Protein-bound Azo Dye in the Liver of Hypophysectomized Rats

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Since Miller and Miller first published their classic paper on the protein-bound azo dye in the liver of animals subjected to this type of experimental carcinogenesis (7), many observations have accumulated to correlate this aspect of the process with the carcinogenicity of the particular azo dye (8, 9). Recently, Griffin and co-workers (5) have shown that removal of the pituitary gland blocks carcinogenesis by the azo dye 3'-methyl-4-dimethylaminooazobenzene (3'-Me-DAB), which under the usual conditions produces liver tumors in only 12–15 weeks. No detailed study of the protein-bound azo dye in hypophysectomized rats has yet appeared, although two laboratories made reference to some studies of this nature at a recent conference on experimental hepatomas (4, 6).

The present study stems mainly from a desire to find some parameter of the azo dye carcinogenesis occurring during the early period which may be used to evaluate the effectiveness of hormone preparations for reinstituting carcinogenesis in the hypophysectomized animal. The results obtained from the chronic administration of preparations such as pituitary gonadotrophins or thyroid-stimulating hormone are always open to question, since build-up of antihormones occurs quite soon. Since build-up of protein-bound dye occurs rapidly (7, 9), it was decided to investigate the possible differences in the rate at which this fraction was built up or removed from the liver of intact and hypophysectomized rats. These experiments were designed to show the rate of build-up and removal under the conditions of ad libitum feeding usually employed by Griffin et al. (5, 10, 11) and then to evaluate effects of dietary restriction on this process.

MATERIALS AND METHODS

The rats used in these experiments were Sprague-Dawley males of approximately 150 gm. body weight at the start of the experiment. Hypophysectomized animals were obtained from Hormone Assay Laboratories, Inc. (Chicago) and were allowed to become acclimatized and to lose virtually all the circulating pituitary hormones in a 2-week period during which they were maintained on fox chow with occasional feeding of fresh oranges and glucose-water. For purposes of this experiment, the hypophysectomized animals were those animals which showed marked atrophy of the testes and adrenals, accompanied by a loss of body weight during feeding of the azo dye, and which, at autopsy, revealed no grossly visible remnants of the pituitary. These criteria are not sufficiently sensitive that the presence of very small pituitary remnants would be detected. Approximately 7 per cent of the rats were discarded, owing to an inability to satisfy these criteria.

Male rats of the Sprague-Dawley strain, approximately 150 gm. in weight, were procured at the start of the experimental feeding period to serve as the intact controls.

In the first series of experiments, rats were fed, ad libitum, a semisynthetic diet (3) containing 0.06 per cent 3'-Me-DAB and 2 mg. riboflavin/kg. The shape of the curve for bound dye vs. time of feeding approximates that obtained with high levels of riboflavin (10 mg/kg of diet) in the original description by Miller and Miller (7) after due allowance is made for the greater rate at which the maximum bound dye concentration is reached with 3'-Me-DAB (9) as the azo dye in the diet. Numbered animals were kept in wire-bottomed cages, five animals per cage, during the feeding period. Room temperature was maintained essentially constant by air conditioning. Animals were sacrificed in numerical order at the various time periods selected.

In the second series of experiments, the hypophysectomized animals were maintained on ad libitum feeding as above, and the average consumption of food for the entire group was calculated daily. The intact controls in this series were kept in smaller cages, two rats per cage, and fed the same quantity of food per rat as consumed by the hypophysectomized animals.
Four animals from each group were sacrificed at 3, 7, 10, 14, 18, 22, and 25 days of feeding in the first series of experiments. To estimate the rate of removal of the bound dye from the liver, subgroups of each were made in which feeding of the azo dye was discontinued after 14 days, the approximate time required to reach maximal bound dye concentrations in the intact animal (9), and feeding with the basal diet continued for the remainder of the experimental period. In this subgroup series, four animals from each were sacrificed at 16, 20, 22, 24, and 25 days on the experiment (equivalent to 2, 4, 6, 8, and 11 days after dye feeding was discontinued).

In the second series of experiments involving the pair-fed intact controls, all animals were fed the azo dye diet for 14 days. Five animals from each group were then sacrificed, the remainder of the animals placed on the basal diet for additional feeding without the azo dye, and five more animals from each group were sacrificed following 3 and 7 days of dye-free diet.

At the time of sacrifice the body weight was determined, the animal killed with ether, and the liver immediately perfused with cold 2 per cent sodium citrate (7). Livers were removed, blotted dry, and weighed. In the hypophysectomized animals an examination of adrenals, testes, and sella turcica was made before the carcass was discarded. The left lateral lobe of each liver was then removed for bound dye analysis. The remainder of the liver was used, in part, for histochemical studies, and for a sequence of the fact that the two groups arrived 2 days apart in order to begin the feeding experiment at the same time. From this chart it is evident that the intact animals fare better on the azo dye diet than do the hypophysectomized animals.

Changes in weight for the animals in the ad libitum-fed groups are shown in Chart 1. The difference in starting weight was an inevitable consequence of the fact that the two groups arrived 2 weeks apart in order to begin the feeding experiment at the same time. From this chart it is evident that the intact animals fare better on the azo dye diet than do the hypophysectomized animals.
Liver weight differed in the two groups of animals, with an over-all average of 7.9 gm. for the intact animals fed the azo dye, and increasing to 10.0 gm. for those animals returned to the basal diet after 14 days. The hypophysectomized animals fed the azo diet had an over-all average of 4.6 gm., and in those hypophysectomized animals returned to the dye-free diet after 14 days there was little change in the liver weight—average, 4.8 gm. The average liver weight did not change appreciably from that obtained in the groups sacrificed early in the experimental period or late, except in those intact animals returned to the basal diet.

Chart 2 summarizes the bound dye values for the ad libitum-fed groups. It is apparent that the hypophysectomized animals had bound dye levels only two-thirds as great as the intact animals, except in the latter part of the experiment. The dashed lines represent those animals taken off the dye-containing diet at 14 days. The bound dye was removed from the liver of the hypophysectomized animals much more slowly than in the intact animals, which may be attributed, at least in part, to the increased liver growth noted in the intact animals.

In Chart 3 the log of the mean values for the animals returned to the basal diet has been plotted versus time off the dye-containing diet. This plot is very nearly linear; thus, the slope may be used to estimate the half-life for the bound-dye protein in the two groups. Such a calculation gives a half-life of 3.3 days for the intact animals and 6.3 days for the hypophysectomized animals. As noted above, however, part of the apparent removal of bound dye in the intact group represents simply a dilution obtained during the liver growth in this group, so the half-life for the intact animals is an underestimate.

An attempt was made to correct for this protein dilution, first by correcting for the new protein formed by multiplying the bound dye concentration by the ratio of the liver weight in the intact animals taken off the dye to the average liver weight of the intact animals maintained on the diet. This amounted to approximately a 20 per cent increase in the absolute value but affected the slope very little, since the half-life thus calculated was 3.4 days for the intact animals. If the bound dye concentration in the entire liver was used, the calculated half-life was 3.3 days. Thus, the dilution effect has not materially altered the slope of the curve as plotted in Chart 3. The reason for this seems to be that the increase in liver weight oc-
The calculated half-life for protein-bound dye of 3.3 days in the intact animals is in good agreement with the half-life of approximately 4 days obtained by Miller and Miller in their original bound dye report (7), and a more recently quoted figure of about 3 days (6).

The "free" dye levels (see "Methods") were not measured until the 10th day on the azo dye diets, but averaged 7.8 mmole/gm of liver in the intact animals and 5.5 mmole/gm of liver in the hypophysectomized animals, the level being fairly constant in all groups examined. After 2 days off the dye diet, the free dye was down to 2.7 mmole/gm liver in the intact animals and 1.7 mmole in the hypophysectomized rats. After 4 days off the dye diet, this level had dropped to less than 0.5 mmole/gm of liver in both groups, and after 6 days none could be detected.

An experiment was carried out to evaluate the effect of dietary intake on the observed removal rates for protein-bound dye by means of pair feeding. This experiment is summarized in Table 1. Food intake and weight averages are given from the start of the feeding period until sacrifice. The time indicated is the days off the azo dye diet after 14 days of feeding. Each group thus gave only 3 points for the estimation of the half-life of the bound dye protein; thus, the half-life values tabulated must be considered very coarse estimates. In spite of these limitations that must be cited in discussing the pair-fed experiment, it is apparent that the level of bound dye obtained after 14 days of feeding was less than in the ad libitum-fed intact animals and that some decrease in the rate of removal of protein-bound dye may be achieved by simply restricting the food intake for the intact animals.

From calculations based on the incorporation of labeled amino acids, liver protein half-life values of 6-8 days have been reported by most investigators (1, 12), although values as low as 4 days have been observed (2). From the half-life figures obtained in the present experiments and the work of others, it may be concluded that the turnover of bound dye protein was not noticeably different in either hypophysectomized or normal animals from total liver protein turnover. Since it is known that the level of protein intake has an appreciable effect on the turnover rates of body protein (14), it is still possible that turnover studies in the same animals, with both labeled amino acids and bound dye levels, could reveal significant differences in the two turnover rates.

We conclude that protein-bound dye levels are not sufficiently altered by hypophysectomy that they offer any promise as a sensitive indicator for changes in the ability of the liver to undergo cancerous transformation, at least in terms of the relationship of the pituitary gland to this process. Our data are in complete agreement with the conclusion of Miller and Miller (6), based on their studies of bound dye levels in hypophysectomized rats, that the formation of bound dye may be a necessary, but not sufficient, requirement for the induction of liver tumors by azo dyes. Both the present study and that of Miller and Miller (6) would thus indicate that, if the formation of bound dye is nec-

### Table 1

**Summary of Paired-Feeding Experiment**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. rats</th>
<th>Time* (days)</th>
<th>Av. wt. change (gm.)</th>
<th>Av. liver wt. (gm.)</th>
<th>Av. food intake to time of sacrifice (gm/day)</th>
<th>Liver bound dye concentration† (mmole/100 mg of liver residue)</th>
<th>Estimated $t_1/2$ of bound dye protein (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypophysectomized</td>
<td>5</td>
<td>0</td>
<td>154±116 (–18)</td>
<td>4.4</td>
<td>5.5</td>
<td>16.8±6.4</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>154±119 (–15)</td>
<td>6.8</td>
<td>5.9</td>
<td>6.2±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7</td>
<td>154±124 (–10)</td>
<td>5.7</td>
<td>6.2</td>
<td>6.2±1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>146±129 (–20)</td>
<td>5.3</td>
<td>5.3</td>
<td>25.0±2.6</td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>5</td>
<td>3</td>
<td>141±132 (–9)</td>
<td>6.6</td>
<td>5.9</td>
<td>9.9±1.5</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7</td>
<td>140±137 (–5)</td>
<td>5.5</td>
<td>6.2</td>
<td>7.1±1.5</td>
<td></td>
</tr>
</tbody>
</table>

* Days off dye-containing diet after 14 days of dye feeding.
† Calculated from start of experiment to time of sacrifice.
‡ Expressed as mmole/100 mg of liver residue (see "Methods"), plus standard deviation of the mean.
necessary for liver tumor production by azo dye, then the blockage of this process by hypophysectomy must occur at some stage subsequent to this.

SUMMARY
Protein-bound dye levels have been determined in the liver of normal and hypophysectomized rats fed ad libitum on a diet containing 0.06 per cent 3'-Me-DAB. Bound dye levels were approximately two-thirds as great in the hypophysectomized animals as in the intact animals for periods up to 25 days. The half-life of the bound dye protein, determined after 14 days of dye feeding, was 3.3 days in intact rats and 6.3 days in hypophysectomized rats, fed ad libitum.

Pair-feeding experiments indicated that the level of bound dye could be suppressed in the intact animal by a food intake equivalent to that of the hypophysectomized rats. At the same time, the lower food intake increased the half-life of the bound dye protein.

ACKNOWLEDGMENTS
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8. ———. In Vivo Combinations between Carcinogens and Tissue Constituents and Their Possible Role in Carcinogenesis. Ibid., 12:547–55, 1952.
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