The Influence of Riboflavin, Pyridoxine, Inositol, and Protein Depletion-Repletion upon the Induction of Neoplasms by Choline Deficiency*

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The production of neoplasms in the livers and in other tissues of rats as a result of prolonged choline deficiency was reported by Copeland and Salmon in 1946 (1). This was confirmed by Engel, Copeland, and Salmon in 1947 (4) and by Staub, Viollier, and Werthemann in 1948 (10). The influence of the vitamin-B complex on the incidence of liver tumors in rats fed carcinogenic dyes has been studied by many laboratories. The observation by Kensler et al. (6) in 1941 that riboflavin suppressed the development of tumors in rats fed p-dimethylaminoazobenzene, has been amply confirmed. Retardation of the production of azo dye-induced tumors by diets low in pyridoxine was suggested by Miner et al. (8) in 1943 and by Miller et al. (7) in 1945. It was shown by Engel (2) that inositol, along with choline, was a necessary dietary constituent for the maintenance of liver fat at normal levels. Recently, it was observed in this laboratory (5) that protein depletion-repletion superimposed upon choline deficiency in adult rats greatly increased liver fat in very short periods.

It is the purpose of this paper to describe the influence of variations in the dietary level of riboflavin, pyridoxine, and inositol, and of protein depletion-repletion upon the incidence of choline deficiency-induced tumors in rats.

EXPERIMENTAL

In the experiments designed to determine the influence of riboflavin, pyridoxine, and inositol on the neoplasm induction by chronic choline deficiency, weanling rats of the Alabama Experiment Station (AES) strain, weighing 40–50 gm., were used. For the protein depletion-repletion studies, female rats weighing 160 gm. and male rats weighing 200 gm. were used. All animals were housed in individual cages and uniformly grouped with respect to number, weight, sex, and litter.

Feed and water were supplied ad libitum. The basal diet contained extracted peanut meal, 30 (3); sucrose, 39.5; extracted casein, 6 (9); minerals, 4.4 (undried) (9); L-cystine, 0.1; cod liver oil, 1; and lard, 19. Vitamins were added in mg/kg of diet, except where otherwise indicated, as follows: thiamine, 2; riboflavin, 4; pyridoxine, 2; calcium pantothenate, 10; niacin, 20; inositol, 200; alpha-tocopherol, 25; alpha-tocopherol acetate, 25; and 2-methyl-1,4-naphthoquinone, 5. The dietary variations of riboflavin, pyridoxine, inositol, and choline, and the numbers of animals per treatment, are given in Tables 1, 2, and 3.

The diet fed during protein depletion was similar to the basal diet described, except that the peanut meal and casein were replaced by sucrose. During the repletion period, the animals were fed the basal diet. The variations in the periods of protein depletion and repletion and the choline chloride supplementation are given in Table 4.

Since the hepato-renal damage is severe and mortality is high in choline-deficient weanling rats, their diets were supplemented with 800 mg. choline chloride/kg for the first 2 weeks, and this was then reduced by 200 mg. at 2-week intervals. Under such a procedure only 2 of 78 choline-deficient rats died of the acute deficiency. Of the fourteen groups fed various low choline diets, four groups received a continued dietary supplement of 200 mg of choline chloride/kg, to determine whether this sub-minimal level would prolong survival and yield a higher tumor incidence.

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### TABLE 1

**Effect of Riboflavin on Liver Cirrhosis and Tumor Incidence in Choline Deficiency**

<table>
<thead>
<tr>
<th>Riboflavin (mg/kg)</th>
<th>Av. Final Diet Variation</th>
<th>Av. Time on Expt. (weeks)</th>
<th>Body Weight (gm.)</th>
<th>Hb. at 40 Weeks Liver (gm. percent)</th>
<th>Cirrhosis* Tumor Incidence</th>
<th>No. and Types of Tumors</th>
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<td>2/4</td>
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<td>0/4</td>
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<tr>
<td>20</td>
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<td>348</td>
<td>15.4</td>
<td>0/4</td>
<td>0/4</td>
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</table>

* Ratio of affected animals/total animals in group.

### TABLE 2

**Effect of Pyridoxine on Liver Cirrhosis and Tumor Incidence in Choline Deficiency**

<table>
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<tr>
<th>Pyridoxine (mg/kg)</th>
<th>Av. Final Diet Variation</th>
<th>Av. Time on Expt. (weeks)</th>
<th>Body Weight (gm.)</th>
<th>Hb. at 40 Weeks Liver (gm. percent)</th>
<th>Cirrhosis* Tumor Incidence</th>
<th>No. and Types of Tumors</th>
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<td>8/8</td>
<td>3/8</td>
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<td>15.7</td>
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### TABLE 3

**Effect of Inositol on Liver Cirrhosis and Tumor Incidence in Choline Deficiency**

<table>
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<tr>
<th>Inositol (mg/kg)</th>
<th>Av. Final Diet Variation</th>
<th>Av. Time on Expt. (weeks)</th>
<th>Body Weight (gm.)</th>
<th>Hb. at 40 Weeks Liver (gm. percent)</th>
<th>Cirrhosis* Tumor Incidence</th>
<th>No. and Types of Tumors</th>
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<td>295</td>
<td>14.3</td>
<td>2/4</td>
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<td>305</td>
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<td>2/4</td>
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<td>360</td>
<td>15.6</td>
<td>0/4</td>
<td>0/4</td>
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<td>82</td>
<td>361</td>
<td>17.7</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* One rat in this group developed diverticulosis of the stomach, not classified as a tumor.

### TABLE 4

**Effect of Protein Depletion-Repletion on Liver Cirrhosis and Tumor Incidence in Choline Deficiency in Young Adult Rats**

<table>
<thead>
<tr>
<th>Choline Supplement (mg/kg)</th>
<th>Feeding Cycle</th>
<th>Av. Final Diet Variation</th>
<th>Av. Time on Expt. (weeks)</th>
<th>Body Weight (gm.)</th>
<th>Hb. at 40 Weeks Liver (gm. percent)</th>
<th>Cirrhosis* Tumor Incidence</th>
<th>No. and Types of Tumors</th>
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<tr>
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<td>0</td>
<td>3</td>
<td>8</td>
<td>47</td>
<td>139</td>
<td>8/8</td>
<td>3 hepatomas</td>
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<td>0</td>
<td>3</td>
<td>5</td>
<td>71</td>
<td>361</td>
<td>0</td>
<td>0/9</td>
<td>1 hepatoma</td>
</tr>
</tbody>
</table>

* One of the eight rats was maintained on the protein depletion-repletion regimen for 5 cycles and thereafter continued on the depletion diet. This rat developed a hepatoma.
† These rats were maintained on the depletion diet after 5 cycles of protein depletion.

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Tissues were saved for routine microscopic studies. Bouin's fixative and hematoxylin and eosin stain were routinely used.

Hemoglobin was determined by the acid hematin method twice during the experiment, at 32 and 40 weeks.

RESULTS

Growth, survival, and hemoglobin levels.—The average final body weight of 76 weanling rats fed diets low in choline, with varying levels of riboflavin, pyridoxine, and inositol, was 226 gm. as compared to 345 gm. for the control rats receiving 0.2 per cent choline chloride. In general, the animals receiving the choline-deficient diets grew moderately well the first 6 months and thereafter declined in weight very slowly with a marked loss just prior to death.

The average survival time of the choline-deficient rats was 68 weeks, with a range of 42–98 weeks. A number of these animals had lung involvement, characterized by râles and hemorrhagic rhinitis. The controls were maintained for an average of 82 weeks. The hemoglobin level of the 68 rats receiving no choline supplement averaged 12.4 gm. per cent. Of these animals, one group of eight rats receiving 20 mg of riboflavin/kg of diet had an average hemoglobin level of 14.9 gm. per cent, which was essentially normal for the control animals fed 0.2 per cent choline chloride. Supplementation of various diets with the low level of 0.02 per cent choline chloride resulted in an average hemoglobin level of 15.8 gm. per cent (Tables 1–3).

The ten adult rats subjected to protein depletion-repletion without choline supplementation survived an average of 47 weeks, with a range of 25–63 weeks. Final body weight of these animals was 136 gm. and ranged from 81 to 194 gm. The nine control rats, similarly treated but receiving 0.2 per cent dietary choline chloride, were maintained on the experiment for an average of 71 weeks and weighed 361 gm. (Table 4).

Incidence of tumors and liver cirrhosis.—In the riboflavin, pyridoxine, and inositol experiments involving a total of 76 choline-deficient rats, 29 had tumors, and 63 had gross cirrhosis of the liver. No tumors or cirrhotic livers were found in 24 control rats receiving 0.2 per cent dietary choline chloride (Tables 1–3). In the protein depletion-repletion studies, 4 of the 10 choline-deficient rats had tumors, and all had cirrhotic livers. The 9 control animals did not develop liver cirrhosis or tumors (Table 4).

Influence of riboflavin.—A strikingly significant result of this series of experiments was the suppression of tumor development by 20 mg of riboflavin/kg of diet. Of fifteen animals that received 1 or 2 mg of riboflavin/kg of diet (Table 1), 40 per cent had tumors, whereas none of the eight rats fed high riboflavin developed tumors. Although gross liver cirrhosis was observed in six of the rats fed high levels of riboflavin, it was much less severe than that noted in the other choline-deficient animals. The hemoglobin level of this group was normal.

Influence of pyridoxine.—The omission of pyridoxine from the basal diet markedly suppressed growth. The average maximum weight attained was approximately 120 gm., and the final body weight averaged 112 gm. (range, 96–151 gm.). Typical pyridoxine-deficiency symptoms were noted. Although growth was greatly suppressed, three of the eight rats developed tumors (Table 2). This group also had the lowest hemoglobin level (8.8 gm. per cent). Of 23 rats receiving 0, 0.5, and 1 mg of pyridoxine/kg of diet, 39 per cent had tumors (Table 2), as compared to 50 per cent incidence in 14 rats receiving 10 and 20 mg. of pyridoxine. Thus, in general, the level of pyridoxine did not influence tumor incidence.

Influence of inositol.—The omission or inclusion of dietary inositol did not affect tumor incidence (Table 3). The diet containing 200 mg of inositol/kg is comparable to diet 43A used in the studies reported by Copeland and Salmon (1). The latter workers found a tumor incidence of 38 per cent, as compared to 44 per cent in this experiment.

Influence of protein depletion-repletion.—Protein depletion-repletion markedly shortened the time required for the development of neoplasms in young rats, as compared to the time required for weanling rats. The adult rats of this series of experiments survived an average of 47 weeks and tumors appeared in 32 weeks, whereas the weanling rats of the previous series of experiments survived an average of 68 weeks and tumors appeared in 42 weeks. All the choline-deficient animals had liver cirrhosis, and 40 per cent had neoplasms (Table 4). Body weight of the deficient animals declined rapidly during protein depletion with partial recovery during repletion. In general, body weight was progressively lowered from an average of 180 gm. at the start of the experiment to 136 gm., final weight. The control rats that received 0.2 per cent dietary choline chloride and were subjected to protein depletion-repletion lost weight during depletion, but more than regained this weight loss during repletion and attained a final body weight of 361 gm.

The results indicated that the protein depletion-repletion treatment need not be so drastic as that
employed for most of the animals in these experiments. One of the eight rats undergoing protein depletion for 2-week periods was maintained on the repletion diet after a total of three depletions. This animal survived for 63 weeks and had a hepatoma. The two rats subjected to protein depletion for 3 weeks were only depleted 3 times and were therefore maintained on the repletion diet. One of the two rats developed a hepatoma.

Histopathology.—Neoplasms were found in 33 of 86 of all the chronic choline-deficient rats in 32–98 weeks. Of these neoplasms, 28 were classified as hepatoma, 2 as mammary carcinoma, and 2 as sarcoma. One thoracic tumor has not yet been identified.

The gross appearance of a typical liver tumor is illustrated in Figure 1. This tumor measured 2.5 cm. in diameter. The liver tumors encountered in this experiment varied from 1 to 4 cm. in diameter and were multiple in some livers.

Microscopic studies revealed that, in addition to tumors, about half of the livers exhibited lesions similar to the one illustrated in Figure 2. This type of lesion was classified as a multiple cyst. These cysts contained small amounts of fibro-purulent exudate and were lined by flattened endothelium-like cells in some cases and cuboidal and columnar cells in others. Considerable interstitial connective tissue and a focal loss of parenchymal cells were characteristic of these areas.

Bile duct proliferation was a common finding and was especially extensive in the livers of the 28 rats that developed hepatomas. A type of cholangioma is shown in Figure 3. This cholangioma developed in the liver of a rat, in addition to a hepatoma. The typical acini formation and adenomatous nature of this lesion, as well as the characteristic connective tissue stroma, is evident.

The hepatomas presented very interesting cell patterns, which are illustrated by a series of photomicrographs. Figure 4 shows a hepatoma characterized by chains of cells and irregular trabeculae. "Gland-like" formations such as these were found in most of the hepatomas. The arrangement of the connective tissue stroma in a liver tumor that has the appearance of an adenocarcinoma is shown in Figure 5. The cells are arranged in primitive fashion, simulating glandular stratified epithelial elements. Numerous mitotic figures were observed, and the anaplastic nature of the tumor is evident.

The gross appearance of a mammary carcinoma is shown in Figure 6. A photomicrograph of a section of this tumor is illustrated in Figure 7. The tumor tissue appears to be invading the skeletal muscle. This tumor was composed of small epithelial cells with round nuclei arranged in gland-like structures. In some areas the cells were closely packed, and the glandular arrangement was lost. These tumors were extremely cellular, and mitotic figures were common.

The cellular nature of a sarcoma is shown in Figure 8. The tumor is composed of closely packed spindle cells. The nuclei are stained dark, have a characteristic oval shape, and contain one or more distinct nucleoli. The cytoplasm is fusiform to stellate and without distinct borders. The cells are arranged in different planes and whorls and often have a combed appearance.

DISCUSSION

The role of choline deficiency in carcinogenesis has been further substantiated. Neoplasms occurred in 33 of 86 rats that were fed choline-deficient diets for 32–98 weeks, while no tumors or liver cirrhosis were found in 33 control rats that received 0.2 per cent dietary choline chloride.

An interesting observation was the indication that high riboflavin suppressed tumor formation in chronic choline deficiency. The absence of tumors in eight rats (from six litters) fed a choline-deficient diet supplemented with 20 mg of riboflavin/kg indicates that this vitamin exerts a protection similar to that shown in the case of the azo dye-induced tumors (6). This is puzzling in view of the fact that choline does not prevent the development of azo dye-induced tumors. High levels of riboflavin in the diet did not afford as complete protection to the liver as choline, since 75 per cent of these rats developed a mild liver cirrhosis.

Varying the level of pyridoxine or inositol did not influence tumor incidence. Of interest was the observation that neoplasms developed in rats fed a diet without pyridoxine and choline supplementation, even though body weight was only 33–50 per cent of normal.

The production of neoplasms in nearly mature rats subjected to protein depletion-repletion in addition to choline deficiency and the complete protection by choline against such a rigorous treatment further emphasize the important role of choline. One would suspect that a procedure which results in caloric restriction such as protein depletion would suppress tumor development; however, with protein depletion-repletion, tumors actually appear in only two-thirds of the time required to produce tumors in weanling rats fed a choline-deficient diet without the depletion-repletion procedure.

The lowering of hemoglobin level in the choline-deficient rats confirms the observation by Engel (8). No correlation could be made between hemo-
Fig. 1.—Photograph showing the typical gross appearance of a hepatoma. This tumor developed in the liver of a rat fed a choline-deficient diet containing 30 mg of pyridoxine/kg of diet for 82 weeks. Reduced to less than one-half actual size.

Fig. 2.—Photomicrograph showing multiple cysts in the liver of a rat fed a choline-deficient diet containing 10 mg of pyridoxine/kg of diet. The cystic spaces are lined with flattened endothelium-like cells. Hematoxylin and eosin stain. × 230.

Fig. 3.—Photomicrograph of a cholangioma of the liver of a rat fed a choline-deficient diet containing no inositol for 85 weeks. Hematoxylin and eosin stain. × 230.

Fig. 4.—Photomicrograph of a hepatoma of the liver of a rat fed a choline-deficient diet containing no inositol for 72 weeks. Notice the "glandlike" formation and typical cellular nature of this hepatoma. Hematoxylin and eosin stain. × 230.
Fig. 5.—Photomicrograph of a liver tumor that has the appearance of an adenocarcinoma. This tumor developed in a rat fed a choline-deficient diet without inositol for 72 weeks. Hematoxylin and eosin stain. × 230.

Fig. 6.—Photograph illustrating the gross appearance of a mammary tumor in a rat fed a choline-deficient diet supplemented with 0.5 mg of pyridoxine/kg of diet for 48 weeks. Reduced to about one-half the actual size.

Fig. 7.—Photomicrograph of a section of the mammary tumor shown in Figure 6. The tumor appears to be invading the skeletal muscle. Hematoxylin and eosin stain. × 230.

Fig. 8.—Photomicrograph of a section of a sarcoma. This tumor developed in a rat fed a choline-deficient diet containing 1 mg of riboflavin/kg of diet for 44 weeks. Hematoxylin and eosin stain. × 230.
REFERENCES


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