Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes*

I. 4-Aminoazobenzene, 4-Dimethylaminoazobenzene, 4′-Methyl-, and 3′-Methyl-4-Dimethylaminoazobenzene

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When the hepatic carcinogen 4-dimethylaminoazobenzene is fed to rats the intracellular composition of the liver is altered considerably (6, 7). After four weeks there is an increase in the desoxypentosenucleic acid and protein contents of the nuclear fraction, and a marked decrease in the amounts of protein, riboflavin, and pentosenucleic acid in the large granules (mitochondria). A decrease in the pentosenucleic acid level also occurs in the small granules (microsomes), and protein-bound aminoazo dye is found in each fraction, with the highest concentration occurring in the supernatant fluid (particles not sedimented at 19,000 × g). These alterations in intracellular composition are even more exaggerated in the hepatic tumors induced by the dye (7).

If any of these changes are related to the carcinogenic activity of the dye, one might expect that other aminoazo dyes which are either more or less active than 4-dimethylaminoazobenzene would produce similar changes to a greater or lesser extent, respectively. On the other hand, if the changes are only manifestations of the general toxic properties of aminoazo dyes, one would expect little or no correlation between carcinogenic potency and the observed changes in intracellular composition. In the present study three aminoazo dyes closely related to 4-dimethylaminoazobenzene were fed to rats and their effects on the intracellular composition of the liver were compared. The three dyes selected, 4-aminoazobenzene, 4′-methyl-4-dimethylaminoazobenzene, and 3′-methyl-4-dimethylaminoazobenzene, have carcinogenic activities of 0, less than 1, and 10 to 12, respectively, as compared with the arbitrarily chosen activity of 6 for the reference compound 4-dimethylaminoazobenzene (5).

METHODS

These experiments were carried out in two separate but identical series. For each series 5 groups of male rats[1] weighing 180 to 200 gm. were fed *ad libitum* a semi-synthetic diet (4, diet 3) containing 1.2 mgm. of riboflavin per kgm. One group was fed the basal diet (no dye added) while the others were fed diets containing 0.06 per cent of 4-dimethylaminoazobenzene or equimolar levels of one of the other dyes. Four weeks after the rats were placed on the experimental diets the group receiving 3′-methyl-4-dimethylaminoazobenzene was killed, and the remaining groups were killed at 2 day intervals in the order of decreasing carcinogenicities of the dyes fed.

For each fractionation the livers of 3 rats were perfused *in situ*, forced through a plastic tissue mincer, and homogenized in 0.88 *M* sucrose as previously described (6, 7). The fractionation procedure was the one used previously (6, 7) except that the large and small granules were sedimented in an improved rotor[2] (designed in collaboration

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[1] Obtained from the Holtzman Rat Company, Madison, Wisconsin.

[2] This rotor fits the spindle on the high speed attachment sold by the International Equipment Co., Boston, Mass. It is made of a magnesium alloy and has an outside diameter of 6½ inches and an over-all height of 4⅜ inches. It holds twelve ⅜ inch × 3⅜ inches plastic tubes, each with a capacity of 13 ml., at an angle of 25° from vertical. The distance from the axis to the geometrical center of the fluid is 6.5 cm. when the tube is filled and the rotor is revolving. It was made by Joseph Grebmeier and Sons, P.O. Box 235, Menlo Park, California.

with Dr. W. C. Schneider). The large granules were still sedimented by applying a force of 19,000 × g for 10 minutes, but since a centrifugal force of 25,000 × g was obtained at the center of the tubes at full speed, 3 hours at the higher centrifugal force were found to be sufficient to sediment the small granules.

Each fraction and the whole homogenate were analyzed for protein, desoxypentosenucleic acid, pentosenucleic acid, riboflavin, and protein-bound

clear protein, but the protein content of this fraction increased by 133 per cent when the more active carcinogen 3′-methyl-4-dimethylaminoazobenzene was fed. The large and small granule fractions from the livers of rats fed 4-dimethylaminoazobenzene contained 32 and 18 per cent less protein than the same fractions of the control livers; even larger decreases of 57 and 24 per cent, respectively, were found when the 3′-methyl dye was fed.

TABLE 1

DISTRIBUTION OF PROTEIN IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4′-ME-DAB†	DAB	3′-ME-DAB
		Milligrams of protein per gram of fresh liver‡			
Whole homogenate	121–123	130–118	111–115	107–106	111–120
Nuclei	15– 16	12– 17	14– 16	18– 16	36– 37
Large granules	39– 41	45– 35	38– 34	26– 29	15– 20
Small granules	16– 18	20– 18	17– 18	12– 16	12– 14
Supernatant fluid	47– 47	51– 46	42– 45	45– 41	41– 49
Recovery	117–122	128–116	111–113	101–102	104–120

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.
† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.
‡ The figures to the nearest whole numbers.

TABLE 2

DISTRIBUTION OF DESOXYPENTOSENUCLEIC ACID IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4′-ME-DAB†	DAB	3′-ME-DAB
		Milligrams of nucleic acid per gram of fresh liver			
Whole homogenate	1.78–1.94	1.79–1.93	1.95–1.75	2.28–2.10	4.78–4.22
Nuclei	1.84–1.89	1.82–1.93	1.85–1.86	2.44–2.01	4.54–3.95
Recovery	1.84–1.89	1.82–1.93	1.85–1.86	2.44–2.01	4.54–3.95
		Milligrams of nucleic acid per gram of protein‡			
Whole homogenate	15– 16	14– 16	18– 15	21– 20	43– 35
Nuclei	123–118	152–113	132–116	135–126	126–107

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.
† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.
‡ The figures to the nearest whole numbers.

aminoazo dye as described previously (6), and the average recoveries of these substances in the fractions were 98, 100, 95, 102, and 98 per cent, respectively, of the amounts found in the whole homogenates.

RESULTS

Protein distribution.—The intracellular distribution of protein in the livers of the rats fed the basal diet was similar to that observed previously (6), and the ingestion of either 4-aminoazobenzene or 4′-methyl-4-dimethylaminoazobenzene did not alter the distribution significantly (Table 1). In these series ingestion of 4-dimethylaminoazobenzene caused little or no increase in the level of nu-

Desoxypentosenucleic acid distribution.—Regardless of the dye fed, the desoxypentosenucleic acid was always found only in the nuclear fraction (Table 2). Neither the ingestion of 4-aminoazobenzene nor 4′-methyl-4-dimethylaminoazobenzene altered the level of this nucleic acid in the nuclear fraction, but the ingestion of 4-dimethylaminoazobenzene caused a slight increase (6) while consumption of 3′-methyl-4-dimethylaminoazobenzene resulted in increases of 169 and 117 per cent in the two series. Similar increased levels of desoxypentosenucleic acid in the livers of rats fed the 3′-methyl dye have been observed by Griffin, Nye, Noda, and Luck (1). In spite of the wide differences between the desoxypentosenucleic acid

contents of the nuclear fractions from the livers of rats fed the various dyes, the level of this nucleic acid per gram of nuclear protein was remarkably constant.

Pentosenucleic acid distribution.—In general, ingestion of any of these dyes resulted in reduced levels of pentosenucleic acid in the large and small granules, but the largest differences were found when the more carcinogenic dyes were fed (Table 3). Thus in the two series the average decreases were 12, 23, 33, and 58 per cent for the large granules and 10, 24, 44, and 43 per cent for the small granules from the livers of rats fed 4-aminoazobenzene, 4'-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, and 3'-methyl-4-dimethylaminoazobenzene, respectively. The only other consistent alterations in the distribution of this nucleic acid were average increases of 144 and 32 per cent, respectively, in the amounts found in the nuclear and supernatant fluid fractions from the livers of the rats fed 3'-methyl-4-dimethylaminoazobenzene. In the cases of the nuclear and large granule fractions the protein and pentosenucleic acid levels were altered to about the same extent, so that in spite of large absolute changes the concentrations of this nucleic acid per gm. of protein were unaltered.

TABLE 3

DISTRIBUTION OF PENTOSENUCLEIC ACID IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4'-ME-DAB†	DAB	3'-ME-DAB
	Milligrams of nucleic acid per gram of fresh liver				
Whole homogenate	5.32–5.70	5.29–4.68	4.09–4.60	4.27–3.57	5.32–4.81
Nuclei	0.58–0.49	0.34–0.49	0.28–0.43	0.47–0.25	1.58–1.06
Large granules	1.74–1.80	1.88–1.24	1.41–1.33	1.31–1.06	0.75–0.76
Small granules	1.78–1.78	1.70–1.52	1.20–1.43	1.04–0.97	1.01–1.04
Supernatant fluid	1.33–1.11	1.38–1.09	1.06–1.08	1.30–0.96	1.72–1.50
Recovery	5.36–5.18	5.30–4.34	3.95–4.27	4.12–3.24	5.06–4.36
	Milligrams of nucleic acid per gram of protein‡				
Whole homogenate	44–46	41–40	37–40	40–34	48–40
Nuclei	39–31	28–29	20–27	26–16	44–29
Large granules	45–44	42–35	37–39	50–37	50–38
Small granules	111–99	85–85	71–79	87–61	84–74
Supernatant fluid	28–24	27–24	25–24	29–23	42–31

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.

‡ The figures to the nearest whole numbers.

TABLE 4

DISTRIBUTION OF RIBOFLAVIN IN THE LIVER FRACTIONS*
(Azo Dye Fed)

FRACTION	NONE	AB†	4'-ME-DAB†	DAB	3'-ME-DAB
	Micrograms of riboflavin per gram of fresh liver				
Whole homogenate	9.9– 9.8	9.8–10.0	9.7–14.4	6.5–7.1	7.0–5.5
Nuclei	0.7– 0.5	0.6– 1.0	1.0– 1.2	0.8–0.6	2.0–1.8
Large granules	5.8– 5.1	4.8– 5.5	5.1– 6.5	3.6–3.9	2.1–1.8
Small granules	1.0– 1.6	1.0– 1.7	1.5–2.2	0.8–1.8	0.9–0.6
Supernatant fluid	1.7– 3.5	2.8– 1.6	2.3– 4.0	1.7–1.4	2.4–1.6
Recovery	9.2–10.7	9.2– 9.8	9.9–13.9	6.9–7.7	7.4–5.8
	Micrograms of riboflavin per gram of protein‡				
Whole homogenate	82– 80	75– 85	87–125	61– 67	63–46
Nuclei	47– 31	50– 59	71– 75	44– 38	56–49
Large granules	149–124	107–157	134–191	139–134	140–90
Small granules	63– 89	50– 94	88–122	67–112	75–43
Supernatant fluid	36– 74	55– 35	55– 89	38– 34	59–33

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† AB = 4-aminoazobenzene.
DAB = 4-dimethylaminoazobenzene.

‡ The figures to the nearest whole numbers.

Riboflavin distribution.—While administration of either 4-aminoazobenzene or 4'-methyl-4-dimethylaminoazobenzene had no consistent effect on the level of riboflavin in any of the fractions, ingestion of either 4-dimethylaminoazobenzene or

its 3′-methyl derivative resulted in decreases of 35 and 65 per cent, respectively, in the level of this vitamin in the large granule fraction (Table 4). The 3′-methyl dye also caused a large increase in the amount of riboflavin in the nuclear fraction. Since in both of these fractions there were approximately proportionate changes in both the riboflavin and protein contents, the concentrations of riboflavin per gm. of protein were not altered.

Distribution of protein-bound dye.—The distribution of protein-bound dye in the livers of rats fed 4-dimethylaminoazobenzene and its 3′-methyl and 4′-methyl derivatives is given in Table 5. In each case 50 to 60 per cent of the bound dye was found in the supernatant fluid fraction, and the proteins of the supernatant fluid and small granules had the highest and second highest concentrations of bound dye, respectively. As observed earlier (2) the bound dye derived from 4-aminoazobenzene absorbed so little light that the necessary correction for non-specific absorption accounted for 30 to 80 per cent of the light absorbed by the extracts from the various fractions. Further, while the bound dyes liberated from the protein contain only 5 to 10 per cent of non-polar dyes in the case of the dimethylamino compounds (2, 3), about 50 per cent of the bound dye derived from 4-aminoazobenzene is non-polar and appears to be 4-aminoazobenzene (2). For these reasons the bound dye analyses on the livers from rats fed the non-methylated dye have not been included in the table.

It is apparent from the table that even the relatively inactive dye 4′-methyl-4-dimethylaminoazobenzene yielded a high level of protein-bound dye. Other studies have shown that, for the dyes studied, the level of bound dye rises to a maximum level and thereafter plateaus or, more often, declines even though the rats continue to ingest dye (3). The rapidity with which the level of bound dye reaches a peak and begins to decline can be correlated with the activities of the dyes. Thus, the maximum levels of bound dye are found in approximately 2, 4, and >16 weeks when 3′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, and 4′-methyl-4-dimethylaminoazobenzene are fed.

DISCUSSION

Our previous work (6, 7) has shown that 4-dimethylaminoazobenzene induces gross changes in the intracellular composition of the rat liver which are continued in an exacerbated form in the neoplastic state. These changes include increases in the amounts of both nucleic acids and of protein in the nuclear fraction, marked decreases in the amounts of protein, pentosenucleic acid, and riboflavin in the large granules, and a fall in the pentosenucleic acid content of the small granules. In the present study the non-carcinogenic dye, 4-aminoazobenzene, and the weak carcinogen, 4′-methyl-4-dimethylaminoazobenzene, did not significantly alter the levels of desoxypentosenucleic acid, protein, or riboflavin in the cell fractions. However, these two dyes did produce moderate decreases in the level of pentosenucleic acid in the large and small granules. The ingestion of the strong carcinogen, 3′-methyl-4-dimethylaminoazobenzene, produced very marked changes in the liver. The levels of both nucleic acids and of protein in the nuclear fraction increased greatly and approached to a considerable degree the levels previously found in the same fraction of liver tumor (7). Furthermore, the levels of pentosenucleic acid, protein, and riboflavin in the large and small granule fractions of these livers were lowered nearly to the levels found in liver tumors (7). While the increase in the level of pentosenucleic acid in the supernatant fluid produced by the highly carcinogenic 3′-methyl derivative was only half of that found in hepatic tumor tissue (7), this is the first dye which has been found to induce this particular change. It is of interest that neither this dye nor any of the other dyes produced any changes which exceeded significantly in magnitude the corresponding changes from normal as found in the tumor tissue.

TABLE 5

DISTRIBUTION OF PROTEIN-BOUND AMINOAZO DYE IN THE LIVER FRACTIONS OF RATS FED VARIOUS AMINOAZO DYES*†

(Azo Dye Fed)

FRACTION	4′-ME-DAB‡	DAB	3′-ME-DAB
	Micromoles×10^2 of dye per gram of fresh liver		
Whole homogenate	1.71–1.82	2.40–3.41	1.91–2.64
Nuclei	0.10–0.05	0.31–0.31	0.41–0.38
Large granules	0.22–0.16	0.38–0.49	0.18–0.14
Small granules	0.22–0.30	0.32–0.62	0.25–0.38
Supernatant fluid	1.01–1.45	1.67–1.88	1.09–1.53
Recovery	1.55–1.96	2.68–3.30	1.93–2.43
	Micromoles×10 of dye per gram of protein		
Whole homogenate	1.55–1.57	2.28–3.22	1.72–2.20
Nuclei	0.85–0.36	1.82–1.90	1.19–1.04
Large granules	0.58–0.48	1.42–1.67	1.08–0.70
Small granules	1.30–1.69	2.76–3.92	2.06–2.68
Supernatant fluid	2.40–3.20	3.76–4.57	2.64–3.14

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† The non-specific absorption in the bound dye extracts was determined by carrying each of the protein samples from the liver fractions of the rats fed the basal diets through the bound dye determination. The absorption of these extracts at 520mμ gave the following corrections to be applied as log $I_0/I \times 10^3$ per 100 mgm. of protein: whole homogenate 57, nuclei 68, large granules 42, small granules 62, and supernatant fluid 35. The corrected values for log I_0/I were converted to micromoles of dye as described previously (3).

‡ DAB = 4-dimethylaminoazobenzene.

There does not appear to be any distinct relationship between the intracellular distribution of the protein-bound dye formed by any of the dyes studied here and their carcinogenic potencies. While it will be necessary to go far beyond these relatively gross morphological boundaries to identify the proteins involved, it is clear that initial cell fractionation will be of great value in any further study.

SUMMARY

1. The livers of rats fed no azo dye or equimolar levels of either 4-aminoazobenzene, 4′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, or 3′-methyl-4-dimethylaminoazobenzene for four weeks were homogenized and separated by differential centrifugation into nuclear, large granule, small granule, and supernatant fluid fractions. The original homogenate and the fractions were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dye.

2. The non-carcinogenic dye, 4-aminoazobenzene, and the weakly carcinogenic dye, 4′-methyl-4-dimethylaminoazobenzene, produced little or no change in the composition of the liver.

3. The highly active carcinogen, 3′-methyl-4-dimethylaminoazobenzene, produced many of the same changes in the composition of the fractions as were observed previously with 4-dimethylaminoazobenzene, a moderately strong carcinogen, except that the changes were greater in magnitude. These changes included increased levels of protein and desoxypentosenucleic acid in the nuclear fraction, decreased contents of protein and pentosenucleic acid in the large and small granules, and a decreased amount of riboflavin in the large granules. In addition this powerful carcinogen increased the level of pentosenucleic acid in the nuclear fraction and the supernatant fluid. Many of the changes induced by feeding 3′-methyl-4-dimethylaminoazobenzene were so great as to make the liver very similar to hepatic tumor tissue in intracellular composition.

4. All of the dyes produced protein-bound aminoazo dye in each fraction of the liver cell. No correlation was evident between the intracellular distribution of bound dye and the carcinogenicity of the dye fed.

REFERENCES

1. Griffin, A. C., Nye, W. N., Noda, L., and Luck, J. M. Tissue Proteins and Carcinogenesis. I. The Effect of Carcinogenic Azo Dyes on Liver Proteins. J. Biol. Chem., **176**: 1225–1235, 1948.
2. Miller, E. C., and Miller, J. A. The Presence and Significance of Bound Aminoazo Dyes in the Livers of Rats Fed *p*-Dimethylaminozobenzene. Cancer Research, **7**:468–480, 1947.
3. Miller, E. C., Miller, J. A., Sapp, R. W., and Weber, G. M. Studies on the Protein-bound Aminoazo Dyes Formed *In Vivo* from 4-Dimethylaminoazobenzene and Its C-Monomethyl Derivatives. Cancer Research, **9**:336–343, 1949.
4. Miller, E. C., Miller, J. A., Kline, B. E., and Rusch, H. P. Correlation of the Level of Hepatic Riboflavin with the Appearance of Liver Tumors in Rats Fed Aminoazo Dyes. J. Exper. Med., **88**:89–98, 1948.
5. Miller, J. A., and Miller, E. C. The Carcinogenicity of Certain Derivatives of *p*-Dimethylaminoazobenzene in the Rat. J. Exper. Med., **87**:139–156, 1948.
6. Price, J. M., Miller, E. C., and Miller, J. A. The Intracellular Distribution of Protein, Nucleic Acids, Riboflavin, and Protein-bound Aminoazo Dye in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. J. Biol. Chem., **173**:345–353, 1948.
7. Price, J. M., Miller, J. A., Miller, E. C., and Weber, G. M. Studies on the Intracellular Composition of Liver and Liver Tumor from Rats Fed 4-Dimethylaminoazobenzene. Cancer Research, **9**:96–102, 1949.