

The Carcinogenicity of *p*-Dimethylaminoazobenzene in Diets Containing Hydrogenated Coconut Oil*†

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The original observation that the carcinogenic potency of *p*-dimethylaminoazobenzene varies with diet (19) has been followed by numerous studies of individual dietary substances on the development of hepatic tumors with this agent. Much emphasis has been placed on the vitamins of the B complex (4, 9, 17) or on certain of these vitamins plus proteins or amino acids (5, 9, 16, 30). Comparatively little attention has been given to the fats, although at least three fatty extracts are reported to reduce the carcinogenic effectiveness of the azo dye when fed with it at levels of 5 per cent of the diet or less: rice bran oil (28), an ether extract of yeast (28), and a similar extract of liver (16). However, the original materials from which these extracts were prepared all happen to be anticarcinogenic by virtue of nonfatty substances such as proteins and B vitamins that they contain. Analyses of rice bran oil for riboflavin and other B vitamins revealed the presence of many water-soluble materials (28), although in amounts that were probably insufficient to account for the entire anticarcinogenic effect observed.

A second group of fats appears to have no significant effect on the development of hepatic tumors due to *p*-dimethylaminoazobenzene. These include cod liver oil at 1 or 2 per cent of the diet (16); olive oil at 3 per cent (10, 19); cottonseed oil (16), corn oil (9, 16), butter fat (16), or wheat germ oil (27) at 5 per cent; and partially hydrogenated vegetable oil (primex) at 20 or 30 per cent of the diet (16). These latter oils, however, were usually fed in experiments designed to

reveal the effects of the nonfatty constituents of the diet, and true differences between the various oils may therefore have been masked. Another complication arises from the fact that as the percentage of fat in the diet increases, the amount of food, and therefore of carcinogen consumed by the animal, decreases. Nevertheless, these "nonprotective" fats represent a considerable range in such chemical characteristics as degree of unsaturation, saponification equivalent, and auto-oxidizability.

György and his associates (7) compared the carcinogenic effectiveness of diets low in protein and high in fat and reported that crisco and butter fat were cocarcinogenic as compared to lard. As an explanation of the protective effect of the lard diet a destruction of the carcinogen *in vitro* was assumed.

The present study deals with the effect of hydrogenated coconut oil on the incidence of liver tumors in rats fed *p*-dimethylaminoazobenzene in synthetic diets. From a chemical point of view this oil represented a considerable departure from the corn oil previously employed for the production of liver tumors in rats fed synthetic diets (17). The hydrogenated coconut oil contained no unsaturated fatty acids, whereas the corn oil was highly unsaturated, linoleic acid constituting approximately 42 per cent of the fatty acids present; the hydrogenated coconut oil was low in antioxidants whereas the corn oil contained appreciable amounts of tocopherol; and, furthermore, most of the fatty acids in the hydrogenated coconut oil were of relatively short chain length. Another reason for the comparison of this particular pair of oils arose from the observation that the formation of hepatic (17) and other (11) types of tumors is diminished on diets low in pyridoxine. In multiple deficient diets pyridoxine and the essential fatty acids appear able to replace one another to a certain extent, for either by itself is sufficient to alleviate most of the symptoms of acute acrodynia (23, 26). The possibility existed that a similar association of these two factors might manifest itself in the formation of hepatic tumors.

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METHODS

As in our previous studies (17), young, mature, albino Sprague-Dawley rats 160 to 190 gm. in weight were fed 0.06 per cent of *p*-dimethylaminoazobenzene for a period of 120 days. The animals were kept in groups of 7 and 8 in screen-bottomed cages; food and water were given *ad libitum*. The dye was incorporated in the rations by dissolving it with heat in the fat of the diets. The rations were mixed in amounts sufficient for 2 to 4 weeks and stored at 0° to 5° C. After 120 days the livers were examined by laparotomy and the animals continued for an additional 2 months on the same diet as before, but minus the azo dye. At 6 months a final examination of the livers was made. The diets fed had the following composition:

Casein (vitamin-free ¹)	120
Cerelose ²	800 or 790
Corn oil or hydrogenated coconut oil	40 or 50
Salts	40
<i>p</i> -Dimethylaminoazobenzene	0.6

¹ Crude casein was washed in cloth bags at room temperature for 5 days with 15 changes of tap water and 2 changes of distilled water (20 gallons for each 10 pounds). After being dried and ground it was finally extracted at 50° C. for 4 days with two changes of ethyl alcohol (4 gallons for each 10 pounds). By the methods indicated each gram of the purified casein contained an average of 0.6 μ gm. thiamin (thiochrome), 0.6 μ gm. riboflavin (fluorometric), less than 1 μ gm. pantothenic acid (growth of *Lactobacillus casei*, determination made by Dr. A. L. Neal), and 0.17 μ gm. pyridoxine (growth of *Saccharomyces carlsbergensis*, determination made by Mrs. E. C. Miller).

² A pure glucose monohydrate obtained from Corn Products Refining Company.

Each rat received one drop of halibut liver oil weekly. Crystalline vitamin B supplements added per kilogram of ration were:

Thiamin chloride	3.0 mgm.
Riboflavin	2.0 mgm.
Calcium pantothenate	7.0 mgm.
Pyridoxine hydrochloride	0.0, 0.2, or 2.5 mgm.
Choline chloride	30.0 mgm.

The hydrogenated coconut oil used in these experiments was a refined oil, hydrogenated in the presence of nickel at 140° C., and deodorized at 180° C. for 5 hours at a pressure of 5 mm. Hg. It was a pasty solid at room temperature and had a melting point of 36.0° to 36.2° C. (Wiley). The iodine numbers of the two lots of oil used for the two series of experiments reported below were 0.33 and 0.40 respectively. No tocopherol was found to be present as determined by a modification of the Furter-Meyer test (22).

Two of the groups receiving the hydrogenated coconut oil were also fed daily supplements of 2 drops (40 mgm.) of ethyl linolate given by dropper. This amount of linoleic acid is much less than that con-

sumed by the animals receiving diets containing 5 per cent of corn oil, but is sufficient for the growth and general well-being of young rats on a fat-free diet (15). The ethyl linolate was prepared according to a modification of the method of Rollet (24). Crystalline 9,10,12,13-tetrabromostearic acid, m.p., 115° C., was isolated from the brominated fatty acids of corn oil, decomposed with zinc in absolute ethanol-HCl, and the resulting ethyl linolate freed of fatty acid and dried. The ester was not redistilled but was sealed *in vacuo* in 5 gm. lots containing 10 μ gm. of synthetic α -tocopherol per gm. Each 5 gm. tube supplied 30 animals for 4 days and was kept in the refrigerator after opening.

The various combinations of corn oil, hydrogenated coconut oil, pyridoxine, and ethyl linolate fed are listed in Table I.

RESULTS

Most of the rats on the various diets maintained their weights well, their general physical condition remained good, and the survival at 4 months was 100 per cent in 5 of the 8 groups (Table I). In the initial series (Table I, groups I to III) a normal tumor production of 53 per cent at 6 months was observed in a diet containing 5 per cent of corn oil and 2.5 mgm. of pyridoxine per kg. of ration. This agrees with previous experience with this diet (17). However, when the corn oil was replaced by 4 per cent of hydrogenated coconut oil either in the presence of 2.5 mgm. of pyridoxine per kg. of diet (group II) or in the absence of added pyridoxine (group III) the animals were almost completely protected against the formation of tumors. Only one animal fed the hydrogenated coconut oil developed a tumor by 6 months, and the livers of the others were nearly normal in appearance. The protective effect of the hydrogenated coconut oil could not be ascribed either to a low caloric intake or to a reduced consumption of carcinogen, for the average daily food consumption in the groups getting hydrogenated coconut oil was as high as, or higher than, that in the control groups getting corn oil.

In a second series (Table I, groups IV to VIII) both corn oil and hydrogenated coconut oil were fed at a level of 5 per cent. The pyridoxine levels were 2.5 and 0.2 mgm. per kg. of ration. In addition ethyl linolate (stabilized with a small amount of synthetic α -tocopherol) was given orally at the level of 40 mgm. per rat daily to one of each of the two groups receiving medium and low amounts of pyridoxine. At 4 months a tumor incidence of 36 per cent was observed in rats fed the corn oil control diet. No tumors were found in any of the rats fed the hydrogenated coconut oil diets irrespective of the levels of pyridoxine or ethyl linolate given. At 4 months the groups fed hydrogenated coconut oil were subdivided; in each group 10 rats continued to receive the dye for the

following 2 months while the remaining 5 rats were fed the dye-free diet as usual. At the six months examination the tumor incidence in the group fed corn oil had risen to 64 per cent while only 2 tumors were found in the four groups that received the dye continuously in diets containing hydrogenated coconut oil. No tumors were observed in the four groups that were given the dye for only 4 months in similar diets; in other words, the hydrogenated coconut oil again exerted a pronounced anticarcinogenic effect. As in the previous series the livers of the protected rats were nearly normal in appearance. The survival was good and the consumption of azo dye was comparable with that of the control group.

linoleic acid . . ." When the latter substance, which was toxic to rats even in the absence of carcinogen, was incorporated in the diet at a level of 16 per cent, a visible destruction of the dye occurred *in vitro* within 5 days. No destruction of the dye was observed in the lard diet, which had proved to be noncarcinogenic, but it was nevertheless "assumed that the liberation of unsaturated fatty acids in the process of digestion opens the way to the decomposition of butter yellow before it reaches the liver cells."

Since there may be considerable discrepancy between the fate of the dye in whole fats in the digestive tract, as contrasted to its behavior in a free acid *in vitro*, it appeared desirable to study the stability of *p*-dimethyl-

TABLE I: INCIDENCE OF HEPATIC TUMORS IN RATS FED CORN OIL OR HYDROGENATED COCONUT OIL

Group *	Fat, %	Pyridoxine hydrochloride, μ gm. per 10 gm. diet	Ethyl linolate, mgm. per rat per day	Average starting weight, gm.	Average weight at 4 months, gm.	Average food consumption, gm. per rat per day	Survival † at		Tumors ‡ at			Cirrhosis
							4 mo.	6 mo.	4 months	6 months		
									dye for 4 mo.	dye for 6 mo.		
I	5 corn oil	25	0	161	166	7.5	15/15	—	2/15	8/15	—	Moderate-severe
II	4 HCNO §	25	0	166	190	9.3	13/15	—	0/13	1/13	—	None or mild
III	4 HCNO	0	0	181	169	7.5	12/15	—	0/12	0/12	—	None or mild
IV	5 corn oil	25	0	191	193	8.0	11/15	—	4/11	7/11	—	Moderate-severe
V	5 HCNO	25	0	185	192	9.8	15/15	12/15	0/15	0/5	1/7	None or mild
VI	5 HCNO	25	40	183	183	10.8	15/15	13/15	0/15	0/5	0/8	None or mild
VII	5 HCNO	2	0	166	157	7.5	15/15	15/15	0/15	0/5	0/10	None
VIII	5 HCNO	2	40	170	159	7.7	15/15	12/15	0/15	0/4	1/8	None or mild

* Groups I to III constitute one series of experiments and are directly comparable; groups IV to VIII comprise a second series of experiments.

† Survival = Number living over number at start.

‡ Tumors = Number with tumors over number surviving at 4 or 6 months.

§ HCNO = Hydrogenated coconut oil.

The stability of p-dimethylaminoazobenzene in the diet and in the gastrointestinal tract.—It has been pointed out previously that the protective effect of diet against the action of *p*-dimethylaminoazobenzene might be exerted through several quite different channels (17) and György and his co-workers (7) placed particular emphasis on the conservation and destruction of the azo dye *in vitro*, before it gets a chance to act in the critical carcinogenic areas. Obviously such protection of the animal would merely be a reflection of a reduced intake of effective carcinogen. A more basic question is whether the observed dietary modification of the effect of *p*-dimethylaminoazobenzene is a cellular process. György and his group fed the dye in rations containing only 6 per cent of casein, and the B vitamins were given as separate supplements. When the diet contained 23 per cent of lard, liver tumors failed to develop, while the presence of crisco or butter fat in the diet "proved to be procarcinogenic." The explanation of this effect was stated as follows: "The answer has been provided by results of still other experiments in which the source of fat was . . .

aminoazobenzene in a series of selected diets known to vary widely in carcinogenicity. For this purpose an accurate and specific method for the determination of *p*-dimethylaminoazobenzene was devised. The dye readily dissolves in strong hydrochloric acid to give a red solution having an absorption maximum at 516 $m\mu$. The red complex is insoluble in ethyl ether and petroleum ether but can be extracted from aqueous solutions with chloroform. Furthermore the intensity of the red color in acid solution is considerably greater than the corresponding yellow color in ordinary organic solvents.

The procedure finally adopted for the analysis of the diets was as follows: 1 gm. of food (approximately 0.6 mgm. azo dye) was extracted with several 5 to 10 cc. portions of petroleum ether and the extracts filtered and made up to 50 cc. In highly rancid diets difficulty was experienced in obtaining complete extraction of the dye as tested by adding 1:1 HCl to the residue. In such cases acetone was used to complete the extraction and was then washed out of the petroleum ether with water. Five cubic centimeters of the combined

extracts were placed in a separatory funnel and extracted twice with 10 cc. portions of 1:1 hydrochloric acid. The acid extracts were made up to a volume of 30 cc. (approximately 60 μ gm. azo dye) with more acid and the concentration, C, of the dye ascertained by measuring the density, L, of this final solution through the 520 m μ . filter of an Evelyn photoelectric macrocolorimeter. The colorimeter constant, K, in the relation $C = KL$ was found to be 2.98 μ gm. dye/cc. With this procedure it was easily possible to measure as little as 1 μ gm. of *p*-dimethylaminoazobenzene in 10 cc. of acid with an accuracy of ± 5 per cent.

coconut oil, but also those containing corn oil in the presence of rice bran concentrate or of mixtures of synthetic B vitamins in amounts somewhat different from those in the control diet (Table II).

There does not appear to be any reason to attribute antioxidant properties to different mixtures of crystalline B vitamins (6). Apparently the experimental procedure of spreading corn oil on a dry diet and exposing it to air at room temperature for 90 days was just sufficient to destroy the antioxidants in a certain percentage of samples, and once autoxidation began, it proceeded rapidly. Should the stabilization of the azo

TABLE II: STABILITY OF *p*-DIMETHYLAMINOAZOBENZENE IN VARIOUS DIETS *In Vitro* AFTER STORAGE IN THE DARK AT DIFFERENT TEMPERATURES

Diet †	Fat, %	Initial azo dye content, %	% change in azo dye content from initial level * after							
			15 days at		30 days at		60 days at		90 days at	
			-5°	25°	-5°	25°	-5°	25°	-5°	25°
Synthetic control	Corn oil 5	0.059	+1.7	-5.1	+6.8	+6.8	—	—	+5.1	-75 (rancid)
2% Rice bran extract ‡ crude casein	Corn oil 5	0.056	+5.4	0.0	+12.5	+7.2	—	—	-3.6	+1.8
HCNO § 2 μ gm. pyridoxine	HCNO 5	0.060	+5.0	0.0	+6.7	0.0	—	—	+8.3	+8.3
HCNO	HCNO 5	0.062	+9.7	0.0	0.0	+3.2	—	—	-4.8	-1.6
2 μ gm. Pyridoxine, 50 μ gm. flavin	Corn oil 5	0.054	0.0	+1.8	+7.4	0.0	+3.7	+1.8	—	—
0.035% Niacin	Corn oil 5	0.055	0.0	+1.8	+3.6	-1.8	0.0	0.0	—	—
Egg white	Corn oil 5	0.057	-5.3	0.0	-1.8	-3.5	-7.0	0.0	—	—

* Except for the change observed in the synthetic control diet at 90 days at 25°, the variations can be ascribed to sampling and to changes in the moisture content of the diets.

† These diets consisted of 12 per cent protein, usually purified casein, and 4 per cent salts, with cerelose as the remaining major constituent. B vitamins included 25 μ gm. pyridoxine hydrochloride, 30 μ gm. thiamin chloride, 20 μ gm. riboflavin, 70 μ gm. calcium pantothenate, and 300 μ gm. choline chloride per 10 gm. diet unless otherwise noted.

‡ "Vitab Rice Bran Concentrate," National Oil Products Co., Harrison, N. J. No crystalline vitamins were added to this diet.

§ HCNO = Hydrogenated coconut oil.

The diets analyzed included the synthetic diets containing corn oil or hydrogenated coconut oil as described in the present paper, and a diet containing 2 per cent of rice bran concentrate and 12 per cent of crude casein, selected because of the unusually high carcinogenicity of this particular combination (16). Other diets were high in riboflavin and low in pyridoxine, high in niacin, or contained egg white as the source of protein. The freshly mixed diets were stored in the dark at room temperature (25° C. \pm) and at -5° C. and analyzed periodically.

The azo dye was found to be very stable in the various diets *in vitro*. An appreciable destruction of the dye was observed in only one sample, the synthetic diet containing corn oil stored at room temperature for 90 days (Table II). This sample had developed a very strong rancid odor. Neither destruction of the dye nor rancidity were observed when the same diet was stored at -5° for 90 days, nor was any loss of the dye detected in any of the other diets. The stable diets included not only those containing hydrogenated

dye prove to be essentially a matter of preventing rancidity, the stability observed in the presence of the rice bran concentrate could be attributed to the antioxidants in the concentrate (6, 7).

Nevertheless, it is apparent that there was no correlation between the stability of the carcinogen in prolonged experiments *in vitro*, and the number of tumors that developed when the various diets were fed to rats. Tumors failed to develop on the diets containing hydrogenated coconut oil, in which the dye was perfectly stable, whereas a high incidence of tumors (50 to 66 per cent) has been observed consistently on the one diet in which the dye failed to survive the drastic conditions imposed *in vitro*. It should be pointed out that in the feeding experiments the diets were routinely stored at -5° C., at which temperature the carcinogen was stable in all the rations. The exposure of diets to room temperature prior to feeding never exceeded one week, whereas even in the control diet the azo dye was stable for at least 30 days at this temperature. Thus it appears most unlikely

that the variations in tumor incidence observed with the different diets were due to a destruction of carcinogen prior to the consumption of the diet by the animals.

Another possibility considered is that part of the azo dye was destroyed in the gastrointestinal tract, and that more destruction occurred in the presence of hydrogenated coconut oil than in corn oil. This possibility, however, does not readily lend itself to experimentation. Measurements can of course be made of the amount of dye disappearing from the digestive tract when the two oils are fed but much of the loss, if not all, represents dye that has been absorbed by the animal. Furthermore, the proportion of dye recovered in the tissues is very small (10). When a fasted rat is fed a few grams of the synthetic diet containing *p*-dimethylaminoazobenzene, the gastric contents appear yellow except for isolated pockets of red near the stomach wall, and virtually all the dye can be recovered from the stomach. Thereafter the amount of dye in the stomach decreases, and small amounts appear in the small intestine. But this latter amount is never large, and even 12 to 24 hours after the ingestion most of the dye still remains in the stomach. Frequently the amount of dye in the small intestine is too small to be measured accurately. Furthermore, a yellow substance is present that turns red in the presence of strong acid, and therefore presumably is related to the original dye, but that differs from the dye itself in that neither the yellow form nor the red is extracted from water with chloroform.

Preliminary results indicated that somewhat more color was present in the lower third of the small intestine when the *p*-dimethylaminoazobenzene was fed in hydrogenated coconut oil than when it was fed in corn oil. The amount remaining in each case, however, was such a small fraction of the total pigment ingested that no conclusions could be drawn as to either the stability or absorbability of the dye from the tract in the presence of these two oils.

DISCUSSION

The striking differences observed in the carcinogenic potency of *p*-dimethylaminoazobenzene with corn oil and hydrogenated coconut oil respectively might be explained in a number of ways. Corn oil might contain a cocarcinogenic ingredient that is lacking in the hydrogenated coconut oil; the coconut oil might contain an anticarcinogenic factor that is lacking in the corn oil; or suitable combinations of cocarcinogenic and anticarcinogenic factors in the two oils might be involved. Present data do not permit identification of the chemical responsible for the great difference in carcinogenicity observed on the two diets, for the oils differ in many particulars such as chain length, unsaturation, and antioxidants, and any of these, or all, might prove to be significant. The experiments *in vitro* seem to indicate that with these

two oils equal amounts of dye are consumed per gram of ration, and since the total amount of food consumed by the rats on the two diets was the same, it would follow that the animals in both groups were ingesting the same amount of carcinogen.

Experiments on the disappearance of dye from the gastrointestinal tract failed to reveal any appreciable difference between the two diets. These experiments were obviously inconclusive, yet in the absence of any supporting evidence there appears to be little reason to impute the anticarcinogenic effect of hydrogenated coconut oil to a reaction within the digestive tract. Accordingly, the different incidences of tumors on the two oils are attributed to reactions within the animal itself.

Fats have previously been reported to influence the development of tumors in two quite different types of experiment. In the first type diets high in fat accelerated the production of skin tumors due to ultraviolet light (2) or to the local application of carcinogenic hydrocarbons (1). Fat also increased the total number of tumors that appeared (12, 29). However, in contrast to the present results with hepatic tumors, all types of fat appeared to be effective in promoting the development of the skin tumors (8). With these latter tumors the action of the fat was partly local (25), presumably resulting in a better penetration of hydrocarbon into the skin. Furthermore, the increased number of tumors observed appeared to be associated with an increased consumption of calories by the animals fed the high-fat diets (13).

Another effect of fat on tumor formation has been that the carcinogenicity of hydrocarbons injected subcutaneously depends upon the nature of the fat in which the carcinogen is dissolved. For example, when dibenzanthracene was injected into fowls in lard solution, many tumors developed, but when the hydrocarbon was administered in egg-yolk fat or in chicken fat, no tumors appeared (20). Benzpyrene, injected subcutaneously into mice, was much more carcinogenic when dissolved in olive oil, in sesame oil, or in arachis oil than when injected in mouse fat, in organic solvents, or as a fine powder (3, 18, 21). The literature has recently been summarized by Dickens and Weil-Malherbe (3). In a very extensive series of experiments Leiter and Shear (14) demonstrated that benzpyrene was much less effective when injected in "lard residue," the portion solid at 38°, than when injected in "lard filtrate," the portion liquid at 38°. Fractionation studies pointed toward the saturated glycerides of high molecular weight as the components responsible for the retarding action.

There is some parallel between the experiments with hydrocarbons in various oils and the experiments with *p*-dimethylaminoazobenzene described in the present paper, because in both types of experiments the essential differences in carcinogenicity depended upon the

nature of the oil used. However, the chemical factors responsible in the two phenomena may prove to be quite dissimilar, since the azo dye differs considerably from the hydrocarbons in its chemical behavior, and somewhat different methods of producing tumors with these respective carcinogens are employed. It is possible that the observed effects of the oils are due to their relation to a specific carcinogen as, *e.g.*, to the rate of diffusion of the hydrocarbon out of an injected oil globule, rather than to the fundamental carcinogenic reaction itself. The effect of hydrogenated coconut oil on hepatic tumors may likewise be mainly on the metabolic fate of the specific azo dye involved.

SUMMARY

When rats were fed *p*-dimethylaminoazobenzene in a synthetic diet containing corn oil, the incidence of hepatic tumors at 6 months ranged from 53 to 64 per cent. However, when the corn oil was replaced by hydrogenated coconut oil the tumor incidence never exceeded 8 per cent, while in most groups it was zero. This effect of the hydrogenated coconut oil persisted when the rats received 25 μ gm. of pyridoxine and/or 40 mgm. of ethyl linolate daily.

By means of an analytical method adequate for the quantitative determination of 1 μ gm. of *p*-dimethylaminoazobenzene, it was demonstrated that the azo dye was very stable in the diets *in vitro*. Attempts to demonstrate differences in the stability of the dye in the digestive tract were unsuccessful. It therefore appeared that the differences in carcinogenicity observed in the two oils were due to changes within the animal itself.

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