The Metabolism of Benzidine in the Rat

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The establishment of the carcinogenic activity of benzidine by Spitz, Maguigan, and Dobriner (9) necessitates a more complete understanding of the mechanism of benzidine metabolism in the animal body. Little investigation has so far been carried out. Goldblatt (5) has suggested that acetylbenzidine is excreted in addition to the base, and the work of Weber and Heideprim (10) indicates that benzidine is excreted partly unchanged and partly as what they describe as an ethereal sulphate and glucuronide. The formation of these conjugates indicates that benzidine may be oxidized in vivo. Adler (1) has demonstrated a dihydroxy derivative of unknown structure in the urine of rabbits fed benzidine, and Baker (2) has demonstrated that oxidation may take place to give 3,3'-dihydroxybenzidine. In recent investigations on synthetic 3,3'-dihydroxybenzidine, Baker (3) noted a marked similarity between results of tumor formation in animals fed this compound and those fed 2-acetylaminofluorene (4, 13). This suggested to him that the metabolism of benzidine, 2-aminofluorene, and 2-acetylaminofluorene, respectively, might follow a similar, if not parallel, course in the rat.

The present study, part of a series of studies on the metabolism and degradation of benzidine, was undertaken to determine the distribution of benzidine in the rat, the extent of conjugation of the aromatic amino groups, and the rate of recovery of benzidine from tissue and other biological materials.

MATERIALS AND METHODS

Animals.—Male Slonaker rats, bred and maintained in this laboratory, were used. At the start of the experiment they weighed 200–250 gm.

Diet.—Prior to the experiments, the animals were maintained on Ministry of Food rat cake 41, supplemented with green vegetables and Adexolin.1

Preparation and administration of chemicals.—Benzidine, m. p. 128° C. (corr.), was prepared by a zinc reduction of nitrobenzene. This was purified by recrystallization and fractional sublimation.

The chemical was dissolved in 1–2 ml. of warm propylene glycol for intraperitoneal injections, and all animals received 100 mg of the chemical/kg of body weight. The toleration of this dose had been previously established. Following injection, the animals were placed in metabolism cages; water was allowed ad libitum, but food was withheld during the experimental period.

Preparation and extraction of tissues.—The method of Gutman, Kiely, and Klein (6), a modification of that of Westfall (11), and the method of Morris and Westfall (8) were used in the preparation and extraction of tissues and biological material throughout this series. These consisted of homogenizing pooled samples of tissue and extracting with acetone. Following evaporation of the solvent, the residues were washed in a measured amount of hot glacial acetic acid, and the wash liquid was made up to 10 ml. with distilled water. After cooling and filtering, the estimation of diazotizable amino groups was carried out.

Determination of benzidine.—The diazotization method of Westfall (11), as modified by Gutman, Kiely, and Klein (6), in which tissue extracts were diazotized with 0.029 M sodium nitrite and coupled with 0.031 M sodium 2-naphthol-3,6-disulphonate in 10 M aqueous ammonium hydroxide, was used for estimating the presence of benzidine. The optical density (log K) of the resultant dye solution was immediately determined at a wave length of 545 mμ (Chart 1), using a Spekker photoelectric absorptiometer. The readings obtained were expressed in μg. of benzidine by reference to a previously established calibration curve which obeyed Beer's law within a range of 0–50 μg.

The values thus obtained are expressed as “free” diazotizable material. The amino groups...
determined after acid hydrolysis according to the method of Westfall and Morris (12) are referred to as "total" diazotizable material. When benzidine was added to tissue homogenates, 80—100 per cent could be recovered by this method.

RESULTS AND CONCLUSIONS

From the results listed in Table 1, it will be noted that the amounts of diazotizable materials, 4 and 12 hours after administration of benzidine, varied considerably from tissue to tissue. After 4 hours, the concentration of total diazotizable aromatic amino groups in rat tissue ranged from 310 µg/gm in stomach to 11 µg/gm in red cells; and after 12 hours, from 185 µg/gm in small intestines to 5 µg/gm in red cells.

Although all the tissues analyzed contained "free" diazotizable material, additional aromatic amino groups became available after acid hydrolysis. This conjugation of benzidine may be of importance in considering whether acetylation is involved in benzidine metabolism, as has been suspected (5); that the rat is able both to acetylate and to deacetylate aromatic amino groups has been shown by Morris et al. (7) working with 2-acetylaminofluorene. One may presume that a similar mechanism is involved here and that acetylbenzidine is a product of benzidine metabolism.

The percental recovery and distribution of benzidine, measured as diazotizable amino groups, are also shown in Table 1. After 4 hours, 93 per cent of the administered amino groups were accounted for after acid hydrolysis, diazotization, and coupling with R-salt. Approximately 24 per cent were in the conjugated form; 29 per cent of the total amino groups recovered were found in the carcass, 25 per cent in the small intestines, and approximately 5 per cent in the liver. Only 0.5 per cent of the recovered amino groups were found in the urine, compared to over 15 per cent in the contents of the gastrointestinal tract.

Twelve hours after administration of the chemical, 68 per cent of the administered amino groups were recovered, 49 per cent being in the conjugated form. The general decrease in the measurable diazotizable aromatic amino groups in tissue after 12 hours is similar to that found by Gutman et al. (6) working with 2-aminofluorene and by Westfall and Morris (12) working with 2-acetylaminofluorene, although with benzidine the extent of conjugation is very much greater. In most of the tissues there was a decrease in the recovery of injected amino groups at 12 hours, the largest decreases occurring in the stomach contents and carcass.

That a significant level of diazotizable material was maintained in the liver for at least a 12-hour period is of interest, since benzidine, like 2-aminofluorene and 2-acetylaminofluorene, induces tumors in this organ.

The amount of diazotizable amino groups found in the urine after 12 hours was 5 times and that in the contents of the gastrointestinal tract about twice as great as at 4 hours. The marked increase in the urinary level indicates that benzidine is excreted by the urinary tract, as are 2-aminofluorene and 2-acetylaminofluorene. In addition, there is evidence that benzidine is also excreted by the intestinal tract to a great extent, since a portion of the products of metabolism is excreted by the bile to the stomach and intestinal tract.

After 4 hours, 93 per cent of the injected amino groups were recovered, after acid hydrolysis, diazotization, and coupling with R-salt, but only 68 per cent of the injected amino groups were recovered after 12 hours. The chemical reaction by which the amino groups of benzidine were rendered nondiazotizable after 12 hours is not known, but it is suspected that this loss may be due to in vivo oxidation. Experiments designed to isolate the products of in vivo oxidation and their identification are under way.
SUMMARY

The distribution of benzidine, expressed as diazotizable amino groups following a single intraperitoneal injection, has been studied in the rat. The aromatic amino groups were present in both the “free” and conjugated form, the latter amounting to 24 or 49 per cent of the dose after 4 or 12 hours.

By 12 hours, a portion of the injected amino groups are rendered nondiazotizable, since only 68 per cent could be accounted for at this time, as compared to 93 per cent 4 hours after injection.

It is suggested, in the light of the similarity of benzidine and 2-aminofluorene metabolism, that this difference in recovery may be due, in part, to in vivo oxidation.

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REFERENCES


TABLE 1

THE CONCENTRATION AND DISTRIBUTION OF FREE AND TOTAL Diazotizable MATERIAL IN RAT TISSUES

Single Intraperitoneal Injection of 100 Mg Benzidine/Kg of Body Weight

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Free</th>
<th>Total</th>
<th>Free</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/gm</td>
<td>µg/gm</td>
<td>µg/gm</td>
<td>µg/gm</td>
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<tr>
<td>Whole blood f</td>
<td>10.2</td>
<td>35.0</td>
<td>1.0</td>
<td>17.5</td>
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<tr>
<td>Plasma t</td>
<td>32.5</td>
<td>57.0</td>
<td>0.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>6.7</td>
<td>11.0</td>
<td>3.6</td>
<td>15.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>21.2</td>
<td>46.2</td>
<td>1.1</td>
<td>12.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>9.2</td>
<td>15.0</td>
<td>1.5</td>
<td>11.5</td>
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<tr>
<td>Stomach</td>
<td>71.8</td>
<td>210.0</td>
<td>60.0</td>
<td>115.0</td>
</tr>
<tr>
<td>Stomach contents</td>
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<td>15.0</td>
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<td>3.5</td>
</tr>
<tr>
<td>Small intestines</td>
<td>81.2</td>
<td>130.0</td>
<td>85.0</td>
<td>111.5</td>
</tr>
<tr>
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<td>14.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
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<td>11.0</td>
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<tr>
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<td>16.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
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<td>1.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Carcass</td>
<td>31.8</td>
<td>98.5</td>
<td>29.3</td>
<td>92.3</td>
</tr>
</tbody>
</table>

| Total             | 69.2 | 93.3  | 19.30 | 72.4  |

* The analyses were performed on the pooled samples of four rats.
† These figures calculated from 2 ml. of pooled blood.
‡ Not estimated for 4 hours.
§ The miscellaneous sample consisted of brain, pancreas, diaphragm, bladder, gonads, esophagus, heart, thymus, lungs, and adrenals.
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