplastic (hyperplastic) nodules, and hepatocellular carcinomas.[1-12] In previous studies, it has been demonstrated that, in the presence of hepatic siderosis produced by dietary overload, all these lesions are resistant to the accumulation of stainable iron.[13-15] This sensitive and objective marker for preneoplastic and neoplastic lesions would be of greater value in carcinogenesis studies if the iron-loading could be accomplished without dietary modification during exposure to a carcinogen. Therefore, hepatic siderosis was produced within 2 weeks by subcutaneous injection of iron dextran [16] so that iron loading could be achieved at the end of carcinogen exposure for the objective demonstration of liver

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lesions. Under these conditions, hepatocellular neoplasms in mice were refractory to iron accumulation.[16]

It therefore became of interest to determine whether lesions in earlier stages, ie, altered foci, were also resistant to iron accumulation when siderosis was induced by this procedure. Altered foci as well as hepatocellular carcinomas induced by N-2-fluorenylacetamide (FAA) in rat liver were found to be resistant to iron accumulation induced by parenteral administration. The same number of altered foci were resistant to iron accumulation following the rapid loading by parenteral administration as displayed this property during dietary iron overload.

A variety of enzyme histochemical reactions such as gamma-glutamyl transpeptidase (GGT), adenosine triphosphatase (ATPase), and glucose-6-phosphatase (G-6-Pase) have been described as valuable markers for the identification of carcinogen-induced rat liver lesions.[17-23] In this study, the foci identified by alterations in these enzymatic properties were found to correspond to those characterized by resistance to iron accumulation. However, considerable phenotypic heterogeneity existed between foci in their enzymatic activities, and none of these properties identified as many foci as were detected by resistance to iron accumulation.

Materials and Methods

Fischer strain male rats were fed a diet containing 0.02% FAA as previously described.[15] Three experimental groups, each of which comprised 6 animals, were studied. The first group received FAA in the diet as well as 0.8% 8-hydroxyquinoline and 2.9% ferrous gluconate to produce iron loading.[13] The second group was injected subcutaneously in the inguinal area with 0.1 ml iron dextran per 40 gm body weight (Imferon, 50 mg elemental iron per milliliter) three times per week, alternating sides for 2 weeks prior to killing.[16] Rats in these two groups were killed at 13 weeks as previously described [15] for the study of altered foci, and the number of histochemical lesions per square centimeter in each lobe was calculated. The third group was continued on carcinogen alone for 20 weeks, then switched to control diet, and held an additional 15 weeks for the development of carcinomas. Iron injection was given by the same method as in the second group.

Serial sections were prepared and reacted for iron by the method of Gömöri [24] and three enzymic activities to make a comparative study between the markers.

Demonstration of GGT was carried out both on fresh frozen and paraffin-embedded sections using the histochemical technique described by Rutenburg et al.[25] Liver slices (1 to 2 mm) were fixed for 2 hours in cold acetone (4 C), dehydrated in acetone at room temperature, cleared in benzene, and embedded in paraffin at 54 C. Absolute alcohol was also substituted for acetone as a fixative for other slices. The histochemical reactions for ATPase and G-6-Pase activities were performed on frozen sections using the methods of Wachstein and Meisel.[26]

Results

The six iron injections over 2 weeks were sufficient to produce a siderosis in rats, as in mice,[16] in which every hepatocyte contained substantial

stainable iron. In spite of the heavy iron loading of hepatocytes, the bile duct epithelium remained free of iron (Figure 1).

Carcinogen-induced hepatocellular tumors in rats were typical trabecular hepatocarcinomas and were resistant to iron accumulation (Figure 2) by this procedure, as with dietary overload.[13-15] Some tumors were completely free of iron, whereas others contained some areas in which apparently neoplastic cells displayed a faint iron reaction. There was no correlation of histologic appearance with this apparent iron uptake.

Rats that received FAA in the diet for 13 weeks plus 8-hydroxyquinoline and ferrous gluconate developed approximately the same incidence of altered foci as those in previous studies (Table 1). In the other group that received carcinogen alone and iron injection prior to sacrifice, the same incidence of iron-resistant foci was identified (Table 1). Some cells in these focal iron-resistant lesions contained a faint blue hue in the cytoplasm with Prussian blue staining. Formalin- and ethanol-fixed tissues worked equally well for the iron reaction, but acetone-fixed tissues failed to give a satisfactory iron reaction (Table 1).

Serial sections reacted for iron and enzyme activities revealed that every iron-resistant altered focus corresponded to one of those exhibiting a positive reaction for GGT or deficiencies in ATPase or G-6-Pase. All discrete GGT-positive foci were also iron-resistant (Figures 3 and 4). Distinct activity of GGT could be seen not only in the cell membranes along the bile canaliculi of hepatocytes in the altered foci but also in the cytoplasm, as described by Kalengayi et al.[22] All ATPase- (Figures 5 and 6) and G-6-Pase- (Figures 7 and 8) deficient foci corresponded to iron-resistant foci in serial sections. The ATPase activity in normal hepatocytes was generally concentrated at the canalicular regions, but when large amounts of reaction product were precipitated, it was deposited throughout the cell (Figure 5). Whatever enzyme abnormalities were present in individual iron-resistant foci were generally uniform throughout each

Table 1—Number of Iron-Resistant and GGT-Positive Foci in Rat Liver After Dietary 2-FAA Exposure for 13 Weeks

	Iron-resistant foci per sq cm		GGT-positive foci per sq cm
Fixative*	Dietary iron overlead	Iron injection	Iron injection
Formalin (10%, buffered)	34 ± 10	39 ± 16	—
Ethanol (absolute)	36 ± 18	40 ± 17	32 ± 13
Acetone	—†	—†	28 ± 10

* Followed by paraffin embedding
† Reaction unsatisfactory

focus, eg, no focus was half GGT-positive and half GGT-negative, although a few ATPase-deficient and G-6-Pase deficient foci contained a minor fraction of cells with residual activity (Figure 5).

More lesions were detected by resistance to iron accumulation than by any one of the enzyme markers (Table 2) due to the fact that each of the enzyme abnormalities was occasionally not present in iron-resistant foci. The iron-resistant foci that were not positive for GGT were frequently basophilic foci (Figures 9 and 10). G-6-Pase deficiency was most frequently not a property of foci (Figures 11 and 12; Table 2). In addition to the inconstant accompaniment of each of the enzyme abnormalities with resistance to iron accumulation, foci were also randomly heterogeneous for the enzyme markers, eg, GGT-positive foci were not always ATPase-deficient (Figures 13 and 14) or G-6-Pase deficient (Figures 15 and 16). The diversity in enzyme activities of foci was not confined to any lobe or region but occurred in adjacent foci (Figures 10, 12, and 16) in all locations.

Discussion

Rapid and intense iron loading by subcutaneous injection was employed so that the resistance to iron accumulation in hepatocellular tumors and preneoplastic lesions could be used as a marker without the necessity of administering an iron-loading diet as in some of our previous studies.[13-15] Using this technique, mouse neoplasms were demonstrated to be resistant to iron accumulation,[16] and in the present study rat hepatocarcinomas were found to be resistant, as in studies involving dietary administration.[13-15] In addition, carcinogen-induced preneoplastic altered foci in rats were found to be resistant to iron accumulation during overload by parenteral administration. The same number of resistant foci were apparent with either dietary overload or parenteral administration. Using parenteral administration, Lipsky et al [27] have shown that carcinogen-induced foci in mice are resistant to iron accumulation. The observation that some cells in the rat liver foci gave a faint blue hue in the cytoplasm

Table 2—Number of Histochemical Foci in Livers Made Siderotic by Iron Dextran Injection Following Dietary 2-FAA Administration for 13 Weeks

Iron-resistant foci per sq cm*	GGT-positive foci per sq cm	ATPase-deficient foci per sq cm	G-6-Pase-deficient foci per sq cm
42 ± 12	33 ± 8 (0.2)†	28 ± 12 (0.1)†	20 ± 7 (0.005)†

* ± SD per section
† Probability that average is lower than that of iron-resistant foci

with iron reaction is consistent with our documentation by electron microscopy that small amounts of ferritin aggregates in the cytoplasm of some cells are, on occasion, present in iron-resistant hepatocellular lesions.[28]

The FAA-induced iron-resistant hepatocellular altered foci studied corresponded to foci characterized by deficiencies of ATPase and G-6-Pase or appearance of distinct GGT activity. This confirms our previous suggestion [14] that the various investigators studying liver lesions identified by different single histochemical abnormalities, eg, glycogen storage [18] and ATPase deficiency,[21] are all in fact describing the same lesion. None of these individual enzyme histochemical methods, however, identifies as many foci as can be detected by their resistance to iron accumulation (Table 2), indicating that iron resistance as a marker for carcinogen-induced hepatocellular foci is more sensitive than any of the other histochemical properties. GGT was found to be next in sensitivity as a marker for foci, correlating with the biochemical studies of Fiala et al [29,30] and Taniguchi et al.[31] The use of absolute ethanol in place of acetone as a fixative for iron-loaded livers for the GGT reaction proved to be essential for a comparative histochemical study because acetone, which causes a considerable disruption of ferritin,[32] was an unsatisfactory fixative for the iron reaction studies. Although GGT activity was closely correlated with resistance to iron accumulation, a significant shortcoming of GGT as a marker was its failure to identify some basophilic foci (Figures 9 and 10). These foci, in particular, are suspected to be precursors for hepatocarcinomas [4,10] and, thus, such a failure would limit the usefulness of GGT in analysis of tumor development. The least reliable enzyme marker was G-6-Pase deficiency. This property in part is the basis for the glycogen retention observed in liver foci and nodules [33]; thus, its inconstant representation in foci correlates with our previous report [15] that only approximately 67% of foci were characterized by glycogen retention.

The enzyme abnormalities in each hepatocellular altered focus were generally uniform throughout each focus, supporting the suggestion of Scherer and Hoffmann [34] that these foci, which are proliferative,[14,34] have a clonal origin. However, these phenotypic enzyme changes varied significantly between foci, as has been described in detail by others.[19,35,36] The present observations on foci at a stage in which the majority can undergo phenotypic reversion on removal of the carcinogen [15,37] strengthen other reports [19] indicating that the heterogeneity in phenotype arises early in the carcinogenic process. Since the properties of foci in this study differed markedly even between adjacent foci, this diversity cannot be easily attributed to local modulating factors. Rather, the phenotypic hetero-

geneity of foci may reflect multiple genotypic changes in the cells of foci.

The heterogeneity between foci was interpreted by Scherer and Emmelot [35] in terms of one-hit kinetics to indicate that the single hit initiating a focus may be in a DNA segment that regulates multiple cell properties. If this were so, some constant linkage of properties would be expected. No such linkage was evident in the foci studied here or by others [36]; therefore, we suggest that the heterogeneity, if genetic, reflects multiple effects. The accumulation of multiple lesions in DNA may be a consequence of the number of DNA hits required on a probability basis before a lesion critical to neoplastic conversion is introduced. Such multiple hits and the resulting phenotypic diversity may be a characteristic of the carcinogenic process initiated by chemicals, as opposed to the uniform changes mediated by viruses which introduce a segment of genetic information into the cell in a highly specific manner.

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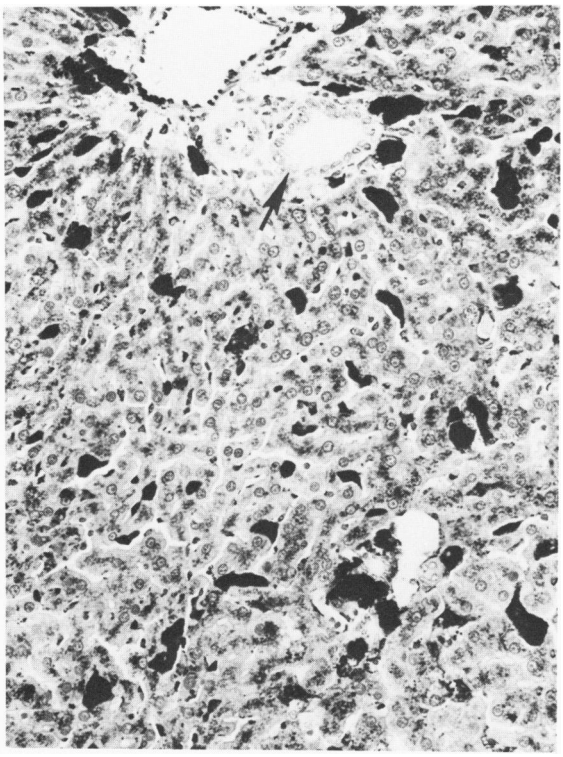
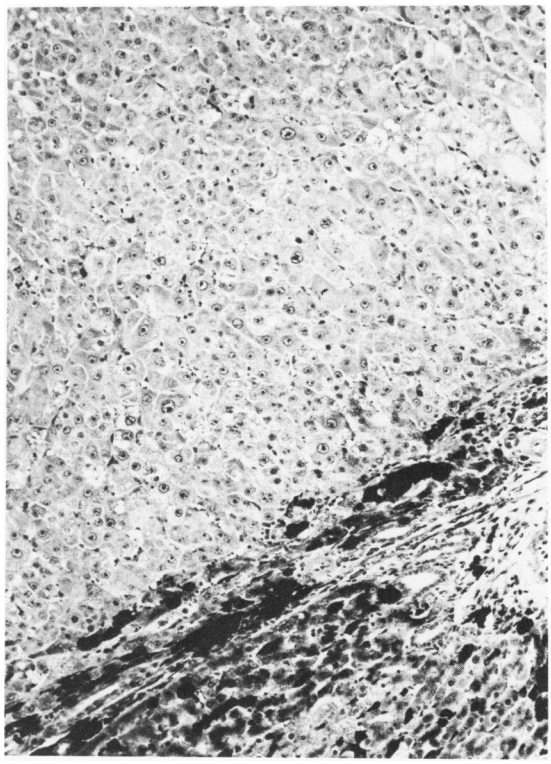
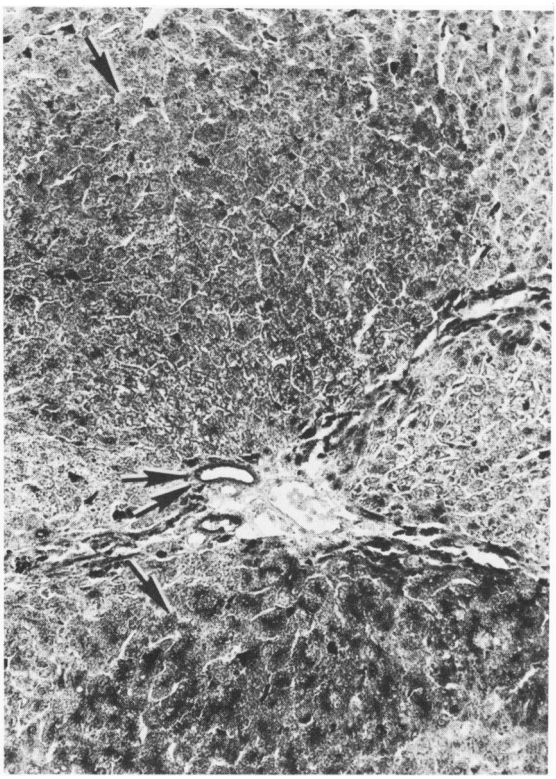






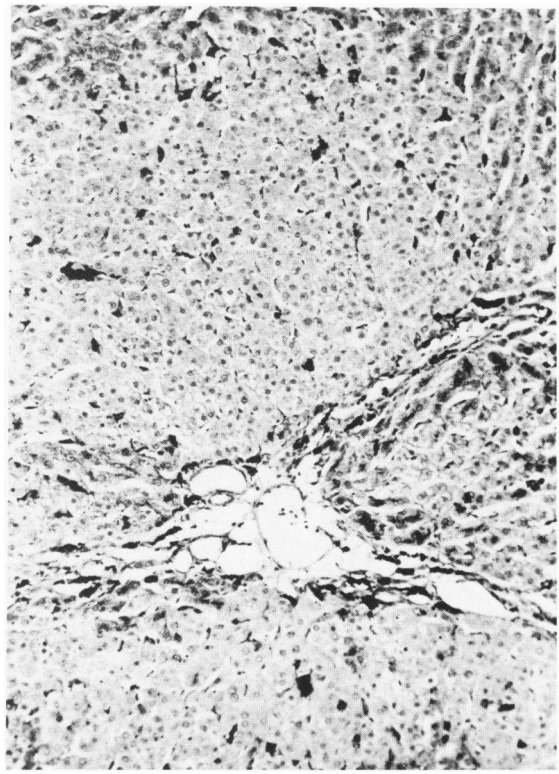
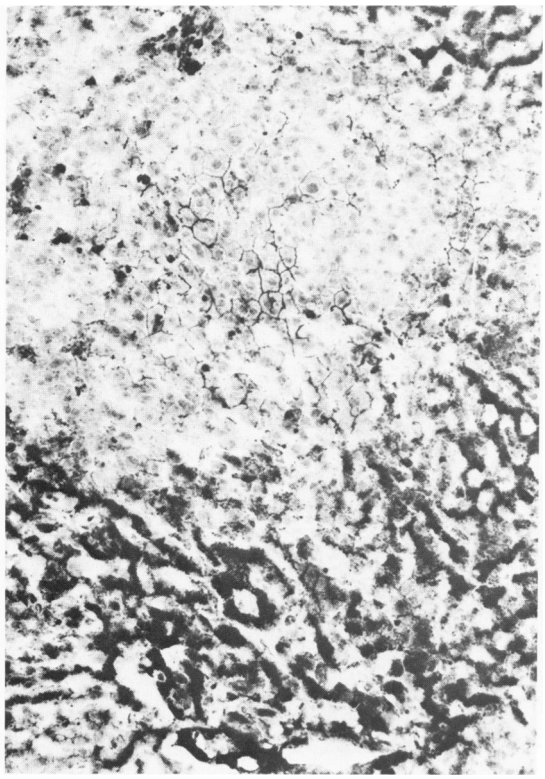
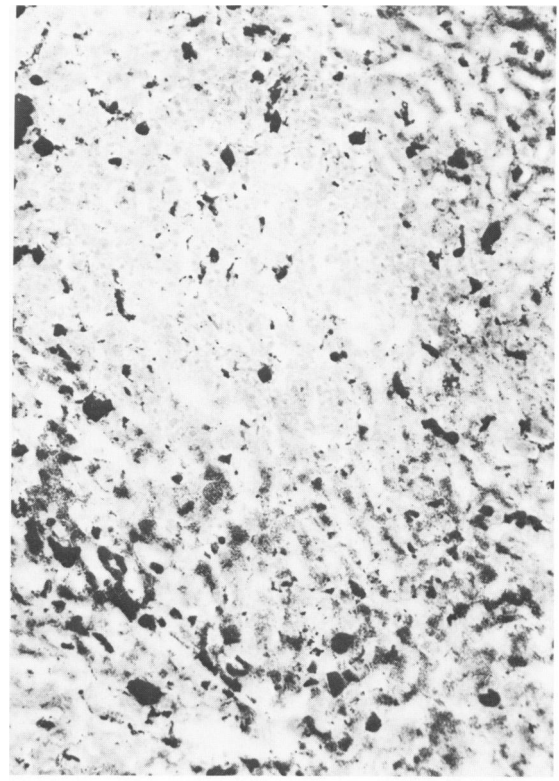
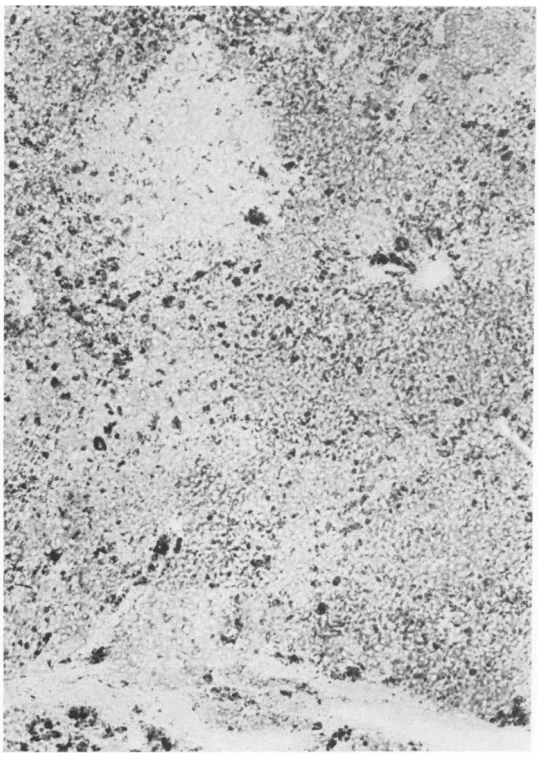


Figure 1—Siderotic liver following six injections of iron dextran over 2 weeks. The bile duct epithelium (*arrow*) is free of iron. (Iron reaction, × 140) **Figure 2**—Hepatocellular carcinomas induced by dietary 0.02% FAA for 20 weeks followed by holding for 15 weeks. The tumor has a trabecular pattern and is devoid of iron accumulation. (Iron reaction, × 120) **Figure 3**—Altered foci induced by dietary 0.02% FAA for 13 weeks in siderotic liver produced dietary overload. Two foci (*single arrows*) can be identified by positive reaction for GGT. A bile duct (*double arrow*) also is positive. GGT reaction, counterstained with hematoxylin. Ethanol-fixed, paraffin section. (× 115) **Figure 4**—Same as Figure 3. Two foci identified by the absence of iron accumulation correspond to GGT foci in Figure 3. Iron reaction counterstained with nuclear fast red. Serial section to that of Figure 3. (× 115)







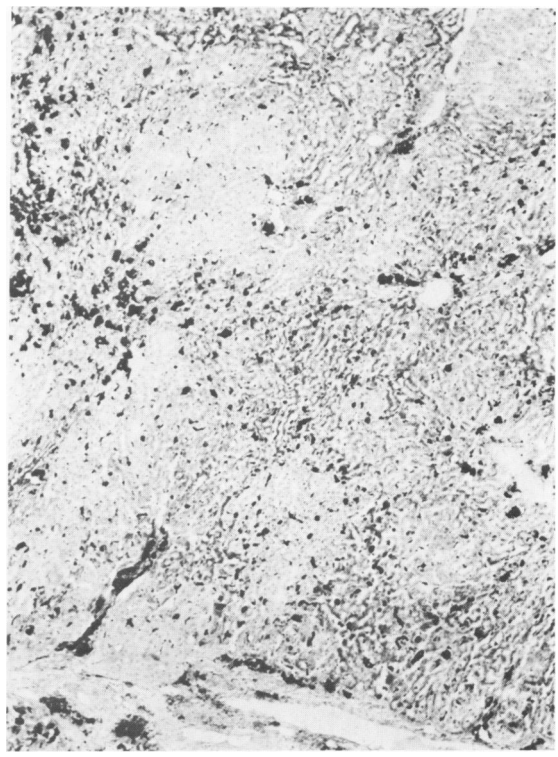
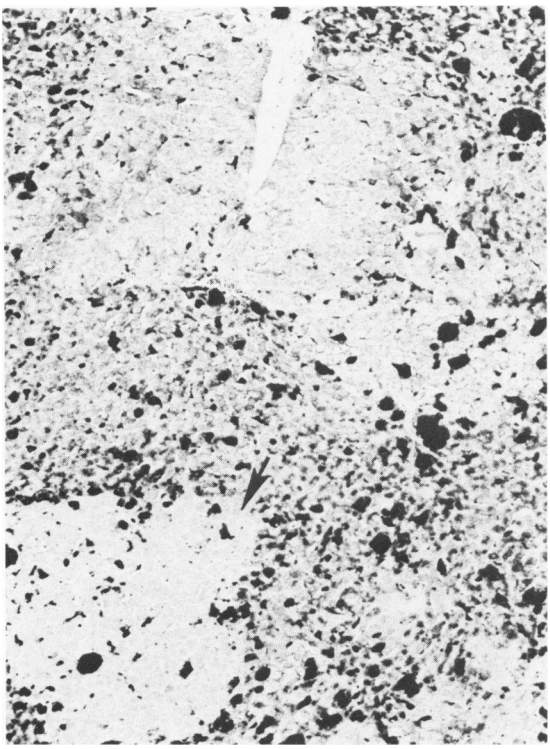
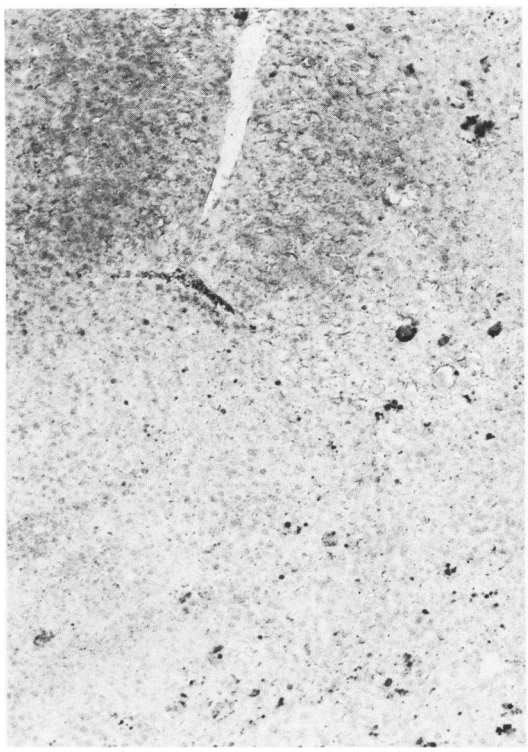


Figure 5—An altered focus induced by dietary 0.02% FAA for 13 weeks. The focus is seen to be deficient in ATPase in the siderotic liver produced by iron dextran injections. ATPase reaction; counterstained with hematoxylin. Frozen section. (× 115) **Figure 6**—Same as Figure 5. The iron-resistant focus corresponds to the ATPase-deficient focus in Figure 5. Iron reaction; counterstained with nuclear fast red. Serial section to that of Figure 5. (× 115) **Figure 7**—Altered foci after dietary 0.02% FAA exposure for 13 weeks. The foci are deficient in G-6-Pase in the siderotic liver produced by iron dextran injections. G-6-Pase reaction, frozen section. (× 43) **Figure 8**—Same as Figure 7. Foci identified by resistance to iron accumulation correspond to those in Figure 7. Iron reaction, counterstained with nuclear fast red; serial section to that of Figure 7. (× 43)





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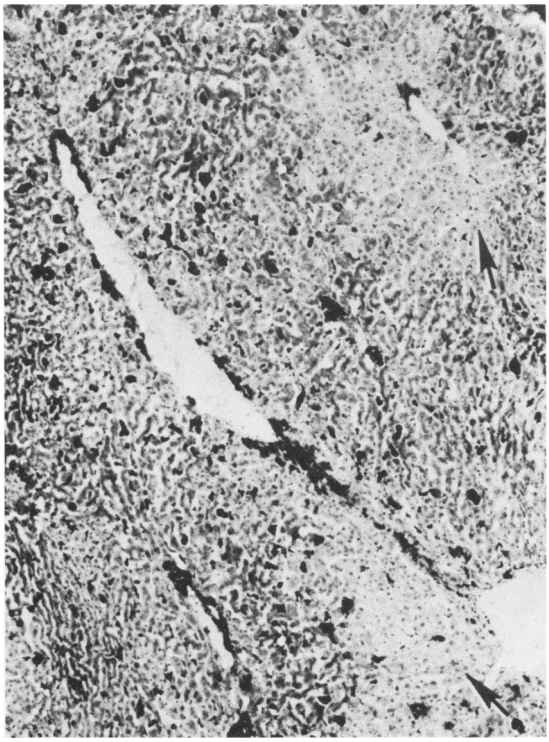

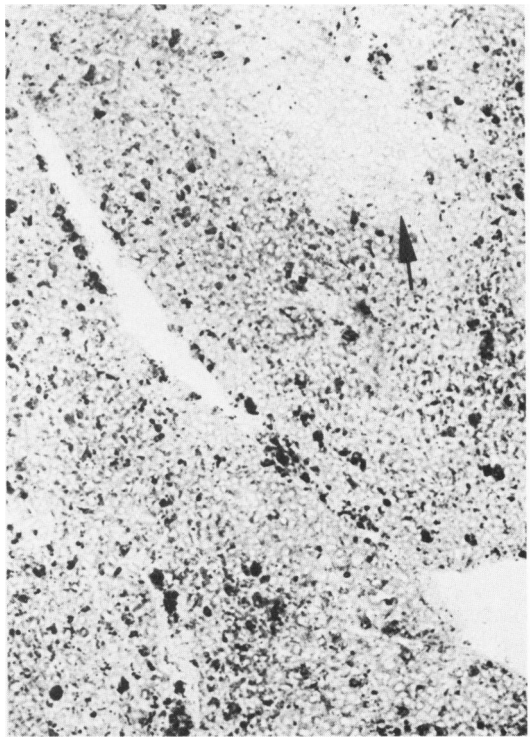

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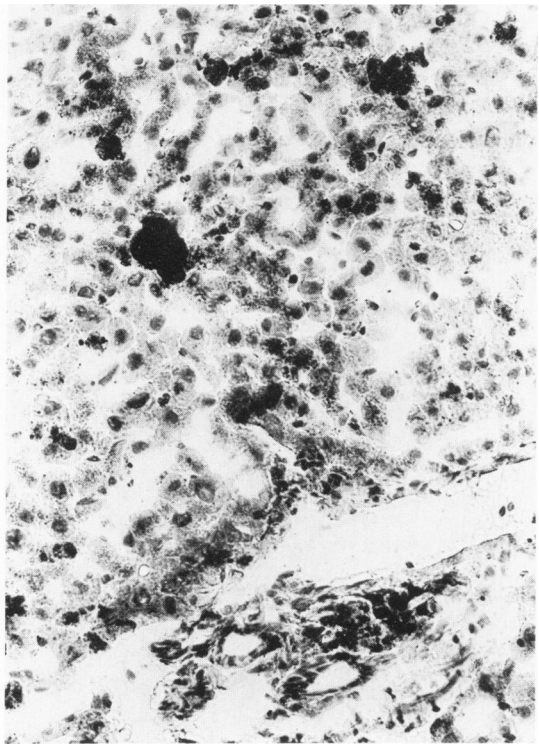
Figure 9—Altered foci induced by dietary 0.02% FAA for 13 weeks. Two iron-resistant foci are present in the siderotic liver produced by iron dextran injections. One of them (*arrow*) is basophilic, as indicated by the strong reaction with nuclear fast red.[15] Iron reaction; counterstained with nuclear fast red. Ethanol-fixed, paraffin section. (× 78) **Figure 10**—Same as Figure 9. One focus is positive for GGT, but hyperbasophilic focus in Figure 9 is negative. GGT reaction; counterstained with hematoxylin; serial section to that of Figure 9. (× 78) **Figure 11**—Altered foci induced by dietary 0.02% FAA for 13 weeks. Two iron-resistant foci are present (*arrows*) in the siderotic liver produced by iron dextran injection. Iron reaction, counterstained with nuclear fast red; serial section to that of Figure 11. (× 60) **Figure 12**—Same as Figure 11. One focus (*arrow*) is deficient in G-6-Pase, but the other is not. G-6-Pase reaction; frozen section. (× 60)



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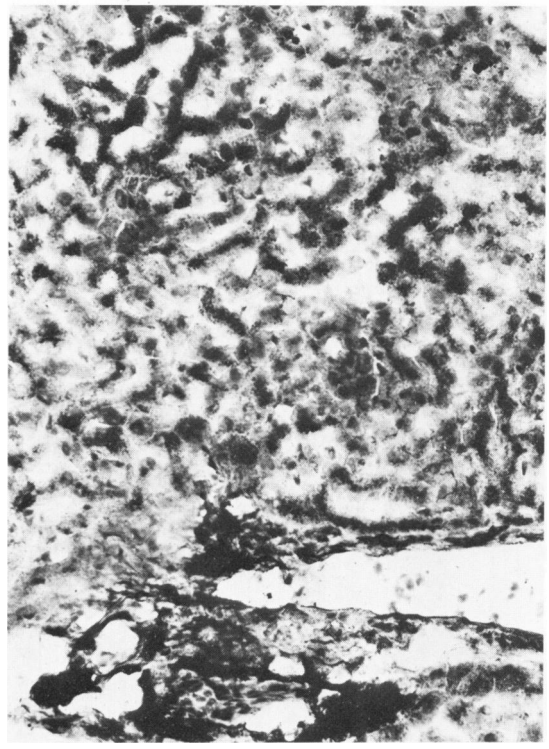

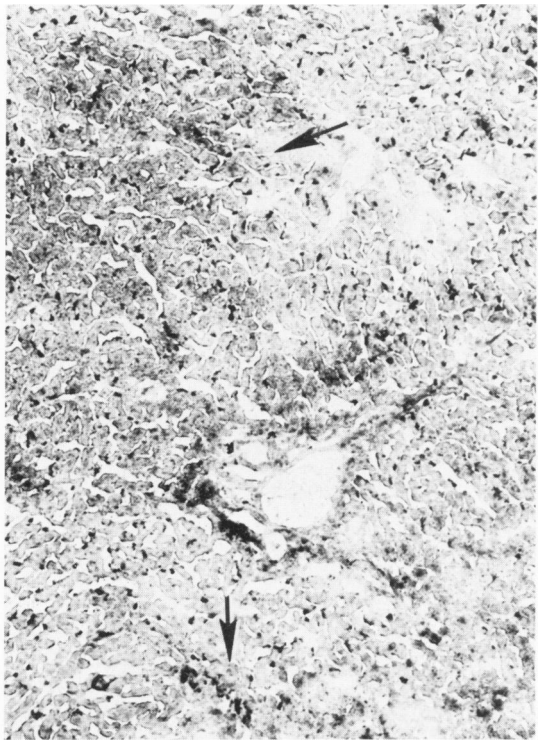

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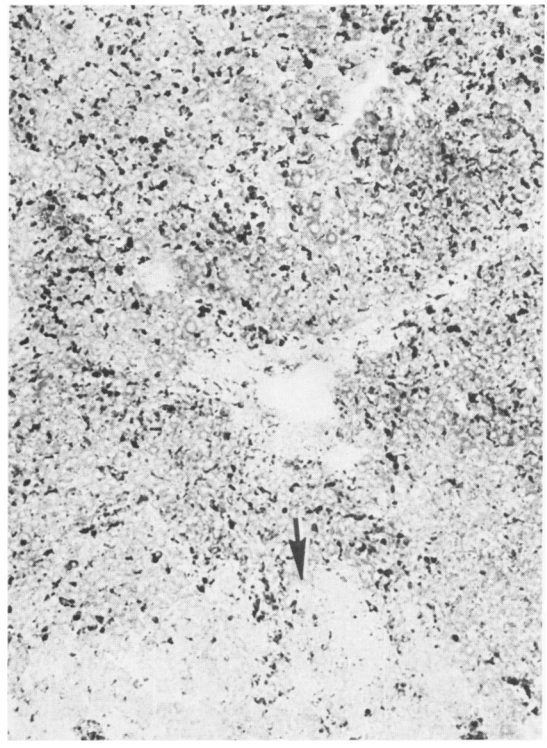

Figure 13—An altered focus induced by 0.02% FAA for 13 weeks. GGT-positive focus is present in the iron-dextran–induced siderotic liver. GGT reaction, counterstained with hematoxylin; frozen section. (× 180) **Figure 14**—Same as Figure 13. The focus showing positive reaction in Figure 13 is not deficient in ATPase. ATPase reaction, counterstained with hematoxylin; serial section to that of Figure 13. (× 180) **Figure 15**—Altered foci induced by dietary 0.02% FAA for 13 weeks. Two foci (*arrows*) are identified by GGT activity in this field of iron-dextran–induced siderotic liver. GGT reaction counterstained with hematoxylin; frozen section. (× 66) **Figure 16**—Same as Figure 15. Only one of the foci in Figure 15 (*arrow*) displays deficiency in G-6-Pase. G-6-Pase reaction; serial section to that of Figure 15. (× 66)