The Levels of Carcinogenic Azo Dyes in the Livers of Rats Fed Various Diets Containing \( p \)-Dimethylaminoazobenzene

Relationship to the Formation of Hepatomas*

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Variations in the composition of the diet influence the rate of appearance of liver tumors in rats fed certain azo dyes. The effective dietary factors and possible mechanisms involved have been reviewed by Rusch, Baumann, Miller, and Kline (14); as yet no generally accepted explanation has been advanced either for the mode of carcinogenic action or for the means by which diet affects it.

It has been shown that the rate of appearance of tumors of the skin or subcutaneous tissues, induced by carcinogenic hydrocarbons, is directly dependent on the dosage of carcinogen applied. Furthermore, there is evidence that the same relationship may be valid for the induction of hepatomas by means of azo dyes. It may be that variations in the composition of the diet increase or decrease the rate of appearance of hepatomas by increasing or decreasing the level of azo dyes in the liver.

Since the azo dyes are fat soluble, one way in which diets might produce differences in dye levels in the liver is through their effect upon the lipid content of the liver. Any diet tending to increase liver lipids might lead to high concentration of azo dyes in the liver, and in this way augment the formation of hepatomas. Consideration of the various dietary conditions known to affect the formation of induced hepatomas suggests that at least some of the diets augmenting tumor formation would tend to increase liver lipids. Indeed, Gyorgy, Poling, and Goldblatt (2) concluded that the action of casein, in protecting against pathological changes induced by \( p \)-dimethylaminoazobenzene, may be due to its lipotropic activity.

The present experiments were designed to examine the relationship between hepatoma formation and the levels of carcinogenic azo dye and liver lipids in rats fed \( p \)-dimethylaminoazobenzene. Diets known to affect different rates of formation of hepatomas were utilized. Each diet was fed to two groups of rats; one group was employed to determine the influence of the diet on tumor incidence, the other the effect of the diet on the level of carcinogenic azo dye and lipids in the liver.

PROCEDURES

The diets are shown in Table I. Diets g 71 and g 72, brown rice diets, differed in that the latter contained 15 per cent brewer's yeast. Diets g 73, g 74, and g 75 differed in the levels of protein (casein), fat (partially hydrogenated oils) and carbohydrate (cornstarch), but were designed to supply equal amounts of salts and vitamins relative to equal caloric intakes. Diets g 73 and g 74, contained equal amounts of fat but differed in protein content and, reciprocally, in carbohydrate. Diets g 74 and g 75 contained equal amounts of protein with respect to equal caloric intakes but differed in the amounts of fat.

Diets g 71 and 72 were utilized because of the known effect of supplemental yeast in retarding the formation of liver tumors induced in rats fed \( p \)-dimethylaminoazobenzene in a rice diet (17). Similarly, available data (5, 12) indicated that the rate

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TABLE I: COMPOSITION OF DAILY RATIONS

<table>
<thead>
<tr>
<th></th>
<th>Brown rice diets</th>
<th>Partially purified diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g71</td>
<td>g72</td>
</tr>
<tr>
<td>Grams of food per rat per day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ground brown rice</td>
<td>15.68</td>
<td>13.28</td>
</tr>
<tr>
<td>Brewer's yeast</td>
<td>2.4</td>
<td>2.4</td>
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<tr>
<td>Olive oil</td>
<td>.24</td>
<td>.24</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>.08</td>
<td>.08</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Calculated data*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein—%</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Fat—%</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Riboflavin µg.m. per gm.</td>
<td>0.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Calories</td>
<td>58</td>
<td>58</td>
</tr>
</tbody>
</table>

* Brewer's yeast—Anheuser Busch's Strain K Yeast; Casein—Borden's Labco Vitamin Free; Salts—Wesson (18); Kremax—Armour's partially hydrogenated cottonseed-soybean oil. The caloric values, the protein and fat, and the riboflavin of the diet were computed from the manufacturers' analysis and from "Tables of Food Composition," U.S. Department of Agriculture, 1945, Misc. Publication No. 572.

The same rations containing p-dimethylaminoazobenzene were also fed to groups of 5 rats for 8 weeks. During the last week, the rats were sacrificed and the livers of the individual animals analyzed for total lipids, cholesterol, lipid phosphorus, and azo dyes. The rats were not deprived of food before being sacrificed. It was assumed that by the eighth week, the livers would have attained a relatively representative or average state with respect to metabolism of liver lipids and the azo dyes, and, on the basis of available reports, would not yet have developed any striking structural changes or neoplasia.

Liver lipids.—The animal to be analyzed was lightly anesthetized with ether and then decapitated; this resulted in fairly effective exsanguination of the hepatic tissue. Pieces of liver weighing 3 to 4 gm. were cut from the several lobes (to insure adequate sampling), weighed to the nearest centigram, then ground with sand and 10 cc. of hot acetone in a mortar. This was transferred quantitatively with the aid of 10 cc. of acetone to a 100 cc. flask where it was further extracted with two successive portions of ethyl ether and once with petroleum ether (Skellysolve F). The successive extracts were filtered through a cotton-plugged funnel into a second 100 cc. flask. The flask containing the combined extracts was covered with a watch glass and placed in a water bath at 65 to 75° C. until the odor of acetone was no longer detectable. One drop of 50 per cent KOH was added to the residue which was then extracted 3 times with 5 to 10 cc. portions of petroleum ether. The combined petroleum ether extracts were collected in a weighed 50 cc. flask and all but the last few milliliters evaporated off on a water bath. The extract was dried to constant weight in a vacuum desic-
cator over P₂O₅, under CO₂ at 40 to 50° C.; the flask was reweighed to determine the total lipids. As soon as possible (generally within 5 minutes after removal from the vacuum desiccator), the lipids were redissolved in petroleum ether. Aliquots of this solution were employed to determine lipid phosphorus by the method of Fiske and Subbarow (1), and cholesterol by the method of Schoenheimer and Sperry (15).

The average deviation of duplicate samples was 5 per cent. With respect to recovery, the overall efficiency was 92 per cent to 96 per cent compared with lengthier and more vigorous procedures such as extraction in a soxhlet with ethanol, chloroform and ether, or ether extraction of the alkali-digested tissue.

**Azo dyes.**—The analysis of the livers for azo dye was based on the following considerations: Miller and his associates (11) have shown that p-dimethylaminoazobenzene (DAB) is demethylated in vivo to p-monomethylaminoazobenzene (MAB) and p-aminoazobenzene (AB). This demethylation also occurs in vitro in the diets themselves (4). DAB and MAB are equally carcinogenic when fed to rats while AB is a relatively feeble carcinogen (8, 16). The analytical method employed in the present experiment determined the total azo dye (DAB, MAB, and AB), and AB alone; the difference between these 2 determinations represents the sum of DAB and MAB, that is, the total carcinogenic azo dye.

DAB, MAB, and AB may be quantitatively extracted from petroleum ether solutions by strong HCl in ethanol or water, forming red to orange-red solutions. The absorption of light by such solutions (containing less than 3 mgm. of the dyes per cc.) follows Beer's Law, both with respect to changing concentrations and to the additivity of the optical densities of the individual dyes, in the light range from 500 to 520 mµ. In equal concentrations DAB and MAB have very nearly equal optical densities (7). AB diazotizes and couples with α-naphthol to form a purple colored bis-azo dye which in alkaline alcohol solutions has maximal light absorption at 580 mµ; at this wave length the light absorption follows Beer's Law. By this reaction AB can be determined without interference by DAB or MAB. These two procedures afford a means of determining the amount of DAB and MAB, the carcinogenic azo dyes, in a solution containing all 3 dyes.

By successive extraction with acetone and ether, the azo dyes added to liver are quantitatively extracted along with the hepatic lipids. The solvents can be removed from this extract and the azo dyes and lipids dissolved in petroleum ether. The azo dyes in this petroleum ether solution cannot be directly determined by HCl extraction or diazotization and coupling because of marked interference by phospholipids: As little as 2 mgm. of phospholipid per cc. of solution reduces the amount of DAB or AB extracted by HCl by more than 50 per cent; a smaller but still considerable interference is observed in the diazotization and coupling reaction for AB. Other petroleum ether soluble substances present in animal tissues did not interfere.

The azo dyes can be quantitatively separated from the lipids (including the interfering phospholipids) by chromatography. When a petroleum ether solution of the azo dyes and liver lipids is filtered through a short column of an acid earth adsorbent, the azo dyes are adsorbed as an orange-red to red crust at the top of the column whereas only small, noninterfering amounts of the lipids remain on the column. The dyes are readily eluted from the column by means of a mixture of ethanol and petroleum ether; the eluate can then be quantitatively analyzed. Extracts of materials low in phospholipid, such as urine or inguinal depot fat, can be analyzed for the azo dyes without use of an adsorption procedure.

The details of the analytical methods employed in the present study were as follows:

**Reagents:**
1. Petroleum ether. Skellysolve F. (b.p., 30-60° C.)
2. 1:1 Petroleum ether-ethanol. Mixture of equal parts 95 per cent ethanol and petroleum ether.
3. Concentrated HCl
4. 1N HCl
5. 0.1% NaNO₂ in water, freshly prepared.
6. 0.05% — Naphthol in 1N NaOH, freshly prepared.
7. Lloyd's Reagent, a fine mesh aluminum silicate, employed as the adsorbant.

**Apparatus:**
1. Colorimeter. Cenco-Sheard spectrophotometer, employing test tubes (11.2 mm. i. d.) as cuvettes.
2. Calibrated centrifuge tubes, 15 ml. size.
3. Adsorption columns. The columns of Lloyd's Reagent were prepared in funnels of the type used with Gooch crucibles; the funnels have a bowl capacity of 30 ml. and stems 6 mm. by 10 cm.; the tip of the stem is constricted to a 2 mm. opening. Columns 8 to 13 mm. high were prepared in the stem by plugging the tip with cotton and tamping down the
Procedure.—4 to 5 grams of liver, cut from the several lobes and weighed to the nearest centigram, was subjected to the treatment described for determination of lipids through the stage of redissolving the dried lipid extract in petroleum ether. The column of Lloyd's Reagent was wetted with petroleum ether and the lipid extract filtered at the rate of 1 to 2 cc. per minute; the column was then washed twice with 10 cc. portions of petroleum ether. While still damp, the column was transferred to a 15 cc. centrifuge tube where it was eluted successively with 5 and 4 cc. portions of 1:1 petroleum ether-ethanol. The eluates were collected in a 15 cc. calibrated centrifuge tube.

The combined eluate was diluted to 9 cc. with 1:1 petroleum ether-ethanol; 3 cc. was transferred to a second calibrated centrifuge tube for determination of AB, while the remaining 6 cc. was used to determine the total azo dye (DAB, MAB, and AB).

The 3 cc. aliquot was diluted to 4 cc. with 1:1 petroleum ether-ethanol; 0.3 cc. of 1N HCl and 0.1 cc. of 0.1 per cent NaNO₂ were added and the tube shaken well. After the phases separated, sufficient ethanol was added to make the alcohol phase 2.4 cc. After 3 to 4 minutes, 0.6 cc. of the solution of α-Naphthol in 1N NaOH was added, the tube shaken again and the phases separated by centrifugation; the petroleum ether phase was discarded. To determine the amount of AB present in the aliquot, the absorption of light by the alkaline alcohol phase was determined at 580 mμ using a reagent blank. Although the solutions darken in air, the light absorption relative to that of the reagent blank remains constant for at least 24 hours.

To the 6 cc. aliquot, 2 cc. of concentrated HCl was added, the tube shaken vigorously and the phases separated by centrifugation; sufficient ethanol was added to make the acid phase 5 cc. in volume, following which the shaking and centrifugation were repeated; the petroleum ether phase was discarded. To determine the amount of DAB, MAB, and AB present in the aliquot, the absorption of light by the acid-alcohol phase was determined at 510 mμ.

The amount of carcinogenic azo dye (DAB plus MAB) was computed on the basis of the predetermined colorimeter constants of the 3 azo dyes in HCl solution and the colorimeter constant of the diazotized and coupled AB. The optical density of the 6 cc. aliquot is the sum of the optical densities of the DAB, MAB, and AB present. The optical density of the AB alone was calculated from its specific measurement in the 3 cc. aliquot. The difference, considering the volumes of the aliquots, is due to the carcinogenic dyes.

Precautions.—1. The exposure to air of the dried lipid extract containing the azo dyes must be kept to a minimum, since under such conditions the dyes are destroyed by autooxidative changes of the lipids. 2. The petroleum ether extract which is filtered through the column must be free of water; even traces of water cause the Lloyd's Reagent to swell, slowing the filtration or stopping it altogether. 3. While no experiments were performed on the effect of temperature on the diazotization and coupling reaction, it was noted that when the room temperature exceeded 26° C. color development was erratic; therefore, when the temperature was above 25° C. the solutions were cooled to 20 to 25° C. in a water bath.

Limitations of the method.—With amounts of DAB and AB ranging from 4 to 10 μgm. in the presence of one another and separately, added to 4 gm. of liver, the recovery of added dye varied from 80 to 100 per cent; average recovery was 90 per cent. By adding the dyes at successive steps in the procedure, the loss was found to be due to a summation of smaller losses. The average deviation of a series of 15 duplicate determinations, for DAB plus MAB in the livers of rats which had been fed DAB, was 7 per cent; with smaller amounts than 1 μgm. per sample (4 duplicates) the deviations ranged from 2 to 23 per cent; between 1 and 5 μgm. per sample (11 duplicates) the deviations ranged from 0 to 10 per cent. The tissue blank,—i.e., apparent azo dye in the livers of animals that had not been exposed to p-dimethylaminooazobenzene,—was less than 0.07 μgm. per gm. of tissue. In presenting the data no correction was made for average losses due to the method or for tissue blank.

The method of extraction is not applicable to analysis for AB in the blood of animals which have been injected with or fed p-dimethylaminooazobenzene. When AB is added to samples of blood, plasma, or liver it is recovered in near quantitative amounts; on the other hand, the extraction procedure described here does not appreciably extract AB from the tissues of animals which have been ingesting DAB. Less than 0.2 μgm. of AB per cc. was extracted from the blood of rats in contrast to 7 to 35 μgm. of AB per cc. extracted from the
blood of the same animals when the extraction procedure described by Miller and Baumann (7) was used.

These authors report AB at levels of 0.07 to 2.0 μgm. per gm. of liver in rats ingesting 0.06 per cent DAB, while our method never detected more than 0.3 μgm. per gm. of liver in comparable animals. Whether this striking difference is due to the relative efficiencies of the methods in extracting AB from the liver itself or from the residual hepatic blood is not known. If it is due to the latter, our extraction procedure may have the advantage of eliminating residual hepatic blood as a possible interfering factor in the determination of AB in liver. At any rate, quantitative extraction of AB is not necessary for the quantitative determination of the carcinogenic azo dyes, DAB and MAB.

RESULTS

Hepatoma formation.—The effects of the diets on body growth, food intake, and hepatoma formation are shown in Table II. At 6 months (the end of the experiment) the incidence of hepatomas in the 5 groups ranged from 25 to 96 per cent. The group fed brown rice supplemented with 15 per cent yeast developed 37 per cent hepatomas in comparison with 96 per cent in the group fed brown rice alone, a statistically significant retardation.

The following differences were found among the 3 groups given the rations prepared from partially purified constituents: Of the two groups fed diets containing moderate levels of B vitamins and 25 per cent fat, the one fed 10 per cent casein developed 58 per cent hepatomas, in comparison with 25 per cent in the group fed 30 per cent casein; the difference is statistically significant. Of the two groups fed diets containing the same amounts of casein, vitamins, and salts, and equicaloric amounts of fat plus carbohydrate, the group fed 2 per cent fat developed 71 per cent hepatomas compared with 58 per cent in the group fed 25 per cent fat.

The rate of appearance and the size of the hepatomas at autopsy in the several groups paralleled the final incidences of hepatomas. Before the experiment was terminated 16 of the animals in group 71 had died from or were sacrificed because of large, usually multiple, hepatic tumors, while no more than 6 rats had died or been sacrificed for the same reason in any of the other groups. While all of the hepatomas observed in group 71 and group 75 were large (1 to 5 cm.), several of the rats in the other groups had hepatomas only 5 mm. in diameter. It may be emphasized that there were no deaths from causes other than hepatomas.

The average daily food intake (and correspondingly the amount of p-dimethylaminoazobenzene) and body growth differed among the groups. Hepatoma incidences in groups 71 and 72 or in groups 73 and 74 were not related to the weight of food and dye ingested; on the other hand, the increase in tumor incidence from 58 per cent in group 74 to 71 per cent in group 75 paralleled the greater (by about 10 per cent) food and dye consumption of group 75.

There was an inverse relationship between the average change in body weight and the incidence of hepatomas. The 2 groups, 71 and 75, developing the higher incidences of hepatomas lost weight while the other 3 groups, 72, 73, and 74, either maintained their weight or grew. The differences in body weight were apparent as early as the fourth week of the experiment.

The differences in food intake and body growth of the several groups were due principally to the p-dimethylaminoazobenzene in the rations rather than to the rations themselves. This was determined by feeding the same diets, but without the dye, to groups of comparable rats for 4 weeks. These rats consumed all their daily rations (58 calories) and the average weight gains for the groups were from 19 to 40 gm. In contrast, the groups fed the dye-containing rations consumed from 47 to 55 calories daily with average weight changes of -34 to +19 gm. for the same period.

Liver lipids and carcinogenic azo dye.—The liv-

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean initial weight, gm.</th>
<th>p-Dimethylaminoazobenzene in diet At 4 weeks</th>
<th>p-Dimethylaminoazobenzene in diet At 4 months</th>
<th>Mean daily food intake</th>
<th>Calories</th>
<th>Rats with hepatomas by 6th month Number Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>g71—Brown rice</td>
<td>280</td>
<td>-10 to -56</td>
<td>70 to -6</td>
<td>13</td>
<td>47</td>
<td>23</td>
</tr>
<tr>
<td>g72—Brown rice, yeast</td>
<td>289</td>
<td>-34 to +24</td>
<td>+6 to +66</td>
<td>15</td>
<td>54</td>
<td>9</td>
</tr>
<tr>
<td>g73—High protein, high fat</td>
<td>288</td>
<td>-18 to +42</td>
<td>+12 to +84</td>
<td>11.5</td>
<td>55</td>
<td>6</td>
</tr>
<tr>
<td>g74—Low protein, high fat</td>
<td>285</td>
<td>-42 to +22</td>
<td>-20 to +14</td>
<td>11.5</td>
<td>55</td>
<td>14</td>
</tr>
<tr>
<td>g75—Low protein, low fat</td>
<td>277</td>
<td>-50 to +6</td>
<td>-58 to +14</td>
<td>13</td>
<td>47</td>
<td>17</td>
</tr>
</tbody>
</table>
Cancer Research

ers of the animals sacrificed during the eighth week for analytical purposes were grossly normal in appearance; in some of the animals fed the brown rice diet, g 71, and the low protein synthetic rations, g 74 and g 75, the livers were slightly paler than normal, and the edges slightly rounded. No association was observed between these changes and any of the analytical findings.

For convenience in considering the data on liver lipids and carcinogenic azo dye, the groups are tabulated in order of increasing incidence of hepatomas. The data on liver lipids are shown in Table III. The only animals revealing increased levels of total lipids were those fed the high fat-low protein ration, g 74. None of the other rations led to any noteworthy increase in hepatic lipids under the conditions of the experiment. Comparable rats fed the same rations for the same period of time, but without \( \text{p-dimethylaminoazobenzene} \), revealed, in general, slightly lower levels of total liver lipids, about 9.5 per cent in those fed the g 74 ration and 3.5 per cent in those fed the other rations.

Comparison of the total liver lipids of the several groups with the incidences of hepatomas, suggests that increased total liver lipids are not essential to increased development of hepatomas. Thus, the retarding effect on the rate of formation of \( \text{p-dimethylaminoazobenzene-induced hepatomas} \), caused by supplementing a brown rice diet with 15 per cent yeast is not dependent on differences in total lipids of the liver. Furthermore, the rats fed g 74, the only ration that produced an increase in total lipids, developed an intermediate percentage of hepatomas. As with total lipids, the liver cholesterol values do not show any association with the formation of liver tumors.

In these experiments the average values for lipid phosphorus appear to be inversely related to the incidence of tumors. The diets resulting in the lowest lipid phosphorus (g 71 and g 75) led to a greater hepatoma development; the ration effecting the lowest incidence of hepatomas (g 73) resulted in an average lipid phosphorus level equivalent to that found in the livers of rats fed a presumably complete ration (Purina fox chow checkers) without \( \text{p-dimethylaminoazobenzene} \).

The levels of carcinogenic azo dye (DAB plus MAB) found in the livers of the 25 individual rats fed the 5 experimental diets are shown in Table IV. The diets resulting in higher average levels of carcinogenic dye in the liver also effected higher incidences of hepatomas; the average values of 0.32, 0.22, 0.46, 0.51, and 0.89 \( \mu \text{gm.} \) of carcinogenic dye per gram of liver correspond to percentage incidences of hepatomas of 25, 37, 58, 71, and 96, respectively. The group mean levels of carcinogenic azo dye differed significantly among themselves (\( F = 3.66 \), \( P \) less than 5 per cent), although there were only 5 rats in each of the groups analyzed for carcinogenic azo dye in the liver, and the individual values overlapped. Thus, in these experiments, there appears to be a direct relationship between the average levels of carcinogenic dye in the liver and the development of hepatomas in rats fed various diets containing \( \text{p-dimethylaminoazobenzene} \).

DISCUSSION

The direct relationship between the level of carcinogenic azo dye in the liver and the rate of hepatoma formation observed in these experiments should be discussed in connection with the data reported by Miller and his associates (9). They have shown that hydrogenated coconut oil, mineral oil, detergents, or high riboflavin in the diet tend to protect rats against the formation of hepatomas induced by \( \text{p-dimethylaminoazobenzene} \). They also examined the effects of these substances on the levels of DAB, MAB, and AB in the livers of rats and concluded that "although the average levels of the dyes in liver and blood were somewhat lower in animals that received protective factors the vari-

**Table III: Liver lipids in rats fed diets containing 0.06% p-dimethylaminoazobenzene for 8 weeks: 5 rats in each group**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean weight of rats</th>
<th>Mean liver weight per 100 gm. rat, gm.</th>
<th>Total lipids, %</th>
<th>Liver lipids based on wet weight</th>
<th>Lipid phosphorus ( \mu \text{gm.} ), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>g73—High protein, high fat</td>
<td>326</td>
<td>3.8</td>
<td>3.8–4.8</td>
<td>.19–28</td>
<td>.26–31</td>
</tr>
<tr>
<td>g72—Brown rice, yeast</td>
<td>310</td>
<td>3.7</td>
<td>3.8–5.3</td>
<td>.15–22</td>
<td>.20–34</td>
</tr>
<tr>
<td>g74—Low protein, high fat</td>
<td>288</td>
<td>3.6</td>
<td>3.8–14.4</td>
<td>.16–24</td>
<td>.33–54</td>
</tr>
<tr>
<td>g75—Low protein, low fat</td>
<td>261</td>
<td>3.3</td>
<td>4.2–5.8</td>
<td>.19–22</td>
<td>.30–45</td>
</tr>
<tr>
<td>g71—Brown rice</td>
<td>270</td>
<td>3.4</td>
<td>3.2–5.7</td>
<td>.16–23</td>
<td>.22–42</td>
</tr>
</tbody>
</table>

* The rats of each group averaged 260 to 270 gm. at the start of the experiment.
ability and overlapping of figures make it difficult to draw any final conclusion”.

In our experiments, the feeding of 5 different diets, each containing 0.06 per cent $p$-dimethylaminoazobenzene, resulted in average hepatic carcinogenic dye levels that differed significantly among themselves. The range of average values—from 0.22 to 0.89 $\mu$g/m. of carcinogenicazo dye per gram of liver—was obtained with a small number of animals, and the individual values overlapped. Nevertheless, if tumor formation in the liver is dependent on the dosage of carcinogen that comes in contact with that organ, it is probable that the wide range of average dye values observed in our experiments was sufficient to effect different rates of hepatoma formation. Actually a relatively direct relationship was observed: the average values of 0.32, 0.22, 0.46, 0.51, and 0.89 $\mu$g/m. of carcinogenic dyes in the liver corresponded to percentage incidences of hepatomas of 25, 37, 58, 71, and 96, respectively.

Although there are a few studies on the dependence of the rate of formation of liver tumors on the dosage of the injected or ingested carcinogen, there is only one (9) in which the hepatic concentration of the dye was also studied. It was found that feeding 2 pairs of rats diets containing 0.06 per cent and 0.03 per cent $p$-dimethylaminoazobenzene, resulted in average hepatic dye levels (DAB plus MAB) of 0.33 and 0.18 $\mu$g/m. respectively—a direct and almost proportional relationship (8). These concentrations of dye in the diet—0.06 per cent and 0.03 per cent—produced 80 and 0 per cent hepatomas, respectively (10).

Evidence points to the dependence of hepatoma formation on the dosage of carcino gen administered. However, if such dependence is to be related to the concentrations of carcino gen in the liver, more basic data on the relationship of dye administered, hepatic dye levels, and rate of hepatoma formation in graded dosage experiments are needed, as suggested by Miller and associates (9). In addition, this should be coupled with further and more extensive work on the effects of varying the diet (all containing the same concentration or amount of carcino gen) on the concentration of carcinogenic dyes in the liver, and subsequent hepatoma formation.

In view of our experiments and those of Miller and co-workers, it appears that in some instances the composition of the diet may affect the rate of appearance of hepatomas through its effect upon the concentration of carcinogenic azo dye in the liver. However, there is no reason to assume that all dietary modifications produce their effects through this means.

The present experiments offer no support to the assumption that total hepatic lipids are a significant factor in the mechanism whereby diet influences the rate of appearance of hepatomas induced by $p$-dimethylaminoazobenzene: Neither the level of carcinogenic azo dye nor the incidences of hepatomas at 6 months were related to the level of total lipids or cholesterol in the liver. There was an inverse relationship between average liver lipid phosphorus and incidence of hepatomas—livers of rats fed diets which induced higher incidences of hepatomas had significantly depressed concentrations of lipid phosphorus.

In confirmation of the results of other investigations, replacing 15 per cent of a brown rice diet with yeast retarded the appearance of hepatomas. Furthermore, the formation of hepatomas was significantly inhibited by increasing the casein content of a partially purified ration from 10 per cent to 30 per cent. Whether increased dietary casein protects against induction of hepatomas by $p$-dimethylaminoazobenzene depends upon several dietary factors such as the levels of casein being compared and the level of B-vitamins, particularly riboflavin (3, 14).

Group g 75 fed the low fat diet developed about the same incidence of hepatomas as group g 74 fed the high fat ration. This is not in agreement with previous conclusions that high dietary fat accelerates hepatoma formation (6, 13). However, the nature of the fat utilized determines the effect; increasing the level of corn oil from 5 to 20 per cent accelerated the appearance of hepatomas, but replacing 5 per cent corn oil with 20 per cent Crisco or lard did not effect any increase in the incidence of hepatomas (6). Our data may be related to these observations since Kremax, a fat similar to Crisco, was used to increase the fat content of the diets.

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**Table IV: "Carcinogenic Dye" (DAB plus MAB) in Livers of Rats Ingesting Diets Containing 0.06% $p$-Dimethylaminoazobenzene for 8 Weeks**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Incidence of hepatomas (Table II)</th>
<th>Micrograms of carcinogenic dye per gram of liver Individual rats</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>g73—High protein, high fat</td>
<td>25%</td>
<td>0.05, 0.14, 0.22, 0.39, 0.80</td>
<td>0.32</td>
</tr>
<tr>
<td>g72—Brown rice, yeast</td>
<td>37%</td>
<td>0.06, 0.16, 0.21, 0.31, 0.33</td>
<td>0.22</td>
</tr>
<tr>
<td>g74—Low protein, high fat</td>
<td>58%</td>
<td>0.06, 0.11, 0.32, 0.20, 1.11</td>
<td>0.46</td>
</tr>
<tr>
<td>g75—Low protein, low fat</td>
<td>71%</td>
<td>0.12, 0.42, 0.54, 0.68, 0.80</td>
<td>0.51</td>
</tr>
<tr>
<td>g71—Brown rice</td>
<td>96%</td>
<td>0.46, 0.67, 1.02, 1.03, 1.25</td>
<td>0.89</td>
</tr>
</tbody>
</table>
SUMMARY

The present investigation was designed to study the possibility that diets affect the formation of hepatomas induced in rats by \( \beta \)-dimethylaminoazobenzene through modifying the level of carcinogenic azo dye in the liver. The possibility that the liver lipid concentration is a significant factor in the dietary modification of carcinogenicity was also considered. A method for the determination of carcinogenic azo dye (\( \beta \)-dimethylaminoazobenzene plus \( \beta \)-monomethylaminoazobenzene) in the livers of rats fed \( \beta \)-dimethylaminoazobenzene is presented.

Using five diets that resulted in incidences of liver tumors ranging from 25 to 96 per cent at 6 months, it was found that neither the percentage of hepatomas nor the level of carcinogenic azo dye in the liver were associated with the level of total lipids or cholesterol of the liver. Hepatic lipid phosphorus was inversely related to the incidence of hepatomas.

In rats fed the five different diets, average levels of carcinogenic azo dye per gram of liver—0.32, 0.22, 0.46, 0.51, and 0.89 \( \mu \text{g} \text{m} \text{g} \) in animals sacrificed at 8 weeks, corresponded to the percentage incidences of hepatomas at 6 months of 25, 37, 58, 71, and 96, respectively. Thus, in these experiments, there appeared to be a direct relationship between the level of carcinogenic dye in the liver (tissue dosage of carcinogen) and the rate of formation of liver tumors.

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Herbert Silverstone

Cancer Res 1948;8:301-308.

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