Effects of a High-Sucrose Diet on the Development of Enzyme-altered Foci in Chemical Hepatocarcinogenesis in Rats

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ABSTRACT

Dietary factors can modify metabolic events involved in the initiation, promotion, or progression of tumors. To determine whether a high-sucrose diet has any effect on the development of enzyme-altered foci during the promotion step of chemical hepatocarcinogenesis in rats, 1-day-old Sprague-Dawley rats were given a single i.p. dose of diethylnitrosamine; controls received an equivalent i.p. volume of 0.9% NaCl solution. At 21 days of age, the rats were weaned, segregated by sex, separated in groups, and fed modified AIN76A diets containing either 65% glucose or 65% sucrose, with or without 0.05% phenobarbital. At the end of a 4-week treatment period, the sucrose-fed control rats of either sex had significantly heavier livers than did those on the glucose diet. Enlarged livers were found also in the sucrose-fed diethylnitrosamine-treated female rats, which developed twice as many γ-glutamyltranspeptidase-positive foci per sq cm of liver section than did those on the glucose diet. Addition of phenobarbital augmented the number of foci 3-fold in the sucrose group and 5-fold in the glucose group. Focus count per sq cm was similar in animals on the two phenobarbital-supplemented diets. Despite the absence of statistically significant liver enlargement, results analogous to those in females were obtained in carcinogen-treated males. Differences between treatments, however, were smaller. In both female and male rats, the DNA-synthesizing activity of hepatocytes in enzyme-altered foci was significantly higher than in the surrounding normal parenchymal cells, as determined by autoradiography. These studies indicate that a high-sucrose diet has a promoting effect during hepatocarcinogenesis induced in the rat by diethylnitrosamine and that this effect is weaker than that of 0.05% phenobarbital.

INTRODUCTION

It has been known for quite some time that both exogenous and endogenous factors can modify the outcome of hepatocarcinogenesis in laboratory animals. Pioneer studies by Bielenschovsky (5) and Miller et al. (16) have indicated that diet and hormones may have a profound effect on the incidence of hepatoma in rodents. The effects of these 2 types of modifiers are frequently intertwined since both can affect a vast array of cellular enzymes. Most of the early studies of dietary effects on experimental hepatocarcinogenesis, however, made no attempt to delineate whether those effects took place during the initiation or the promotion step, the general assumption being that the diet may exert its influence through modification of enzyme activities responsible for the metabolism and activation of chemical carcinogens.

Among the 3 major dietary components, i.e., carbohydrate, fat, and protein, the latter 2 have been implicated more recently in the promotion of liver cancer (1, 15, 23, 31). Little is known, however, about a similar role for carbohydrates on tumor development. Specifically, the possibility that certain carbohydrates at high dietary levels may have a promoting effect in hepatocarcinogenesis after initiation by DEN has not been examined heretofore. Studies by Bender et al. (3) have demonstrated that rats fed a high-sucrose diet (70%) for only 4 weeks had heavier livers due to hyperplasia of parenchymal cells while rats fed a similar concentration of glucose showed no comparable response. Because of its ability to cause liver enlargement, it would seem plausible that a high-sucrose diet may modulate the promoting phase of hepatocarcinogenesis (27). Accordingly, the present study was undertaken to evaluate such an effect of sucrose in rats treated previously with a small initiating dose of DEN. The development of GGTase-positive foci was used to quantitate the postulated dietary effects. The sequential relationships of these lesions with hyperplastic nodules and hepatocellular carcinomas have been demonstrated with a number of chemicals (7, 22, 35). Since PB is a potent promoter in experimental hepatocarcinogenesis (19, 29), it was also included in this study to determine whether a synergistic effect with a high-sucrose diet could be demonstrated.

MATERIALS AND METHODS

Animals and Treatments. Pregnant Sprague-Dawley rats (Zivic-Miller, Allison Park, PA) were received 1 week before parturition and housed individually in plastic cages in a 23°C constant-temperature room with a 12-h light, 12-h dark illumination cycle. They were given free access to a standard pelleted laboratory diet (Purina Formula 5008; Ralston Purina Co., St. Louis, MO) and water. Twenty-four h after delivery, newborn rats were used as in the "neonatal rat protocol" described by Peraino et al. (21) and one half of the total number of offspring were each given an i.p. injection of a solution of DEN (Sigma) in 0.9% NaCl solution (saline), at a dosage of 0.15 μmol/g body weight. The other half were used as control animals, and each was given an injection of an equivalent volume of saline. The rats were weaned at 3 weeks of age, segregated with respect to sex, and housed individually in stainless steel cages with wire mesh bottoms. Both males and females from either the carcinogen-treated or 0.9% NaCl solution control groups were then randomized by weight into 4 subgroups which were fed ad libitum one of the 4 test diets described below and weighed twice weekly to monitor growth. All animals were sacrificed 4 weeks postweaning, i.e., at 7 weeks of age. Two hours before sacrificing at 9 a.m., each animal received an i.p. injection of [3H]dThd (specific activity, 30 μCi/mmol; ICN Pharmaceuticals, Inc., Irvine, CA) and two h later an i.p. injection of 0.05% phenobarbital. At sacrifice, livers were removed and weighed, liver sections were cut, and the development of GGTPase-positive foci was used to quantitate the postulated dietary effects. The sequential relationships of these lesions with hyperplastic nodules and hepatocellular carcinomas have been demonstrated with a number of chemicals (7, 22, 35). Since PB is a potent promoter in experimental hepatocarcinogenesis (19, 29), it was also included in this study to determine whether a synergistic effect with a high-sucrose diet could be demonstrated.

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Irvine, CA) at a dosage of 50 μCi/100 g body weight. The rats were killed by cervical dislocation, and the livers were quickly excised, washed twice in saline, blotted dry with filter paper, and weighed. Serial samples from each hepatic lobe were taken by a standardized procedure (12) and either fixed in 10% buffered formaldehyde for routine hematoxylin and eosin staining or frozen in isopentane immersed in liquid nitrogen for GGTPase staining and autoradiographic studies.

Diets. The basal diet used, from which the 4 test diets were derived, was the AIN76A semipurified rat-mouse diet (American Institute of Nutrition, recommendation of ad hoc Committee, March 1980). The pelleted test diets (United States Biochemical Corp., Cleveland, OH) with or without 0.05% PB (Sigma) contained 65% of either glucose or sucrose as their only carbohydrate source.

Staining Procedures. Frozen liver sections were processed for histological demonstration of GGTPase as described by a modification of the procedure of Ogawa et al. (18). Basically, tissue samples were fixed in chilled acetone for 2 h followed by rinsing in 2 changes of acetone (1 h each). Liver sections were then treated with 2 changes of cedarwood oil for 16 to 24 h followed by clearing in 3 changes of benzene 20 min apart. After 2 h infiltration in paraffin (Tissue Prep, M.P. 56.5; Fisher Scientific, Fair Lawn, NJ), they were embedded in paraffin. Tissue blocks were cut into 5 μm-thick sections and stained histochemically for GGTPase according to the method of Rutenburg et al. (24) using γ-glutamyl-4-methoxy-2-naphthylamide (Vega Biochemicals, Tucson, AZ) as substrate. The sections were then thoroughly rinsed in distilled water, mounted in buffered glycerol, and stored at 4°C until analyzed.

Quantitation of Enzyme-altered Foci. The total number of GGTPase-positive foci from each histochemically stained slide was counted microscopically under ×40 magnification. Only clearly demarcated groups of 6 or more cells were regarded as foci and included in the present studies (30). All slides were coded in order to avoid bias during the counting procedures. To measure the area of a focus, slides were placed on a microslide projector (Ernest Leitz GMBH, Wetzler, Federal Republic of Germany) with the image projected on a screen. The actual magnification of each section was determined with a micrometer scale; routinely, a ×27 enlargement was used. The perimeters of the liver section and of each focus were then accurately traced out on paper and later transcribed by a Numonic Electronic Digitizer (Numonic, Lansdale, PA) into actual area after scale adjustment. The range of representative liver tissue area scanned for foci was from 2.22 to 5.93 sq cm/animal.

DNA Labeling Index. Five-μm liver sections were cut, deparaffinized, and stained histochemically for GGTPase, as described above. After thorough rinsing with distilled water, autoradiography was carried out on the same sections by the "dipping technique" using Kodak NBT photographic emulsion (Eastman Kodak, Rochester, NY) as described (25). The labeling index for normal hepatocytes was determined by counting graphic emulsion (Eastman Kodak, Rochester, NY) as described (25).

RESULTS

Body Weight and Liver Weight Distribution. There was no significant difference in body weight distribution at any time among female or male rats in either the carcinogen-treated or the control groups fed each of the 4 dietary regimens (Tables 1 and 2). Dietary modulation of the development of hepatic GGTPase-positive foci therefore cannot be attributed to a general effect.

Although animals were randomized by weight, the relative liver weight per 100 g of body weight was used as a better index for comparison. The relative liver weights were significantly higher in sucrose-fed than in glucose-fed female rats, both in the control and in the carcinogen-treated groups (Table 1). Addition of PB caused further liver enlargement and eliminated these weight differences between diets. On the other hand, when the same dietary treatment was applied (i.e., glucose or sucrose), there were statistically significant differences depending on the presence or absence of PB. Any hepatic toxicity due to the carcinogen was not reflected by the size of the liver, since both the absolute and relative weights of this organ in rats given a single i.p. dose of DEN were comparable to those of their respective 0.9% NaCl solution controls.

Male rats in the 0.9% NaCl solution control group responded to the various dietary treatments in a manner similar to that of the females (Table 2). Animals fed the sucrose diet had significantly heavier livers than did those fed the glucose diet. Addition of PB to the carbohydrate diets caused responses similar to those in females. In DEN-treated males, the mean relative liver weight of the group fed 65% sucrose, although larger, was not statistically different from that fed the 65% glucose diet. Supplementation of these carbohydrate diets with PB resulted in mean relative liver weight differences which were highly significant between the sucrose groups ($P < 0.01$) but not significant between the glucose groups.

Number and Area of GGTPase-positive Foci. Occasional foci were found in the 0.9% NaCl solution control group, but only in animals given the PB-supplemented diets. GGTPase-positive foci, on the other hand, occurred in all animals given injections of DEN. Although they were randomly distributed, a midzonal localization in the hepatic lobule was predominant (Figs. 1 and 2). The response was more intense in females than in males. Similar findings have been documented by Peraino et al. (19, 20) and by Deml et al. (8). DEN-treated female rats kept on the high-sucrose diet generated twice as many GGTPase-positive foci per sq cm of liver area scanned as did those on the high-glucose diet (Table 3). Addition of PB to the 2 sugar diets augmented the response 5-fold in the glucose and 3-fold in the sucrose diet group. No significant difference in the number of foci was found between animals fed either of the PB-supplemented diets. There were no significant differences in the average size of GGTPase-positive foci among any of the 4 groups.

Male animals were generally less responsive to the present treatment protocol than were females (Table 4). Among those treated with DEN, a significantly higher number of foci was produced in sucrose-fed than in glucose-fed groups. Inclusion of PB seemed to further enhance the production of foci in both glucose- and sucrose-fed rats since application of the one-tailed test showed significant differences ($P < 0.05$). When these results were analyzed with the 2-tailed test, however, the differences were not statistically significant at the $P = 0.05$. Thus, probably because of the small sample size, the data gave only a weak confirmation of the null hypothesis. Similarly, there were no significant differences between the number of foci counted in rats fed either of the 2 PB-supplemented diets. Average sizes of the GGTPase-positive foci produced by each of the 4 dietary treatments were not significantly different. The size distribution of foci was analogous to that observed in females.
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Table 1
Liver weights in female rats at the termination of the study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals in each group</th>
<th>Liver wt (g)</th>
<th>Body wt (g)</th>
<th>Liver wt (g)/100 g body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution i.p.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65% glucose</td>
<td>4</td>
<td>8.48 ± 0.62a</td>
<td>218.3 ± 10.4</td>
<td>3.89 ± 0.22b, c</td>
</tr>
<tr>
<td>65% sucrose</td>
<td>4</td>
<td>11.20 ± 3.49</td>
<td>211.7 ± 20.4</td>
<td>5.23 ± 1.05d, e</td>
</tr>
<tr>
<td>65% glucose + 0.05% PB</td>
<td>4</td>
<td>12.40 ± 2.27</td>
<td>219.6 ± 12.6</td>
<td>5.68 ± 1.36f, g</td>
</tr>
<tr>
<td>65% sucrose + 0.05% PB</td>
<td>5</td>
<td>15.40 ± 2.08</td>
<td>228.2 ± 22.7</td>
<td>6.35 ± 0.37h, i</td>
</tr>
<tr>
<td>DEN i.p.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65% glucose</td>
<td>5</td>
<td>8.60 ± 1.57</td>
<td>227.2 ± 22.7</td>
<td>3.78 ± 0.34i, j</td>
</tr>
<tr>
<td>65% sucrose</td>
<td>6</td>
<td>10.10 ± 1.33</td>
<td>222.0 ± 17.8</td>
<td>4.56 ± 0.43k, l</td>
</tr>
<tr>
<td>65% glucose + 0.05% PB</td>
<td>6</td>
<td>11.93 ± 2.06</td>
<td>232.9 ± 8.6</td>
<td>5.13 ± 0.89m, n</td>
</tr>
<tr>
<td>65% sucrose + 0.05% PB</td>
<td>7</td>
<td>12.87 ± 2.28</td>
<td>217.8 ± 14.7</td>
<td>5.91 ± 0.89o, p</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

b Differences between means with like superscripts were as follows: b, c, d P < 0.05; e, f not significant; f P < 0.005; g P < 0.01; h, i, j not significant.

Table 2
Liver weights in male rats at the termination of the study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals in each group</th>
<th>Liver wt (g)</th>
<th>Body wt (g)</th>
<th>Liver wt (g)/100 g body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution i.p.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>65% glucose</td>
<td>5</td>
<td>13.5 ± 2.03</td>
<td>278.4 ± 36.5</td>
<td>4.86 ± 0.47b, c</td>
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<tr>
<td>65% sucrose</td>
<td>5</td>
<td>17.9 ± 3.74</td>
<td>299.5 ± 17.8</td>
<td>5.95 ± 0.92d, e</td>
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<tr>
<td>65% glucose + 0.05% PB</td>
<td>5</td>
<td>16.0 ± 1.69</td>
<td>238.0 ± 10.0</td>
<td>6.72 ± 0.67f, g</td>
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<tr>
<td>65% sucrose + 0.05% PB</td>
<td>6</td>
<td>19.8 ± 2.69</td>
<td>285.9 ± 15.6</td>
<td>6.92 ± 0.64h, i</td>
</tr>
<tr>
<td>DEN i.p.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65% glucose</td>
<td>3</td>
<td>13.0 ± 1.67</td>
<td>280.0 ± 18.0</td>
<td>4.66 ± 0.49i, j</td>
</tr>
<tr>
<td>65% sucrose</td>
<td>3</td>
<td>15.1 ± 1.39</td>
<td>281.3 ± 48.9</td>
<td>5.43 ± 0.54k, l</td>
</tr>
<tr>
<td>65% glucose + 0.05% PB</td>
<td>3</td>
<td>16.6 ± 1.97</td>
<td>293.9 ± 18.7</td>
<td>5.67 ± 0.98m, n</td>
</tr>
<tr>
<td>65% sucrose + 0.05% PB</td>
<td>3</td>
<td>22.0 ± 1.35</td>
<td>302.2 ± 34.7</td>
<td>7.31 ± 0.44o, p</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

b Differences between means with like superscripts were as follows: b P < 0.05; e, f, g not significant; h P < 0.01; i, j not significant.

Table 3
Effect of treatments on the number and size of GGTPase-positive foci in female rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>No. of foci/sq cm liver</th>
<th>Size of foci (sq mm x 10^-2)</th>
<th>Volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution i.p.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65% glucose</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>65% sucrose</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>65% glucose + 0.05% PB</td>
<td>4</td>
<td>0.37</td>
<td>1.65</td>
<td>0.006</td>
</tr>
<tr>
<td>65% sucrose + 0.05% PB</td>
<td>5</td>
<td>0.003</td>
<td>1.09</td>
<td>0.00003</td>
</tr>
<tr>
<td>DEN i.p.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65% glucose</td>
<td>4</td>
<td>5.52 ± 1.02e, f, g</td>
<td>1.54 ± 0.37f, g</td>
<td>0.085 ± 0.025h, i</td>
</tr>
<tr>
<td>65% sucrose</td>
<td>5</td>
<td>11.3 ± 2.51b, d</td>
<td>1.72 ± 0.34f, h</td>
<td>0.198 ± 0.064i, j</td>
</tr>
<tr>
<td>65% glucose + 0.05% PB</td>
<td>6</td>
<td>29.5 ± 6.79e, k</td>
<td>2.25 ± 0.65g, l</td>
<td>0.664 ± 0.025h, i</td>
</tr>
<tr>
<td>65% sucrose + 0.05% PB</td>
<td>7</td>
<td>35.8 ± 13.1f, k</td>
<td>1.93 ± 0.64h, i</td>
<td>0.652 ± 0.026m</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

b Differences between means with like superscripts were as follows: b, i, p < 0.01; c, f, k, m not significant.

Histological Examination. In agreement with previous observations (25), foci examined 7 weeks after the single dose of DEN were relatively small. Areas surrounding them resembled morphologically the livers of control animals on the corresponding diets. No histological evidence of inflammation, cell necrosis, or bile duct cell proliferation was present. In contrast to animals on the glucose diet, however, there was increased lipid content in the hepatocytes of those on the sucrose diet (Fig. 2). These early changes were mostly periporal, unlike those seen in the livers of choline-deficient rats (in which lipid deposition occurs first in the central zone of the hepatic lobule). PB induced hepatic fatty changes in all animals, irrespective of the type of carbohydrate diet they were on.

DNA Synthesis of GGTPase-positive Foci. The [3H]dThd labeling indices of GGTPase-positive hepatocytes from female rats are shown in Table 5. Male rats responded in a similar manner (data not shown). These enzyme-positive cells had a 2- to 3-fold higher proliferative activity than did surrounding normal hepatocytes, regardless of dietary treatments. The maximum [3H]dThd mean labeling index of GGTPase-positive hepatocytes, which was attained in animals fed the PB-supplemented high-sucrose diet, was only 2.5%. Mean labeling indices of normal hepatocytes in any of the DEN-treated groups were comparable to those in saline controls (Table 5, Column 4), indicating that increased labeling in the GGTPase-positive foci is not the apparent consequence of a depression of DNA synthesis in intervening normal hepatocytes. On the other hand, hepatocytes from carcinogen-treated animals...

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Differences from corresponding normal hepatocytes were as follows: * P < 0.02; ** P < 0.005.
those in the foci of controls, might have decreased further had the animals been sacrificed a few weeks later because DNA synthesis and cell proliferation in foci appear to be susceptible to some kind of feedback regulation (26, 28).

In spite of the hepatic hyperplasia, labeling indices not unlike those seen in controls were observed in normal hepatocytes of both noninitiated and initiated rats fed 65% sucrose. This is not an unexpected finding since it has been shown, with all hepatic tumor promoters tested, that uninterrupted long-term treatment causes liver DNA synthesis to decline almost back to control values (28). Under the homeostatic conditions prevailing among normal hepatocytes, a feedback mechanism (26) probably prevented excessive DNA synthesis in the presence of continuous mitogenic stimulation by high sucrose.

Diets containing 65% glucose, used as controls in this study, elicited practically no foci. When rats were initiated with DEN and then fed the control diet, however, a small but significant number of foci were generated. Although a weak promoting effect of high glucose could be postulated to explain this, the fact that dietary glucose at concentrations comparable to those of sucrose produces neither liver hyperplasia nor similar metabolic or functional alterations (27, 33) militates against it. Most likely, the dosage of DEN used for tumor initiation was higher than necessary. This is confirmed by the identification of GGTPase-positive foci in numbers equivalent to ours in rats receiving the same dose of DEN at 1 day of age and kept on a 30% casein diet (which does not have promoting effects) for 4 weeks after weaning (30).

Addition of 0.05% PB equalized the number of foci in the glucose and sucrose groups, both of which were increased to a 2 to 3 times more potent PB stimulation. This is inconclusive.

In the case of high sucrose, the tumor-promoting effect may be related to the disaccharide molecule per se, the combination of its products of intestinal hydrolysis (glucose and fructose), or fructose alone. This carbohydrate dietary regimen can alter various hepatic lipogenic enzymes involved in the metabolism of triglycerides, cholesterol, and phospholipids (4, 7, 11, 13, 14, 32). Whereas increased triglyceride synthesis may account for the fatty metamorphosis evidenced histologically in the present studies, either changes in cholesterol or phospholipids could be implicated in a hypothetical promotion mechanism. Thus, increased cholesterol levels in liver (33) can affect the physical properties of cell membranes by reducing the cooperativity of gel-liquid crystal transitions and by increasing the fluidity of the acyl moiety of phospholipids (34). Alternatively, the increase in liver phospholipids observed after feeding a high-sucrose diet (6, 33) could play a role in promotion by modifying membrane structure and composition. Changes in the properties of cell membranes by perturbation of hepatocyte membrane lipids have been suggested as the mechanism of promotion by PB- and choline-deficient diets (29, 36–38). Recently, a great deal of attention has been paid to the turnover of a special group of membrane phospholipids, the inositol phosphatides, in connection with activation of the phospholipid-dependent enzyme protein kinase C during promotion by phorbol esters (17). It is conceivable that the resulting modifications of membrane structure or function, or both, could inhibit transmission of intercellular regulatory signals (36, 39, 40). Initiated cells could thus escape modulation of proliferation by surrounding parenchyma and give rise to foci with increased mitotic indices. Future studies should clarify whether and how the above effects of promoters on these different membrane lipids are related.

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