Studies in Carcinogenesis with Azo Compounds

I. The Action of Four Azo Dyes in Mixed and Pure Strain Mice*

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It has been known since 1932 (23) that 4'-amino-2,3'-azotoluene will induce liver tumors in rats receiving the dye orally. Later, Kinosita (10) demonstrated the superior carcinogenic activity of an isomericazo dyes, N,N-dimethyl-p-aminazobenzene, toward the rat liver; this superiority was revealed both in the shorter minimal feeding period and in the shorter latent period. Both these azo dyes are methyl derivatives of the base, p-aminazobenzene, which was long regarded as noncarcinogenic (8, 10, 14, 21, 23, 25). Consideration of the fact that 4'-amino-2,3'-azotoluene was carcinogenic although the amino group was not substituted, and also of the inhibitory action of p-phenylenediamine, one of the products of reductive fission of p-aminazobenzene, upon certain enzyme systems (8, 9, 20) led the present author to feed p-aminazobenzene in a modified diet to rats for a long period. Unequivocal liver-cell carcinomas were found after 17 months' feeding, with metastases in one animal (11). Thus it appears that p-aminazobenzene, as well as its 2,3'-dimethyl and N,N-dimethyl derivatives, is carcinogenic for the liver of rats; methylation does not confer, but does greatly enhance, carcinogenic power, provided the methyl groups enter certain positions.

Yoshida (25), and Sasaki and Yoshida (21) report having fed 2'-amino-4,5'-azotoluene for periods up to 476 days to rats without eliciting any liver tumors. Although Sasaki and Yoshida provide a structural formula for their compound that would show it to be 5'-amino-2,2'-azotoluene, and Cook and Kennaway (5) depict it as 4'-amino-2,2'-azotoluene, Miura (16), summarizing the experiments of Sasaki and Yoshida, depicts the compound as 2'-amino-4,5'-azotoluene; this identity is accepted by Hartwell (7).

The only reference to tests with p-aminazobenzene in mice is made by Yoshida (24), who gave subcutaneous injections of a 10 per cent solution in olive oil, but as no mice seem to have survived beyond 23 days this was hardly a test for carcinogenic activity; 2'-amino-4,5'-azotoluene does not seem to have been tested in mice. This paper deals with experiments in which azo dyes were injected subcutaneously into stock mice of mixed colors and, in the case of 2 azo dyes, into mice of the Cba and C57 black strains. The 4 azo dyes are shown in Fig. 1.

METHODS AND MATERIALS

The stock mice used were either purchased as required from a dealer or were bred in this laboratory from mice previously bought from the same dealer. The large range of coat color indicates considerable heterozygous constitution; the suitability of these mice for testing for the presence or absence of carcinogenic activity in chemical compounds has been emphasized in a previous paper from this laboratory (19). Spontaneous tumors are rare, although no precise data are available.

The Cba strain was bred in this laboratory from mice of this strain originally obtained from Dr. Greenwood, Edinburgh. The C57 black mice were bred from a pair given to us by Dr. Grüneberg, of Guy's Hospital, London.

The earliest experiment concerned only 2'-amino-4,5'-azotoluene; this dye was obtained from British Drug Houses, Ltd., and used without further purification. Subsequent work involving the other 3 dyes was carried out with materials purified by chromatography. p-Aminazobenzene was obtained from British Drug Houses, Ltd.; after passage in benzene solution...
through activated alumina, and subsequent crystallization by adding 2 volumes of petroleum ether (b.p., 60° to 80°) to the concentrated filtrate, the orange needles melted at 123° C. N,N-dimethyl-p-azoazobenzene was obtained from British Drug Houses, Ltd. under the name “Dimethyl Yellow, Analar”; a quantity of the same substance under the name “Waxoline Yellow, A.D.S.” was given to us by I.C.I., Ltd., Dye-stuffs Group. Both samples, after purification as above, gave orange-yellow needles melting at 117° C. 4'-Amino-2,3'-azotoluene was bought from British Drug Houses, Ltd.; purification as above yielded cerise needles melting at 101.5° C. (sintering at 99° C).

![Structural formulas for azo compounds used in these experiments.](image)

More than 1 type of diet was used in conjunction with each dye, but as the experiment with 2'-amino-4,5'-azotoluene differed from the experiment involving the other 3 dyes the details will be given under the appropriate headings.

**Experiments with 2'-Amino-4,5'-Azotoluene**

Experimental data.—This series was begun early in 1941 and only stock mice of mixed colors were used. The dye was dissolved in olive oil and injections were made subcutaneously in the flank once a fortnight. At first 0.25 ml. of a 1 per cent solution was injected per mouse, but the strength was increased to 2 per cent in Groups I and II after 266 days and in Groups III and IV after 238 days. The dose was further increased, by injecting 0.5 ml. of the 2 per cent solution, in Groups I and II after 366 days and in Groups III and IV after 338 days.

All 4 groups received a basal diet of rat cake (22) and water ad libitum. Group I were given only the basal diet, and served as controls. The other 3 groups were designed to test whether yeast had any protective action against this dye as it had been shown to have in rats injected with N,N-dimethyl-p-azoazobenzene, and, if so, whether the active constituent was extractable with water or not. Group II therefore were fed powdered rat cake (85 per cent) plus dried baker’s yeast (15 per cent). Group III received rat cake made to a stiff paste with a concentrated aqueous extract of similar yeast, while the residue from the extraction was given with rat cake powder, 1:3, to Group IV. Twelve mice of both sexes were used in each group except Group IV, where 6 more were added after 6 weeks to replace early losses.

Results.—As no mice in Group I developed tumors at any site, the possible protective action of yeast could not be assessed. As no tumors were seen in any groups, it can be concluded that 2'-amino-4,5'-azotoluene is noncarcinogenic for stock mice, at least when administered by the subcutaneous route, up to 472 days and to a total dosage of 67.5 mgm. The pathological reports showed that this azo dye is not without action on the liver; at least a quarter of the mice had focal necroses there, while others had massive necrosis. There was little evidence of the regenerative changes that precede carcinogenesis in livers damaged by N,N-dimethyl-p-azoazobenzene (17), nor was bile duct proliferation seen. A degree of periportal lymphocytic infiltration was recorded for several animals. Not all kidneys were examined, but the damage in those studied was only slight, and the acute or chronic toxic nephritis found in mice injected with N,N-dimethyl-p-azoazobenzene, or even azobenzene (see below) was never seen.

**Experiments with p-Aminoazobenzene**

Experimental data.—Only stock mice of mixed colors were used for this series, which was begun in March, 1943. By this time, Miller, Miner, Rusch, and Baumann (15) had shown that hepatic tumors could be induced in rats fed dimethylaminooazobenzene in the following restricted diet:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (per rat per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude casein</td>
<td>9 to 12 per cent</td>
</tr>
<tr>
<td>Cereal</td>
<td>80 &quot; 77 &quot; &quot;</td>
</tr>
<tr>
<td>Salts</td>
<td>4 &quot; &quot;</td>
</tr>
<tr>
<td>Cotton seed oil</td>
<td>5 &quot; &quot;</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>2 &quot; &quot;</td>
</tr>
<tr>
<td>Vitab (0.2 gm.)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1.—Structural formulas for azo compounds used in these experiments.
Cottonseed oil, however, was in very short supply in Britain at this time, and neither cereose, dextrin, nor starch was available. A partially purified diet was therefore adopted as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (Glaxo extracted)</td>
<td>10%</td>
</tr>
<tr>
<td>Boiled potatoes</td>
<td>75%</td>
</tr>
<tr>
<td>Salt mixture (Glaxo, LD6)</td>
<td>4%</td>
</tr>
<tr>
<td>Yeast (D.C.L. dried, baker's)</td>
<td>2%</td>
</tr>
<tr>
<td>Arachis oil</td>
<td>8%</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>1%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

The mice were divided into 2 groups, one of which was fed the full diet of rat cake whereas the other received the restricted diet. In this way it was hoped to discover whether the carcinogenic action of azo dyes in mice is subject to dietary influence or not.

The dye was incorporated in arachis oil, and 0.25 ml. was injected per mouse once a fortnight. A 3 per cent solution was used for the first 10 mice, but as half of them died within 48 days the strength was reduced to 2 per cent for all subsequent injections of the survivors and of other mice started later. Thus the amount injected per fortnight was 5 mgm. per mouse.

**Results.**—Of mice receiving the restricted diet, only 8 of 29 survived for more than 100 days; 3 lived more than 300 days, 2 females died at 391 and 431 days respectively, and 1 male lived 626 days. The amounts of dye received by the 3 mice last named were 117.5 mgm., 140.0 mgm., and 192.5 mgm. respectively; the kidneys of the first 2 showed no gross abnormality, but the male had chronic toxic nephritis. Mice dying earlier usually had some degree of acute toxic nephritis. No tumors were seen in the liver in any mouse; necrosis was frequent, both focal and otherwise, but fatty degeneration was not. The female dying at 431 days had leukemic infiltration of the liver and spleen, a condition seen in several mice dying much earlier. The skin invariably showed cystic spaces at the site of injection, with little reaction; the last survivor had a local foreign body giant cell reaction. In the spleen, the reticuloendothelial cells were usually very numerous and loaded with golden-brown pigment; in some cases the germinal centers or the entire malpighian bodies were degenerated.

Among the 7 mice fed on rat cake that were examined post mortem, 4 had survived 300 days' treatment; 2 males had each received a total of 98 mgm.
of dye, 1 female 113 mgm., and 1 male 120 mgm. Evidence of toxic nephritis was usually present. The skin showed only cystic spaces at the site of injection, and no neoplastic changes were found in the liver. The spleens had pigmented reticuloendothelial cells, but in 2 cases showed a peculiar hyaline degeneration around the malpighian bodies that did not stain for amyloid. Very similar lesions have been described recently by Parsons in mice treated with pentose nucleotides (18).

No unequivocal signs of even early cirrhosis were found in the liver of any mouse on either diet, and it seems safe to say that p-aminoazobenzene does not induce cirrhosis in stock mice. Moreover, no liver tumors were found, although only 1 mouse survived other flank once a fortnight. The same volume and frequency of injection was employed for 4'-amino-2,3'-azotoluene, which was used at a strength of 2 per cent, in arachis oil. Thus 7.5 mgm. of N,N-dimethyl-p-aminoazobenzene or 5 mgm. 4'-amino-2,3'-azotoluene was injected per mouse on each occasion.

**Results.**—1. **N,N-dimethyl-p-aminoazobenzene series.**—Table I shows (a) the number of mice used in each series that were examined post mortem; (b) the number of these that survived for 250 days or more; and (c) the days of experimentation that passed before any individual was found post mortem to have either sarcoma or a liver tumor (animals with large tumors were sacrificed).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mixed</th>
<th>Cba</th>
<th>C57 black</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\varnothing)</td>
<td>(\varnothing)</td>
<td>(\varnothing)</td>
</tr>
<tr>
<td>No. mice examined post mortem</td>
<td>9</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>No. surviving 250 days</td>
<td>9</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Sarcoma found at, days</td>
<td>344</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Liver tumor found at, days</td>
<td>429*</td>
<td>438</td>
<td>(511)‡</td>
</tr>
<tr>
<td></td>
<td>432</td>
<td>552, 556</td>
<td>530, 615</td>
</tr>
<tr>
<td>Retarded</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. mice examined post mortem</td>
<td>5</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>No. surviving 250 days</td>
<td>1</td>
<td>2</td>
<td>6+</td>
</tr>
<tr>
<td>Sarcoma found at, days</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Liver tumor found at, days</td>
<td>&quot;</td>
<td>382</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* Survived 443, 470, and 474 days respectively.

† Last died at 436 days.

‡ Nodular hyperplasia and angiomatous cystic spaces.

The following conclusions emerge from these findings:

1. Sarcoma and liver tumor were never found in the same animal.
2. Liver tumors are commoner than sarcomas in mixed and in pure strains.
3. The time required for sarcoma formation was much less than that required for liver tumor formation, both in the mixed strain mice and in C57 black.
4. In the mixed strain mice, tumors were found in 4 out of 8 males surviving more than 250 days, but in only 1 out of 3 females surviving for the same minimum period. In C57 black mice, only the females developed tumors, either sarcoma or liver tumors, although 3 males survived for 433, 470, and 474 days respectively and the livers in all showed pericellular and/or perilobular cirrhosis.
5. Cba mice seem especially resistant to the induction of subcutaneous tumors, 7 male mice on full diet and 6 on restricted diet living to or beyond the age at which the other strains developed sarcomas, without any sign of reaction at the site of injection.

II. **4'-Amino-2,3'-azotoluene.**—Table II records the corresponding data for the 4'-amino-2,3'-azotoluene experiments. The following conclusions may be drawn from a comparison of Tables I and II:
1. The effect on the liver of the compound having its methyl groups linked to carbon is much greater than that of the isomer in which the methyl groups are linked to nitrogen.

2. On the contrary, 4'-amino-2,3'-azotoluene appears to be the weaker sarcogen of the two dyes, though it is not entirely devoid of this power, and the latent period for the one sarcoma found was actually shorter than for those induced by the other dye.

3. The latent period for hepatoma formation is much less in the case of 4'-amino-2,3'-azotoluene; this is well shown in the Cba mice, where there is no overlap at all between the 2 experiments in the times elapsing before liver tumors were found. It is shown just as clearly by the C57 black females and the heterozygous males, but the females of the mixed strain show no difference.

4. Whereas the C57 black mice show a clear-cut sex difference in their susceptibility to N,N-dimethyl-p-aminoazobenzene, this is not true for 4'-amino-2,3'-azotoluene, which actually induced period required for sarcoma development in mice of the other strains, but none of them showed any significant reaction at the site of injection. The lesions in the mice of mixed origin were spindle cell sarcomas; that in the C57 black female mouse was a fibro- and mixed-cell sarcoma.

Liver lesions.—Three males and 5 females of the C57 black strain survived 250 days, but whereas none of the livers of the males showed more than a slight pericellular and/or periporal increase in reticulum and, in 1 mouse, some degree of anisocytosis, the livers of 4 females showed cirrhosis and hepatoma formation, amounting in 1 dying at 330 days to primary liver cell carcinoma. This result demonstrates the pronounced

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mixed</th>
<th>Cha</th>
<th>C57 black</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. mice examined post mortem</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. surviving 250 days</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Sarcoma found at, days</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Liver tumor found at, days</td>
<td>233, M</td>
<td>408</td>
<td>259, 326</td>
</tr>
<tr>
<td></td>
<td>384 M</td>
<td>423 M</td>
<td>327, 403</td>
</tr>
<tr>
<td></td>
<td>452</td>
<td>379</td>
<td>333, 427</td>
</tr>
<tr>
<td></td>
<td>460</td>
<td>432</td>
<td>343, 437 M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>432, 500</td>
</tr>
</tbody>
</table>

M signifies that metastases from the primary tumor were found.

1. hepatic tumors by 515 days in all of 6 males, compared with 3 out of 5 females. The sarcomas induced by either compound were both in female mice.

5. Metastases were found from five 4'-amino-2,3'-azotoluene-tumors, as compared with none in the other experiment (see Fig. 10).

Lesions in Mice Receiving N,N-Dimethyl-p-Aminoazobenzene

Sarcoma.—Only a very few sarcomas were found in mice injected with N,N-dimethyl-p-aminoazobenzene. Law, who also used C57 black mice (12), obtained sarcomas in just over 20 per cent of the mice of this strain. As can be seen from Table I, in these experiments 1 out of 5 female C57 black mice died with a sarcoma at the site of injection after 321 days; the 3 males survived longer than this, but none developed a sarcoma. Among the mice of mixed origin, 2 out of 9 males died at 344 and 379 days respectively, with sarcomas at the site of injection, while none of 3 females developed such a tumor. Several of the male mice of the Cha strain lived much longer than the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mixed</th>
<th>Cha</th>
<th>C57 black</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. mice examined post mortem</td>
<td>8</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>No. surviving 250 days</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Sarcoma found at, days</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Liver tumor found at, days</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>366 M</td>
<td></td>
<td>515</td>
</tr>
<tr>
<td></td>
<td>381 M</td>
<td>483</td>
<td></td>
</tr>
</tbody>
</table>

sex difference in susceptibility in this strain of mouse to the induction of liver tumors by N,N-dimethyl-p-aminoazobenzene; this difference appears to be the same for sarcoma induction, also. But the result here described does not accord with the results either of Law (12) or of Andervont and Edwards (2), who obtained no liver tumors by a similar technic; i.e., subcutaneous injection of an oily solution. However, the difference is probably due to the difference in dosage. Law's mice received a total of 10 mgm., Andervont and Edwards' 45 mgm., whereas the tumor-bearing C57 black mice in the experiments reported here received totals of 172.5, 187.5, 210.0, and 210.0 mgm. respectively. Moreover, Andervont and Edwards killed all their mice at 1 year, whereas hepatoma due to N,N-dimethyl-p-aminoazobenzene was not seen before 382 days in any of our groups. The induction of liver tumors was not confined to the C57 black strain, although no female Cha mice survived more than 208 days and showed only anisocytosis of liver cells. Of 14 male Cha mice surviving 250 days, 3 developed hepatomas (after an average period of
Figs. 5-8.—Livers of stock mice receiving injections of 4'-amino-2,3'-azotoluene:

Fig. 5.—Mixed hepatoma and angiomata in male Chu mouse dying at 515 days after receiving 232.5 mgm. of N,N-dimethyl-p-aminoazobenzene by subcutaneous injection. Mag. X 100.

Figs. 6-8.—Livers of stock mice receiving injections of 4'-amino-2,3'-azotoluene:

Fig. 6.—Male mouse dying at 384 days; total dye, 115 mgm. Extensive bile duct proliferation, and group of pale, xanthoma-like cells. Mag. X 170.

Fig. 7.—Female dying at 84 days; total dye, 30 mgm. Adenomatous type of hepatoma. Mag. X 170.

Fig. 8.—Female dying at 452 days; total dye, 130 mgm. Extensive cholangioma. Mag. X 170.
540 days and total injections of from 232.5 to 247.5 mgm.), and 1 of these had hemangioma mixed with the hepatoma (Fig. 5); a fourth had nodular hyperplasia and hemangiomatous cystic spaces. Others in this group showed anisocytosis and apparent regeneration of liver cells. A fine degree of cirrhosis, either pericellular or perilobular, was a frequent but not essential feature. In the group of mice of mixed origin, both sexes had survivors beyond 250 days and both were prone to liver tumors. Of 10 males and 5 females, 2 of each sex developed hepatoma, the average induction period being 420 days for either cell sarcoma containing giant cells after a total of 75 mgm. of dye had been injected, at 252 days.

Liver lesions.—Few mice of any group surviving 250 days failed to develop liver tumors. A fine, usually pericellular, increase of reticulum appeared early in the livers of mice of all groups and on either diet; in some mice dying later it was considerable, in others absent. Cirrhosis, in fact, was a common but by no means invariable feature, and was not even always present in mice dying with liver tumors.

All except 1 male of the mice of mixed origin surviving 250 days, and also 1 male dying at 233 days, sex. In all cases the amount of dye injected totalled 187.5 mgm. Cirrhosis, pericellular and/or perilobular, was found in nearly all these mice, but in no case was the hepatoma considered malignant, as no metastases were found.

Other sites.—Toxic nephritis, either acute or chronic, was a common feature in all groups of mice receiving this dye. One male mouse of mixed origin, dying at 432 days, had a lung adenoma.

Lesions in Mice Receiving 4'-Amino-2,3'-Azotoluene

Sarcomas.—No sarcomas were found in any of the mice of mixed origin or of the CBA strain. Male C57 black mice likewise failed to develop this type of lesion, but 1 female of this strain developed a spindle cell sarcoma containing giant cells after a total of 75 mgm. of dye had been injected, at 252 days.

Liver lesions.—Few mice of any group surviving 250 days failed to develop liver tumors. A fine, usually pericellular, increase of reticulum appeared early in the livers of mice of all groups and on either diet; in some mice dying later it was considerable, in others absent. Cirrhosis, in fact, was a common but by no means invariable feature, and was not even always present in mice dying with liver tumors.

All except 1 male of the mice of mixed origin surviving 250 days, and also 1 male dying at 233 days,

Fig. 9.—Cavernous angioma in liver of a control C57 black male mouse dying at 497 days. Mag. X 90.

Fig. 10.—Secondary liver cell carcinoma in a perirenal vein in a female stock mouse dying at 381 days; total dye (4'-amino-2,3'-azotoluene), 115 mgm. Mag. X 170.
the bile ducts was seen in both sexes (Figs. 6 to 8) and amounted to cholangioma in 1 female dying at 452 days after 130 mgm. of the dye had been injected (Fig. 8); in no instance was metastasis found, but neoplastic bile ducts merged into neoplastic liver cells with no sharp boundary.

Only 1 Cba mouse receiving the restricted diet survived more than 250 days and its liver exhibited only anisocytosis and a macroscopic,multilocular cyst protruding above the liver surface and filled with clear fluid. Such cysts were seen in 2 male Cba mice fed a full diet and injected with the same dye. Much larger cysts, identical in appearance, have frequently been seen here in the livers of Wistar rats fed N,N-dimethyl-p-aminoazobenzene in either the restricted diet described in this paper or in that described as "low protein" by Miller and his group (15); they were also associated with notable cholangiomatosis and were almost certainly cystic bile ducts that had pushed up under the capsule. The same is probably true in these Cba mice, as moderate bile duct proliferation was seen in several with or without the macroscopic cysts. Of the 5 male and 5 female mice fed rat cake and surviving for 250 days, all the males and 4 of the females developed hepatomas but no secondaries were found. The type of hepatoma varied somewhat from a simple type illustrated in Fig. 4 to a foamy cell type (Fig. 3), or a type recalling embryonic growth (Fig. 2); moreover, the tumors tended to be angiomatous. Increase in reticulum tended to be perilobular rather than perilobular but, where present at all, it was never advanced.

No female and only 1 male C57 black mouse receiving the restricted diet survived more than 250 days. The male died at 515 days with definite hepatoma formation; no secondaries were seen, but there was a lymphosarcoma in the retroperitoneal tissues that had invaded the pancreas. Three of 5 female mice of this strain receiving a full diet died with obvious hepatomas associated with a pericellular increase in reticulum; in the case of the mouse dying at 407 days, the hepatoma was mixed with hemangiomatous cysts. All 5 male mice surviving 250 days on the full diet died with hepatomas; 1, dying at 427 days, had a very large, hemorrhagic liver cell carcinoma, and another, dying at 437 days, had secondary deposits of liver cell carcinoma in 1 kidney and the corresponding ureter. The last male, dying at 500 days, had angiomata mixed with hepatoma in the liver, and also a hemangioendothelioma behind the left kidney. Pericellular cirrhosis was present in all these livers, but bile duct proliferation was not seen.

Other sites.—Acute to chronic toxic nephritis was commonly found in the mice of all strains. One female of mixed origin, dying at 366 days, had adenoma of the lung; another, dying at 423 days, had secondary liver cell carcinoma and also hemangioendothelioma in the lung.

The Influence of the Restricted Diet

The restricted diet proved rather unsatisfactory, relatively few mice surviving more than 250 days. This is also true for control mice that were fed this diet but given no injections. Toxic nephritis was a common finding in all groups on either dye on either diet, but necrosis of the liver was commoner in mice receiving the restricted diet, and the earlier deaths were probably due to a deficiency in the diet that permitted this type of lesion. In any event, there is no evidence that this particular restricted diet promoted the production of liver tumors.

Control Mice on the Restricted Diet

Stock mice as well as those of the Cba and C57 black strains were maintained on the restricted diet as controls. Of 5 male and 5 female stock mice coming to autopsy, 3 males died at 157 days with abscesses in the cecum and septic lesions in the liver and stomach. Five out of 6 dying between 210 and 223 days showed some degree of subacute toxic nephritis, but the last mouse, a male, dying at 342 days, showed no abnormality of the kidney nor of the stomach, liver, or spleen. The livers were generally normal; 3 showed congestion. Stomachs were normal except that of 1 male mouse, dying at 214 days, which showed extensive hyperkeratosis and papillomatosis, one papilloma in the fundus being visible to the naked eye.

Only 2 male Cba mice came to autopsy. The stomachs were normal in both. The one dying at 153 days had a normal liver, and only cloudy swelling in the kidney; the other, dying at 252 days, had normal kidneys; some liver cells showed fatty degeneration, and the spleen contained prominent megakaryocytes.

The C57 black control mice were more interesting. Seven were examined post mortem; 3 males died before 100 days, and 2 of these, dying at 84 days, showed a fine, pericellular cirrhosis. The stomachs and kidneys of these 3 mice were normal. No abnormality of the stomach was seen in the other 4, and it appears that the restricted diet did not induce stomach lesions in either Cba or C57 black mice; the lesions seen in 2 out of 9 stock mice appear to indicate some difference in reaction of the squamous epithelium of these mice, but one C57 black mouse receiving injections of N,N-dimethyl-p-aminoazobenzene, showed a definite multiple papilloma in the forestomach (Fig. 11). Kidney lesions usually did not exceed cloudy swelling; 1 female, dying after 482 days, had granular debris in the tubules and cystic dilation of the first convoluted tubules. The latter mouse also showed collections of...
polymorphonuclear leukocytes in the liver, and presumably had some sort of infection.

Two C57 black mice showed benign spontaneous liver tumors. A male, dying at 456 days, had a cavernous angioma that caused a nodular appearance and led to hemorrhage under Glisson’s capsule, but the growth was very orderly (Fig. 9). The other, a female dying at 523 days, had hepatoma in all lobes; gross examination showed nodules of flabby, vascular tissue a little paler than the normal liver, while the microscopic picture was that of well differentiated, vascular hepatomatous masses with fatty degeneration in many cells. An interesting feature of the hyperplastic nodular zone was the absence of the fibrous capsule from all the blood vessels, which were not arranged in any normal pattern. Little, Murray, and Cloudman (13) reported 5 liver tumors in a total of 875 C57 black mice, of which 2 were carcinomas (in breeding females) and 3 were adenomas (in males). None of these animals died earlier than 542 days. As no illustrations were given, nor any description of the tumors, it is impossible to compare our results with those of Little and his associates. These hepatic lesions differed both macroscopically and microscopically from any seen in the livers of mice receivingazo dye injections, which leads us to believe that all the tumors reported in this paper were induced by the azo dyes administered and were not spontaneous.

**SUMMARY AND CONCLUSIONS**

1. Stock mice injected subcutaneously with 2'-amino-4,5'-azotoluene in olive oil solution, up to 472 days and a total dosage of 67.5 mgm., developed no neoplastic lesions.

2. Similar mice injected subcutaneously with p'-aminooazobenzene in arachis oil solution, up to 626 days with a total dosage of 192.5 mgm., developed no tumors at any site, either on an adequate diet or on a diet restricted in protein and probably in riboflavin.

3. 4'-Amino-2,3'-azotoluene and N,N-dimethyl-p'-aminooazobenzene have been injected subcutaneously in arachis oil solution into stock mice, Cba mice, and C57 black mice of both sexes. Hepatoma was induced with either dye in mice of either sex of all three genetic types (except male stock mice receiving the latter dye). Sarcoma at the site of injection was rare in stock and C57 black mice, and was never seen in Cba mice. Lung adenoma was found in only 1 female stock mouse. Hemangioendothelioma was found in a few stock and C57 black mice; simple hemangioma also was found.

4. In mice of mixed origin and also in mice of the Cba and C57 black strains, 4'-amino-2,3'-azotoluene proved much more carcinogenic than N,N-dimethyl-p'-aminooazobenzene for the liver, when administered in oily solution by the subcutaneous route.

5. No sex difference in susceptibility was observed either in stock mice or in Cba mice with these latter dyes, or in C57 black mice with 4'-amino-2,3'-azotoluene; liver tumors were obtained in female C57 black mice, and not in males, with N,N-dimethyl-p'-aminooazobenzene.

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