The Intracellular Distribution of Protein, Nucleic Acids, and Riboflavin in the Livers of Mice and Hamsters Fed 4-Dimethylaminoazobenzene

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Previous studies have shown that the ingestion of the hepatic carcinogen 4-dimethylaminoazobenzene by rats causes alterations in the levels and intracellular distribution of several constituents of the liver (9-11). Thus, there was an increase in the desoxypentosenucleic acid and protein contents of the nuclear fraction, a marked decrease in the amounts of protein, riboflavin, and pentosenucleic acid in the large granule (mitochondria) fraction, and a large increase in the pentosenucleic acid content of the small granule (microsome) fraction. These changes in the morphological fractions were found long before neoplasms were detectable by gross or microscopic examination of the organ and tended to make the composition of the liver resemble that of the malignant hepatic tumors eventually produced by the dye (11-13).

The number of tumors induced by 4-dimethylaminoazobenzene can be altered markedly by changes in diet, the structure of the dye, or the species used (2, 5, 6, 15), and these variations have been used to outline the relationship of the intracellular changes to the carcinogenic process. Thus, when this dye was fed to rats in a diet containing a level of riboflavin high enough to inhibit strongly the development of liver tumors, no changes from normal were found in the nuclear fraction (9). However, the alterations in the levels of protein and pentosenucleic acid in the large and small granules were the same whether a high or low level of riboflavin was fed with the dye. Similarly, analyses were made on the livers of rats fed a series of seven aminoazo dyes closely related in structure to 4-dimethylaminoazobenzene but with carcinogenic activities ranging from 0 to 12 (activity of 4-dimethylaminoazobenzene = 6 [11, 12]). In general, the effects of these aminoazo dyes on the composition of rat liver were qualitatively the same as those of the parent compound, and the degree of change was approximately proportional to the carcinogenic potency of the derivative fed.

In the present paper these comparisons are extended to the livers of two species, the mouse and the golden hamster, which are highly resistant to the carcinogenic action of 4-dimethylaminoazobenzene. With this agent mice develop tumors only slowly (5, 15) and hamsters not at all (7); hence, one might expect that this aminoazo dye would have a slight to moderate effect on the composition of mouse liver and little or no effect on the composition of hamster liver. Since the reported intracellular distribution of desoxypentosenucleic acid in the livers of C3H mice (18) differs from that obtained in these experiments with an unknown strain of albino mice, comparative data on the fractionation of livers from these two strains are also presented.

METHODS

Adult female albino mice1 and adult male hamsters (Golden Syrian)2 were fed a semi-synthetic diet (6, diet 3) containing 12 per cent of casein and 1.2 mg riboflavin/kg, with or without the addition of 4-dimethylaminoazobenzene, for 4 months. The diet for the hamsters contained 0.06 per cent of dye, the level fed to rats in previous experiments (9, 11), while the diet for the mice contained only 0.045 per cent, since the mortality is high when greater amounts are fed (2). The adult C3H mice3 were of mixed sex and were maintained on a stock diet4 without the addition of dye.

Duplicate or triplicate fractionations were made in each case, and six to nine mice or three hamsters were used for each fractionation. The animals were killed with ether, and their livers were immediately perfused in situ with 0.14 M NaCl. The pere-
fusion and all subsequent steps prior to analysis were carried out at 0°–5° C. The livers were pooled, forced through a plastic tissue mincer, and homogenized in 0.88 M sucrose solution (9). Nuclear, large granule, small granule, and supernatant fluid fractions were prepared by differential centrifugation (9, 11).

The nucleic acids were determined according to the method of Schneider (16), and protein was determined gravimetrically (9). Riboflavin was assayed by the acid production of Lactobacillus casei (14). Protein-bound aminoazo dye was determined as previously described (5). For histological study small blocks of each liver were fixed in Mossman's fluid (3), and sections were stained with hematoxylin and eosin (4). In addition, some sections were stained for desoxypentosenucleic acid by the Feulgen method or for iron by the method of Tirmann and Schmelzer (4).

RESULTS

Protein distribution.—The livers of mice fed 4-dimethylaminoazobenzene contained about 30 and 19 per cent more protein in the nuclear and supernatant fluid fractions, respectively, and about 34 per cent less protein in the large granules than was found in the corresponding fractions of the normal livers (Table 1). The alterations in the protein contents of the nuclear and large granule fractions were similar to those found in the livers of rats fed the carcinogen (9, 11), while no change in the level of protein in the supernatant fluid was observed when 4-dimethylaminoazobenzene was fed to rats. Ingestion of the dye had no significant effect on the levels or intracellular distribution of protein in the hamster livers (Table 1).

Distribution of desoxypentosenucleic acid.—There appeared to be little or no change in the desoxypentosenucleic acid content of either mouse or hamster liver following the ingestion of 4-dimethylaminoazobenzene (Table 2). However, the values obtained for the livers from the dye-fed mice are somewhat equivocal, since the figures for the nuclear fractions were always about 25 per cent higher than those for the whole homogenate.

The cause of these anomalous results is not known, but it may be related to the pigment which was contained in the trichloroacetic acid extracts of the nucleic acids from these livers. No pigment has been encountered in the samples from normal livers.

Pentosenucleic acid distribution.—All the cytoplasmic fractions from the livers of mice fed 4-dimethylaminoazobenzene contained less pentosenucleic acid than the same fractions from the livers of control mice (Table 3). These reductions averaged 55 per cent in the case of the large granules, 31 per cent for the small granules, and 19 per cent for the supernatant fluid. The changes in the composition of the granules after dye-feeding were

TABLE 1

<table>
<thead>
<tr>
<th>LIVER FRACTION</th>
<th>ALBINO MOUSE LIVERS*</th>
<th>HAMSTER LIVERS†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Basal+dye</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>131 (123–141)</td>
<td>133 (125–142)</td>
</tr>
<tr>
<td>Large granules</td>
<td>55 (54–55)</td>
<td>53 (50–55)</td>
</tr>
<tr>
<td>Small granules</td>
<td>16 (15–16)</td>
<td>15 (15–16)</td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td>58 (50–57)</td>
<td>63 (61–67)</td>
</tr>
<tr>
<td>Recovery</td>
<td>127 (119–139)</td>
<td>131 (127–140)</td>
</tr>
</tbody>
</table>

* Average and range of three fractionations.
† Each figure refers to one fractionation.

TABLE 2

<table>
<thead>
<tr>
<th>LIVER FRACTION</th>
<th>ALBINO MOUSE LIVERS*</th>
<th>HAMSTER LIVERS†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Basal+dye</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>2.57 (2.43–2.70)</td>
<td>2.48 (2.33–2.77)</td>
</tr>
<tr>
<td>Nuclei†</td>
<td>2.50 (2.19–2.70)</td>
<td>3.05 (2.88–3.27)</td>
</tr>
</tbody>
</table>

* See footnotes to Table 1.
† The other fractions did not contain detectable amounts of this nucleic acid.
similar to those found in rats fed the dye. No decrease in the level of pentosenucleic acid in the supernatant fluid was found following ingestion of the carcinogen by rats (9, 11). The recoveries of pentosenucleic acid in the four fractions from the livers of mice fed the dye averaged only 87 per cent, while an average recovery of 95 per cent was obtained with the livers of normal mice. These the unrationed tissue. However, while this ratio in each fraction of rat liver was essentially unaltered by ingestion of the dye, the ratio of pentosenucleic acid to protein was reduced in all the cytoplasmic fractions of the livers from mice fed the carcinogen. The ratio of pentosenucleic acid to protein in the nuclear fraction from the hamster livers was somewhat higher than the cor-

<table>
<thead>
<tr>
<th>LIVER FRACTION</th>
<th>WHOLE HOMOGENATE</th>
<th>NUCLEI</th>
<th>LARGE GRANULES</th>
<th>SMALL GRANULES</th>
<th>SUPERNATANT FLUID</th>
<th>RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg. of nucleic acid per gram of fresh liver</td>
<td>6.54 (5.55-7.37)</td>
<td>0.76 (0.45-0.91)</td>
<td>1.83 (1.48-1.81)</td>
<td>1.80 (1.48-2.00)</td>
<td>2.05 (1.95-2.21)</td>
<td>6.28 (5.59-6.71)</td>
</tr>
<tr>
<td>(%) recovery</td>
<td>4.64 (4.90-5.58)</td>
<td>0.83 (0.57-1.06)</td>
<td>1.79 (0.56-0.90)</td>
<td>1.22 (1.05-1.35)</td>
<td>1.66 (1.45-1.89)</td>
<td>4.45 (4.15-5.00)</td>
</tr>
</tbody>
</table>

**TABLE 3**

**DISTRIBUTION OF PENTOSENUCLEIC ACID IN THE LIVER FRACTIONS**

**TABLE 4**

**DISTRIBUTION OF RIBOFLAVIN IN THE LIVER FRACTIONS**

lower recoveries in the case of the livers from the dye-fed mice may be the result of interference with the orcinol reaction for pentose by the pigment discussed above. The pentosenucleic acid content of hamster liver and its intracellular distribution were not altered by feeding 4-dimethylaminobenzene (Table 3). The ratio of pentosenucleic acid to protein in the fractions from normal mouse liver was similar to that found in normal rat liver (11, 12). In both species the ratio of pentosenucleic acid to protein was lower in the nuclear and supernatant fluid fractions and higher in the small granules than in responding fraction from the livers of the other two species, while the ratio of these two constituents in the cytoplasmic fractions tended to be lower in the case of hamster liver than for the other species.

**Riboflavin distribution.**—The livers from mice fed 4-dimethylaminobenzene contained only 66 per cent as much riboflavin per gram of fresh tissue as normal mouse liver (Table 4). As in the case of rats ingesting the carcinogen (9, 11), the greatest decrease in riboflavin content occurred in the large granule fraction. With the hamsters, on the other hand, there was no reduction in the amount of

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*See footnotes to Table 1.
† Sample lost.
hepatic riboflavin following ingestion of the dye. In fact, the large granules from the livers of hamsters fed the dye appeared to contain more riboflavin than the same fraction from normal livers.

With both of these species and the rat (11, 12), the ratio of riboflavin to protein was greater in the large granule fraction and much lower in the supernatant fluid than in the unfractionated tissue. However, unlike the findings with the rat, the reduction of the riboflavin content of the large granule fraction of mouse liver under the influence of the carcinogen was relatively greater than was the reduction in protein content, so that the ratio of riboflavin to protein was reduced in this fraction.

Protein-bound dye content of livers.—In confirmation of earlier results (7), no protein-bound dye was found in the livers or in any liver fraction from hamsters fed the dye. While a low level of bound dye appeared to be present in the livers of mice fed 4-dimethylaminoazobenzene, the nonspecific absorption of the extracts containing the aminoazo dyes released from the protein was so great that the analyses had no quantitative significance. This nonspecific absorption may have been derived from the pigment seen in the livers microscopically, and the increase in the dye-feeding period from 2 months (5) to 4 months may account for the greater interference in this study.

Histological observations.—On microscopic examination the livers from the mice fed the basal diet appeared essentially normal. However, in a few sections a slight fatty infiltration was seen throughout the lobules, and occasionally slight round cell and polymorphonuclear leukocytic infiltrations were found in the portal areas. Each of these changes occurred to a greater degree in the livers from mice fed the dye. In addition, there was a slight proliferation of the bile ducts following dye-feeding; these ducts appeared normal except that they were larger than the normal ducts of the portal triad. About one-half of the livers from the mice fed the carcinogen showed no necrotic areas; small areas of necrosis, which involved up to about 5 per cent of the parenchymal mass, were found in the other sections. In some instances the necrotic cells, though morphologically intact, had lost all the basophilic staining qualities of the cytoplasm and nucleus, and took a uniform pink stain with eosin. In other areas the cells had undergone partial to complete dissolution, leaving a reticulum framework which held an inflammatory exudate with cellular debris and polymorphonuclear leukocytes. While the nuclei of normal mouse liver varied considerably more in size than those of either normal rat or hamster livers, the variation in nuclear diameter was even greater in the livers from mice fed the aminoazo dye. An occasional nucleus in the normal mouse livers contained small acidophilic inclusion bodies, and similar bodies were found in far greater numbers in the nuclei of the livers from mice fed the carcinogen. These bodies were round, generally hyaline in appearance, and varied greatly in size. Some were as small as 1 μ in diameter, but others were noted which nearly filled the nucleus (Fig. 1). Occasionally, three or four were seen in a single nucleus. The bodies were Feulgen-negative, and did not take the Feulgen stain in the control sections where the acid hydrolysis was omitted. They did not contain histochemically detectable iron.

The livers of all the mice fed the carcinogen contained considerable quantities of brownish, intracellular, granular pigment. This pigment could be seen in nearly every lobule (chiefly in the periphery); it was localized largely in the Kupffer cells, although some was found in the cytoplasm of the parenchymal cells as well. An occasional cell was nearly filled with this pigment, which was probably responsible for the dark brown color of the perfused livers from mice fed the dye and for the difficulties encountered in some of the analyses. This pigment contained histochemically detectable iron (Fig. 2). Neither the pigment nor iron-staining granules were found in the livers from mice fed the basal diet. This iron-containing pigment is apparently similar to the one found by Edwards and White (1) in the livers of rats fed 4-dimethylaminoazobenzene, but it was present in far larger quantities in the livers of mice fed this carcinogen.

No alterations from normal were found in the microscopic appearance of the livers from hamsters fed 4-dimethylaminoazobenzene.

Distribution of nucleic acids and protein in the livers of C3H mice.—In the above experiments with albino mice essentially all the desoxypentosenucleic acid was found in the nuclear fraction, while Schneider, Hogeboom, and Ross (18) found a significant amount of the desoxypentosenucleic acid from C3H mouse livers in the large and small granules. To determine whether this difference might be related to the strain of mouse employed, two fractionations were made of the livers from C3H mice. As seen in Table 5, essentially all the desoxypentosenucleic acid was again found in the nuclear fraction. Apparently, then, the discrepancy in results must be related to differences in technic.

The distribution of protein and nucleic acids in
the livers of C3H mice was similar to that in the livers of the albino mice fed the basal diet (Tables 1–3, 5). However, in these fractionations relatively more pentosenucleic acid was found in the nuclear and large granule fractions and relatively less in the small granule fraction than was reported by Schneider et al. (18). We have also differed from these workers in finding more pentosenucleic acid in the large granules of rat liver. While Schneider and Hogeboom (17) have suggested that the difference may be due to a contamination of the large granule fraction with the small granules in the fractionations carried out in this laboratory, other factors must also be considered. These include differences in the experimental diets, in whether or not the livers are perfused and forced through a tissue mincer to remove most of the nonparenchymal tissue prior to homogenization, and in the concentration of sucrose used.

**DISCUSSION**

Some correlation is evident between the susceptibilities of the livers of rats, mice, and hamsters to the carcinogenic action of 4-dimethylaminoazobenzene and the effects of this dye on the intracellular distribution of protein, nucleic acids, and riboflavin in the livers. Thus, the composition of the livers of hamsters, which are resistant to the carcinogenic action of the dye, was essentially unaltered following ingestion of the dye. On the other hand, 4-dimethylaminoazobenzene caused several changes in the composition of mouse liver, and many of these alterations were similar to those produced by the carcinogen in the liver of the rat, a much more susceptible species (9, 11). Thus, the increase in the protein content of the nuclear fraction and the decrease in the protein content of the large granules were almost as great for mice fed the dye as for rats. Unlike rat livers, however, the livers of mice ingesting the dye contained more protein in the supernatant fluid than livers from mice fed the basal diet. The decrease in the pentosenucleic acid content of the large and small granules following administration of the aminoazo dye was similar to that observed with rats; however, in the case of mouse liver the supernatant fluid also contained reduced quantities of this component when the carcinogen was fed. In both species there was a striking reduction in the riboflavin content of the large granule fraction following the ingestion of the carcinogen. Similarly, the levels of vitamin B₃ in the large granule and supernatant fluid fractions of rat and mouse livers were decreased following the feeding of 4-dimethylaminoazobenzene (10). Thus, following the ingestion of the carcinogen by the mouse, some changes, especially those in the composition of the large granules, were as great as those found in rat liver. However, the mice were fed the carcinogen for 4 months,

<table>
<thead>
<tr>
<th>LIVER FRACTION</th>
<th>PROTEIN</th>
<th>DESOXYPENTOSENUCLEIC ACID</th>
<th>PENTOSENUCLEIC ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>148</td>
<td>2.46</td>
<td>2.41</td>
</tr>
<tr>
<td>Nuclei</td>
<td>51</td>
<td>1.82</td>
<td>1.75</td>
</tr>
<tr>
<td>Large granules</td>
<td>40</td>
<td>1.64</td>
<td>1.66</td>
</tr>
<tr>
<td>Small granules</td>
<td>13</td>
<td>1.74</td>
<td>2.03</td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td>57</td>
<td>1.40</td>
<td>1.78</td>
</tr>
<tr>
<td>Recovery</td>
<td>141</td>
<td>2.27</td>
<td>2.58</td>
</tr>
</tbody>
</table>

* Each figure refers to one fractionation.

while the changes noted for rats were obtained after only 1 month of dye feeding.

The intracellular distribution of protein, nucleic acids, and riboflavin in the normal livers of the three species is quite similar. The desoxypentosenucleic acid was found exclusively in the nuclear fractions of all three species, and the absolute levels were similar. The hamster livers contained a lower level of pentosenucleic acid in the unfractonated tissue than either rat or mouse liver, but the intracellular distribution was similar.

It might have been preferable to express the data in terms of units per cell or per nucleus (8). However, some of the experiments in this report were carried out before nuclear counts were routinely made, and the rest of the data in this series have been reported in terms of units per gram of fresh tissue.

**SUMMARY**

1. Sucrose homogenates of the livers from albino mice or from hamsters fed either a semi-synthetic diet or the same diet plus the hepatic carcinogen 4-dimethylaminoazobenzene were separated by differential centrifugation into nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. Homogenates of the livers of C3H mice fed a stock diet were similarly fractionated. The unfractonated homogenates...
and the four fractions were analyzed for protein, nucleic acids, and riboflavin.

2. The livers from hamsters, which are refractory to the carcinogenic action of this aminoazo dye, were unaffected following ingestion of the compound for 4 months, except that there was an increase in the riboflavin content of the large granule fraction.

3. Mouse liver, which is slightly susceptible to the carcinogenic action of 4-dimethylaminoazobenzene, was altered considerably by the ingestion of this aminoazo dye for 4 months. There were decreased levels of protein, pentosenucleic acid, and riboflavin in the large granules comparable to those previously found in rat liver. However, the increase in the protein content and the decrease in the pentosenucleic acid content of the supernatant fluid were not found in liver from rats fed the dye.

4. The intracellular distribution of protein, nucleic acids, and riboflavin was essentially the same in normal mice, rats, and hamsters.

REFERENCES


12. ——. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. II. 3'-Methyl-, 2'-Methyl-, and 2-Methyl-4-dimethylaminoazobenzene, 3-Methyl-4-monomethylaminoazobenzene, and 4'-Fluoro-4-dimethylaminoazobenzene. Ibid., 10: 15-27, 1950.


Fig. 1.—Typical liver section from mice fed 4-dimethylaminoazobenzene for 4 months. Many nuclear inclusion bodies are present; five inclusion bodies are evident in this field, and one of these nearly fills a nucleus. Hematoxylin and cosin stain. X 600.

Fig. 2.—Mouse liver section showing the distribution of iron-containing pigment (black deposits in photograph) in the cells of animals fed the dye. Tissue stained by the method of Tirmann and Schmelzer (4). X 180.
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