The Histochemical Localization of Alkaline Phosphatase during Carcinogenesis in Rats Fed 
\textit{p}-Dimethylaminoazobenzene*

BJARNE PEARSON, M.D., ALEX B. NOVIKOFF, PH.D.,
and THOMAS G. MORRIONE, M.D.

(From the Department of Pathology, University of Vermont
College of Medicine, Burlington, Vermont)

Greenstein in 1942 found a high content of alkaline phosphatase in transplanted rat hepatoma 31 (9). This tumor arose originally in the liver of a male Osborne-Mendel rat and was induced by the basal diet of White and Jackson (16) containing 0.06 per cent \textit{p}-dimethylaminoazobenzene (DAB) with intermittent addition of 0.5 per cent cystine and 0.5 per cent methionine (17). The enzyme activity was determined with disodium phenylphosphate as substrate. Woodard in 1943 studied the alkaline phosphatase activity of livers of rats fed a diet containing 20 cc. of 3 per cent DAB mixed with 1,000 gin. brown rice and supplemented with carrots (19). Sodium \(\beta\)-glycerophosphate was used as substrate (20). It was noted that the average alkaline phosphatase activity, in units per gram of tissue, was 10 times higher in hepatic tumors than in normal liver. An intermediary stage following dye feeding, designated as precancerous, also showed a significant rise in alkaline phosphatase activity above normal liver, but not as high as that of hepatic tumors.

The histochemical demonstration of alkaline phosphatase activity has been reported by White, Dalton, and Edwards (17) for rat hepatoma 31 transplants and by Kabat and Furth (5) for primary hepatic carcinoma in the rat. The purpose of the present study was to ascertain where the increased phosphatase activity was localized in the liver of rats fed DAB and to follow the localization at intervals during the carcinogenic process. A preliminary report was presented in 1948 (14).

The taxonomy of liver tumors induced by DAB has been studied by several investigators, utilizing different basal diets (10, 11, 13, 17, 18). These tumors are complex histologically and are apparently influenced by diet. Further studies, such as those of Opie (10, 11), to determine the effect of diet variations on the histological appearance of the liver and tumor might be of great value in resolving some of the differences reported by various investigators.

MATERIALS AND METHODS

Seventy-seven male Sherman rats were used in this study. They were placed on the following diet: casein ("Vitamin-free"), 120 gm.; glucose, 790 gm.; corn oil, 50 gm.; salt mixture (modified Phillips-Hart mixture), 40 gm.; and \textit{p}-dimethylaminoazobenzene, 0.6 gm. This was supplemented, per kilogram of ration, by: thiamin chloride, 3.0 mg.; riboflavin, 2.0 mg.; pyridoxine hydrochloride, 2.5 mg.; calcium pantothenate, 7.0 mg.; choline chloride, 30.0 mg.; and cod liver oil, one drop/rat/month.

This diet has been worked out by Miner et al. (9) and by Miller et al. (8). Miller et al. (8) reported a tumor incidence of between 90 and 100 per cent, after 4 months on DAB.

Groups of animals were sacrificed after 1, 2, 3, and 4 months on the diet. The remaining animals were taken off the dye at 4 months and continued on the same diet without the dye for 1 month, at which time they were examined at autopsy. Sections from the liver were fixed in ice-cold acetone and processed according to the method of Gomori (2). Successive slides were incubated in sodium \(\beta\)-glycerophosphate at 37°C for 5 minutes, 30 minutes, 12 hours, and 24 hours. Controls, in which the substrate was omitted, were run for each slide. No counter stain was used. Routine hematoxylin and eosin preparations were also made.

DESCRIPTION AND RESULTS

The 5- and 30-minute incubation periods serve to bring out the structures with intense phospha-
tase activity, while eliminating diffusion phenomena such as described by Martin and Jacoby (6). The slides incubated for 12 hours were chosen for the photographs, because they reveal all the cells on the slide. However, it is not possible to assert that structures staining only lightly after 12-hour incubations do so because of low but intrinsic enzyme activity, for their color may be due in part, or completely, to adsorption of materials from intensely colored areas. Apparent diffusion effects are considerably more pronounced after 24 hours of incubation.

The photographs show representative sections of: (a) livers of control animals (Figs. 1–3); (b) livers of animals on dye for 1, 2, 3, and 4 months (Figs. 4–8); (c) various portions of a complex liver tumor (Figs. 9–14); and (d) some special features of the tumors (Figs. 13 and 14).

Normal liver.—Essentially similar results were observed with fed animals and those on fast for 24 hours. Only the endothelium of the capillaries surrounding the bile ducts and an occasional mononuclear or polymorphonuclear leukocyte stained after 5–30 minutes of incubation. After 12 hours of incubation (Figs. 1–3), capillaries surrounding the bile ducts, the bile canaliculi, and the bile duct nuclei are intensely colored. In the parenchymal cells the nucleoli, chromatin, nuclear membrane, and cell boundaries stand out clearly. The cytoplasm exhibits a fine granular stippling. Often the cells nearer the portal areas are darker than those nearer the central vein (cf. Deane, [1]). To what extent diffusion from the portal area accounts for this picture is not clear. The same uncertainty holds for the more intense phosphatase activity of the hepatic cells, which is observed near the junction of bile canaliculi and bile ducts (Fig. 3).

Liver after 1–4 months of DAB feeding.—The most conspicuous alteration in livers of animals fed DAB is the marked increase in numbers of biliary and vascular epithelial cells.

In the sections incubated for 5 minutes only the infiltrating mononuclear and polymorphonuclear leukocytes stain darkly. The capillaries around the bile ducts show color, but the rest of the section is barely visible. In the 30-minute sections the vascular endothelium, as well as the leukocytes, is intensely stained. The nuclei of the biliary epithelium, and to a lesser extent those of the parenchyma, are slightly darkened.

Figures 4–8 are of slides incubated for 12 hours, showing typical areas of biliary, vascular, and fibroblastic proliferation seen in livers of animals on dye for 1 month (Figs. 5 and 6), 2 months (Fig. 4), 3 months (Fig. 7), and 4 months (Fig. 8). Within these proliferative areas are visible many small ducts of varying size, with lumina of a few micra in the smallest ones, to almost 20 μ in the larger ones. When the bile ducts reach the size where the lumina are 15–20 μ wide (ca. 2 months), as much as half their external circumference is surrounded by closely approximated, newly formed vascular channels. These give an intense alkaline phosphatase reaction. As the ducts enlarge, these juxtapositional vessels become more organized and contain blood (2–3 months). In some instances, these vessels push the bile ducts into irregular channels, contributing to cystadenoma formation (3–4 months).

Everywhere around the bile ducts is epithelium, presumably biliary, composed of small cells indistinguishable from those of the ducts. Proliferation of fibroblasts also occurs; their nuclei show a less intense color than do the biliary cells. After 3 or 4 months of dye feeding these fibroblasts begin to organize into areas of cholangiofibrosis (Fig. 8). Not until the connective tissue becomes fairly dense do the collagen fibers show any significant phosphatase coloration.

Very early, the hepatic parenchyma cells give evidence of degenerative changes as a consequence of DAB feeding. This is particularly true in the region surrounding and ahead of the advancing column of newly formed bile ducts, but it is also, although less frequently, found in the cells surrounding the central veins. The cell boundaries give evidence of disintegration, and there is granular debris in the cytoplasm. The normal architecture of the liver lobules is lost as biliary epithelium, bile ducts, and new vessels invade them. By the end of the second or third month this invasion has extended to all parts of the lobule. Quite frequently, this isolates areas of parenchymal cells into “islands” (Fig. 8) (cf. Orr [13]). The cells are often distinctly larger than those of normal liver. These parenchymal cells give little or no evidence of alkaline phosphatase activity in the cytoplasm; the nuclei, and especially the nucleoli, are quite dark. The contrast between the light parenchymal islands and the intensely dark proliferative tissue surrounding them is striking.

Occasionally, within the parenchymatous tissue, there are foci of cells which give a more intense phosphatase reaction in both cytoplasm and nuclei.

Cholangiofibrosis becomes quite frequent by the end of the fourth month on the dye. The bile ducts, with lumina reaching 20–40 μ in diameter, are surrounded by connective tissue. The cells of the ducts show much less color than they did earlier in carcinogenesis. The most intense phosphatase reaction is given by the crescentic vascular
Figs. 1-3.—Liver of normal fed rat

Fig. 1.—Low Power. Note intense color in capillaries surrounding bile ducts and in bile ducts. Mag. ×154.

Fig. 2.—High Power. Note intense reaction of vessels surrounding bile duct. Mag. ×490.

Fig. 3.—Reaction of parenchymal nuclei and cell boundaries. In lower quadrant is a cross section of a small bile duct, probably at the junction with bile canaliculi; adjacent portions of parenchymal cells show more color. Mag. ×780.

Fig. 4.—Liver of rat #80 after 2 months on DAB. Note intense phosphatase reaction in region of proliferating biliary epithelium. Mag. ×154.
Fig. 5.—Liver of rat #44 after 1 month on DAB. Note marked proliferation of biliary epithelium, surrounding a dilated branch of the portal vein. Intense stain in polymorphonuclear leukocytes and biliary nuclei. Distinct lumen formations are visible. Mag. ×154.

Fig. 6.—Region of same area under higher magnification. Note intense phosphatase reaction in newly formed vessel adjacent to bile duct. Bile duct nuclei dark; fibroblast nuclei (elongated) lighter. Mag. ×490.

Fig. 7.—Liver of rat #51 after 3 months on DAB. Note small duct formations in area of biliary epithelium. Most intense color shown by vascular endothelium and infiltrating leukocytes. Mag. ×280.

Fig. 8.—Liver of rat #64 after 4 months on DAB. Note parenchymal "island." Above it, there is intensely stained biliary epithelium; below it, a beginning area of cholangiofibrosis. Mag. ×154.
endothelium surrounding the ducts. Many of the duct lumina are closed, apparently as a consequence of pressure from the outside and from intra-luminal proliferations of the duct itself. These intra-luminal proliferations show a more intense color than the duct wall from which they arise.

When cholangiofibrosis is present cystadenomatous invariably occur. The pathogenesis of cystadenoma formation, as it appears in this material, is: (a) stasis by external compression by the fibrous and vascular stroma and (b), perhaps more important, excessive vascularization of the ducts with infoldings of epithelium.

**Tumors.**—Three of six animals killed at the end of 3 months of dye feeding showed the beginning of tumors composed of closely packed cells similar to the proliferating biliary cells of this and earlier stages.

Of the thirteen animals fed the dye for 4 months, nine showed the presence of well-defined large tumors. Without an exhaustive study of serial sections of these tumors, an adequate description of their cell types is not possible. Nevertheless, it is of interest to record the results seen on some six or more sections of each of the nine tumors. Four tumors were composed exclusively of cells like those of the biliary epithelium, four contained areas of parenchyma-like cells in addition to areas of biliary cells, and one showed only parenchyma-like cells.

The complex and varied nature of the mixed tumors is demonstrated by Figures 9–14, all different regions of the same tumor, from slides incubated for 12 hours. The cells in Figures 10 and 11 are of biliary origin. In Figure 9 the biliary cells may be seen merging imperceptibly with cells which appear to be parenchymatous (and which would generally be referred to as hepatoma, in its strict sense).

Biliary carcinoma, adenocarcinoma, and cyst-adenocarcinoma are illustrated. The cells, while varying in detailed anatomic architecture, are considerably smaller than hepatic parenchymal cells and have a much larger nucleus-to-cytoplasm ratio. The cell membranes do not show up as distinctly as do those of parenchymal cells with this technic. But the cuticular border, in cells lining adenomata and cysts, shows clearly. In certain areas, the solid, unorganized masses of biliary epithelium show an intense color (Fig. 11), in striking contrast to the organized cells lining cavities. What little color the organized cells show is present in nuclear membrane and chromatin. Scattered among the masses of biliary epithelium, typical bile duct formations may be found.

In areas where there is a dense connective tissue stroma, the fibroblast nuclei and collagen fibers are darkly colored (Fig. 10). This is in contrast to the lack of color in the areas of early connective tissue. In many regions, the stroma takes the form of stalk-like formations containing many vessels, over which the tumor cells are arranged, generally stratified in several layers (Fig. 10). Where there is only one layer of cells, the cells resemble those of normal adult bile ducts.

The parenchymatous region ("hepatoma") of the tumor is composed of larger cells, with more abundant cytoplasm and more clearly visible cell membranes. They frequently show the large nucleoli characteristic of the normal liver parenchymal cells. There is little fibrous tissue among these cells, but there is a marked growth of small blood vessels, which show intense phosphatase activity (Fig. 9).

Adjacent to this "hepatoma" area is a typical cystadenoma formation (Fig. 12). Here, too, the cuticular border shows a positive reaction while the cell membranes between adjacent cells do not. The cells are usually arranged in a single column of cuboidal layers where the lumen is large, and stratified into two or three layers where the lumen is small. The interior of the stalk-like formations gives an intense phosphatase reaction, with the newly formed vascular spaces, biliary epithelium, and connective tissue darkly colored.

Figures 13 and 14 illustrate some other features of the tumors. In the bile duct tumors one frequently sees masses of cells within the lumen which can be traced to hyperplasia of the neoplastic bile ducts. These cells show intense color. Some appear to have swollen and dropped off into the lumen. Similar hyperplasias into the lumen of adenomata may occur in the diffuse "hepatoma." In the midst of the apparent "hepatoma" may be darkly staining small neoplastic ducts. These are similar to the non-neoplastic bile ducts seen in the liver after 1–3 months of DAB feeding.

In the sections of tumors incubated for 5 or 30 minutes, intense phosphatase activity is shown by: (a) the mononuclear and polymorphonuclear leukocytes which may be present, (b) the vascular endothelium, (c) the areas of dense connective tissue, and (d) areas of necrosis. The areas of unorganized biliary epithelium which stain very intensely after 12 hours of incubation are also considerably darker than the organized epithelium in the 5–30-minute preparations.

**DISCUSSION**

These studies demonstrate intense alkaline phosphatase activity in the regions of rapidly proliferating biliary epithelium which constitutes the
most impressive feature of the rat liver following the feeding of DAB. The activity seems to be especially high in the vascular sprouts which proliferate with the biliary epithelium and in the infiltrating white cells.

It is the progressive increase of this proliferative tissue which undoubtedly accounts for the increasing alkaline phosphatase activities which Robertson and Kretchmer (15) found by direct chemical assay of samples of the same livers we have described here. Similarly, it is these tissues which probably account for the high enzyme activity reported by Woodard (19) for "precancerous" livers.

On the basis of our findings, the level of alkaline phosphatase activity of a tumor appearing as a result of DAB feeding would be expected to vary with the extent of vascular and connective tissue stroma, infiltrated leukocytes, unorganized biliary epithelium, and necrosis. The more parenchymal-like cells that are present in the tumor, the more the phosphatase level would be expected to be like normal. It is of interest to note that Greenstein (3, 4) found that in the tumors of mouse liver, in which the tumor cells resemble normal liver parenchymal cells, and where biliary epithelium proliferation is not very prominent, there is no increased level of alkaline phosphatase activity. In the rat, Greenstein (3), Woodard (19), and Robertson and Kretchmer (15) found increased activity. The latter authors (personal communication) found the extent of this increase to be very variable from sample to sample.

At the completion of our studies, we became aware of a significant publication by Mellors and Sugiura (7). These authors report changes in the basophilia of liver cells following DAB feeding such as previously described by Opie (12). They found that in most instances the degree of basophilia was correlated with the intensity of alkaline phosphatase activity.

In our material, areas of regenerating parenchyma cells such as described by Opie and by Mellors and Sugiura are quite uncommon. Where they are seen, intense basophilia is not often present, if one may judge by the degree of hematoxylin staining. In the few instances where such basophilia was observed, intense color was also seen in the corresponding cells on the alkaline phosphatase preparations. To what extent this may be due to the high enzyme activity of adjacent bile canaliculi is not clear. A closer study of the basophilia-alkaline phosphatase activity relationship in our material is in progress.

It is difficult to judge what role, if any, increased alkaline phosphatase plays in tumor formation following DAB feeding. It is highly concentrated in the areas of non-neoplastic proliferating cells early in carcinogenesis. In the final tumors, it is highly concentrated only in the ancillary stroma and in the unorganized biliary epithelium. "Hepatomas" and the organized epithelium of biliary adenomatous formations are low in activity.

CONCLUSIONS

1. The increased alkaline phosphatase activity which occurs during the process of carcinogenesis in the liver of rats fed DAB is localized in the areas of rapidly proliferating biliary epithelium: vascular sprouts and infiltrating leukocytes show most intense activity.

2. In the tumors produced after 4 months of DAB feeding, the enzyme activity is localized in the ancillary stroma, infiltrating leukocytes, necrotic tissue, and areas of unorganized biliary epithelium. Both the organized epithelium of biliary adenomatous formations and "hepatomas" are low in activity.

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REFERENCES


11. ——. The Pathogenesis of Tumors of the Liver Produced by Butter Yellow. Ibid., pp. 231–46, 1944.

12. Mobilization of Basophile Substance (Ribonucleic Acid) in the Cytoplasm of Liver Cells with the Production of Tumors by Butter Yellow. Ibid., 84:91–105, 1946.


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Bjarne Pearson, Alex B. Novikoff and Thomas G. Morrione


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