The Significance of Staining Reactions of Preneoplastic Rat Liver with Fluorescein-Globulin Complexes*

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Investigations to date have failed to elucidate the mechanism of action of any of the chemical carcinogens. There is, however, considerable evidence in favor of a hypothesis that deletion of a growth-controlling protein is an important factor in carcinogenesis.

As a result of their experiments on aminoazo dye binding by cellular protein(s), Miller and Miller (27) advanced a hypothesis of specific protein deletion as being of causal significance in the carcinogenic process initiated by aminoazo dyes.

Weiler (40, 41), using serological and fluorescence antibody technics, claimed that during 4-DAB carcinogenesis there is a progressive loss of an “organ specific antigen” from nests of liver parenchymal cells. He found that, by using particular fractions prepared from rat liver as an antigen for injection into rabbits, an antiserum could be produced which, after extensive adsorption with particles of fresh rat kidney, still reacted in relatively high titer with rat liver particulates in complement fixation test but gave no reaction in complement fixation reaction with rat kidney particulates. Such an antiserum, if coupled with fluorescein isocyanate, was found to stain the cytoplasm evenly but not stain the nuclei of normal rat liver parenchymal cells.

The cells of hepatomas and cholangiomas and the stromal elements of normal rat liver were not stained. During feeding of 4-DAB, Weiler found that groups of liver parenchymal cells, at first topographically related to blood vessels but later distributed in random fashion, failed to stain. He found these islands of nonstaining cells to persist, following sustained 4-DAB feeding, for periods of at least 270 days after cessation of staining. Weiler (42, 43) has also recorded similar findings coincident with the development of estrogen-induced renal tumors in the hamster.

Hughes, Louis, Dineen, and Spector (18), while able to demonstrate the staining phenomena described by Weiler (41), were unable to produce an “organ specific antiserum” by using his methods. Adsorption of antirat-liver rabbit globulin with rat kidney particulates gave a parallel reduction in the titers to rat liver and rat kidney particulate antigens in complement fixation reaction. Furthermore, identical differential fluorescence staining could be produced with any fluorescein-conjugated rabbit globulin fraction whether or not the rabbit had previously been immunized with rat tissues. The normal rabbit globulins used in these conjugations did not fix complement in the presence of rat liver or kidney antigens.

Livers of rats examined during the feeding of 4-DAB contained islands of cells showing loss of affinity for the fluorescein-conjugated globulin stain. These islands appeared to be of three types:

1. Islands composed of cells which morphologically appeared to be intact liver parenchymal cells (Figs. 1–4).

2. Islands of degenerative or necrotic cells usually related topographically to blood vessels.

3. Islands of inflammatory foci, bile duct-like epithelial cells, and other stromal elements (Figs. 5, 6).

In rats sacrificed some weeks after the course of dye feeding was terminated, the second and third types of nonstaining islands were no longer prominent. The first type of island persisted for periods of at least 2–3 months after cessation of dye feeding. This time interval would allow recovery of parenchymal cells damaged as a result of the hepatotoxic as opposed to the carcinogenic action of the dye. The persistence of these areas of nonstaining indicates that they might be of significance in the carcinogenic process.

It is the purpose of this paper to present evidence of the incidence of those persistent islands of parenchymal cells showing loss of fluorescence-staining capacity with the feeding of compounds of varying carcinogenicity and to indicate the possible correlation between this loss and the

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decrease of certain basic cytoplasmic proteins. In this protein complex are included most of the dye-binding protein(s) investigated by Miller and Miller (27), the deletion of which has been suggested to be of causal significance in carcinogenesis.

MATERIALS AND METHODS

Preparation and sources of compounds.—Since the extensive studies at Wisconsin (11, 26, 29—31, 33) have shown that the total known range of carcinogenic activity is exhibited by those derivatives of 4-DAB substituted in the nondiamine ring, only some of these derivatives, 4-monomethylaminobenzene (4-MAB) and 4-aminomethylbenzene (4-AAB), will be considered here.

Apart from 4-aminomethylbenzene (4-AAB), 4-DAB, and some 3'-methyl-4-dimethylaminobenzene (3'-Me-4-DAB), m.p. 117° C., all azo dyes used were synthesized in the laboratory. 4-Mono-methylaminobenzene (4-MAB) was synthesized by the method of Miller and Baumann (29) and, after recrystallization from benzene-petroleum ether, had a m.p. of 87° C.

3'-Bromo-4-dimethylaminobenzene (3'-Br-4-DAB), m.p. 93° C., was prepared as described by Kühn and Quadebeck (19). The other dyes, all monosubstituted derivatives of 4-DAB, were made according to the general methods of Miller and Miller (30). The resulting crude dyes were filtered and recrystallized from ethanol, in the case of the methyl and trifluoromethyl substituted derivatives, and from benzene-petroleum ether mixtures for the halogenated dyes. After recrystallization the following melting points were recorded:

- 2'-methyl-4-dimethylaminobenzene (2'-Me-4-DAB) m.p. 65° C.
- 3'-methyl-4-dimethylaminobenzene (3'-Me-4-DAB) m.p. 98° C., was prepared as described by Kuhn and Quadbeck (19).
- 4'-methyl-4-dimethylaminobenzene (4'-Me-4-DAB) m.p. 117° C.
- 2'-fluoro-4-dimethylaminobenzene (2'-F-4-DAB) m.p. 106° C.
- 3'-fluoro-4-dimethylaminobenzene (3'-F-4-DAB) m.p. 108° C.
- 4'-fluoro-4-dimethylaminobenzene (4'-F-4-DAB) m.p. 151° C.
- 2'-chloro-4-dimethylaminobenzene (2'-Cl-4-DAB) m.p. 106° C.
- 3'-chloro-4-dimethylaminobenzene (3'-Cl-4-DAB) m.p. 104° C.
- 4'-chloro-4-dimethylaminobenzene (4'-Cl-4-DAB) m.p. 158° C.
- 3'-trifluoromethyl-4-dimethylaminobenzene (3'-CF3-4-DAB) m.p. 77° C.
- 4'-trifluoromethyl-4-dimethylaminobenzene (4'-CF3-4-DAB) m.p. 77° C.
- S'-Bromo-4-dimethylaminobenzene (S'-Br-4-DAB) m.p. 77° C.
- S'-chloro-4-dimethylaminobenzene (S'-Cl-4-DAB) m.p. 104° C.
- S'-trifluoromethyl-4-dimethylaminobenzene (S'-CF3-4-DAB) m.p. 77° C.

The melting point of 2'-Me-4-DAB is considerably lower than that recorded by Miller, Sapp, and Miller (28) but agreed with that found by von MecheI and Stauffer (22).

Of the amine salts used in the above syntheses o-toluidine, p-toluidine, o-chloroaniline, m-chloroaniline, p-chloroaniline, N-methylamine, N,N-dimethylamine, m-toluidine, 4-fluoroaniline, p-fluoroaniline, m-bromoaniline, and m-trifluoromethylamine were obtained or obtained commercially.

1 Obtained from Townsend and Mercer (Vic.), Pty. Ltd., Melbourne, Australia.
2 Presented by Imperial Chemical Industries of Australia and New Zealand Limited, Melbourne, Australia.
4 Presented by Eli Lily International Corporation, Indianapolis, U.S.A.

Fluorescein isocyanate was prepared by the method of Coons and Kaplan (5). The isomer designated fluorescein amine I by Coons and Kaplan was used. Globulin fractions were prepared, by precipitation with half-saturated ammonium sulfate from the serums of nonimmunized rabbits. After dialysis, to remove excess ammonium sulfate, the globulin fractions were conjugated with fluorescein isocyanate (5) with the use of 0.06 mg. of isocyanate, weighed as amine, per mg. of protein. The fluorescent serums were dialyzed against phosphate-buffered saline for 5 days with daily changes of dialysate. Just prior to use, the fluorescein-globulin serum was extracted twice with ethyl acetate (8) to remove free fluorescein derivatives.

Care of animals.—Adult rats, descended from Holtzman-Sprague-Dawley stock, weighing 150—200 gm., were fed ad libitum a basal semisynthetic diet (17) containing 2 mg riboflavin/kg. The above-mentioned aminoazo dyes were fed to individual batches of rats by their inclusion in the basal diet, at a level of 0.06 per cent. Other groups of rats were placed on the basal diet plus 0.06 per cent 3'-Me-4-DAB and either 0.0083 per cent 20-methylcholanthrene, 0.03 per cent 2-acetylaminofluorene, or 0.0008 per cent selenium dioxide.

Thioacetamide was administered to another group of rats fed the basal diet by weekly intraperitoneal injections of a 2 per cent aqueous solution. The dosage employed was 100 mg thioacetamide/kg body weight/week.

Carbon tetrachloride was given intragastrically to another group of rats (of Wistar strain) once a week for 8 weeks. The dosage used was 0.3 ml. of a 50 per cent solution of carbon tetrachloride in liquid paraffin/100 gm body weight.

Preparation and examination of liver sections.—At selected times the rats were anesthetized with ether and exsanguinated by the severing of their carotid and jugular vessels. Thin slices of their livers, taken principally from the median lobe, were dropped into a tube of isopentane, precooled by immersion in a dry ice-ethanol mixture. The pieces of tissue were fixed, without thawing, by being frozen onto a precooled microtome chuck. Sections were cut at 5-μ thickness with a rotary microtome (M.S.E.) installed in a convenient deep freeze unit. By use of the modifications introduced by Louis (21), satisfactory sections were obtained without the use of a guide attachment to the microtome knife. After treatment with absolute ethanol at —10° C. for 10 minutes, the sections were dried in a cold room at 1—2° C. with the help of a fan. The next day the sections were stained for 20 minutes at room temperature with the fluorescein-globulin and then washed with three changes in buffered saline, pH 7.3, for 15 minutes.
For examination of the liver sections, a Leitz fluorescence microscope was used. An infrared filter and two 4-mm. blue filters (B.G. 12) were interposed between the carbon arc and condenser. UV protection and Wratten G15 gelatin filters were placed in the ocular. For photography, Pan F (Ilford) film was used with an exposure time of 30 seconds.

Because the livers of rats, if examined during or soon after the administration of hepatotoxic substances, frequently showed islands of nonstaining cells owing either to inflammatory or degenerate foci, the following procedure was adopted: Rats of each group, after requisite times on the various regimens, were placed on the basal diet for a further period of 3 weeks before their livers were examined.

Considerable variation, both from rat to rat even when on the same diet and between sections cut from the same block of tissue, was observed in the frequency of occurrence of islands of morphologically normal cells with decreased affinity for the fluorescein-globulin complex stain. Because of this, at least twelve sections were examined from each block of tissue. When islands of nonstaining were found, adjacent serial sections, stained with hematoxylin and eosin, were examined to observe the morphology of the cells concerned.

Examinations to determine the active component of the stain.—Controlled studies (18) have shown that the differential staining observed by Weiler (41) is independent of any demonstrable antibody-antigen reaction. An attempt has been made to elucidate the nature of this staining. Frozen sections of normal and preneoplastic liver and hepatoma were stained with:

a) Portions of fluorescein rabbit globulin conjugate which had been adjusted to pH 3.5, 4.5, 5.8, 6.8, 7.3, 8.6, and 10.2, respectively, by addition of 0.1 N HCl or 0.1 N NaOH.

b) Portions of fluorescein rabbit globulin conjugate buffered at pH 7.3 with phosphate which had been adjusted to ionic strength 0.15, 0.30, 0.50, and 1.00, respectively, by the addition of sodium chloride.

c) Portions of fluorescein rabbit globulin conjugate buffered at pH 7.3, ionic strength 0.15, to which were added glycine to give concentrations of 35, 70, and 105 gm/l.

d) Fluorescein amines I and II; each isomer was dissolved in a small quantity of acetone and then water added to give dilutions of 1/100, 1/1000, 1/100,000, 1/1,000,000, and 1/10,000,000. Solutions were tested freshly prepared and after aging.

e) Fluorescent rat serums; normal rat serum and rat serum obtained from rats 48 hours after partial hepatectomy by the method of Brues, Drury, and Brues (2) were conjugated with fluorescein isocyanate without any preliminary fractionation of the serums.

f) Fluorescein isocyanate; fluorescein isocyanate, prepared from amine I, was dissolved in a small quantity of acetone and then water added to give dilutions of 1/1000, 1/10,000, 1/100,000, 1/1,000,000 and 1/10,000,000. Solutions were tested freshly prepared and after aging.

RESULTS

It has been found that there is considerable variation from rat to rat in the same group as to whether or not persistent islands of morphologically normal, nonstaining, parenchymal cells can be found. This variability also extends to individual sections cut from the same block of tissue. It is often necessary to examine several sections before finding such an area. The size of these islands may vary from two or three cells up to an area occupying most of a liver lobule.

Most of the rats fed 3'-Me-4-DAB, and to a lesser extent those fed 3'-F-4-DAB, showed a zone of nonstaining cells at the periphery of each lobule after 2-4 weeks of dye administration. However, because these cells resembled stromal cells more closely than parenchymal cells, sections showing this feature were not regarded on this appearance alone as containing islands of nonstaining parenchymal cells. Incidentally, Price, Harman, Miller, and Miller (34) have described excessive bile duct proliferation during the early phases of feeding 3'-Me-4-DAB and the subsequent transformation of these cells into parenchymal cells.

The results obtained from the examination of the livers of rats administered the various compounds are summarized in Table 1. An animal has been regarded as showing "islands of nonstaining parenchymal cells" if any of the sections examined...
show such an island. It has been noted that, even after prolonged feeding, all the sections prepared from the liver of an individual rat fed one of the most potent of the carcinogenic dyes may not show such areas; such, however, is unusual. On the other hand, these islands were not found in the livers of rats fed the noncarcinogenic dyes and only rarely and after prolonged feeding with the less carcinogenic compounds. By adopting the relative carcinogenic ratings of Miller and Miller (31), it can be seen that there is a good over-all correlation between the likelihood of subsequent tumor development and the appearance of these areas with dye feeding.

This correlation also extends to the relative carcinogenic effects of combinations of carcinogens. Thus, the synergistic combination 3′-Me-4-DAB and 2-AAF (24) gives a high incidence of rats showing such areas relatively early in feeding, whereas the inhibitory effect of 20-methylcholanthrene on 3′-Me-4-DAB carcinogenesis (28, 35) is reflected by the failure to observe nonstaining parenchymal cells. Carbon tetrachloride, reported as noncarcinogenic for rat liver (14), and thioacetamide, a weak carcinogen but a potent hepatotoxic substance (10, 12, 13), likewise produced no such areas, although with both gross increases in nonstaining areas of stromal cells were observed.

The inhibitory effect of selenium on azo dye carcinogenesis (3) is reflected in a possibly significant delay in the occurrence of nonstaining parenchymal cells.

Investigations into staining action.—

a) Variation of pH of the stain resulted at both extremes of pH in rapid deterioration of histological detail. The washing procedure restored the pH to alkalinity before microscopy, thus giving conditions for maximum fluorescence. Good staining was obtained in the pH range 5.8–8.6; much less staining was observed at pH 3.5 and 10.2.

b) Good staining was obtained at all ionic strength variations of the stain tested.

c) Addition of glycine, to render the stain 1 M or 1.5 M in respect to glycine, decreased the intensity of staining. However, considerable residual staining remained, and this was of a differential nature similar to that produced by the original stain.

d) Solutions of fluorescein amine did not stain the sections in any of the dilutions tested.

e) In concentrations greater than 1 in 1,000,000, fluorescein isocyanate stained all tissues vividly. At a dilution of 1 in 1,000,000, a fair degree of differential staining was achieved if the staining time was reduced to 1 minute. Aqueous solutions of fluorescein isocyanate, 1 in 100,000, lost their

### Table 1

<table>
<thead>
<tr>
<th>Dye feeding time in weeks</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
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<td>0</td>
<td>0/4</td>
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<td>6</td>
<td>0/3</td>
<td>2/4</td>
<td>2/4</td>
<td>2/4</td>
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<tr>
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<td>0/15</td>
<td>5/14</td>
<td>3/6</td>
<td>4/5</td>
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<tr>
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<td>10-12</td>
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<td>3/6</td>
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<td>2/4</td>
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<td>1/4</td>
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* Relative to 4-DAB = 6 (reference compound). Data obtained from Ref. nos. (9), (10), (14), (19), (24), (28), (31), and (35).

† Number of livers showing islands of loss of staining/total number of livers examined.
aging of fluorescein isocyanate solutions have which was insufficient to stain, and studies of the residual concentration of fluorescein isocyanate shown that such solutions lose their staining ca-
tained at the edge of a hepatoma. However, the stained rat liver sections, and, in high dilutions, some degree of differential staining could be ob-
the other hand, free fluorescein isocyanate readily
cein isocyanate itself give similar differential stain-
itions of the free amine do not stain rat liver. On
be implicated as the significant agent, since solu-
olution) failed to stain any part of the tissue.
In varying the washing medium, it was found
that there was less residual staining if the medium contained 1.0 m glycine. Once again, however, the staining observed was of a differential nature.
Good fluorescence staining was found with sec-
tions of rat heart, kidney, and spleen and with mouse and human liver.

DISCUSSION
A correlation was found between the time of carcinogen administration necessary for the appearance of persistent islands of morphologically normal liver parenchymal cells with a diminished affinity for fluorescein rabbit globulin conjugates and the carcinogenicity of the compounds given. At the same time, with any particular carcinogen, individual rats show a wide variation in their capacity to develop these islands.
The persistence of these islands long after admin-
istration of the carcinogen has ceased, their possession of fluorescence staining characteristics similar to frankly hepatomatous cells, and the correlation between their rates of development with the carcinogenicity of the various dyes fed suggest strongly that they are premalignant foci.
In attempting to elucidate the mechanism of this staining, various points have to be considered. The interpretation of Weiler (41) that it depends upon an organ-specific antigen-antibody reaction is untenable, because fluorescein-normal rabbit globulin conjugates and, to a lesser extent, fluorescein isocyanate itself give similar differential staining.
Free fluorescein amine in the stain could not be implicated as the significant agent, since solutions of the free amine do not stain rat liver. On the other hand, free fluorescein isocyanate readily stained rat liver sections, and, in high dilutions, some degree of differential staining could be obtained at the edge of a hepatoma. However, the method of preparation of the stain resulted in a residual concentration of fluorescein isocyanate which was insufficient to stain, and studies of the aging of fluorescein isocyanate solutions have shown that such solutions lose their staining ca-
pacity much more rapidly than do fluorescein globulin complexes. Thus, the unconjugated isocyanates do not play any significant part on the staining phenomena.
The conditions of conjugation of aromatic iso-
cyanates to protein have been thoroughly investi-
gated (6, 7, 16). Conjugation takes place at a slightly alkaline pH through carbamido linkages. Since conjugation of free fluorescein isocyanate occurs only to an appreciable extent in alkaline conditions, the observation that good staining occurred in the acid pH range excludes conjugation occurring on the slide as the means of staining and supports the above conclusions.
Another possibility is that the rat and rabbit have a common protein component in serum; this may, in some way, react with receptors in the normal cell and so control growth, but cannot react with a malignant cell which is deficient in hypothetical receptors. If such were the case this growth-controlling circulating protein should be greatly reduced after partial hepatectomy. It should be manifest by decreased fluorescence after the use of a stain prepared from such a serum. This is not supported by the experiments using fluorescent serum prepared from rats after partial hepatectomy.
On the other hand, there is considerable evi-
dence to suggest the importance of protein-protein interaction in the staining reaction. Cohn (4) indicated that the formation of salt-like complexes between proteins of opposite net charge is usual in systems containing mixtures of proteins. The effect of such protein-protein interactions in reduc-
the solubilities of both components in a binary mixture over a broad range of pH between the isoelectric points of the two proteins has long been recognized. Dissociation of such complexes may be achieved by alteration of pH so that both components have similar net charges, by increasing the dielectric constant of the medium. A convenient way of increasing the dielectric constant is by the addi-
tion of dipolar ions such as glycine.
Electrophoretic studies (9, 15, 20, 38) have revealed that, concomitant with the onset of neo-
plastic change, there is a significant alteration in the spectrum of cytoplasmic proteins in which the basic proteins are greatly reduced. The major portion of the azo dye-binding protein(s) investigated by Miller and Miller (27) is part of this basic protein complex (38). It is thus one of the most basic proteins of rat liver. Such basic protein(s) could therefore be expected to enter into protein-
protein complex formation with plasma proteins, over a wide pH range, more readily than the less
basic tumor proteins. Extreme ranges of pH decreased but did not inhibit staining, and the rendering of the washing fluid 1 m. with respect to glycine decreased staining.

Electrophoretic studies have shown that, in a variety of tumors investigated (27), there is a gross alteration in the soluble cytoplasmic protein spectrum when compared with that of non-neoplastic tissue. This change is in the direction of a decrease in the more basic components. Therefore, it is to be expected that tumor cytoplasmic proteins in general would have a decreased capacity to form complexes with fluorescein-globulin conjugates over a wide pH range. In several human tumors examined, Louis (23) has found such to be the case. This loss of affinity for fluorescein-globulin conjugates is not merely a function of active cellular proliferation, since actively regenerating liver stains well (22).

Since sections of other rat organs and liver of other species stain well with fluorescein-globulin conjugates and yet have no capacity to bind aminoazo dyes (27), the staining cannot be attributed to "stain-protein"-protein interactions involving the dye binding protein(s) alone. Rather, it is suggested that the differential staining of non-neoplastic and neoplastic tissue is a reflection of an altered protein spectrum of the cell. In particular, this may be related to a decrease in the basic proteins of the cell among which is found most of the carcinogen-binding protein(s).

SUMMARY

1. During aminoazo dye carcinogenesis, islands of morphologically normal parenchymal cells could be found in the livers of most rats which had lost their affinity for fluorescein rabbit globulin complexes. This staining characteristic persisted long after the carcinogen was discontinued.

2. In any group of rats on a particular aminoazo carcinogen, there is a wide variation in the rate at which these areas appear. However, in an investigation with a variety of dyes, a correlation was found between the rate of appearance of these islands and the carcinogenicity of the particular dyes fed.

3. This staining reaction depended not upon an antibody-antigen reaction but probably upon protein-protein interactions. If this is so, the more basic of the soluble cytoplasmic proteins, which include most of the carcinogen-binding protein(s) investigated by Miller and Miller (27), could be implicated. The observations made of this phenomenon thus lend indirect support to the protein deletion hypothesis.

4. Since differential staining of neoplastic and non-neoplastic tissues could be observed with fluorescein-conjugated normal rabbit globulin, any interpretation of the results obtained by fluorescent antibody technics will require great caution and very careful control staining before changes can be attributed to an antibody-antigen reaction.

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43. ———. Antigenic Differences between Normal Hamster Kidney and Stillboestrol-Induced Kidney Carcinoma: Histological Demonstration by Means of Fluorescing Antibodies. Ibid., pp. 560-68.

**Fig. 1.**—Fluorescence photomicrograph of a section of liver of a rat fed 4-DAB for 12 weeks followed by normal diet for 3 weeks. There is a small island of loss of fluorescence staining in the liver parenchyma. X240.

**Fig. 2.**—The same section as shown in Figure 1 stained with hematoxylin and eosin. This shows that the fluorescing and nonfluorescing cells are indistinguishable morphologically in ordinary circumstances. X240.

**Fig. 3.**—Fluorescence photomicrograph of a section of liver similar to that shown in Figures 1 and 2, in which a large island of nonfluorescing tissue is demonstrated. X160.

**Fig. 4.**—The same section as in Figure 3, stained with hematoxylin and eosin to show uniform morphological appearances of tissue. X160.

**Fig. 5.**—Fluorescence photomicrograph of a section of rat liver after a feeding period of 12 weeks with 4-DAB. An area of nonfluorescence is present. X400.

**Fig. 6.**—The same section as in Figure 5 stained with hematoxylin and eosin showing that the nonfluorescing area consists of cells of "biliary" type. X400.
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