The fact that benzidine, in the commercial form of the free base, the sulphate, or the hydrochloride, exerts a powerful carcinogenic activity upon the human bladder has been accepted owing to the clinical evidence reported during the past 20 years (4, 7, 9, 13, 16).

However, the importance of ruling out any other compound, or combination of compounds, as the carcinogen in cases of bladder tumors found among workers in benzidine industries, necessitated evidence other than clinical. Attempts to induce experimental tumors with benzidine during that period had been without success (11), and it was not until 1950 that evidence was produced to show that benzidine was carcinogenic in experimental animals. Spitz et al. (17) produced neoplastic changes in rats injected with both pure and technical-grade benzidine, including hepatoma, squamous-cell carcinoma of the sebaceous glands adjacent to the external auditory canal, and adenocarcinoma of the colon. No bladder changes were noted, except in one dog.

In a previous communication, Baker (2), working with a suspected metabolite of benzidine, 3,3'-dihydroxybenzidine, produced liver and bladder tumors in mice, including hepatoma and squamous-cell carcinoma of the sebaceous glands of the external auditory canal, and adenocarcinoma of the colon. No bladder changes were noted, except in one dog.

MATERIALS AND METHODS

Strain.—Male heterozygic rats, derived from the Slonaker Colony and of 12–18 weeks of age at the beginning of the experiment, were housed four to a cage and fed up to a weight of approximately 175 gm. before administration of the chemical began. Sex differences were not studied. Four rats from a group of sixteen used in this experiment were separated for control purposes.

Diet.—A normal basic diet of Ministry of Food rat cake 41 was used throughout this experiment. This was supplemented from time to time with cabbage and cod liver oil. The M.O.F. rat cake 41 consists, roughly, of whole meal flour, 46 per cent; S.S. oats, 40 per cent; fish meal, 5 per cent; dried skimmed milk, 3 per cent; and 1 per cent each of dried yeast, cod liver oil, and salt. Water was allowed ad libitum. The control animals on this diet gained weight and were in excellent condition at the end of 1 year.

Methods of chemical administration.—The synthetic dihydroxybenzidine under investigation was supplied as the hydrochloride by Ciba, Basle, and after conversion to the free base was purified by repeated recrystallisation from absolute alcohol until the melting point was stable at 286° C. This compound oxidizes readily and is not stable above 286° C.

M.O.F. rat cake 41 was ground to a powder in a rotary ball mill and the chemical added to a concentration of 0.125 per cent by weight. This preparation was mixed with sufficient olive oil to mould it into a pellet, and each animal was fed roughly 10–15 gm. once per day. To counteract any disturbance, and since it was desirable that the experiment should run for the maximum length of time, occasional rest periods on a diet free of the chemical were allowed. These were during the 22nd, 27th, and 37th weeks, respectively. On the 24th and 25th weeks the animals were given the diet every other day, and the diet was terminated on the 41st week. The estimated average daily intake of the diet containing the chemical per animal, was of the order of 5–8 gm. per day.

RESULTS

Control series.—The four control animals survived for more than 52 weeks and in the end were sacrificed. No gross pathological changes were noted, and serial sections of all the major organs, including the sebaceous glands of the external auditory canal, were made. No evidence of neoplastic changes was found.

Experimental series.—Survival of the twelve animals under investigation was good, seven rats surviving more than 30 weeks. All these animals carried one or more of the five types of tumors which occurred in the experimental series (Table 1). These were hepatoma, adenocarcinoma of the colon, carcinoma of the sebaceous glands adjacent to the external auditory canal, squamous-cell
carcinoma of the forestomach, and bladder carcinoma. In addition, cirrhosis of the liver, nodular regeneration, and bile duct proliferation were found. Metastases were noted in lung, spleen, kidney, and in adrenal, thyroid, and lymphatic tissue.

Changes in the liver.—Of the seven rats surviving 30 weeks or more, all seven showed evidence of histological changes in the liver. In all the nine experimental animals, fed 3,3'-dihydroxybenzidine, which survived more than 12 weeks, bile duct proliferation, nodular regeneration, and cirrhosis were found. Seven hepatomas were observed in the experimental group, the earliest noted in an animal treated for 20 weeks. These were usually multiple, soft, greyish-white tumor nodules, about 2–3 cm. in diameter. However, in two cases these were the size of a walnut. Histologically, the structure of the malignant hepatoma varied, the cells being irregular and often very large with giant nuclei. Acidophilic inclusion and vacuoles were commonly noted in the cytoplasm of the neoplastic cells and occasionally in the nuclei (Figs. 1, 2). The histological appearance of the metastases found in the lung, kidney, and spleen of some animals reflected the structure of the primary tumors (Fig. 3).

Tumors of the sebaceous glands adjacent to the external auditory canal.—Three of the twelve animals fed 3,3'-dihydroxybenzidine were found to have tumors of the sebaceous glands adjacent to the external auditory canal. These developed after 31, 33, and 41 weeks, respectively, and the animals themselves survived but 2–3 weeks after the first appearance of the tumor. In each case the tumor ulcerated through the skin and into the external auditory canal. When these were dissected out, it was found that they had involved the deep muscles of the neck, which were grossly infiltrated with neoplastic tissue (Fig. 4). Histologically, these were mature keratinized squamous-cell carcinomas (Fig. 5) and appeared to be similar to those reported in the results of investigations with acetylaminofluorene (5) and benzidine (17).

Adenocarcinoma of the colon.—Six animals were found to have intestinal tumors. These tumors were noted in animals dying after 16, 20, 31, 33, 40, and 41 weeks. Histologically, they were typical adenocarcinomas of the colon. As in the case of tumors arising from the sebaceous glands adjacent to the external auditory canal, the occurrence of intestinal tumors coincides with the results of investigations with acetylaminofluorene (5) and benzidine (17).

Gastric tumors.—Histological changes of the forestomach were noted in five of the seven animals surviving for 30 weeks or more. These changes ranged from hyperplasia of the viscus to an invading keratinizing tumor mass. In two animals a keratinizing squamous-cell carcinoma was found which invaded the viscus and involved the serosa of the stomach wall (Fig. 6).

TABLE 1
Type and Location of Tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Rats started</th>
<th>Chemical Hepatoma*</th>
<th>Carcinoma of the colon</th>
<th>Carcinoma of the forestomach</th>
<th>Bladder carcinoma</th>
<th>Sebaceous gland tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental series</td>
<td>12</td>
<td>0.125 per cent 3,3'-dihydroxybenzidine</td>
<td>7(5)†</td>
<td>6(2)‡</td>
<td>2(0)</td>
<td>3(0)</td>
</tr>
<tr>
<td>Control series</td>
<td>4</td>
<td>none</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Figures in brackets indicate number of rats showing one or more metastasis.
† Metastases to lung, kidney, spleen, and lymphatic tissue.
‡ Metastases to kidney and adrenals.
§ Metastasis to thyroid.

DISCUSSION

Tumors of the urinary bladder.—The urinary bladders were completely distended with "Susa" fixative by means of injection and serial sections made. Of the seven rats surviving 30 weeks or more, five were found with evidence of histological changes in the urinary bladder. These ranged from simple hyperplasia of the bladder mucosa to invasive carcinoma of the bladder wall. The hyperplasia varied in intensity but generally proved to be more marked in the animals which survived the greatest length of time. Bladder tumors were found in three of the seven experimental animals under review. These appeared in animals surviving 33, 40, and 41 weeks. Histologically, the appearance of these three tumors differed. One tumor was a simple sessile papilloma (Fig. 7); the other tumors were found to be invasive carcinomas. These tumors, which were invading the bladder mucosa and infiltrating into the bladder wall, were diagnosed as keratinized squamous-cell carcinoma of the bladder (Figs. 8, 9).

The neoplasms reported by Spitz and co-workers (17) indicate that benzidine is a carcinogen, as distant tumors were observed in animals re-
The fate of the benzidine in the animal body has not been fully established, and more work will be required before it is proved that it is benzidine, as such, or a product of its metabolism which is the carcinogenic in cases of tumors of the urinary bladder in man. However, the fact that, of the seven rats in this series surviving 30 weeks or more, three developed bladder tumors, two of which were carcinomas, and two developed gastric carcinoma after prolonged administration of dihydroxybenzidine is of importance. In addition, the animals also developed liver, intestinal, and auditory tumors similar to those reported by Spitz et al. (17), working with benzidine.

As the two gastric tumors may be an indication of its carcinogenicity at the site of administration, we are undertaking further experiments to discover if sarcoma can be produced at the injection site with dihydroxybenzidine.

The similarity of the results obtained by Wilson et al. (20) and Bielschowsky (5), working with 2-acetylaminofluorene, and by Spitz et al. (17), working with benzidine, to the results of this series are all the more striking when one notes that acetylaminofluorene, benzidine, and dihydroxybenzidine all react on the liver, colon, and sebaceous glands of the external auditory canal and, in the case of acetylaminofluorene and dihydroxybenzidine, on the bladder (5, 20, 21).

The metabolism of 2-acetylamino fluorene has been investigated by the usual biochemical techniques as well as with the tracer labeling technique, and it has been established that, in the rat, 2-acetylamino fluorene is deacetylated (19). The work of Morris et al. (15) also suggests that the process of deacetylation is reversible. A metabolite, 2-acetylaminom-7-hydroxyfluorene, has been isolated from rat urine (6).

It has been suggested that the benzidine is partly acetylated and partly hydroxylated (9). Baker (2) has also suggested that oxidation may take place to 3,3'-dihydroxybenzidine.

It is possible that in the rat, with a comparatively short life span compared to that of the human, benzidine metabolites, even with the maximum dosage, would not have time to affect the urinary bladder. First, benzidine may be acetylated and excreted as such to a great extent, and the subsequent oxidation of the acetylbenzidine may take place so slowly or to such a small extent as to preclude a possible effect on the urinary bladder during the life span of the animal. However, dihydroxybenzidine introduced as such, in large amounts, over a long period of time, seemed to exert a more direct influence against the urinary bladder than benzidine. One might conclude it was because of a reduction in the necessary stages of the metabolism that the resulting carcinogenic activity, against the urinary bladder, was brought within the life span of the animal.

2-Acetylamino fluorene, which, when ingested, is subsequently metabolized to 2-acetylaminom-7-hydroxyfluorene, affects the urinary bladder of rats (5, 20) and mice (1). Although 2-acetylamino-7-hydroxyfluorene failed to produce bladder tumors when fed to albino rats (12), it is still possible that it is the process of deacetylation, acetylation, and oxidation which renders 2-acetylamino fluorene carcinogenic to the urinary bladder.

Truhaut (18) feels that “the selective action of bladder carcinogens may well represent the action of urinary metabolites,” and it is interesting to note that two hydroxy derivatives of beta-naphthylamine, 6-hydroxy-2-acetylnaphthylamine and 1-hydroxy-2-aminonaphthol, have been isolated and reported in literature. The carcinogenic activity of the latter compound has been established by Bonser et al. (8). This compound was isolated from the urine of both humans and dogs assimilating beta-naphthylamine.

Leuenberger (14) attributed the carcinogenic activity of aromatic amines to the fact that hydroxylated derivatives result from their metabolism, and it seems that in the case of beta-naphthylamine, benzidine, and possibly 2-acetylamino fluorene, either the hydroxy derivatives themselves are carcinogenic, or the processes involved in their formation. Thus, one may suspect that in vitro oxidation is a prerequisite in the establishment of a condition, or a compound, which results in tumors of the urinary bladder both in experimental animals and the human.

The possibility that oxidation of these aromatic amines in vitro is important to the setting up of a urinary carcinogen should not be overlooked, and it is suggested that thorough and complete investigation of the mechanism of benzidine metabolism should be the next most logical step in the investigation of this carcinogen, which, in many cases, is the causative agent of carcinoma of the bladder.
(16) found in dye-workers and allied trades using or manufacturing benzidine.

SUMMARY

Synthetic 3,3'-dihydroxybenzidine was fed to rats of the Slonaker strain at a concentration of 0.125 per cent in a normal basic diet of Ministry of Food rat cake 41.

Histological changes in the liver, including cirrhosis and hepatomas, adenocarcinoma of the colon, carcinoma of the sebaceous glands adjacent to the external auditory canal, and squamous-cell carcinoma of the stomach were present in a number of animals under investigation. In addition, neoplastic changes in the bladders were observed in three of seven animals surviving 30 weeks or more. These tumors of the urinary bladder consisted of one sessile papilloma and two keratinized squamous-cell carcinomas of the bladder wall.

These findings are discussed in relation to the results of Spitz and co-workers, and the possibility that in vivo oxidation is a prerequisite to carcinogenic activity within the bladder is suggested, in the light of the similarity of results of this series and those reported with 2-acetylaminofluorene and 1-hydroxy-2-aminonaphthol.

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REFERENCES


Fig. 1.—Malignant hepatoma arising in a rat fed S,3'-dihydroxybenzidine for 96 weeks. H & E X100.

Fig. 2.—High power photomicrograph of the hepatoma shown in Figure 1. Note the acidophilic inclusion and vacuoles in both the cytoplasm and nuclei of the malignant cells. H & E X450.

Fig. 3.—Metastases arising in the lung of a rat fed S,3'-dihydroxybenzidine for 36 weeks. In addition, hepatoma and adenocarcinoma of the colon were found. H & E X10.

Fig. 4.—Cross section of the head of a rat bearing a squamous-cell carcinoma arising in the sebaceous gland adjacent to the external auditory canal. Note the hypertrophy of the otherwise normal sebaceous gland on the left (arrow). H & E X10.

Fig. 5.—High power photomicrograph of the tumorous area shown in Figure 4. H & E X450.

Fig. 6.—Carcinoma of the forestomach of a rat fed S,3'-dihydroxybenzidine for 36 weeks. H & E X160.

Fig. 7.—Papilloma of the urinary bladder found after feeding a rat for 40 weeks with S,3'-dihydroxybenzidine. H & E X10.

Fig. 8.—Carcinoma of the bladder arising after 41 weeks of feeding S,3'-dihydroxybenzidine. H & E X400.

Fig. 9.—Squamous-cell carcinoma of the urinary bladder. Rat fed S,3'-dihydroxybenzidine for 38 weeks. H & E X400.
The Carcinogenic Activity of Dihydroxybenzidine Further Investigations

R. Kenneth Baker

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