Studies in Carcinogenesis with Azo Compounds

II. The Action of Azo Compounds in Mice, and the Bearing Thereof on Theories of Azo Dye Carcinogenesis*

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INTRODUCTION.

INVESTIGATIONS WITH AZO COMPOUNDS:

A. LIVER LESIONS (SEE TABLE I)
1. p-Aminoazobenzene (AAB)
2. 2'-Amino-4,5'-azotoluene (pAAT)
3. 2,3'-Azotoluene (AT)
4. Azonaphthalenes (AN)
5. 4'-Hydroxy-2,3'-azotoluene (oHAT)
6. N,N-Dimethyl-p-aminoazobenzene (DAB)
7. 4'-Amino-2,3'-azotoluene (oAAT)

B. TUMORS AT THE SITE OF INJECTION (SEE TABLE II)
C. LUNG TUMORS (SEE TABLE III)
D. ENDOTHELIOMAS (SEE TABLE IV)
E. LIVER LESIONS OTHER THAN LIVER CELL TUMORS

STRAIN SUSCEPTIBILITY.

THEORIES OF THE MECHANISM OF TUMOR INDUCTION BY AZO COMPOUNDS

INTRODUCTION

The results of earlier investigations of the carcinogenic action of azo dyes in rats were summarized by Kinosita in 1937 and 1940 (22, 23), and by Hartwell in 1941 (17). Since then much work has been done involving various types of controlled, restricted diets and a theory has been evolved (19) to account for the activity of certain dyes and the inactivity of others. A short review of this work appeared in a recent paper by Miller and Baumann (29), who criticized the theory on the basis of their own experimental findings. A considerable amount of work on mice has been carried out with azo compounds since the earlier reviews appeared, but no attempt has been made to examine it critically and to determine its bearing on the "split product" theory of carcinogenesis.

In this paper, in a series of tables, there has been collated the information available in the literature regarding the production of neoplastic growths in mice by such azo compounds as have been shown to have any effect by any route. It will be seen that a number of pure strains have been used by various workers, and that these provide a fair degree of checking upon each other. 4'-Amino-2,3'-azotoluene has usually been employed, but investigations have also been made with N,N-dimethyl-p-aminoazobenzene by four groups of workers other than the present author, with 2,3'-azotoluene by two groups of workers, and 4'-hydroxy-2,3'-azotoluene and 1,1'- and 2,2'-azonaphthalenes in one case each, only. These azo compounds have been fed, painted on the skin, or injected subcutaneously; for the latter route either the solid or an oily solution has been used, leading to some significant differences in response. While liver tumors have been the predominating interest, growths at the site of injection, pulmonary neoplasms (other than metastases), and endotheliomas of various types and at various sites have been observed by several workers. No tumors at the site of painting or in the alimentary tract, with two exceptions (10, 25), have been reported. The data are discussed in the text for each azo compound and also from the points of view of the lesions induced and the response by any particular strain of mouse that has been used.

Certain clear-cut facts emerge from the data now available and their bearing on the "split product" theory and on the older "rearrangement" theory of Cook and his group (10), recently extended by Elson and his co-workers (12, 13), is discussed in a later section.

INVESTIGATIONS WITH AZO COMPOUNDS

A. LIVER LESIONS (SEE TABLE I)

1. p-Aminoazobenzene (abbreviation: AAB).—Yoshida reported (49) having injected mice subcutaneously with a 10 per cent solution of AAB in olive oil, but none of them survived more than 23 days and
hence no data were provided on the carcinogenicity of this dye for mice. The experiments reported in the preceding paper (25) included 2 mice surviving about 400 days and 1 that survived 626 days under experimentation. No tumors were seen in any of these mice, but it has to be borne in mind that 500 days were required to induce liver tumors in rats fed this dye at a high level (24) and the possibility therefore remains that tumors might arise in mice fed AAB or receiving it by subcutaneous injection for a very long time. Against this is the fact that, although necrosis and other signs of damage were frequently found in the livers of these mice, neither regenerative hyperplasia nor cirrhosis was observed; these latter lesions, or at least regenerative hyperplasia, seem to be an essential premalignant reaction to other azo compounds.

The only other organ suffering consistently was the kidney, in which some degree of toxic nephritis was usually found. Renal changes were reported by Maruya in rats fed this azo dye, or 2,4'-diaminoazobenzene, or even the simple parent compound, azobenzene (28).

2. 2′-Amino-4,5′-azotoluene (abbreviation: pAAT).—This azo dye was reported by Sasaki and Yoshida (41) to have no carcinogenic action when fed to rats up to 476 days. It has also been described as 5′-amino-2,2′-azotoluene (41) and 4′amino-2,2′-azotoluene (11), but Hartwell (17) accepts the formulation of Miura (25). The latter found no tumors in stock mice injected subcutaneously with an olive-oil solution of this dye up to 472 days; necrosis of the liver was frequent, but cirrhosis and the premalignant regenerative hyperplasia were not seen. Moreover, pAAT did not apparently damage the kidneys.

Tests by other routes do not appear to have been carried out, and it is just possible that this azo dye might prove to be a weak carcinogen if presented suitably and long enough.

3. 2,3′-Azotoluene (abbreviation: AA).—This azo compound and the following two are of special interest, as they contain no amino group and therefore cannot be broken down to yield a diamine of the type required for the “split product” theory, although they all could undergo a benzidine type of rearrangement. AT has been reported to cause bladder papilloma when fed to rats in a rice diet (39), a finding that has been confirmed by Cook (9), who also investigated the effect on mice receiving the compound orally or by subcutaneous injection; the mice developed no significant lesion.

Two other investigations with AT have been carried out in mice. Seligman and Shear (42) injected the pure compound, which is an oil, subcutaneously into female mice of strain A, but even after 18 months no tumors had developed; it will be seen from Table I that strain A mice of either sex are easily susceptible to tumor induction, both in lung and in liver, by the p-amino derivative of AT, namely, 4′-amino-2,3′-azotoluene. Law (26) gave 3 subcutaneous injections of AT, 5 mgm. total, in olive oil during 2 months; he also reports introducing a 5 mgm. pellet at 4 months of age, but as AT is a liquid it is not clear whether or not he gave a fourth dose in the case of this compound. His dba mice developed neither sarcoma nor liver tumor; but among 20 C57 black mice 2 developed

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**KEY TO TABLES I TO IV**

- pAAB  —  p-aminoazobenzene.
- pAAT  —  2′-amino-4,5′-azotoluene.
- oAAT  —  4′amino-2,3′-azotoluene.
- BY  —  N,N-dimethyl-p-aminoazobenzene.
- AT  —  2,3′-azotoluene.
- 1,1′-AN  —  1,1′-azonaphthalene.
- 2,2′-AN  —  2,2′-azonaphthalene.
- pHAT  —  p-hydroxyazotoluene.
- 1. Hepatomas unless otherwise indicated.
- 2. Adenoma; annular cirrhosis.
- 3. After 340 days.
- 4. Unspecified.
- 5. Focal necrosis; fatty degeneration.
- 6. After 11 months.
- 7. "Characteristic changes . . . which precede malignancy."
- 8. Law gives no figures for survival of mice not bearing tumors in any of his groups.
- 9. After 11 months: 3 mice had received liver supplements and 2 of these had normal livers, but all had sarcomas.
- 10. Mice bearing tumors are in all cases expressed as a fraction of the total mice surviving 250 days or longer.
- 11. Hemangioendothelioma, unless otherwise stated.
- 12. Hartwell (17) refers to lung tumors, but quotes no figures.
- 14. After 42 weeks.
- 15. After 50 weeks.
- 16. After 44 weeks.
- 17. After 32 weeks.
- 18. After 38 weeks.
- 19. At 52 weeks.
- 20. At 42 weeks.
- 21. At 41 weeks: 1 at 50 weeks.
- 22. At 41st week.
- 23. 1 at 42 weeks: 1 at 52 weeks.
- 24. 1 at 51 weeks: 1 at 52 weeks.
- 25. At 344 and 379 days respectively.
- 26. At 252 days.
- 27. At 321 days.
- 28. Adenoma: 2 other female mice had secondary liver cell carcinoma.
- 29. 1 reticuloendotheloma; 2 hemangioendotheloma.
- 30. 1 hemangioendotheloma behind left kidney; 1 lymphoma, invading pancreas, in retroperitoneal tissues.
- 32. Nearly all cholangiomas.
- 33. 4 females had cirrhotic livers.
### Table: Effect of Azo Compounds on the Liver in Mice

<table>
<thead>
<tr>
<th>Author and Reference</th>
<th>Azo compound</th>
<th>Solvent</th>
<th>Dose</th>
<th>Route</th>
<th>Experimental period</th>
<th>Incidence of liver lesions&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Mixed</th>
<th>M</th>
<th>A</th>
<th>P-B</th>
<th>C</th>
<th>IH</th>
<th>Y</th>
<th>dba</th>
<th>Cba</th>
<th>CST black</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yoshida (49)</td>
<td>oAAT</td>
<td>Olive oil 10%</td>
<td></td>
<td>SubQ</td>
<td></td>
<td>Liver&lt;sup&gt;4&lt;/sup&gt; changes</td>
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<tr>
<td>Kohiyama (37)</td>
<td>oAAT</td>
<td>0.3 mgm. per gm. diet</td>
<td>Per os</td>
<td>400 days</td>
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<td>Minuta and Matsuya (33)</td>
<td>BY</td>
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<tr>
<td>Boyland and Bruns (8)</td>
<td>oAAT</td>
<td>Benzene 0.3%</td>
<td>About 4.5 mgm. per gm. diet</td>
<td>Skin painting</td>
<td>104 days</td>
<td>Liver&lt;sup&gt;4&lt;/sup&gt; changes</td>
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<tr>
<td>Sherr (43)</td>
<td>oAAT</td>
<td>Solid used</td>
<td>7x10 mgm.</td>
<td>SubQ</td>
<td>14 mo.</td>
<td></td>
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<td>13/16^2</td>
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<tr>
<td>Mensenkskaya (36)</td>
<td>oAAT</td>
<td>Olive oil</td>
<td>0.5 mgm. per gm. diet</td>
<td>Per os</td>
<td>587 days</td>
<td>Liver&lt;sup&gt;4&lt;/sup&gt; changes</td>
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<tr>
<td>Maruya (35)</td>
<td>oAAT</td>
<td>Benzene 0.6%</td>
<td>Sunflower seed oil</td>
<td>Painting</td>
<td>549 days</td>
<td>Liver&lt;sup&gt;4&lt;/sup&gt; changes</td>
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<tr>
<td>Baumann, Jacobi, and Rashi (7)</td>
<td>oAAT</td>
<td>0.5 mgm. per gm. diet</td>
<td>Per os</td>
<td>400 days</td>
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<td>16/10^2</td>
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<tr>
<td>Andervont (1)</td>
<td>oAAT</td>
<td>Solid used</td>
<td>11x10 mgm.</td>
<td>SubQ</td>
<td>1 yr.</td>
<td></td>
<td></td>
<td>7/8</td>
<td></td>
<td>14/16</td>
<td>6/9</td>
<td>10/10</td>
<td>9/9</td>
<td>2/7</td>
<td>3/6</td>
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<tr>
<td>Cook, Hewett, Kennaway, and Kennaway (10)</td>
<td>BY 1, 2—AN</td>
<td>Olive oil or buter benzene 0.6%</td>
<td>Skin painting</td>
<td>549 days</td>
<td>2/20^2</td>
<td>Liver&lt;sup&gt;4&lt;/sup&gt; changes</td>
<td></td>
<td>0/4</td>
<td></td>
<td>7/23</td>
<td>7/19</td>
<td>7/25</td>
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<tr>
<td>Law (26)</td>
<td>oAAT</td>
<td>Olive oil solid subl</td>
<td>5 mgm. per wk.</td>
<td>Per os</td>
<td>587 days</td>
<td>Liver&lt;sup&gt;4&lt;/sup&gt; changes</td>
<td></td>
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<tr>
<td>Sigman and Sten (42)</td>
<td>AT</td>
<td>Liquid used</td>
<td>0.05 ml.</td>
<td>SubQ</td>
<td>18 mo.</td>
<td></td>
<td></td>
<td>0/20</td>
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<tr>
<td>Law and Stewart (3, 17)</td>
<td>BY</td>
<td>Solid used</td>
<td>7x10 mgm.</td>
<td>SubQ</td>
<td>15 mo.</td>
<td></td>
<td></td>
<td>0/10</td>
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<tr>
<td>Turn and Muliken (47)</td>
<td>oAAT</td>
<td>Corn oil</td>
<td>at least 130 mgm.</td>
<td>SubQ</td>
<td>over 1 yr.</td>
<td></td>
<td></td>
<td>0/10</td>
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<tr>
<td>Andervont, Grady, and Edwards (5)</td>
<td>oAAT</td>
<td>Solid used</td>
<td>100 mgm.</td>
<td>SubQ</td>
<td>1 yr.</td>
<td></td>
<td></td>
<td>7/7</td>
<td></td>
<td>0/20</td>
<td>15/20</td>
<td>0/9</td>
<td>1/8</td>
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<tr>
<td>Andervont and Edwards (5)</td>
<td>oAAT</td>
<td>Olive oil 2%</td>
<td>45 mgm.</td>
<td>SubQ</td>
<td>1 yr.</td>
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<td>none</td>
<td>none</td>
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<tr>
<td>Andervont and Edwards (4)</td>
<td>oAAT</td>
<td>Crystals used</td>
<td>10 mgm.</td>
<td>SubQ</td>
<td>32 wk.</td>
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<td>0/20</td>
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<td>18/21</td>
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<tr>
<td>Andervont, White, and Edwards (6)</td>
<td>oAAT</td>
<td>Max. 220 mgm.</td>
<td>About 300 mgm.</td>
<td>Per os</td>
<td>223 days</td>
<td></td>
<td></td>
<td>8/20</td>
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<td>24/31</td>
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<tr>
<td>Kirby (25)</td>
<td>oAAB</td>
<td>Olive oil 1 or 2%</td>
<td>Amoeba oil 2%</td>
<td>SubQ</td>
<td>472 days</td>
<td></td>
<td></td>
<td>9/8</td>
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<tr>
<td>Kirby (25)</td>
<td>oAAT</td>
<td>0%</td>
<td>Up to 130 mgm.</td>
<td>SubQ</td>
<td>515 days</td>
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<tr>
<td>Kirby (25)</td>
<td>BY</td>
<td>Amoeba oil 3%</td>
<td>Up to 241.5 mgm.</td>
<td>SubQ</td>
<td>615 days</td>
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<sup>4</sup> Changes in liver histology, including bile ductule proliferation, focal necrosis, and cirrhosis.
hepatoma at 585 and 639 days respectively, and 5 developed sarcomas at the site of injection at a mean age of 361 days. Spontaneous liver tumors in C57 black mice were reported by Little, Murray, and Cloudman (27) after 540 days, and were seen by Kirby (25) in a male dying at 436 days and a female dying at 523 days. Hence the liver tumors seen by Law in mice of this strain receiving AT may have been spontaneous. But this would hardly apply to the sarcomas, which are rare at any age. Andervont and Edwards (3) concluded that sarcoma arose more easily when 4'-amino-2,3'-azotoluene was injected in oily solution than as a solid. Law's positive results in C57 black mice and Seligman and Shear's negative results in strain A mice may therefore be due to difference in strain, or to the use of an oily vehicle by Law, or to both differences. It seems clear that AT is not carcino- genetic for the liver of the mouse.

4. (a) 2,2'-Azonaphthalene (abbreviation: 2,2'-AN).

The only investigations with azonaphthalenes in mice were carried out by Cook, Hewett, Kennaway, and Kennaway (10), who found definite hepatic lesions following the administration of 2,2'-AN either by feeding, painting or subcutaneous injection. These lesions were almost always cholangiomatous, with only an occasional hepatoma; this is in sharp contrast with the picture usually seen in the livers of mice receiving N,N-dimethyl-p-aminobenzene or 4'-amino-2,3'-azotoluene, which rarely induce cholangioma (25), but is in harmony with the histology of liver lesions induced by the "benzidine-type rearrangement" product of 2,2'-AN, namely, 2,2'-diamino-1,1'-dinaphthyl (10), and by the deamination product of the latter, namely, 3,4,5,6-dibenzcarbazole (2, 8, 45). The possible significance of the type of liver lesion associated with these compounds is discussed in a later section of this paper.

Tumors of the lung were also seen in mice receiving 2,2'-AN, including some very large adenomas; whether or not this was after administration by all three routes is not mentioned.

(b) 1,1'-Azonaphthalene (abbreviation: 1,1'-AN).

This isomer had a slight action on the liver when fed or injected; the few survivors after painting showed no liver lesions. The type of liver tumor was cholangioma in 5 out of the 6 mice affected; the sixth mouse showed a doubtful hepatoma. A squamous cell carcinoma was found in the stomach of 1 mouse that had been fed 1,1'-AN. Another, injected subcutaneously, had a malignant spindle cell and giant cell sarcoma at the site of injection after 441 days; numerous metastases were found in the peritoneal cavity.

5. 4'-Hydroxy-2,3'-azotoluene (abbreviation: oHAT).

This compound, unlike the preceding two, is substituted in one para-position, but the substituent is a hydroxy and not an amino group; hence it could not yield a p-diamine by reductive fission. The only experimental work reported with oHAT was carried out by Law (26), who injected a total of 5 mgm. in olive oil solution followed by a 5 mgm. pellet of the solid azo compound, all subcutaneously in the same zone. Slight activity against the liver was indicated by the finding of hepatoma at 629 days in a mouse of dba strain, in which spontaneous hepatoma is very rare; a similar lesion in a C57 black mouse at 604 days may have been spontaneous. No sarcomas were found in dba mice, but 11 out of 30 C57 black mice developed fibrosarcoma at the site of injection at a mean age of 350 days; i.e., 290 days after the first injection. In Law's experiments this is almost as high an incidence as he obtained with the 4'-amino-derivative of AT, but the latent period was about one-third longer. Law observed no tumors in the bladder, alimentary tract, or lung, and no hemangioendothelioma.

6. N,N-Dimethyl-p-aminobenzene (abbreviation: DAB).—Five groups of workers have investigated the effect of this dye in mice. The earliest report, that by Mizuta and Maruya (35), records "changes in the liver" according to Hartwell (17); the dye was fed in the food, but details of dosage or of the duration of the experiment are not available (17).

Law (26) administered DAB to mice of the dba and of the C57 black strains, 5 mgm. in olive oil followed by a 5 mgm. pellet, subcutaneously. Liver changes were apparently only minor, since no tumors of this organ were seen in C57 black mice and only 1 dba mouse, dying at 581 days, developed hepatoma. Out of 106 dba mice injected by Law with one or other of 4 azo compounds, only 1 developed fibrosarcoma at the site of injection and this, dying at 476 days, had received a total of 10 mgm. of DAB. On the other hand, while 20.7 per cent of the C57 black mice treated with DAB developed fibrosarcoma at the site of injection, this was only approximately half the incidence Law obtained in this strain by injecting oHAT or oAAT. Law's results indicate that DAB is a stronger sarcogen than carcinogen.

According to Hartwell (17) and to Andervont and Edwards (3), Shear and Stewart injected up to 70 mgm. of DAB in the form of crystals into mice of the dba and A strains, without eliciting any tumors at any site up to 15 months after the first implantation. The period employed was shorter than that of Law (26), whose dba mice yielded no tumors until more than 15 months had elapsed. Moreover, crystals were used, whereas Law injected first an oily solution, which probably favored sarcogenesis.

Andervont and Edwards (3) injected subcutaneously a total of 45 mgm. of DAB dissolved in olive oil into mice of both sexes of strains A, C, and C57 black.
In spite of the increased dose, compared with that of Law, none of these mice showed even microscopic liver changes. No tumors were found at the site of injection in strain A or strain C mice, but 2 females of the C57 black strain that died during the 41st week of the experiment (after about 280 days) had fibrosarcomas related to the site of injection. There was therefore further evidence that DAB can act as a saccrogen in at least one strain of mouse, but no evidence that it can act as a carcinogen for the liver or other tissues.

This noncarcinogenicity of DAB for mouse liver stood in sharp contrast with the powerful carcinogenic action it is known to have on the liver of rats. In the latter species, however, administration has almost invariably been per os. Special interest, therefore, lies in the experiments of Andervont, White, and Edwards (6), who fed DAB to strain C mice of both sexes at the usual level of 0.06 per cent in a low-protein:high-fat diet containing 0.5 per cent cystine; all these dietary modifications are known to favor DAB carcinogenesis in rat liver (33, 38, 48). They obtained no liver lesions in the 8 male mice that survived 310 days and appear to have consumed at least 300 mgm. of DAB each. Of 6 female mice, which presumably would consume less food and therefore less dye than the males, 4 had cirrhotic livers and 1 had 3 hepatomas. Experiments with restricted diets in other animals (16) suggest that the cirrhosis might have been entirely due to the unbalanced nature of the diet and/or to restricted intake consequent upon the unpalatable dye, but the presence of hepatomas in 1 mouse liver indicated that DAB might have a definite carcinogenic effect, at least when administered orally. Unfortunately, only strain C mice were used in these experiments and this strain has not been employed by other workers.

That DAB can produce severe and neoplastic lesions in the livers of mice has been shown by Kirby (25), who injected the dye, 7.5 mgm. in arachis oil, once a fortnight, subcutaneously into stock mice of mixed genetic constitution and into mice of the Cba and C57 black strains. Among the stock mice, 2 out of 10 males and 2 out of 5 females (surviving 250 days) developed hepatoma; these 4 animals had each received a total of 187.5 mgm. of DAB, and the average latent period was 420 days. Only male Cba mice survived more than 208 days, but 3 out of 14 died with definite hepatomas, 1 having hemangioma mixed with hepatoma; total dye received by these 3 mice ranged from 232.5 to 247.5 mgm., and the average induction period was 540 days. The males of the C57 black strain developed no liver tumors, although an early increase of reticulum was usually present. Four out of 5 females surviving 250 days developed both cirrhosis and hepatoma; in 1 dying at 530 days the liver-cell tumor was histologically malignant. The amounts of dye administered to these 4 mice were 172.5, 187.5, 210.0, and 210.0 mgm. respectively. Hence it is clear that the livers of all 3 types of mouse are susceptible to DAB given by the subcutaneous route. But in view of the quantities of dye administered in these experiments and, moreover, the duration of the experiments, it is not comprehensible that positive results should have been obtained when Law and likewise Andervont and his associates found no liver lesions when using the same route of presentation. Kirby also found sarcomas at the site of injection of DAB (see section 3 of this paper).

The cancer-producing activity of DAB is clear for the subcutaneous tissues from the work of Law, of Andervont and his co-workers, and of Kirby, but it is not great and some strains, notably Cba, A, and C, seem to be entirely resistant. The action of DAB on liver seems to be definite also; here again it is relatively weak and in only one instance (a female C57 black mouse) was the liver lesion considered to be definitely malignant; no metastases have been reported. In C57 black mice, and possibly in C strain mice also, the females appear to be more susceptible than the males, but this is doubtful for stock mice of mixed origin. Kirby also reports that toxic nephritis was a common feature in all his mice receiving DAB (25).

7. 4'-Amino-2,3'-azotoluene (abbreviation: oAAT).—By far the largest bulk of information on the action of an azo compound in mice concerns oAAT. It begins with the early observation of Yoshida (49) that annular cirrhosis and adenoma were present in mice (presumably of mixed origin) that had been injected with a 10 per cent olive oil solution of this dye; the total dose administered and the duration of the experiment are not available. Three years later Nishiyama (37) found hepatoma in 90 per cent of mice, also probably of mixed origin, fed the dye at a level of 0.03 per cent up to 400 days. The third normal route of presentation, namely, painting, was employed by Boyland and Brues (8), who used a 0.3 per cent solution in benzene on stock mice and mice of the Simpson strain. Out of 20 mice, 4 survived 95 to 104 days and received about 4.5 mgm. of dye; 3 had focal necroses, and 1 degenerative changes in the liver, but none showed any hyperplastic lesion.

In the same year Shear (43) reported liver carcinoma in 13 out of 16 mice injected with crystals of oAAT, totalling 70 mgm., and surviving 11 to 14 months; whether any of these were in strain M (leaden) mice is not clear, but most of them must have been in strain A mice, although the sexes are not reported separately. Boyland and Brues' negative results were thus shown to be probably due to the insuffi-
cent duration of the experiment. This was confirmed by results that began to appear 2 years later.

Early in 1939 Baumann, Jacobi, and Rusch (7) reported "changes which precede malignancy" in all of 16 strain C mice surviving 8 months on various diets containing 0.05 per cent of oAAT; no differentiation was made between the sexes.

According to Hartwell, Morosenskaya (36) painted stock mice with a benzene solution of oAAT, administering not more than 0.1 mgm. in the course of 7 to 8 months, and obtained liver tumors of an unspecified nature in 22 out of 40 mice. Morosenskaya found liver tumors in 14 out of 30 mice fed a total of 0.5 mgm. of oAAT in olive oil during 7 to 8 months. He also obtained similar lesions in 2 out of 17 stock mice injected with as little as 10 mgm. of the dye in sunflower seed oil and surviving 7 to 8 months. Moreover, this worker employed all three routes to administer oAAT to mice of the P.-B. strain, which led in each case to liver tumors in an unspecified proportion of animals. Hence the capacity of this dye to cause hypertrophic lesions of the liver in mice, even in very small doses if given sufficient time, was clearly demonstrated.

Before the end of the year Andervont (1) reported results obtained by the implantation of crystals into mice of 5 strains. Hepatoma in 7 out of 8 males of strain A confirmed Shear's findings. Similarly, all of 9 C3H males showed liver tumors, as did all of 14 C females. However, in strain I the incidence among males was only 66 per cent, compared with 100 per cent for females; in strain Y the figure for males was 28 per cent and for females 50 per cent. Thus there came to light here for the first time the difference in susceptibility between the two sexes in certain strains that was to be more clearly revealed in later studies from the same laboratory.

After an interval of nearly 2 years Law (26) reported hepatoma in 7 out of 30 dba mice after 434 days and in 1 out of 30 C57 black mice at 465 days, after subcutaneous injections of oAAT in olive oil followed by pellets of the dye. The next year Turner and Mulliken (47) found a 60 per cent incidence of liver lesions after 11 months in 10 strain C mice injected subcutaneously with the dye dissolved in corn oil. Law administered 10 mgm. in all per mouse, while Turner and Mulliken gave at least 130 mgm. Neither group referred to any sex difference in incidence, although Law states that he used "equal numbers of . . . both sexes . . . in each experiment." Meanwhile, Andervont, Grady, and Edwards (5), in an attempt to discover the mechanism of inheritance of susceptibility to tumor induction in certain strains of mice, repeated and extended Andervont's work. Injecting solid dye subcutaneously up to 1 year (100 mgm. total) they obtained liver tumors in 36 per cent of C3H male mice surviving the full period. However, in strains A, C, and C57 black they used males and females, and obtained decided differences in the incidences for the two sexes. Thus in strain A, males yielded 45 per cent and females 100 per cent; in strain C, males 8 per cent, and females 100 per cent; C57 black mice resembled strain C, with males yielding 9 per cent and females 100 per cent. The following year Andervont and Edwards (3), comparing the activities of this dye and of DAB, used the same 4 strains, but administered a total of only 45 mgm. in olive oil solution. In this experiment strain C males yielded no tumors, compared with 75 per cent in females; C57 black mice were similar. The yield of liver tumors was low in C3H mice, but 1 female out of 8 was positive compared with none out of 9 males. Strain A males were not used, but 100 per cent incidence was found in the females of this strain. Andervont, White, and Edwards (6) extended the comparison of the two azo dyes to the feeding route, using only strain C mice. They induced hepatomas, lung tumors, and hemangioendotheliomas (especially of the lungs) with oAAT in most of the females and many of the males also. The sex difference observed when the dye was injected subcutaneously was found in the case of oral administration also, but it was not so definite.

Andervont and Edwards (4) also investigated the effects of graduated doses of oAAT-injected subcutaneously as crystals into female mice of strain A. This revealed that 10 mgm. of dye was sufficient to induce hepatomas in 30 per cent of the mice receiving that dose by 1 year, whereas a 40 mgm. dose led to hepatoma in 10 per cent at 27 weeks and in 50 per cent by 52 weeks; 65 per cent had hepatoma by 1 year after a 60 mgm. dose. Thus the effect of the dye was to a definite degree proportional to the dose given, and the results of Law and of Morosenskaya, both of whom used 10 mgm. doses, were supported.

While Kirby (24) found a definite sex difference in the susceptibility of C57 black mice to the action of DAB on the liver, his results in this strain, and also in CBA and stock mice, with oAAT show no such difference. In the case of stock mice, 2 out of 3 surviving 233 days developed hepatoma (1 had proved secondaries), while 7 out of 7 females surviving 250 days had hepatoma (secondaries found in 3), the earliest in a female mouse being at 366 days. Cba mice showed no sex difference; 5 out of 6 males and 4 out of 5 females surviving 250 days died with liver tumors, although no metastases were found in this strain. But in C57 black mice, 6 out of 6 males and only 3 out of 5 females surviving 250 days had hepatoma, 1 male showing secondary deposits. Not more than 130 mgm. of oAAT was administered to any one
mouse, and, as Andervont and his group (5) used 100 to 110 mgm., it would seem that massive dosing is not responsible for the difference in results. The use of C57 black mice is common to, and makes comparable, the experiments of Law (26), of Kirby (25), and of Andervont and his associates (3, 5). Andervont, Grady, and Edwards (5) used solid dye, Andervont and Edwards (3) an olive oil solution, Kirby (25) used an arachis oil solution, whereas Law (26) used an olive oil solution plus solid dye. The results of Andervont and his co-workers would seem to indicate that the presence or absence of a solvent makes no difference to the unequal sex susceptibility, and it whether the dyes be administered orally or by subcutaneous injection. Moreover, the total evidence available shows that oAAT is the most powerful carcinogen for mice of all the azo compounds yet tested. Tests with 2 other azo dyes are very desirable: first, the new carcinogen for rats, m'-methyl-p-dimethylaminoazobenzene (15, 29) and secondly, the hitherto unknown N,N-dimethyl-4'-amino-2,3'-azotoluene.

The advantage of the 2,3'-azotoluene molecule over the azobenzene molecule in mice is shown by the activity of 4'-hydroxy-2,3'-azotoluene which, in the hands of Law (26), yielded almost as many fibrosarcomas in C57 black mice as did oAAT; even the parent

| TABLE II: EFFECT OF AZO COMPOUNDS AT THE SITE OF INJECTION IN MICE |
|---------------------------------|-----------------|---------|---------|---------|---------|---------|
| Author                          | Azo compound    | Route   | Mixed   | A       | C       | C3H     | dba     | Cba       | C57 black |
| Shear (43)                      | oAAT            | SubQ    | 0/19    |         |         |         |         |           |           |
| Cook, Hewett, Kennaway, and     |                 |         |         |         |         |         |         |           |           |
| Kennaway (10)                   |                 |         |         |         |         |         |         |           |           |
| Law (26)                        | oAAT            |         |         | 0/30    | 13/30   | 6/29    | 11/30   | 5/20      |           |
|                                  | BY              |         |         | 1/30    | 0/24    |         |         |           |           |
|                                  | oHAAT           |         |         | 0/22    |         |         |         |           |           |
|                                  | AT              |         |         |         |         |         |         |           |           |
| Seligman and Shear (42)         | AT              |         |         | 0/10    |         |         |         |           |           |
| Shear and Stewart (3, 17)       | BY              |         |         | 0/10    |         |         |         |           |           |
| Turner and Mulliken (47)        | oAAT            |         |         | 8/10^9  |         |         |         |           |           |
| Andervont and Edwards (3)       | oAAT            |         |         | none    | 1/20^19 | 1/8^20  |         |           | 3/21^21  |
|                                  | BY              |         |         | none    |         |         |         |           |           |
|                                  | pAAT            |         |         | none    |         |         |         |           |           |
|                                  | pAAAB           |         |         |         |         |         |         |           |           |
|                                  | oAAT            |         |         |         |         |         |         |           |           |
|                                  | BY              | 2/9^56  |         |         |         |         |         |           | 1/5^57   |

is unlikely that a change of solvent would favor equality of susceptibility in the sexes. Law’s low incidence (3 per cent) is presumably due to his low dosage. The explanation seems to lie in the time factor. The tumorous male mice in Kirby’s C57 black series all died at an age later than that to which Andervont and his group allowed any of their mice to live. The sex difference in susceptibility observed by Andervont and his co-workers may therefore be one of latent period and not of susceptibility; this is certainly indicated by Kirby’s results, but a different result might follow prolonged experimentation with smaller doses.

Summing up the position from the point of view of the azo compounds, one can say that the 3 groups of workers who have carried out comparative experiments with 2 or more azo compounds (3, 6, 25, 26) are agreed that 4'-amino-2,3'-azotoluene is much more carcinogenic for the livers of mice, either of mixed or pure strain, than is N,N-dimethyl-p-aminoazobenzene, compound, 2,3'-azotoluene, yielded a higher percentage of these tumors than did DAB. On the other hand, the 4,5'-azotoluene molecule, at least when the amino group is in the 2'-position, seems to be inert; similarly, p-aminoazobenzene seems to be inactive in mice.

Whereas 2,3'-azotoluene seems to have no effect on the mouse liver, 2,2'-azonaphthalene causes tumors in a high proportion of animals, cholangioma being seen as early as 32 days after the initial dose was given (10). But 1,1'-azonaphthalene had only a slight action, and the 1,2'-isomer none at all.

B. TUMORS AT THE SITE OF INJECTION (SEE TABLE II)

The early workers, including Shear (43), reported no tumors at the site of injection, and it was not until Law published his results (26) that there was any reason to believe that azo dyes were carcinogenic. Cook and his associates (10) had reported a very malignant
spindle and giant cell sarcoma 441 days after injections of 1,1'-azonaphthalene, but Law found a fibrosarcoma in a C57 black mouse injected subcutaneously with oAAT after only 45 days. Since Shear used oAAT in his experiments and, moreover, injected 7 times the amount used by Law per mouse, the effect of the solvent and/or strain was brought out very clearly; Shear used 70 mgm. crystals, Law an olive oil solution until 5 mgm. dye had been injected and then a 5 mgm. pellet. The first fibrosarcoma appeared before the pellet was introduced. It is interesting that Morosenskaya, who injected 10 mgm. oAAT dissolved in sunflower seed oil, observed no sarcomas at all, even at 8 months. The effect of the solvent was also brought out by the work of Andervont and his co-workers. Andervont (1) used crystals in his first experiment, and reported no sarcomas. Similarly, no sarcomas were reported by Andervont, Grady, and Edwards (5), who also used the solid dye. In one series (3) Andervont and Edwards injected oAAT in olive oil and then 3 out of 21 female C57 black mice died with sarcoma at the site of injection at or before 52 weeks; the same lesion was found in 1 female of strain C and in 1 of strain C3H. Even then, Andervont and Edwards found no sarcomas in mice of strain A. Kirby (25), using an arachis oil solution, obtained a spindle cell sarcoma containing giant cells at 252 days in a female of C57 black strain, but none in stock mice or in the Cba strain. On the other hand, Turner and Mulliken (47), using a corn oil solution of oAAT, found sarcoma in 8 out of 10 strain C mice at about 1 year.

While Shear and Stewart are reported (3, 17) to have found no sarcomas in strain A mice injected with crystals of DAB, and Andervont and Edwards (3) found none in mice of strains A or C injected with an olive oil solution of this dye, the latter authors found sarcoma in 2 out of 21 females of C57 black strain. The sarcogetic action of DAB was confirmed by Kirby (25), who found a mixed cell fibrosarcoma in a female C57 black mouse dying at 321 days after a total injection 127.5 mgm. of dye. Whereas no male C57 black mice developed a tumor at the site of injection (although 10 survived 250 days and Law obtained first fibrosarcoma at about 50 days), 2 male stock mice developed spindle cell sarcomas related to the injections; no female stock mice showed such a lesion. The 2 male stock mice had received 157.5 and 172.5 mgm. of dye respectively, and the tumors were found at 344 and 379 days. Female mice of the Cba strain did not survive 208 days, but 12 males survived periods ranging from 374 to 565 days without showing any neoplastic reaction at the site of injection. Hence Cba mice were resistant both to DAB and to oAAT.

Law, using DAB in olive oil, found fibrosarcomas in C57 black mice (20 per cent), while AT was at least as effective a sarcogen, and oHAT was nearly as powerful as oAAT.

The consensus seems to favor oAAT as the most powerful sarcogetic for mouse subcutaneous tissues. Law's work places oHAT second, with DAB only half as powerful as oAAT. Other findings would indicate that DAB is much more nearly equal in power to oAAT. The result of Turner and Mulliken is remarkable, exceeding even the high activity found by Law for oAAT. In view of the favorable nature of corn oil for the development of liver tumors due to DAB fed to rats (31), it is tempting to speculate whether it specially favors sarcogetic action at the site of injection.

C. LUNG TUMORS (SEE TABLE III)

One of the earliest workers with azo dyes, Nishiyama (37), observed lung tumors in 1 out of 7 mice fed oAAT, and stated that the nodules were hemangioendotheliomas. Kirby found this lesion in the lung of a female stock mouse injected with oAAT in arachis oil; this mouse also had secondary hepatoma deposits. Andervont, White, and Edwards (6), who fed oAAT to strain C mice, obtained pulmonary tumors in 20 out of 29 males and in 27 out of 31 females; of these 47 lesions, 20 were hemangioendotheliomas. Similarly, Andervont, Grady, and Edwards (5), who injected oAAT as crystals subcutaneously into mice of strains A, C, C3H, and C57 black, found pulmonary tumors, including hemangioendotheliomas, in 13 out of 24 strain C mice and in 18 out of 22 mice of strain A. The same 4 strains were employed by Andervont and Edwards (3), who injected oAAT in olive oil solution; lung tumors were found in 6 out of 7 strain A mice and in 14 out of 33 strain C. These authors also injected DAB in olive oil into A, C, and C57 black mice; C57 black strain mice never developed lung tumors, and the incidence in the A and C strains was not greater than the normal spontaneous figures. Law reported no lung tumors with any of the compounds he used, but Morosenskaya (36) found these lesions in an unrecorded proportion of P.-B. mice receiving oAAT orally, cutaneously, or subcutaneously. Kirby found 1 adenoma in a female stock mouse after injections of oAAT, and in 1 male stock mouse after injections of DAB; mice of the Cba strain were free of any lung tumors.

The conclusion may be drawn that azo dyes have little capacity to provoke pulmonary tumors in mice, except in strains that are liable to spontaneous tumors of the lung. Andervont, Grady, and Edwards (5) concluded that "the response of the lungs of mice to o-aminoazotoluene injections was similar to that produced by hydrocarbons. In addition, the pulmonary..."
tumors induced by the compound were indistinguishable from those produced by other carcinogens."

**D. ENDOTHELIOMAS (see Table IV)**

Two types have been recorded in mice receiving oAAT; DAB appears not to induce this lesion at all even in strains very susceptible to oAAT. Law (26) reported 1 reticuloendothelioma in a C57 black mouse injected with oAAT, and 1 in a mouse of the same all, but in a later experiment (4) these authors found a 30 per cent incidence in females of strain A receiving 60 mgm., compared with zero per cent in females injected with 10 mgm. oAAT. Andervont, White, and Edwards (6), who fed oAAT, found as great an incidence of hemangioendothelioma in strain C mice as Andervont, Grady, and Edwards had found after subcutaneous implantation; female mice of this strain were always much more susceptible than males.

**Table III: Induction of Lung Tumors in Mice by Azo Compounds**

<table>
<thead>
<tr>
<th>Author</th>
<th>Azo compound</th>
<th>Route</th>
<th>Mixed</th>
<th>A</th>
<th>C</th>
<th>C57 black</th>
<th>Cha</th>
<th>P.-B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nishiyama (37)</td>
<td>oAAT</td>
<td>Per os</td>
<td>1/7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Morosenskaya (36)</td>
<td>oAAT</td>
<td>Skin</td>
<td></td>
<td></td>
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<td></td>
<td>painting</td>
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<td></td>
</tr>
<tr>
<td>Andervont, Grady, and</td>
<td>oAAT</td>
<td>SubQ</td>
<td>18/22</td>
<td><strong>13</strong></td>
<td><strong>24</strong></td>
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<tr>
<td>Edwards (5)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Andervont and Edwards (3)</td>
<td>oAAT</td>
<td>SubQ</td>
<td>6/7</td>
<td><strong>19</strong></td>
<td><strong>33</strong></td>
<td>0/42</td>
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<td></td>
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<tr>
<td>Andervont, White, and</td>
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<td>BY</td>
<td>20/29</td>
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<td><strong>31</strong></td>
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<tr>
<td>Edwards (6)</td>
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<tr>
<td>Kirby (25)</td>
<td>oAAT</td>
<td>SubQ</td>
<td>0/2</td>
<td><strong>2</strong></td>
<td><strong>7</strong></td>
<td>none</td>
<td></td>
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</tbody>
</table>

**Table IV: Induction of Endothelioma in Mice by Azo Compounds**

<table>
<thead>
<tr>
<th>Author</th>
<th>Azo compound</th>
<th>Route</th>
<th>Incidence of endothelioma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nishiyama (37)</td>
<td>oAAT</td>
<td>Per os</td>
<td>1/7</td>
</tr>
<tr>
<td>Law (26)</td>
<td>oAAT</td>
<td>SubQ</td>
<td>1/7</td>
</tr>
<tr>
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<td>SubQ</td>
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<tr>
<td>Edwards (5)</td>
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<td>oAAT</td>
<td>By</td>
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</tr>
<tr>
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</tr>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>1/7</td>
</tr>
</tbody>
</table>

Thus oAAT seems to be unique among the azo compounds tested in mice in being able to induce hemangioendotheliomas at a large range of sites in certain strains, and occasionally in other strains.

**E. LIVER LESIONS OTHER THAN LIVER-CELL TUMORS**

Two forms of liver lesion other than hepatoma call for some comment. Under the section on azonaphthalenes it was stated that stock mice developed bile duct proliferation and cholangioma almost exclusively (10). Law (26) states that in his dba and C57 black mice "there was little or no proliferation of fibrous connec-
tive tissue nor was there extensive proliferation of bile duct epithelium toward cholangioma formation." This presumably refers to the effects of oAAT, oHAT, DAB, and AT; but adenomatosis of bile ducts was apparently found in 2 dba mice receiving oAAT. Andervont and his group, using oAAT (1, 3-6), found very little bile duct proliferation in mice of the A, C, C3H, or C57 black strains. Cholangioma was not reported by other workers except Kirby (25), who found following injection of oAAT considerable bile-duct proliferation with the formation of subcapsular cysts in Cba mice, and proliferation amounting to cholangioma in several stock mice of mixed origin. The latter result suggests that mice are not unable to react in this way to oAAT, and that a pure line might be found that was very susceptible to bile duct proliferation under the stimulus of oAAT. But the generalization remains that mouse liver shows a sharp divergence in reaction to azonaphthalenes on the one hand, and to oAAT and DAB at least, on the other.

The work of Opie (38) makes it clear that DAB can cause liver tumors in rats without cirrhosis at any stage, although the proportion of animals developing tumors increases with the severity of cirrhosis when it is present. Cirrhosis would appear to indicate a dietary deficiency either from the diet as ingested, or arising as the result of the removal, by one means or another, of some constituent of the diet in the alimentary canal or inside the body itself, especially in the liver. Deficient diets have been devised that induce cirrhosis; the picture for mice is not notably different from that for the rat. A focal increase in reticulum was usually seen following administration of oAAT, even when a full diet was given (1, 3, 5, 6, 25), and this was true for DAB (25). Andervont and his co-workers refer to the "cirrhotic appearance" of their mouse livers but say "there was no extensive fibrosis" (5); with this Law and Kirby agree. All strains of mice, as well as heterozygous mice, seem to show the same kind of lesion.

### STRAIN SUSCEPTIBILITY

Apart from mice of mixed origin, 10 strains of mice have been used in experiments with azo compounds.

1. **M (leaden).**—A few of these were injected by Shear (43), but it is not clear from his paper whether any of the lesions reported were in mice of this strain.

2. **P-B.**—These were used by Morosenskaya (36), and showed themselves to be susceptible to liver tumor and lung tumor induction by means of oAAT by any of the 3 routes employed. Unfortunately, the proportion of animals actually developing tumors is not stated (17).

3. **dba.**—Mice of this strain were employed by Law (26), and by Shear and Stewart (3, 17). The latter found no tumors in males when DAB was given in solid form; Law found sarcoma in 1 out of 30 injected with DAB in olive oil, but no tumors at the site of injection with oAAT, oHAT, or AT. Law reported a very low incidence of hepatic tumors with DAB, oHAT, and AT also, and only 7 out of 30 with oAAT. This strain may therefore be regarded as rather resistant.

4. **Cba.**—This strain has been used only by Kirby (25). Liver tumors were found in most males and females injected with oAAT in oil; DAB in oil gave liver tumors in 3 out of 14 males, females not having been adequately tested. No sarcomas, lung tumors, or endotheliomas were found, and this strain appears to be susceptible only to liver tumor induction, although this includes definite bile duct proliferation.

5. **I.**—Strain I mice were used by Andervont (1), who injected solid oAAT. Two-thirds of the males and all the females developed liver tumors, but no other neoplasms were recorded.

6. **Y.**—Andervont also injected mice of this strain with solid oAAT. The results indicate a greater resistance than that possessed by the other 4 strains employed, namely, A, C, C3H, and I.

7. **C3H.**—Three series of experiments by Andervont and his co-workers concern strain C3H mice. Andervont's original experiment (1) yielded 100 per cent of liver tumors in males, but Andervont, Grady, and Edwards (5), who injected the same amount of dye, also as the solid, found only 5 out of 14 males surviving a year to have liver tumors. When the total dose was decreased to 45 mgm. and injected in olive oil, Andervont and Edwards (3) found no liver tumors in males and only 1 in 8 females; DAB in oil produced no liver tumors. The reasons for these differences are not apparent. Injections of oAAT caused 1 sarcoma and 1 endothelioma among all the mice used (about 50). Lung tumors were never found in this strain.

8. **A.**—Seven investigations have been made with mice of this strain; 3 by Shear and his collaborators (3, 17, 42, 43) and 4 by Andervont and his group (1, 3-5). AT and DAB caused no tumors at any site. On the other hand, 2 out of 22 mice in Andervont, Grady, and Edwards' experiments with solid oAAT developed hemangioendotheliomas; oAAT also caused lung tumors in a very large percentage of mice, far in excess of the spontaneous incidence in these animals. Liver tumors following oAAT administration were very common. The experiment of Andervont and Edwards (4), who injected graded doses of crystals into female mice, indicated a rise in incidence from 6 out of 20 after 10 mgm. to 13 out of 20 after 60 mgm.
Other experiments showed 100 per cent incidence among females receiving 100 mgm. oAAT. Males were usually more resistant, and it seems that females develop liver lesions more readily although the ultimate incidence may be the same. Subcutaneous injection of oAAT or DAB, even in oil, failed to evoke sarcoma in this strain.

9. C.—Baumann, Jacobi, and Rusch (7) fed oAAT and Turner and Mulliken (46) injected oAAT in corn oil, using strain C mice. Baumann and his associates reported premalignant changes in 100 per cent of their mice, and Turner and Mulliken found that 60 per cent developed liver tumors. Andervont’s work shows a high degree of susceptibility to liver tumor induction by oAAT; this applies much more to females, 1 even developing hepatomas when fed DAB, than to males. Here again the sex difference may be one of latent period only. Turner and Mulliken found sarcoma in 80 per cent of their mice injected with oAAT, but Andervont and Edwards (3) saw this lesion in only 1 out of 20 females, using about one-third of the dosage given by the former workers. The work of Andervont and his group makes it clear that strain C mice respond very readily to oAAT with lung tumors and endotheliomas, the females again being more susceptible than the males up to 1 year. DAB elicited no tumors outside the liver.

10. C57 Black.—Law (26) encountered liver tumors in mice of this strain following injections of oAAT, oHAT, or even AT, though in the latter case the time was so long that spontaneous tumors cannot be excluded. Andervont and his collaborators (3, 5) found a much higher percentage of animals developing hepatomas, especially females. Kirby, also, obtained hepatomas in a high percentage of C57 black mice; DAB revealed a much greater susceptibility among females, but oAAT attacked male livers just as readily as female, though rather later. It is worth noting that Kirby saw no cholangiomatous lesions in this strain (25).

Law found sarcomas at the site of injection in this strain following oAAT, DAB, oHAT, and AT; in nearly 50 per cent in the case of oAAT. Andervont and Edwards (3) saw these lesions after injection of oAAT or of DAB, and Kirby obtained the same result but only in females. All three groups of workers agree that endotheliomas are caused by oAAT but not by DAB. No lung tumors were found by any investigator.

This review of strain susceptibility shows that the carcinogenic (or sarcogenic) power of an azo compound is not absolute, but depends on the strain of mouse employed in the test. Thus the sarcogenic power of oAAT, for example, would never have been discovered had investigations been confined to mice of strains A, Cba, and dba. On the other hand, all strains investigated have shown some degree of liver tumor formation, but low doses may fail to evoke such lesions when higher ones would succeed. The only strain that developed liver, lung, connective tissue, and endothelial tumors was the C strain, but a full test for any azo compound would probably best be carried out by (a) feeding, and (b) subcutaneous injection in mice of strains A, C, and C57 black.

THEORIES OF THE MECHANISM OF TUMOR INDUCTION BY AZO COMPOUNDS

A. The “Split-Product” Theory of Liver Carcinogenesis by Azo Dyes

The work of Kinosita (22) established that DAB is more carcinogenic for the liver of rats than the isomeric oAAT. Since the latter dye was shown by Hashimoto (18) to be metabolized in N,N'-diacetyl-\(p\)-toluenediamine when fed to rabbits, Kinosita suggested that DAB also would be split at the azo link by rats, and excreted as aniline and N,N-dimethyl-\(p\)-phenylenediamine. The work of Stevenson, Dobriner, and Rhoads (44) confirmed the suggestion that reductive fission occurs, but the products excreted were \(p\)-aminophenol or its N-acetyl derivative, and \(p\)-phenylenediamine or its N,N'-diacetyl derivative. Hence demethylation occurred at some stage, but there was no evidence whether this took place before or after the azo link had been reduced and broken. Meanwhile Kensler, Sugiura, and Rhoads (20) had reported that the diphosphopyridine nucleotide content of rat livers was reduced during carcinogenesis by DAB and absent from the neoplastic tissue. Kensler, Dexter, and Rhoads (19) proceeded to show that a DPN system was inhibited by the partial oxidation products of various \(p\)-diamines, which could be regarded as deriva-

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this property, and both would yield the same fission product. Moreover, although the solubility of methyl orange in water might account for its noncarcinogenicity, this can hardly explain the absence of activity in 2(N,N-dimethyl-p-aminophenylazo)-naphthalene; both of these should yield N,N-dimethyl-p-phenylenediamine. Miller and Baumann have also found that N-methyl-p-aminoazobenzene is as strong a carcinogen for the liver of rats as is the fully methylated dye, and this is confirmed by Kensler and his associates (46), although from the relative inhibitory powers of the fission products, N-methyl-p-phenylenediamine and N,N-dimethyl-p-phenylenediamine respectively, one would have predicted only half the carcinogenic activity for the monomethyl compound. It must be borne in mind, however, that Miller, Miller, and Baumann (32) have shown the body capable both of methylating the monomethyl to the dimethyl compound and, conversely, of demethylating the dimethyl to the monomethyl compound, and the closely similar activities might be accounted for in this way.

Much more serious criticism arises from the findings of Miller and Baumann (29) with the o', m', and p' methyl derivatives of DAB. These would all yield N,N-dimethyl-p-phenylenediamine, yet the o'-compound has a carcinogenic activity for rat liver about one-third that of the parent dye and the p'-compound seems to be nearly as weak a carcinogen as is p-aminoazobenzene, whereas the m'-compound is even more active than DAB itself. It thus appears that the carcinogenic activity of azo dyes cannot be strictly correlated with the enzyme inhibitory powers of the diamines produced by reductive fission. Miller, Miller, and Baumann (32) further considered that demethylation may precede reductive fission, so that the highly inhibitory dimethyl-p-phenylenediamine would never be formed in vivo. If this is so, then p-aminoazobenzene and the dimethylated dye would both yield the same fission product, p-phenylenediamine, and the carcinogenic activities would bear no relation at all to the enzyme inhibitory power of the diamine.

B. The "Benzidine Rearrangement" Theory

The great carcinogenic and sargogenic power of 3,4,5,6-dibenzcarbazole for the skin of mice and for the subcutaneous tissues of mice and rats was demonstrated in 1937 by Boyland and Brues (8). The occurrence of bladder epithelioma among aniline dye workers, especially among those handling naphthylamines, led Cook, Hewett, Kennaway, and Kennaway (10) to investigate the effect of the azonaphthalenes administered to mice by the usual 3 routes—oral, cutaneous, and subcutaneous. Though no bladder tumors were obtained, 2,2'-azonaphthalene was shown to cause liver tumors, 1,1'-azonaphthalene being slightly active and the 1,2'-isomer inactive. Cook and his collaborators argued that as azo compounds could undergo reductive fission in the body, hydrazo compounds are presumably formed and that the latter might also follow the alternative path of rearrangement to a benzidine type of compound. They therefore also tested the dinaphthyls carrying two amino groups ortho to the "benzidine type" bond that could arise by such a rearrangement of the 3 azo naphthalenes. Activity was confined to 2,2'-diamino-1,1'-dinaphthyl, which would be derived from 2,2'-azonaphthalene. As de-amination of this compound occurs very readily in vitro to yield 3,4,5,6-dibenzcarbazole, which is known to be a powerful carcinogen, Cook and his group suggested that 2,2'-azonaphthalene acts on the liver of mice because it is there converted to 3,4,5,6-dibenzcarbazole; the same process would explain the activity of the intermediate dinaphthyl.

In 1944 Elson and Warren (13) reported that they had isolated aniline from the urine of rats injected intraperitoneally with an arachis oil solution of azobenzene. This indicated that part of the azobenzene underwent reductive fission at the azo link. However, other extraction of the acidified urine yielded benzidine, which they regarded as formed during the acidification stage from a soluble derivative of hydrazoazobenzene. The evidence showed that very small quantities of benzidine were present in the livers of rats receiving azobenzene. But preliminary results with rats receiving DAB showed that the actual "benzidine rearrangement" takes place more readily than in the case of azobenzene, and that a compound giving a color reaction, which they used as a test for "benzidine type" compounds, was present in the alkaline or neutral urine. The indications, then, are that in the rat azo compounds may, and DAB in particular does, follow 2 metabolic paths; one leads to the cleavage of the molecule at the azo link to provide amines such as aniline, while the other leads to benzidine type derivatives. Since Elson and Hoch-Ligeti (12) have found that "benzidine rearrangement" products of the latter type, when suitably oxidized, are strong inhibitors of urease and succinoxidase systems, it is clear that both metabolic routes can provide the means of enzyme inhibition, and hence of favoring carcinogenesis if Potter's views are accepted.

As the comparative carcinogenic activities of DAB and oAAT are reversed in mice as compared with rats, a theory that explains the greater activity of DAB in rats can hardly explain the greater activity of oAAT in mice. It may be suggested that reductive fission predominates in rats and leads to diamines known to have a certain order of inhibitory powers against rat
and plant enzymes. On the other hand, in mice the other path of “benzidine rearrangement” might predominate, and lead mainly to compounds having a different order of inhibitory powers against enzyme systems. Three questions need to be answered before a decision can be made. First, have the diamines tested by Kessler and his associates (19, 21) and Potter (40) the same order of toxicity toward mouse enzymes as they have toward the 2 rat and the 2 plant enzymes tested so far? Secondly, what are the metabolic products excreted by mice receiving azo compounds; are these the same as those excreted by rats and, if so, are the relative amounts excreted the same in rats and mice? Thirdly, have the “benzidine rearrangement” products the same order of toxicity toward enzymes as the “split product” diamines derived from the same azo compounds?

Meanwhile it is possible to postulate that the existence of the “split product” and “benzidine rearrangement” paths of metabolism is responsible not for the difference between the activities of DAB and oAAT in rats and in mice, but for the difference in the nature of the liver response to various azo compounds. The hypothesis of Cook and his group (10), that 2,2′-azonaphthalenes in mice, and of oAAT in stock, Cba, and stock mice of mixed origin, are active against mouse liver by virtue of “benzidine rearrangement” and subsequent de-amination, implies that the type of tumor seen is linked with this metabolic pathway. Actually, cholangioma was the almost exclusive liver neoplasm seen after the administration of 2,2′-azonaphthalene. In rats both hepatoma and cholangioma follow administration of DAB or of oAAT, although cholangioma is less frequent with the latter dye (14); this may indicate a fairly equal importance of the 2 metabolic pathways following DAB administration, and a lesser importance of the “benzidine rearrangement” pathway for oAAT metabolism in the rat. In mice receiving DAB only hepatoma has been reported; this may be construed as indicating that reductive fission predominates. When oAAT is given C57 black mice, only hepatoma formation is seen and hence, possibly, the same pathway predominates. But stock mice of mixed origin and Cba strain mice, injected with oAAT, were shown by Kirby to be liable to develop cholangioma; in these mice the second, “benzidine rearrangement,” pathway might be assumed to be utilized to a relatively greater extent. Until data regarding the actual metabolism of azonaphthalenes in mice, and of oAAT in stock, Cba, and C57 black mice, are available, this hypothesis must remain a speculation.

SUMMARY

1. The investigations into the action of azo compounds in mice reported in the literature are reviewed from the points of view of the azo compounds used, the lesions evoked, and the strains of mice employed.

2. The relative carcinogenicities of N,N-dimethyl-p-aminoazobenzene and 4′-amino-2,3′-azotoluene are shown to be reversed in mice compared with rats. Theories explaining the order of carcinogenicities in one species will not suffice for the other species.

3. The predominance of cholangioma in the livers of mice receiving azonaphthalenes, and other data for rats and mice, may indicate that metabolism of azo compounds to “benzidine type” derivatives favors bile duct proliferation, while “reductive fission” favors hepatoma formation.

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Studies in Carcinogenesis with Azo Compounds. II. The Action of Azo Compounds in Mice, and the Bearing Thereof on Theories of Azo Dye Carcinogenesis

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