

Liver and Lung Tumors in Mice Exposed at Birth to 4-Dimethylaminoazobenzene or Its 2-Methyl or 3'-Methyl Derivatives^{1,2}

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SUMMARY—Male and female Swiss mice received, by subcutaneous injection on each of the first 5 days of life, 200 μ g 4-dimethylaminoazobenzene (DAB), 2-methyl-4-dimethylaminoazobenzene (2-methyl-DAB), or 3'-methyl-4-dimethylaminoazobenzene (3'-methyl-DAB). A control group was similarly treated with the vehicle, 0.02 ml arachis oil. Between 50 and 56 mice in each group survived until the experiment was terminated 1 year later. The incidence of liver cell adenomas (benign hepatomas) was significantly higher in male mice given any 1 of the 3 test compounds (63–92%) than in the control males given arachis oil only (10%), and the incidence in males given DAB (92%) was significantly higher than in males given 2-methyl-DAB (67%) or 3'-methyl-DAB (63%). None of the treatments significantly increased the incidence of liver tumors in female mice. The incidence of lung tumors in control males was 10% and in control females 4%. DAB itself had little or no effect on the incidence of lung tumors (σ^7 = 8%, ϕ = 16%), but 3'-methyl-DAB significantly increased lung tumor incidence in both sexes (σ^7 = 34%, ϕ 50%) and 2-methyl-DAB did so in females (σ^7 = 11%, ϕ = 42%). The interpretation of these findings is discussed in the light of the results of experiments based on the exposure of partially hepatectomized adult rats to the same compounds, the results of studies on germfree mice, and of postulated metabolic pathways.—*J Nat Cancer Inst* 47: 593–601, 1971.

MANY CHEMICALS, if injected subcutaneously during early neonatal life, increase the risk of liver tumor development in mice, particularly in males. Such chemicals include certain carcinogenic polycyclic hydrocarbons (1, 2), 6-aminochrysene (3), 4-aminobiphenyl and 3 of its hydroxylated derivatives (4), *o*-aminoazotoluene (5), griseofulvin (6), maleic hydrazide (7), *N*-2-fluorenylacetamide (8, 9), dimethylnitrosamine (10, 11), and β -propiolactone (12). Where comparable data are avail-

able, newborn mice seem to be more sensitive than adult mice (12–15).

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Some of the same substances can induce liver tumors when given to rats, but information is sparse on the relative sensitivity of neonates and adults of this species. Weisburger and colleagues (16) showed that infant rats are more susceptible than weanling rats to the hepatocarcinogenic activity of intragastrically administered *N*-2-fluorenylacetamide.

Most agents that increase the risk of development of liver tumors in newborn mice also increase the risk of development of lung tumors in the same animals. In general, it is more difficult to increase lung tumor incidence in rats than in mice, and there is no evidence that any chemical listed above can do so, irrespective of the age of the animals at first exposure.

Partial hepatectomy enhances liver carcinogenesis in both mice and rats (17-19). This was first evidenced in studies on rats (17). The hepatocarcinogenicity of 2-methyl-4-dimethylaminoazobenzene (2-methyl-DAB) was first revealed in a study of the response of partially hepatectomized rats given this substance in their diet (17).

The present paper is concerned with the response of mice to 4-dimethylaminoazobenzene (DAB) and its 2-methyl and 3'-methyl derivatives (2-methyl-DAB and 3'-methyl-DAB), given during early neonatal life. Compared with adult rats, adult mice are relatively insensitive to the hepatocarcinogenic activity of *N*-methylated aminoazo dyes given orally (20). Della Porta and Terracini (15) reported a raised incidence of liver cell tumors in mice given DAB by intraperitoneal injection during early neonatal life, but there is no information on the response of newborn mice to 2-methyl-DAB or 3'-methyl-DAB.

MATERIALS AND METHODS

Chemical agents.—DAB (I) was a commercial product obtained from British Drug Houses, Poole, Dorset, England. It was percolated through a column of alumina and recrystallized from ethanol. 2-Methyl-DAB (II) and 3'-methyl-DAB (III) were prepared and purified by standard methods (21). The chemical structures of the 3 compounds are shown in text-figure 1.

Mice.—Litters from Swiss females, derived from a pathogen-free unit, were grouped at random for

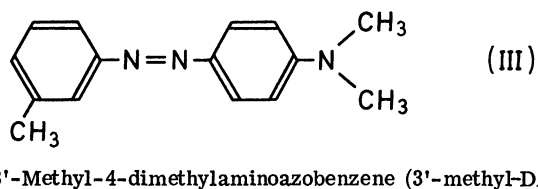
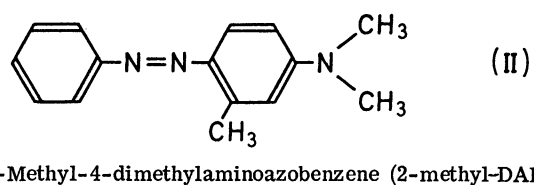
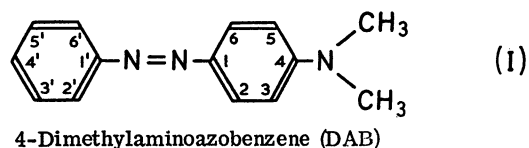
treatment: Group A received 200 μ g DAB in 0.02 ml arachis oil by subcutaneous injection on each of the first 5 days of life (total dose, 1 mg); group B was treated similarly with 2-methyl-DAB in arachis oil; group C was treated similarly with 3'-methyl-DAB in arachis oil; and group D (control) was given similar treatment but with arachis oil only.

For injections, a fine-gauge needle was inserted near the root of the tail under the skin to deliver the injected material in the interscapular region.

After being weaned at 3 weeks of age, males and females were caged separately. They were fed a standard laboratory cubed diet of formulation 41B, obtained from Messrs. Dixon, Ware, Hertfordshire, England, and were given water *ad libitum*. Mice were kept in zinc boxes on wood shavings. The number of mice alive at weaning is shown in table 1.

Mice were examined daily for their general state of health and more closely weekly for tumors and other lesions.

The experiment was ended when the mice were between 52 and 54 weeks old. A postmortem ex-



TEXT-FIGURE 1.—Chemical structures of DAB, 2-methyl-DAB, and 3'-methyl-DAB.

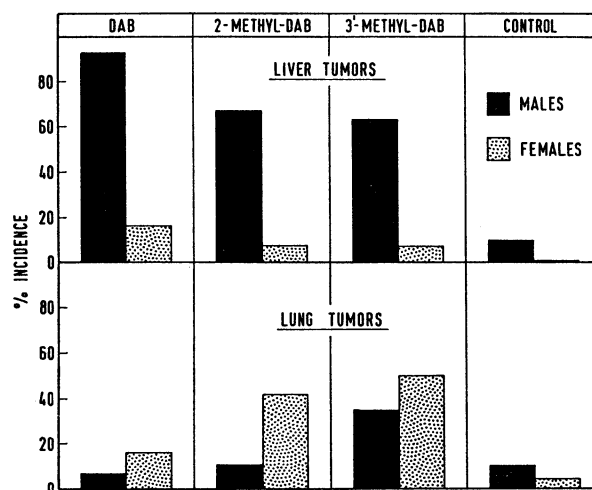
TABLE 1.—Treatment of mice and survival in different groups

Group	Treatment (200 μ g in arachis oil on each of first 5 days of life)	Number of litters	Number of infant mice treated	Number of mice alive at time of weaning	Number of mice alive at 1 year
A	DAB	7	64	29 ♂ 27 ♀	28 ♂ 25 ♀
B	2-methyl-DAB	6	62	28 ♂ 27 ♀	27 ♂ 24 ♀
C	3'-methyl-DAB	6	61	41 ♂ 17 ♀	38 ♂ 14 ♀
D	Arachis oil only	6	61	31 ♂ 25 ♀	31 ♂ 25 ♀

amination, which included distension of the urinary bladder with fixative but not examination of the brain or spinal cord, was performed. Recorded were the number of lesions thought to be neoplasms or possible neoplasms and the sizes of the largest of such lesions in each organ affected. All tissues with such lesions were examined histologically. Fixed bladders were bisected sagittally and examined with a lens for tumors. Although no tumors were seen, 5 bladders were selected at random from each treatment group for histological study. Tissues were fixed in Bouin's solution; 5- μ paraffin sections were prepared and stained with hematoxylin and eosin.

RESULTS

There were 1 male and 2 females in group A, 1 male and 3 females in group B, and 3 males and 3 females in group C that died or were killed because they became sick, between weaning and the end of the experiment; no premature deaths occurred among the control mice in group D. In group C, 1 male died from generalized malignant lymphoma after 40 weeks. No neoplasms were seen in the other mice that died or were killed before 1 year (mainly quite early in the experiment); but in 6 animals postmortem examination was not possible because of advanced decomposition (2 females in group A, 1 male in group B, and 2 males and 1 female in group C). Since it is unlikely that lung or liver tumors caused any deaths of mice between weaning and 1 year of age, no bias was introduced if the mice that died prematurely were ignored in the assessment of incidence rates of lung or liver tumor at the termination of the experiment.



TEXT-FIGURE 2.—Percentages of mice killed between 52 and 54 weeks of age that bore liver or lung tumors.

The incidence of liver and lung tumors in mice killed at the end of the experiment is shown in tables 2 and 3 and summarized in text-figure 2. Statistical analysis of the results indicated significant heterogeneity between groups, both in liver tumor incidence in males ($P < 0.001$)⁵ and lung tumors in mice of both sexes (males, $P < 0.01$; females, $P < 0.01$). Thus treatment with DAB or its 2 methylated derivatives significantly increased the incidence of both liver and lung tumors in mice killed at 1 year.

The next analyses were undertaken to determine whether there was any significant heterogeneity

⁵ It could be supposed that the significance levels are exaggerated, because whole litters of mice of higher than average sensitivity to liver or lung tumor induction were allocated to particular groups. However, comparison of the tumor incidence rates within and between litters revealed no systematic variation.

TABLE 2.—Liver cell neoplasms in mice killed at end of experiment

Group	Treatment	Sex	Number killed at end of experiment	Number (percent) with liver tumors	Number (percent) with multiple liver tumors	Average No. of liver tumors per survivor
A	DAB	♂	28	26 (92.3)	24 (85.7)	2.0
			25	4 (16.0)	3 (12.0)	0.3
B	2-methyl-DAB	♂	27	18 (66.7)	12 (44.4)	2.0
			24	2 (8.3)	1 (4.2)	0.1
C	3'-methyl-DAB	♂	38	24 (63.2)	18 (47.4)	1.6
			14	1 (7.1)	0 (0)	0.1
D	Arachis oil only	♂	31	3 (9.7)	2 (6.5)	0.3
			25	0 (0)	0 (0)	0

in the responses of mice to the 3 treatment agents. Significant heterogeneity was found among males in groups A, B, and C in respect to liver tumors ($P < 0.01$) and among mice of both sexes (combined) in the 3 groups in respect to lung tumors ($P < 0.01$). In both cases, DAB was found responsible for the heterogeneity, and it produced significantly more liver tumors and significantly less lung tumors than its 2 methylated derivatives.

All liver tumors were of parenchymal cell origin. Most appeared to be well-differentiated lesions clearly demarcated from surrounding liver tissue. Several showed marked fatty change (fig. 1). A few neoplasms were less well differentiated (fig. 2) but, although some appeared to be locally invasive, they did not metastasize to distant sites. Liver cell tumors in the test group and those in the control group did not differ morphologically.

All the lung tumors, except one, were benign or locally invasive adenomatous tumors of alveolar cell or bronchiolar cell origin (fig. 3). Morphologically, lung tumors in test and control mice were essentially similar. In 1 mouse in group C, there was evidence of metastasis of a lung tumor

within the lobe in which it originated, but no extrapulmonary metastases were seen.

None of the mice killed at the end of the experiment had neoplasms other than of the lung or liver. In group A, 1 female had a simple cyst of the liver and another female had a similar lesion in the pancreas. In group A, 1 male had chronic pyelonephritis and another male had chronic nephritic changes involving both tubules and glomeruli. In group C, 1 mouse had early nephritic lesions of a similar nature. In group D, 1 control male had pyelitis and cystitis. No neoplastic or other significant change was seen on microscopic examination of urinary bladders from 5 randomly selected males and females from each group (see "Materials and Methods").

DISCUSSION

The results clearly show that all 3 test substances increased the incidence of liver cell tumors in male mice and that DAB was significantly more effective in this respect than either 2-methyl-DAB or 3'-methyl-DAB. The data are insufficient to be sure

TABLE 3.—Lung tumors in mice killed at end of experiment

Group	Treatment	Sex	Number killed at end of experiment	Number (percent) with lung tumors	Number (percent) with multiple lung tumors	Average No. of lung tumors per survivor
A	DAB	♂	28	2 (7.7)	1 (3.7)	0.1
			25	4 (16.0)	1 (4.0)	0.2
B	2-methyl-DAB	♂	27	3 (11.5)	3 (11.5)	0.4
			24	10 (41.7)	2 (8.3)	0.7
C	3'-methyl-DAB	♂	38	13 (34.2)	5 (13.2)	0.7
			14	7 (50.0)	2 (14.3)	1.5
D	Arachis oil only	♂	31	3 (9.7)	0 (0)	0.1
			25	1 (4.0)	0 (0)	0.04

whether any agent increased the incidence of liver cell tumors in female mice.

In contrast, the incidence of lung tumors was significantly raised by exposure of mice at birth to 2-methyl-DAB (females only) or 3'-methyl-DAB (both males and females), whereas treatment with DAB was without effect.

In the interpretation of any study of the effects of chemical agents on animals, both the characteristics of the animal system and the administered substances must be considered.

Liver Tumors in Mice

We may first consider the nature of mice in relation to liver tumor incidence and induction, a subject we recently reviewed (22). Mice of different strains vary widely in their liability to the spontaneous development of liver cell tumors. Close to 100% of both male and female mice of certain sublines of the C3H strain, if allowed to live out their lifespan, spontaneously develop liver cell tumors. In other strains, spontaneous liver tumors are rare. In high liver-tumor strains, males tend to be far more susceptible than females. High susceptibility is manifest by the development of tumors early in life and by the development of multiple tumors. Ovariectomy and the administration of testosterone to female mice increase their susceptibility to liver tumor development. A high calorie diet favors liver tumor development, and germfree status inhibits it. This is the natural background against which the effects of the various chemical agents that increase the risk of liver tumor development in mice must be considered. At present, there is no means of knowing whether the chemicals increase liver tumor development by *inducing* new tumors or by promoting tumor development in a biological system in which *induction has already occurred or is constantly occurring* as a result of the presence of undetected carcinogens in the general environment.

Additional problems arise when chemical substances are injected subcutaneously into newborn mice. 1) The immaturity of the neonate with regard to immune mechanisms and enzymic capacity may result in a different response to administered substances from that of older animals. 2) Chemicals given subcutaneously tend to pass through the

lungs before reaching the liver, whereas chemicals given orally usually pass through the liver first.

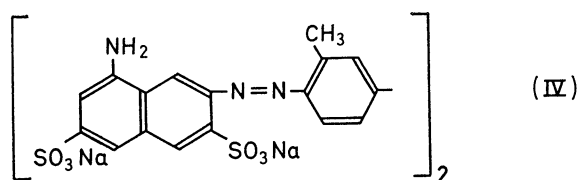
3) Substances given orally are liable to attack by enzymes produced by the microbiological flora of the gastrointestinal tract, whereas this can only happen secondarily after the subcutaneous administration of chemical agents if they reach the gut lumen via the bile or because of direct secretion through the gut wall. In relation to the first of these problems, there is evidence that the livers of newborn animals are deficient in certain important drug-metabolizing enzymes (23-26). On the other hand, sulfate-conjugating mechanisms in the liver, which may be involved in the metabolism of DAB and its derivatives, are functional at birth or shortly afterward (26).

Lung Tumors in Mice

Strains vary widely in susceptibility to the spontaneous development of lung tumors, but in both high lung-tumor strains and low lung-tumor strains there is little difference in incidence between males and females. Germfree status inhibits the development of lung tumors in mice given 7,12-dimethylbenz[*a*]anthracene at birth, and a high casein diet favors lung tumor development under similar circumstances (22). This is the background against which the effects of a wide variety of substances which increase lung tumor incidence in mice have to be considered. As with liver tumors, the question: "Do such substances exert their effects by inducing lung tumors or by promoting the development of tumors induced by other agents associated with the genome or present in the environment?" remains unanswered. The additional problems associated with the use of neonates are similar to those discussed above in relation to liver tumors.

Response to DAB, 2-Methyl-DAB, and 3'-Methyl-DAB

Irrespective of whether the liver and lung tumors in the treated groups of the present experiment were due to induction or promotion by the test substances, it is interesting to speculate as to why liver tumor incidence was lower and lung tumor incidence higher in response to the 2-methyl and 3'-methyl



TEXT-FIGURE 3.—Chemical structure of trypan blue.

derivatives of DAB than to DAB itself. The presence or absence of ring methyl groups determines other important biological activities of azo compounds. Thus trypan blue (IV) (text-fig. 3) is an effective trypanoside, whereas the demethylated compound is not (27). Ring methyl groups in phenylazo compounds can modify metabolism, either because they alter the rate of reduction of the azo linkage (28) or because they undergo oxidation to alcohols or acids (29). Easier reducibility of the azo linkage may be associated with lower carcinogenic activity and oxidation of azo compounds to more polar compounds might influence distribution in the body.

In the present experiments, the test substances were injected subcutaneously, so that they or their metabolites passed through the lungs before they reached the liver. A greater ability to react with lung tissue could explain both the higher lung-tumor incidence and the lower liver-tumor incidence in the groups treated with 2-methyl-DAB or 3'-methyl-DAB as compared with the group treated with DAB.

Special Sensitivity of the Neonate

It is attractive to suggest that the susceptibility of the newborn mouse to the induction of liver tumors, especially by 2-methyl-DAB, relates to the high rate of liver cell proliferation shortly after birth and to a special vulnerability of dividing cells to chemicals such as 2-methyl-DAB. However, if this is so, it is not clear why only males are susceptible (30), unless the sex hormones are involved in the metabolism of the chemicals or in a later stage of tumor development. Also of possible relevance is that, in the high spontaneous liver-tumor strain, C3H/Mza, partial hepatectomy decreases rather than increases the risk of liver tumor development (31). On the other hand, undoubtedly in mice, as in rats, partial hepatectomy enhances liver tumor

development in response to chemicals, provided the chemicals are given during the rapid liver cell proliferation that follows the operation (18, 19, 32, 33).

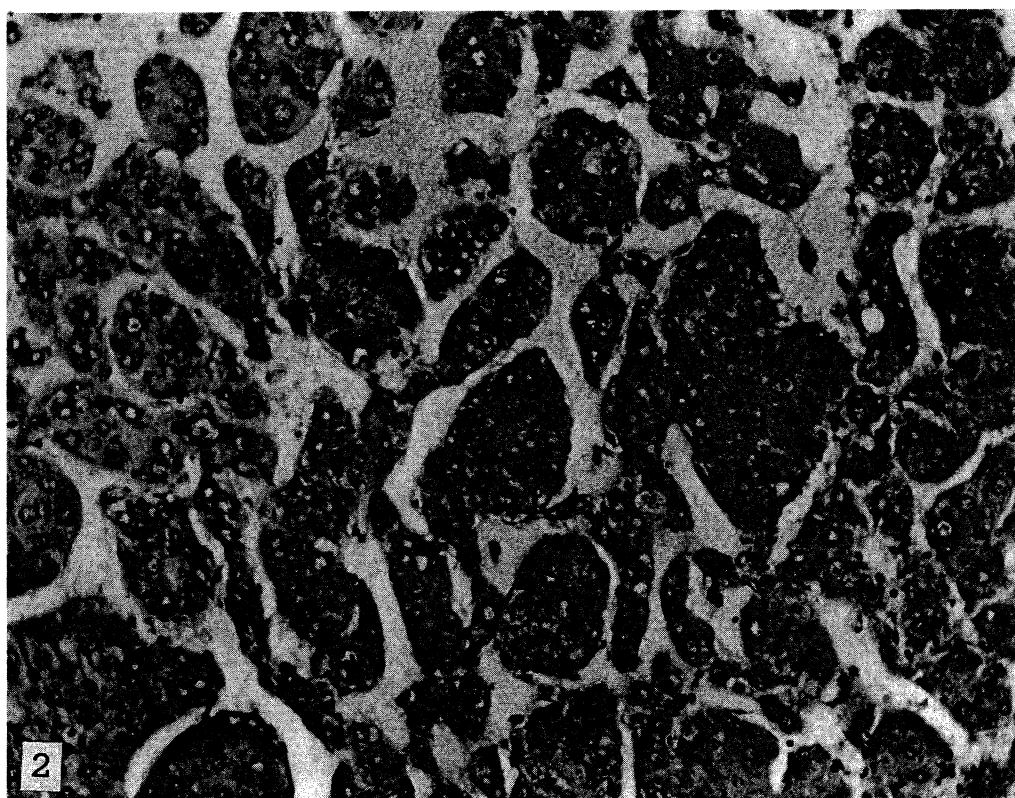
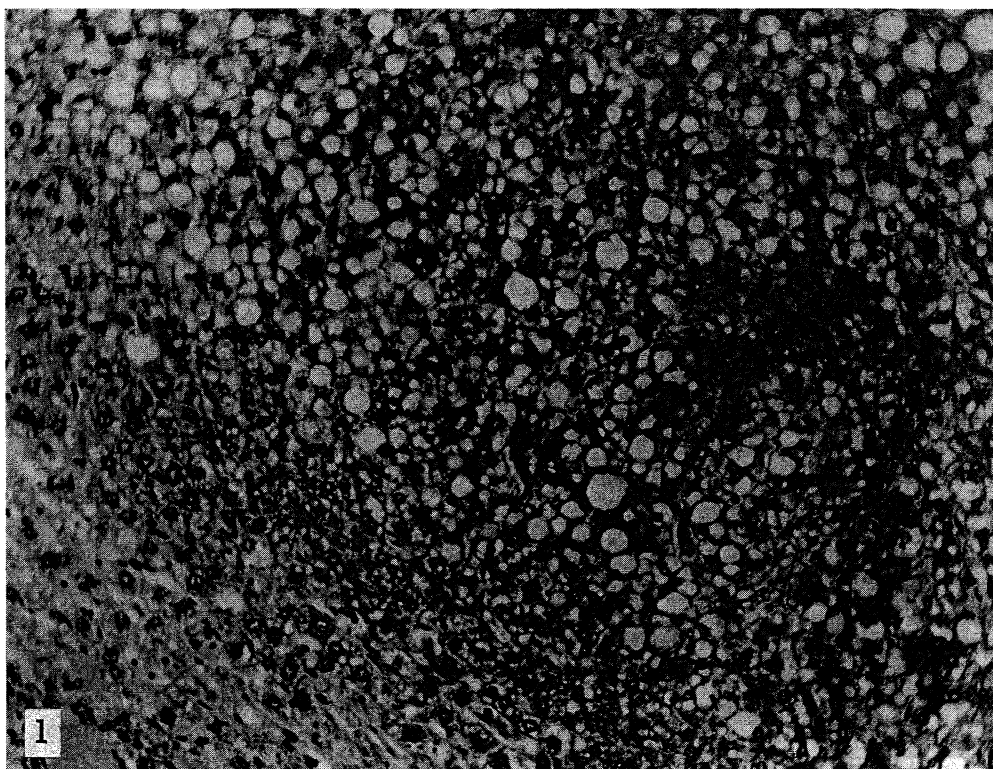
Conclusion

Better information is urgently needed on the etiology of liver and lung tumors in mice. It is important to know whether substances, such as DAB, 2-methyl-DAB and 3'-methyl-DAB, increase the incidence of these tumors by a carcinogenic or by a cocarcinogenic process. Only against the background of such knowledge will it be possible to assess the role of cell proliferation and other factors on the incidence of these tumors and to judge whether agents that increase the incidence of these tumors are likely to exert similar effects on tumor incidence in other species, including man. In this connection, more comparative studies in rats and mice are needed. It would be interesting to know how many agents that increase liver tumor incidence on administration to newborn mice do so in intact or partially hepatectomized adult rats or newborn rats. Discordant results in such comparative studies would tend to discredit liver tumor induction in mice as an indicator of carcinogenicity.

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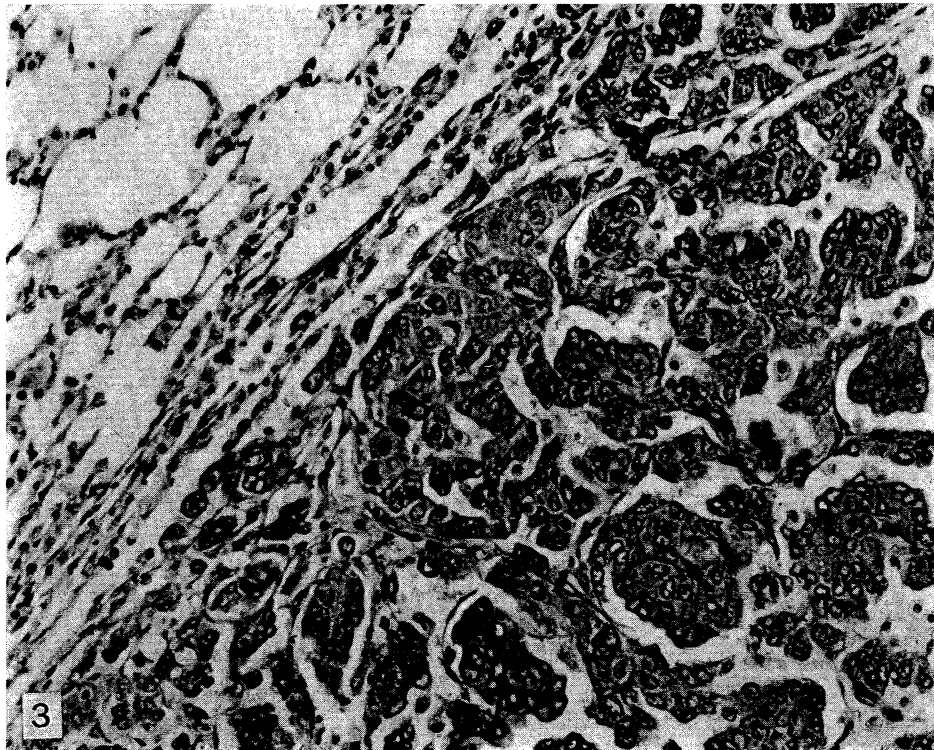


FIGURE 1.—Well-differentiated hepatoma, showing considerable fatty change, from mouse aged 54 weeks, exposed to 2-methyl-DAB at birth. Normal liver tissue is at *lower left corner*. Hematoxylin and eosin. $\times 200$

FIGURE 2.—Moderately well-differentiated hepatoma from mouse aged 54 weeks, exposed to 3'-methyl-DAB at birth. Hematoxylin and eosin. $\times 200$

FIGURE 3.—Pulmonary adenoma from mouse aged 53 weeks, exposed to 3'-methyl-DAB at birth. Hematoxylin and eosin. $\times 200$

