PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **181**, 498–506 (1986)

Influence of Dietary Fat on the Promotion of Diethylnitrosamine-Induced Hepatocarcinogenesis in Female Rats[1] (42283)

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Abstract. The effect of varying the amount and type of dietary fat on the promotion of γ-glutamyltranspeptidase (GGT)-positive foci and hepatocarcinomas in female rats was studied. In the first study, two-thirds of the rats were first intubated with diethylnitrosamine (DEN, 10 mg/kg) 20 hr after partial hepatectomy; 1 week later, rats were fed one of three purified diets (a low-fat diet similar to the AIN-76 diet, a high saturated fat diet, or a high polyunsaturated fat diet) with or without 0.05% phenobarbital in the diet for 10 months. Increasing the fat level of the diet did not increase the number of GGT-positive foci arising spontaneously or induced by DEN alone. When phenobarbital was present in the diet, feeding the high polyunsaturated fat diet slightly increased the number of GGT-positive foci and the incidence of tumors. The low-fat diet, however, increased the incidence of fatty liver. We therefore reexamined the effect of diet on promotion by phenobarbital, using an additional low-fat diet with cornstarch rather than sucrose as the carbohydrate source. In this experiment, both high-fat diets slightly enhanced the induction of GGT-positive foci; the carbohydrate source had no effect. The incidence of tumors was not affected by diet in this experiment, but the incidence of fatty liver was again enhanced by feeding a diet high in sucrose. We conclude that increasing the fat level of the diet does not promote the development of DEN-initiated GGT-positive foci or carcinomas in female rats. Increasing the dietary fat level, however, may enhance promotion of liver foci by phenobarbital. Finally, increasing the sucrose content of the diet increases the incidence of fatty liver.

Dietary fat has been shown to influence the development of cancer in both epidemiological and experimental studies. In humans, an increase in the fat content of the diet has been correlated with high incidences of cancer of the breast, colon, rectum, prostate, and ovary (1). In studies using experimental animals, increasing the fat content of the diet enhances the development of chemically induced tumors in several tissues, including the colon, breast, skin, and pancreas (1–5).

In the liver, the development of tumors, nodules, or enzyme-altered foci has been shown to be altered both by the quantity of fat in the diet and by its saturation level. Increasing the fat level of the diet enhances the development of p-dimethylaminoazobenzene (DAB)-, 2-acetylaminofluorene (AAF)-, and aflatoxin B_1 (AFB)-induced γ-glutamyltranspeptidase (GGT)-positive foci (6, 7) or tumors (8–10) in rats, and the development of spontaneous hepatomas in C3H mice (11). In addition, the development of DAB- and AAF-induced liver tumors is enhanced by feeding a diet which contains a greater proportion of polyunsaturated fatty acids (12–14). In most of the above studies (8–10, 12–14), however, the carcinogen was administered at the same time as the diet, making it difficult to determine which stage of carcinogenesis was affected.

In this study, we examined the effect of dietary fat on the promotion phase of diethylnitrosamine (DEN)-initiated, two-stage rat liver carcinogenesis using the initiation-pro-

[1] This work was supported by DHHS PHS National Research Service Award 5 T32 ES-07175 from the National Institute of Environmental Health Sciences (H.P.G. was an NIEHS Postdoctoral Trainee); Grants CA-07175 and CA-22484 from the National Cancer Institute; and Contract NO1-ES-3-5024 from the National Toxicology Program.

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0037-9727/86 $1.50

motion protocol of Pitot and associates (15–19). Rats were fed one of three diets: a low-fat (LF) diet, a high saturated fat (HSF) diet, or a high polyunsaturated fat (HPUF) diet. Diets were fed during the promotion period to determine whether increasing the fat level of the diet could promote an increase in DEN-initiated γ-glutamyltranspeptidase-positive foci or in benign or malignant neoplasms. The diets were also fed with 0.05% phenobarbital (PB) to determine whether dietary fat alters promotion by PB.

Materials and Methods. *Chemicals.* DEN was obtained from Eastman Organic Chemicals, Rochester, New York; phenobarbital sodium was from Mallinckrodt, Inc., Paris, Kentucky.

Animals and diets. Female CD rats (80 to 100 g) were obtained from Charles River Laboratories, Wilmington, Massachusetts. Rats were housed in stainless-steel, hanging wire cages, and were given food and water *ad libitum.* All rats were allowed to adjust for 1 week before an experiment was started. Rats were fed the NIH-07 diet during this time. The NIH-07 diet was obtained from Ziegler Brothers, Garners, Pennsylvania. Palm oil was a gift from Dr. J. Edward Hunter, The Procter and Gamble Company, Cincinnati, Ohio. All other diet ingredients were obtained from Teklad Test Diets, Madison, Wisconsin. The palm oil and safflower oil used in the study were analyzed for their fatty acid contents by Procter and Gamble.

Experiment 1. Rats were subjected to a 70% partial hepatectomy (20); 20 hr later, two-thirds of the rats were given a single intragastric dose of DEN (10 mg/kg). One week later, rats were transferred from the NIH-07 diet to one of three purified diets: a low-fat diet similar to the AIN-76A diet (21, 22) with 5% of the calories derived from fat (safflower oil); a high saturated fat diet with 47% of the calories derived from palm oil; and a high polyunsaturated fat diet with 47% of the calories from safflower oil. The composition of these diets is shown in Table I. Palm oil contained 51% saturated fatty acids and 8% polyunsaturated fatty acids, whereas safflower oil contained 11% saturated fatty acids and 74% polyunsaturated fatty acids. One-half of the rats on each diet were fed 0.05% PB in the diet.

Rats were fed the experimental diets for 4 or 10 months. Rats were then killed by decapitation and frozen liver samples were prepared as previously described (15) and then stored at −70°C. Additional liver samples were fixed in Formalin and processed for histological analysis. Histologic diagnoses of neoplastic nodules and hepatocellular carcinomas were made based on previously reported criteria (23). Frozen liver sections were prepared and stained for GGT as described previously (15). The number of GGT-positive foci per liver and the volume of liver occupied by the foci were determined as described previously (15, 24).

TABLE I. COMPOSITION OF DIETS IN EXPERIMENT 1 (PERCENTAGE OF DIET)

	Low fat	High saturated fat	High polyunsaturated fat
Casein	20.0	25.8	25.8
Methionine	0.3	0.4	0.4
Choline	0.2	0.3	0.3
Cellulose	5.0	6.5	6.5
Vitamin mix[a]	1.0	1.3	1.3
Mineral mix[a]	3.5	4.5	4.5
Cornstarch	15.0	19.3	19.3
Sucrose	53.0	16.1	16.1
Safflower oil	2.0	—	25.8
Palm oil	—	25.8	—
Percentage of kcal from fat	5	47	47

[a] See Refs. (21, 22).

Data for the number of foci per liver and the focal volume were analyzed nonparametrically at each time point. Data were ranked and the ranks were then converted to rankits, which were then analyzed using three-way analysis of variance (25). Data for rat weights and rat liver weights were analyzed directly by three-way analysis of variance. If a significant effect or interaction was produced by diet, the data were further analyzed by Bonferroni t statistics. Differences brought about by either DEN or PB treatment could be detected by the analysis of variance, if no significant interactions between the DEN and PB treatments were seen. Tumor incidence data were analyzed by χ^2 analysis. In all analyses, a confidence level of 95% or greater was considered significant.

TABLE II. COMPOSITION OF DIETS IN EXPERIMENT 2 (PERCENTAGE OF DIET)

	Low fat			
	High cornstarch	High sucrose	High saturated fat	High polyunsaturated fat
Casein	20.0	20.0	25.8	25.8
Methionine	0.3	0.3	0.4	0.4
Choline	0.2	0.2	0.3	0.3
Cellulose	5.0	5.0	6.5	6.5
Vitamin mix[a]	1.0	1.0	1.3	1.3
Mineral mix[a]	3.5	3.5	4.5	4.5
Cornstarch	68.0	15.0	35.4	35.4
Sucrose	—	53.0	—	—
Safflower oil	2.0	2.0	—	25.8
Palm oil	—	—	25.8	—

[a] See Refs. (21, 22).

Experiment 2. All rats were subjected to partial hepatectomy followed by intubation with DEN (10 mg/kg) 20 hr later, as described above. One week later, rats were transferred from the NIH-07 diet to one of four purified diets: a low-fat diet with sucrose as the primary carbohydrate source; a low-fat diet using cornstarch as the sole carbohydrate source; a high saturated fat diet; or a high polyunsaturated fat diet (Table II). All diets contained 0.05% PB. Rats were fed the experimental diets for 10 months. At this time, all rats were killed, and the GGT-positive foci in frozen liver sections were quantitated as described above.

Data for the number of foci per liver and the focal volume were analyzed using nonparametric statistics (26). Data from rats fed the high-sucrose, high saturated fat, and high polyunsaturated fat diets were compared to data from rats fed the high cornstarch diet using a two-sided multiple comparison test. Data for rat weights and rat liver weights were analyzed by one-way analysis of variance (25). If significant f values were detected, means were compared to the mean of the high cornstarch control group using Dunnett's t test. Tumor incidence data were analyzed using a χ^2 test. In all analyses, a confidence level of 95% or greater was considered significant.

Results. In the first experiment, a three-factorial design was used to ascertain the effects of varying the level and type of dietary fat during the promotion period of DEN-initiated, PB-promoted liver carcinogenesis. Rats were fed the diets for 4 or 10 months. The number of induced GGT-positive foci per liver and the volume those foci occupied as a percentage of liver volume are shown in Tables III and IV. A typical analysis of variance of the data is shown in Table V. Although this table shows only the analysis of variance for the number of GGT-positive foci at 10 months (Table IV), the analyses of variance of all of the data in Tables III and IV were identical in levels of significance. Because the foci data were not normally distributed and because treatment variances were heterogeneous, conventional analysis of variance could not be used (25, 26). Data were therefore ranked and the ranks were converted to rankits, which follow a normal distribution. The rankits were then analyzed

TABLE III. EFFECT OF DEN, PB, AND DIETARY FAT ON THE NUMBER AND VOLUME OF GGT-POSITIVE FOCI PER LIVER IN EXPERIMENT 1 (4 MONTHS)

Dietary treatment	No. of GGT$^+$ foci/liver		Liver volume occupied by GGT$^+$ foci (%)	
	−PB	+PB	−PB	+PB
No DEN				
LF	0	234 ± 145[a]	0	0.01 ± 0.008
HSF	84 ± 52	11 ± 11	0.007 ± 0.007	0.005 ± 0.005
HPUF	100 ± 65	81 ± 37	0.0004 ± 0.0002	0.002 ± 0.001
+ DEN				
LF	479 + 158	2669 ± 1392	0.04 ± 0.02	0.10 ± 0.04
HSF	327 ± 184	6661 ± 2620	0.009 ± 0.006	0.19 ± 0.05
HPUF	504 ± 81	3690 ± 1249	0.01 ± 0.006	0.10 ± 0.05

[a] Data are expressed as means ± SEM.

TABLE IV. EFFECT OF DEN, PB, AND DIETARY FAT ON THE NUMBER AND VOLUME OF GGT-POSITIVE FOCI PER LIVER IN EXPERIMENT 1 (10 MONTHS)

Dietary treatment	No. of GGT$^+$ foci/liver		Liver volume occupied by GGT$^+$ foci (%)	
	−PB	+PB	−PB	+PB
No DEN				
LF	58 ± 39[a]	10 ± 6	0.02 ± 0.01	0.01 ± 0.01
HSF	58 ± 44	48 ± 25	0.002 ± 0.001	0.003 ± 0.002
HPUF	42 ± 42	100 ± 47	0.0004 ± 0.0004	0.013 ± 0.008
+ DEN				
LF	761 ± 186	3274 ± 1127	0.07 ± 0.02	1.02 ± 0.45
HSF	773 ± 288	3597 ± 801	0.10 ± 0.02	0.89 ± 0.36
HPUF	743 ± 174	8248 ± 1707	0.12 ± 0.07	2.33 ± 0.68

[a] Data are expressed as means ± SEM.

by three-way analysis of variance. For both the number of foci per liver and the focal volumes at both 4 and 10 months, the administration of either DEN or PB induced a statistically significant effect; a significant interaction between DEN and PB was also present. This demonstrates that PB had an effect only when DEN was administered previously. Diet did not induce a significant effect on the number of GGT-positive foci per liver or on the focal volume at either 4 or 10 months. In rats which did not receive DEN, very few foci were induced. In rats which received DEN but not PB, a larger number and volume of foci were induced, but the values were still low and clearly were not altered by diet. In rats which received both DEN and PB, however, the number and volume of foci were increased about twofold in rats fed the HSF diet at 4 months and in rats fed the HPUF diet for 10 months. The increase at 4 months was caused mainly by a very high number of foci in one rat (there were six rats in the group). At 10 months, the much greater response to DEN and PB administration, coupled with the lack of an effect of dietary fat in rats which did not receive both DEN and PB, probably masked the effect seen in the rats which received both DEN and PB.

TABLE V. ANALYSIS OF VARIANCE OF THE NUMBER OF GGT-POSITIVE FOCI AT 10 MONTHS IN EXPERIMENT 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	f
PB	1	6.2	6.19	22.1**
DEN	1	44.2	44.16	157.7**
Diet	2	0.8	0.41	1.5
PB × DEN	1	3.3	3.30	11.8**
PB × diet	2	0.9	0.45	1.6
DEN × diet	2	0.5	0.23	0.8
Three-way interaction	2	0.0	0.00	0.0
Error	88	24.6	0.28	

** The source of variation is significant ($P < 0.01$).

The incidence of tumors at 10 months in rats initiated with DEN is shown in Table VI. Only rats which were intubated with DEN developed tumors; only rats which were intubated with DEN and then fed PB in the diet developed an appreciable number of tumors. Rats which were fed the HPUF diet had about twice the tumor incidence as those fed one of the other two diets. Most of this difference was due to a higher incidence of neoplastic nodules.

The final rat weights and rat liver weights were altered by diet (data not shown). Rats fed the HPUF diet weighed significantly more than those fed either of the other two diets. Rats fed PB also weighed significantly less than those not fed PB. Rats which were fed the LF diet had higher liver weights than those fed the HSF diet; all other dietary comparisons were nonsignificant. Neither DEN nor PB treatment altered rat liver weights.

One explanation for the increased liver weights in rats fed the LF diet is that those rats had a higher incidence of fatty liver than rats fed the other two diets (Table VII). The diagnosis of fatty liver was made when an estimated 20% or more of the hepatocytes ex-

TABLE VI. INCIDENCE OF TUMORS IN RATS INITIATED WITH DEN IN EXPERIMENT 1 (10 MONTHS)

Dietary treatment	Percentage of rats with		
	Neoplastic nodules	Hepatocellular carcinomas	Total
− Phenobarbital			
Low fat	0	0	0
High saturated fat	17 (2/21)[a]	0	17 (2/12)
High polyunsaturated fat	9 (1/11)	0	9 (1/11)
+ Phenobarbital			
Low fat	29 (2/7)	14 (1/7)	43 (3/7)
High saturated fat	17 (2/12)	17 (2/12)	34 (4/12)
High polyunsaturated fat	50 (6/12)	25 (3/12)	75 (9/12)

[a] Number of rats with tumors divided by the number of rats is in parentheses.

hibited visible fat vacuoles in the light microscope.

From this experiment, it was apparent that increasing the fat content of the diet did not in itself promote the development of DEN-initiated tumors or GGT-positive foci in rat liver: very few tumors were induced and the number and volume of foci were unchanged. Increasing the PUF content of the diet did appear to alter promotion by PB: the tumor incidence and the number and volume of foci were doubled. This effect, however, was not statistically significant, in part because of the experimental design which was used; the larger responses induced by DEN and PB administration and the lack of a response to the dietary manipulations in rats which did not receive both DEN and PB may have prevented a statistically significant response from being seen.

TABLE VII. INCIDENCE OF FATTY LIVER AT 10 MONTHS IN EXPERIMENT 1

Dietary treatment	Incidence of fatty liver (%)	
	−Phenobarbital	+Phenobarbital
No DEN		
LF	67 (4/6)[a]	20 (1/5)
HSF	0 (0/6)	0 (0/5)
HPUF	17 (1/6)	0 (0/6)
+ DEN		
LF	83 (10/12)	43 (3/7)
HSF	33 (4/12)	17 (2/12)
HPUF	9 (1/11)	0 (0/12)

[a] The number of rats with fatty liver divided by the total number of rats is in parentheses.

In addition, a larger number of rats per group may have been needed to detect a response. We therefore repeated the portion of the experiment examining the effect of dietary fat on PB promotion, using a larger number of rats in each dietary treatment. By simplifying the experimental design and by increasing the number of rats per group, we hoped to increase the power to detect a response (25). In addition, we fed one additional low-fat control diet, to lower the incidence of fatty liver seen in the rats fed the LF diet and to ensure that the fatty liver was not the cause of the diet-induced differences.

In the second experiment, all rats were initiated using the PH/DEN method described earlier; all rats also received 0.05% PB in the diet during the promotion period. We fed one additional LF diet, in which all carbohydrates came from cornstarch, in addition to the LF diet fed earlier, in which most of the carbohydrates were derived from sucrose.

After 10 months, increasing the level of dietary fat again slightly enhanced the development of GGT-positive foci (Fig. 1). In rats fed the HSF diet (as compared to rats fed the LF, high-cornstarch diet) the number of foci per liver was increased by about 60% and the focal volume was increased about twofold; neither of these differences was statistically significant. Rats which were fed the HPUF diet had about 65% more foci per liver, but this difference was also not statistically significant. The carbohydrate source had no effect on the induction of GGT-positive foci. The incidence of tumors and fatty liver is shown in Fig. 2.

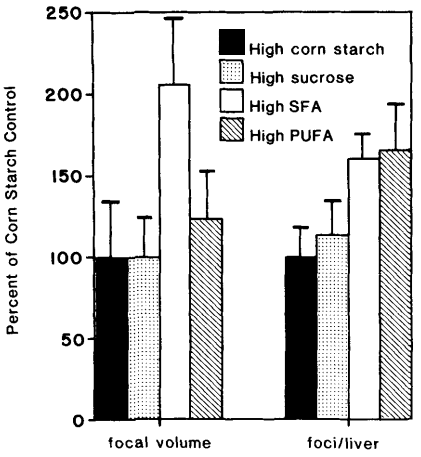

FIG. 1. Influence of diet on the induction of GGT-positive foci in experiment 2. All rats were intubated with DEN (10 mg/kg) 20 hr after PH. One week later, they were fed one of four purified diets containing 0.05% PB for 10 months. The number of GGT-positive foci per liver and the percentage of liver volume occupied by GGT-positive foci were quantitated. Data are expressed as a percentage of the values for the low-fat, high cornstarch diet, which are as follows: 7021 ± 1354 foci/liver; 1.67 ± 0.56% of liver volume occupied by foci. High cornstarch: low-fat, high cornstarch diet; high sucrose: low-fat, high-sucrose diet; high SFA: high saturated fat diet; high PUFA: high polyunsaturated fat diet.

The incidence of neoplastic nodules or hepatocellular carcinomas did not differ between groups, that of the latter being very low. Rats fed the high sucrose diet had a much higher incidence of fatty liver. Rats which were fed the HSF diet also weighed more than rats fed the control diet, and, as in the previous study, rats fed the high-sucrose diet had a higher liver weight (data not shown).

Discussion. These experiments show that, while increasing the fat content of the diet does not in itself promote DEN-initiated foci or tumors, it may enhance the promoting action of PB. Altering the fat content of the diet did not promote hepatocarcinogenesis: the number and volume of GGT-positive foci were unchanged and very few tumors were induced. But when PB was present in the diet, both experiments showed an effect when the fat content of the diet was increased. In the first experiment, increasing the polyunsaturated fat content of the diet for 10 months slightly enhanced the production of GGT-positive foci and tumors; in the second experiment, increasing the concentration of either saturated fat or polyunsaturated fat in the diet slightly increased the number of GGT-positive foci. The first and second experiments differ in that, in the first study, the HPUF diet increased the number of foci and tumors while the HSF diet had no effect, whereas in the second experiment both the HSF and HPUF diets slightly increased the number of foci without affecting the incidence of tumors.

The difference in the two experiments can possibly be attributed to a caloric effect: in both experiments the group which gained the most weight also developed the largest number and volume of foci. The body weight gain could have been increased either by a greater food intake or by more efficient utilization of calories in rats fed the high-fat diets (2). According to Boutwell *et al.* (2), increases in skin carcinogenesis brought about by dietary fat could be accounted for entirely by more efficient utilization of fat. Alternatively, an increase in food intake would also result in an increased intake of PB, which could conceivably be responsible for the increase seen. However, Goldsworthy *et al.* (15) have reported that increasing PB intake over 0.05% did not increase the production of GGT-positive foci. In addition, the addition of PB to the diet decreased final body weights; this could be caused either by a lower food intake or a less efficient utilization of calories.

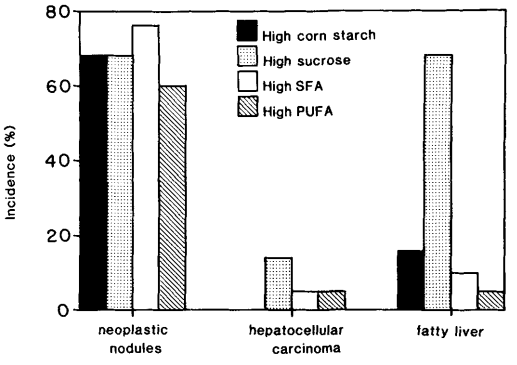

FIG. 2. Influence of diet on the induction of tumors and fatty liver in experiment 2. All rats were intubated with DEN (10 mg/kg) 20 hr after PH. One week later, they were fed one of four purified diets containing 0.05% PB for 10 months. The incidences of neoplastic nodules, hepatocellular carcinomas, and fatty liver were determined. Legend is the same as for Fig. 1.

Increasing the dietary fat level may also increase the number of foci by enhancing the action of PB. Rats which are fed a HPUF diet have a higher activity of PB-induced microsomal cytochrome *P*-450 than rats which are fed a fat-free diet or a diet containing only highly saturated fats (27). The metabolism of several chemicals, including hexobarbital, aniline, ethylmorphine, benzo[*a*]pyrene, and dimethylnitrosamine, is also enhanced by feeding diets high in polyunsaturated fatty acids (27–31).

Previously published work has shown an increase in the production of chemically induced liver tumors, but in a majority of the studies the carcinogen was fed in the diet or administered at the same time (8–10, 12, 13). In these studies, it is not possible to determine whether the nutritional variations altered the initiation or the promotion phase of hepatocarcinogenesis. In the study by Newberne *et al.* (14), diets containing either 20% beef fat or corn oil were fed during and after, or only after the administration of AFB. Feeding corn oil increased the incidence of tumors (as compared to feeding beef fat) when the diets were fed during and after AFB administration, but not when the diets were fed only after AFB administration. This study implies that unsaturated fats enhance tumorigenesis by acting on the initiation phase of hepatocarcinogenesis. In the study by Misslbeck *et al.* (7), the dietary fat content was varied after the administration of 10 doses of AFB. Increasing the dietary fat level in this study increased the number of GGT-positive foci after 12 weeks, although the number of foci induced was very low. This study implies that dietary fat is acting during the promotion phase, although the multiple doses of the complete carcinogen, AFB, probably exerted significant promoting action before the dietary manipulations were started. In a similar study, Baldwin *et al.* (6) found that increasing the dietary fat level during and after AFB administration also increased the number of GGT-positive foci, although not significantly.

Rats which were fed a diet containing high amounts of sucrose also developed fatty liver and an increased liver weight in both experiments. Dietary sucrose has previously been shown to increase the lipid content of hepatocytes and to increase liver weight (32–35); the increase in liver weight has been attributed to hyperplasia (32, 33). In our study, altering the sucrose content of the diet did not affect the appearance of tumors or GGT-positive foci in the presence or absence of PB: when PB was present in the diet, using sucrose rather than cornstarch as the carbohydrate source did not alter the appearance of tumors or GGT-positive foci in Experiment 2; when PB was not in the diet (Experiment 1), the number of tumors and GGT-positive foci was very low for all diets fed. These results contrast somewhat with those of Hei and Sudilovsky (34), who, using a somewhat different protocol and a different statistical analysis, found that rats fed sucrose rather than glucose as the main carbohydrate source after DEN administration developed more GGT-positive foci when PB was not fed in the diet.

In this study, we quantitated altered hepatic foci using the enzyme marker GGT. In previous studies we have found that when only the initiator DEN is administered, GGT is equivalent in scoring foci to the other two markers commonly used: glucose 6-phosphatase (G6Pase) and canalicular ATPase (ATPase) (17). When PB is administered as the promoter, however, GGT is the most efficient marker: 80–90% of foci are positive for GGT (16, 17). In this study, many of the liver sections—especially those from rats fed diets high in sucrose—exhibited mild to severe fatty metamorphosis. In these sections, it was difficult, if not impossible, to accurately quantitate the negative markers G6Pase and ATPase, although reading the positive stain GGT presented no problems. In random sections not exhibiting fatty metamorphosis, we confirmed previous results: GGT was the predominant marker in rats fed PB, and GGT was equivalent to G6Pase and ATPase in scoring foci in rats receiving DEN but not PB. Nevertheless, a selective increase in ATPase or G6Pase cannot be unequivocally ruled out, particularly since GGT has been shown to be an inadequate marker under certain experimental conditions (36, 37).

The results of this study show that dietary fat probably plays only a minor role in the promotion process of hepatocarcinogenesis. Although increasing the fat content of the diet or altering the proportion of polyunsaturated fat did not promote liver carcinogenesis, it may

have the potential for altering the promoting abilities of other chemicals. A role in tumor initiation also seems possible, based on the results of other studies and the lack of promoting ability seen in this study.

We thank Dr. J. Edward Hunter of The Procter and Gamble Company for supplying the palm oil and for analyzing it and the safflower oil used in the study for their fatty acid compositions; Dr. Harold Campbell for his assistance in the use of the computer to quantitate foci; Jane Weeks, Mary Folz-Erbs, and Susan Carlson for preparing the histological slides; Susan Moran and Wendy Kennan for excellent technical assistance; Kevin Moran, Chris Beckman, and Michael Judkins for animal care; and Mary Jo Markham for typing the manuscript.

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Received July 29, 1985. P.S.E.B.M. 1986, Vol. 181.
Accepted December 2, 1985.