Effect of DibutylNitrosamine and Saccharin on Glutamyl Transpeptidase-Positive Foci and Liver Cancer

by Michael A. Pereira,* Sydna L. Herren,* and Alfred L. Britt†

An attempt was made to evaluate whether the simultaneous administration of the urinary bladder tumor promoter, saccharin, and the substance being tested for carcinogenicity could be developed into a rapid and efficient bioassay for bladder carcinogens. Dibutylnitrosamine (DBN) a bladder and—to a lesser extent—liver carcinogen, was used as the test substance. The attempt evaluation failed because of the high incidence of liver cancer in the rats that simultaneously received DBN and saccharin. The simultaneous administration of 5% sodium saccharin in the diet and 0.02% DBN in the drinking water of rats for 26 weeks resulted in an 81% DBN in the drinking water of male rats for 26 weeks resulted in an 81% incidence of both hyperplastic nodules and hepatocellular carcinomas. A much lower incidence of 17 and 3% for hyperplastic nodules and hepatocellular carcinomas, respectively, was present in the rats that received only DBN, and no tumors were present in the liver of rats that received only saccharin. The liver of the rats that received both DBN and saccharin compared to those that received only DBN also had an increased incidence of \(\gamma\)-glutamyltranspeptidase (GGTase)-positive foci at 4, 8 and 26 weeks of treatment. The presence of GGTase activity in hyperplastic nodules and hepatocellular carcinomas and the association of the increased incidence of GGTase-positive foci with the increased incidence of tumors are consistent with a precursor relationship of foci to hyperplastic nodules and hepatocellular carcinomas. Saccharin did not increase the size of GGTase-positive foci, indicating that saccharin is not an hepatic tumor promoter. The increased incidence of DBN initiated GGTase-positive foci and tumors that resulted from the simultaneous administration for saccharin, implicates an hepatic cocarcinogenic activity for saccharin.

Areas of GGTase-positive hepatocytes were induced in zone 1 by DBN, saccharin and DBN and saccharin at 4 and 8 but not 26 weeks. These zonal areas of induced GGTase activity are different from GGTase-positive foci. The induction of zonal GGTase activity by saccharin indicated that saccharin at the high dose of 5% in the diet can cause hepatotoxicity and/or bile ductule proliferation. It is proposed that a regenerative response to the hepatotoxicity could result in an increased efficacy of DBN initiation by increasing the fixation of DBN adduct in DNA.

Introduction

\(\gamma\)-Glutamyltranspeptidase (GGTase)-positive foci are proposed to be preneoplastic lesions in rat livers (1-3). The incidence of GGTase-positive foci has been proposed to be associated with the extent of the initiation of the neoplastic progression and with the ultimate incidence of hyperplastic nodules and hepatocellular carcinomas (1-3). The screening of chemicals for tumor promoting and cocarcinogenic activity by demonstration of their ability to enhance the incidence of GGTase-positive foci is predicated on the capability of the incidence of foci to predict the cancer incidence. A tumor promoter would act after initiation to stimulate the growth of the foci so that there is an earlier increase in foci and tumor incidence. A cocarcinogen would act during initiation to increase the efficacy of initiation as indicated by an increased number of GGTase-positive foci.

Saccharin 1, 2-benzisothiazol-3(2H)-one-1,1-dioxide, (CAS No. 81-07-2) is a weak urinary bladder carcinogen in rats (1-4). Chemical carcinogenesis in the urinary bladder has been divided into the two stages of initiation and promotion. Sodium saccharin ad-
ministered in the diet has been shown to promote the incidence of urinary bladder cancer in rats that were previously initiated with a subcarcinogenic dose of either methyl nitrosourea administered intravesically (4-6), FANFT, N-4-(5-nitro-2-furyl)-2-thiazolyiformamide administered in their diet (7) or N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in their drinking water (8). In mouse skin (9,10) and urinary bladder (9), tumor promoters have been shown also to possess cocarcinogenic activity. The simultaneous administration of the carcinogen and the promoter resulted in an enhanced and more rapid appearance of tumors than occurred in the two stage bioassay consisting of the carcinogen followed by the promoter. The study reported in this communication describes an attempt to develop a rapid and efficient bioassay for bladder carcinogens that consists of administering the substance being tested for carcinogenicity simultaneously with the bladder tumor promoter, saccharin. This communication also describes the effect of saccharin concurrently administered with dibutyl nitrosamine (DBN) on the relationship of DBN induced GGTase-positive foci to hyperplastic nodules and hepatocellular carcinomas.

Materials and Methods

Chemicals

Sodium saccharin synthesized by the Maumee Procedure was purchased from Sigma Chemical Company (St. Louis, MO) and added at a concentration of 5% by weight to AIN-76 semipurified diet purchased from ICN Nutritional Biochemicals (Cleveland, OH). The sodium saccharin as expected for Maumee synthesized saccharin, was determined by gas chromatography to contain undetectable levels of either o-tolunesulfonamide or p-toluenesulfonamide. Dibutyl nitrosamine was purchased from Eastman Kodak Company (Rochester, NY) and N-γ-L-glutamyl-4-methoxy-2-naphthylamide from Bachem (Torrence, CA).

Animals

Male Fischer 344 rats were purchased from Charles River Laboratories, Inc. (Portage, MI) and were 7 to 8 weeks of age at the start of the experiment. The animals were maintained in accordance with the standards set forth in the literature (11). They received their drinking water and food ad libitum.

Experimental Design

The experimental design consisted of four treatment groups. Group A received the control AIN-76 diet; Group B, AIN-76 diet and 0.02% DBN in their drinking water; Group C, 5% sodium saccharin in the AIN-76 diet; and Group D, 5% sodium saccharin in the AIN-76 diet and 0.02% in their drinking water. Rats were sacrificed from each of the four groups at 4, 8, and 26 weeks of treatment. The experiment was terminated at 26 weeks because of the high mortality rate in Group D.

Histopathology

At the termination of the experiment, the rats were killed by decapitation and necropsied. The urinary bladder was inflated with 10% buffered formalin and the inflated bladder and liver fixed in 10% buffered formalin. After the tissues were embedded in paraffin, sections obtained from each of four different lobes of the liver and a longitudinal section from each of the two halves of the urinary bladder were examined.

For evaluation of GGTase activity, tissues blocks approximately 10 x 10 x 2 mm were taken from three different lobes, rapidly frozen in optimum cutting temperature (OCT) compound on dry ice and stored at -80°C for up to 6 months. Cryostat sections (8 μm) were mounted on slides, air-dried, and stained for γ-glutamyltranspeptidase activity according to the method described by Rutenburg et al. (12). Nuclei were counterstained with hematoxylin. Only GGTase-positive foci of nine cells (nuclei) or greater were scored. Figure 1 depicts a

![Figure 2. Average body weights of rats in the four treatment groups from week 0 to 26 of the experiment: (x) Group A, controls; (O) Group B, DBN; (+) Group C, saccharin; (▲) Group D, DBN and saccharin.](image-url)
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Results

Toxicity

The effect of treatment on the body weights of the rats is presented in Figure 2. The rate of gain in body weight over the course of the experiment was not affected by DBN, but sodium saccharin caused a decreased rate of gain. The body weight of the rats that simultaneously received saccharin and DBN were the lowest of all the groups and appeared to decrease after 20 weeks, during which time a large number of rats died. This high mortality rate in Group D resulted in the termination of the experiment at week 26.

The amount of drinking water consumed by the rats of each group was determined on a biweekly basis. Over the 26-week course of the experiment the mean percentage ± standard error in drinking water consumption determined as mL/100 mg body weight relative to control (Group 4) was for Group B 97 ± 10, group C, 135 ± 10, and Group D, 134 ± 12. The consumption of water by Group D was therefore on the average 37% greater than Group B.

The liver weights and their percentage of the body weight are presented in Table 1. DBN did not alter the percentage of the body weight represented by the liver, while sodium saccharin decreased the contribution of the liver to the body weight. Upon histological evaluation, the livers from the control and DBN treated rats contained hepatocytes with fat vacuoles that were diffusely dispersed, mainly in zone 1 of Rappaport. The livers from saccharin-treated rats (Group C) lacked these fat vacuoles which might explain their lower percent of the body weight. The livers of the rats from Group D represented an apparent, but not statistically significant, higher percentage of the body weight. This apparent increase in the relative weight of the liver probably resulted from the presence of the numerous large tumors. The rats in Group D also had a complete lack of body fat.
Initiation of GGTase-Positive Foci

The incidence of GGTase-positive foci was higher in Group D when compared to Group A, B or C (Table 2). At 26 weeks, Group B also had an increased incidence of foci compared to Groups A or C; however, throughout the course of the study the incidence of foci in Group D was much greater than Group B. At no time during the experiment did sodium saccharin administered by itself (Group C) significantly increase the incidence of foci. Therefore, DBN induced the formation of GGTase-positive foci and the coadministration of saccharin greatly enhanced the response to DBN.

The size distribution of the GGTase-positive foci was determined at 26 weeks for Group B and D (Table 3). The size distribution of the foci was divided into logarithmic intervals from 0.0-0.03 to >3 mm². The foci in Groups B and D had the same size distribution. Even the percentage of foci greater than 3 mm², which probably represents hyperplastic nodules, was the same in Groups B and D. Therefore, the concurrent administration of saccharin with DBN did not increase the rate of growth of GGTase-positive foci, so that saccharin did not appear to promote DBN hepatocarcinogenesis.

Tumorigenicity

Tumors were found only in the liver of rats from Groups B and D (Fig. 3 and Table 2). The coadministration of sodium saccharin in the diet greatly increased the incidence of hyperplastic nodules and hepatocellular carcinomas that were induced by DBN. The hyperplastic nodules and hepatocellular carcinomas were positive for GGTase activity. The carcinomas were of the trabecular type. In Group B, animals which received only DBN, there were 16

| Group | Treatment | N^a | Body weight, g^b | Liver weight, g^b | Liver weight, % of body weight |
|-------|-----------|-----|------------------|------------------|-----------------------------|
| A     | Control   | 11  | 391 ± 8.8       | 12.6 ± 0.85      | 3.21 ± 0.17                |
| B     | DBN       | 29  | 367 ± 8.3       | 11.4 ± 0.41      | 3.10 ± 0.07                |
| C     | Saccharin | 36  | 337 ± 4.2       | 9.47 ± 0.21      | 2.81 ± 0.04*               |
| D     | Saccharin | 21  | 282 ± 10.7      | 10.7 ± 0.73      | 3.78 ± 0.20**              |

^a N represents the number of animals at the termination of the study.
^b Values are means ± SE.
* Different from Group A by the Student t test with p = 0.0017.
** Different from Group A by the Student t test with p = 0.085.

Table 2. Induction by DBN and saccharin of GGTase-positive foci and tumors in rat liver.

| Group | Treatment | 4 weeks^a | 8 weeks^a | 26 weeks^a | GGTase-positive foci/cm² | Hyperplastic nodules | Carcinoma |
|-------|-----------|-----------|-----------|------------|--------------------------|----------------------|-----------|
|       |           |           |           |            |                          | Animals^b | %         | Animals^b | %         |
| A     | Control   | 0.45 ± 0.30(14) | 1.63 ± 0.81(10) | 0.31 ± 0.31(10) | 1.63 ± 0.81(10) | 0       | 0        | 0         | 0        |
| B     | DBN       | 1.86 ± 0.45(12) | 2.19 ± 0.72(8)  | 5.03 ± 1.06(29)*| 2.19 ± 0.72(8)  | 5       | 17       | 1         | 3        |
| C     | Saccharin | 1.95 ± 0.61(13) | 1.57 ± 0.54(9)  | 0.23 ± 0.23(36) | 1.57 ± 0.54(9)  | 0       | 0        | 0         | 0        |
| D     | DBN + saccharin | 6.26 ± 1.69(11)*+ | 9.19 ± 1.84(9)*+ | 31.31 ± 4.65(21)*+ | 9.19 ± 1.84(9)*+ | 17      | 81       | 17        | 81       |

^a Foci results are mean ± standard error for the number of animals in parentheses.
^b Results are the number of animals at 26 weeks with tumors among the animals observed for foci at 26 weeks.
* Results are different from Group A by the Student t test with p<0.01.
+ Results are different from Group B by the Student t test with p<0.01.

Table 3. Size distribution of GGTase-positive foci at 26 weeks.

| Group | Treatment | N^a | Size distribution of GGTase-positive foci, % |
|-------|-----------|-----|-------------------------------------------|
|       |           |     | 0.0-0.03 mm² | 0.03-0.1 mm² | 0.1-0.3 mm² | 0.3-1 mm² | 1-3 mm² | 3 mm² |
| B     | DBN       | 44  | 14±       | 41±        | 30±        | 9±        | 5±      | 2±     |
| D     | DBN + Saccharin | 244 | 14±       | 43±        | 29±        | 8±        | 2±      | 3±     |

^a N equals the number of foci evaluated.
hyperplastic nodules among five of 29 (17%) rats and two carcinomas in one of 29 (3%) rats, and in Group D animals which received both DBN and saccharin, there were 106 hyperplastic nodules among 17 of 21 (81%) rats and 81 carcinomas among 17 of 21 (81%) rats. Therefore, DBN and not saccharin possessed the ability to initiate tumors and the coadministration of saccharin with DBN greatly enhanced the tumorigenic potency of DBN.

Urinary bladder tumors, either benign or malignant, were not observed in any of the rats of the four groups. There was simple focal/and/nodular-papillary hyperplasia of the bladder transitional epithelium in the animals that received either DBN (Group B) or saccharin and DBN (Group D; Table 4). The hyperplastic response was associated with the administration of DBN and not the administration of the sodium saccharin. The coadministration of sodium saccharin increased both the incidence and severity of the hyperplastic response to DBN in the urinary bladder.

Areas (Zone 1) of GGTase-Positive Hepatocytes

During the course of this study, we observed an induction in Groups B, C and D of GGTase activity in hepatocytes in Zone 1 of the liver as defined by Rappaport (Fig. 4). These zonal areas of GGTase-positive hepatocytes were distinct from foci in that they appeared to radiate out from most, if not all, triads of a section, while foci were dispersed randomly in all three zones. The percentage of the liver containing GGTase activity in zone 1 hepatocytes is presented in Table 5. At 4 and 8 weeks, an increase in GGTase activity was observed in Groups B, C and D. The order of efficacy was DBN + saccharin > saccharin > DBN. The coadministration of DBN and saccharin appeared to be synergistic, that is, greater than the addition of the potencies of DBN.

Table 4. Lesions of the urinary bladder.

| Group | Treatment     | N | Simple Focal | Nodular and Papillary | Total |
|-------|---------------|---|--------------|-----------------------|-------|
| A.    | Control       | 10| 0            | 0                     | 0     |
| B.    | DBN           | 20| 3(15)        | 4(20)                 | 7(35) |
| C.    | Saccharin     | 25| 1(4)         | 0                     | 0     |
| D.    | Saccharin + DBN | 10| 3(30)        | 4(40)                 | 7(70) |

* N represents the number of bladders examined by light microscopy. The remaining bladders were used for histochemical and electron microscopic examination.

* Results are the number of animals examined that exhibited the lesions. Animals that exhibited both nodular and single focal hyperplasia were scored for the more advanced nodular lesion. The numbers in parentheses are the percentage of the animals that exhibited the lesion.

Table 5. Induction by DBN and saccharin of GGTase-positive hepatocytes.

| Group | Treatment     | 4 weeks | 8 weeks | 26 weeks |
|-------|---------------|---------|---------|----------|
| A.    | Control       | 0.66 ± 0.24(14)* | 0.19 ± 0.10(10) | 0 ± 0(10) |
| B.    | DBN           | 2.6 ± 0.53(12)  | 2.9 ± 0.73(9) | 0.05 ± 0.03(29) |
| C.    | Saccharin     | 7.4 ± 1.4(13)   | 5.1 ± 1.2(9) | 0 ± 0(36) |
| D.    | Saccharin + DBN | 13.7 ± 1.4(11)| 16.3 ± 1.8(9) | 0.92 ± 0.43(21) |

* Results are means ± standard error for the number of animals in parentheses.
and saccharin. Even in the presence of continuous treatment with DBN and saccharin, by 26 weeks, the zonal areas of GGTase activity had regressed. GGTase-positive foci on the other hand, continued to increase in number and size.

**Discussion**

The first step in the neoplastic progression of chemical carcinogenesis is the initiation of the target cell. Initiation is believed to start with the covalent binding of the carcinogen or one of its metabolites to DNA. Replication of the DNA then completes the initiation step by fixation of the alteration in the genetic code of the daughter strand. DNA repair prior to fixation of the alteration prevents initiation. Cellular replication of the genetically altered and initiated cell results in a focus of cells expressing an altered phenotype. The acquisition of GGTase activity is one of the altered phenotypes used to identify these foci of transformed hepatocytes (1-3). The incidence of GGTase-positive foci would, therefore, be an indication of the extent to which a carcinogen has initiated carcinogenesis.

Dibutynitrosamine induced GGTase-positive foci, hyperplastic nodules and hepatocellular carcinomas. The nodules and carcinomas also contained GGTase activity. When saccharin was administered concurrently with DBN, there was an increased incidence of foci, nodules and carcinomas. This is consistent with a precursor relationship of GGTase-positive foci to hyperplastic nodules and hepatocellular carcinomas. Therefore, it appears that the induced incidence of GGTase-positive foci by a chemical would predict the hepatocarcinogenicity of the chemical.

Tumor promoters act after initiation has been completed to stimulate the neoplastic progression so that tumors appear earlier. The appearance of GGTase-positive foci occurs at the end of the initiation step so that the effect of promoters would be to enhance the growth and progression of foci to cancer. The concurrent administration of saccharin with DBN did not increase the size of GGTase-positive foci indicating that saccharin does not promote
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that, activity diet regenerative cacy increased fixation of DBN-induced urinary bladder tumors. DBN did induce simple focal, nodular and papillary hyperplasia and the concurrent administration of saccharin increased both the incidence and severity of the lesions. The concurrent administration of sodium saccharin in the diet with N-butyl-N(4-hydroxybutyl)-nitrosamine (BBN) in the drinking water enhanced the hyperplastic and tumor response to BBN (8). In our investigation and Nakanishi's (8) study, the 37% and 50% increase in drinking water consumption resulting from saccharin administration obscured the interpretation that saccharin is a cocarcinogen. Therefore, further evaluation of the possible cocarcinogenic activity of saccharin is necessary.

Summary

Consistent with the proposed precursor relationship of GGTase-positive foci to hepatocarcinogenesis, the induction of foci by DBN was associated with the induction of hyperplastic nodules and hepatocellular carcinoma and the concurrent administration of sodium saccharin in the diet with DBN in the drinking water increased the tumorigenic response in the liver to DBN.

The authors gratefully acknowledge the excellent technical assistance of Ms. Yvonne M. Aube. The work reported in this article was carried out in part under contract No. 68-03-2870 with the U.S. Environmental Protection Agency.

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REFERENCES

1. Farber, E. The sequential analysis of liver cancer induction. Biochim. Biophys. Acta 605: 149-166 (1980).
2. Pitot, H. C., and Sirica, A. E. The stages of initiation and promotion in hepatocarcinogenesis. Biochim. Biophys. Acta 605: 191-216 (1980).
3. Pereira, M. A. Rat liver foci bioassay. J. Am. Coll. Toxicol. 1: 101-118 (1982).
4. Hicks, R. M., and Chowaniec, J. The importance of synergy between weak carcinogens in the induction of bladder cancer in experimental animals and humans. Cancer Res. 37: 2943-2949 (1977).
5. Hicks, R. M. Wakefield, J. St. J., and Chowaniec, J. Cocarcinogenic action of saccharin in the chemical induction of bladder cancer. Nature 243: 347-349 (1973).
6. Hicks, R. M., Wakefield, J. St. J., and Chowaniec, J. Evaluation of a new model to detect bladder carcinogens or cocarcinogens; results obtained with saccharin, cyclamate, and cyclophosphamide. Chem. Biol. Interact. 11: 225-233 (1975).
7. Cohen, S. M., Arai, M., Jacobs, J. B., and Friedell, G. H. Promoting effect of saccharin and 4-f-tryptophan in uri-
nary bladder carcinogenesis. Cancer Res. 39: 1207-1217 (1979).
8. Nakanishi, K., Hirose, M., Giso, T., Hasagawa, R., Arai, M., and Ito, N. Effects of sodium saccharin and caffeine on the urinary bladder of rats treated with N-butyl-N(4-hydroxybutyl)-nitrosamine. Gann 71: 490-500 (1980).
9. Van Duuren, B. L., and Goldschmidt, B. M. Effects of sodium saccharin and caffeine on the urinary bladder of rats treated with N-butyl-N(4-hydroxybutyl)-nitrosamine. Gann 71: 490-500 (1980).
10. Van Duuren, B. L. Tumor promoting and co-carcinogenic agents in chemical carcinogenesis. Amer. Chem. Soc. Monograph 173: 24-51 (1976).
11. NRC Guide for the Care and Use of Laboratory Animals. Institute of Laboratory Animals Resources, National Research Council, Washington, DC, 1972.
12. Rutenburg, A. M., Kim, H., Fishbein, I. W., Hanker, S. S., Wasserkug, H. L., and Seligman, A. M. Histochemical and ultrastructural demonstration of gamma-glutamyl-transpeptidase activity. J. Histochem. Cytochem. 17: 517-526 (1969).
13. Ito, N., Tatematsu, M., Imaida, K., Hasegawa, R. and Murasaki, G. Effect of various promoters on the induction of neoplastic nodules in rat liver. Gann 71: 415-416 (1980).
14. Druckrey, H., Preussman, R., Ivarkovic S., and Schmahl, D. Organotrope carcinogene Wirkung bei 65 verschiedene N-Nitros Verbindungen an BD-Ratten. Z. Krebsforsch 69: 103-201 (1967).
15. Kunze, E., and Schauer, A. Enzymhistochemische and autoradiographische Untersuchungen as Dibutylnitrosamine-induzierten Harnblasenpapillomen der Ratte. Z. Krebsforsch 75: 146-160 (1971).
16. Kunze, E., Schauer, A., and Calvoer, R. Zur Histochemie von Harnblasenpapillomen der Ratte, induziert durch Dibutylnitrosamine. Naturwissenschaften 56: 639 (1969).
17. Kunze, E., Schauer, A., and Spielmann, J. Autoradiographische Untersuchungen über den RNS-Stoffwechsel während der Entwicklung von Dibutyl-α-nitrosamin-induzierten Harnblasentumoren der Ratte. Z. Krebsforsch 76: 236-248 (1971).
18. Ito, N. Experimental studies on tumors of the urinary system of rats induced by chemical carcinogens. Acta Pathol. Japan 23: 87-109 (1973).
19. Sander, J., Burkle, G. and Burkley, V. Induktion von Lungentumoren und Harnblasentumoren bei Ratten durch Di-N-butylNitrosamin in Dimethylsulfoxide (DMSO). Z. Krebsforsch 82: 83-89 (1974).
20. Vlasov N. N., Dzhioev, F. K. and Pliss, G. B. On peculiarities of a carcinogenic action of dibutyl nitrosamine. Vop. Onkol. 19: 55-65 (1973).
21. Chowaniec, J., and Hicks, R. M. Response of the rat to saccharin with particular reference to the urinary bladder. Brit. J. Cancer 39: 355-375 (1979).