THE INFLUENCE OF DIETARY FAT UPON THE CARCINOGENIC ACTIVITY OF PARA DIMETHYLAMINOAZOBENZENE IN RATS

by

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INTRODUCTION

Miller and Miller (1) have investigated the carcinogenic activity of a large number of compounds which either were similar structurally or were possible metabolites of para dimethylaminoazobenzene, hereafter called DAB. They suggested that two conditions appear to be essential if an aminoazo dye is to have high activity: 1) At least one methyl group must be attached to the amino group, and 2) The rings must bear no substituents or carry only certain substituents in the 3' position.

In the rat DAB may follow two metabolic paths. Kinositu (2) has indicated that it would be split during metabolism at the azo linkage. This has been confirmed through the work of Stevenson, Dobriner, and Rhoads (3). They found that reductive fission occurred, and that the products excreted were para aminophenol or its N-acetyl derivative and para phenylene diamine or its N,N'-diacetyl derivative. Demethylation has occurred either before or after fission of the azo link. It has also been shown (4) that small quantities of benzidine were found in the livers of rats fed azobenzene, indicating that a benzidine rearrangement may have occurred. This benzidine rearrangement takes place readily in rats receiving DAB (5).

It also appeared that metabolism of the benzidine type favored bile duct proliferation while reductive fission favored heptoma formation. The mechanism of tumor induction in each case may result from inhibition of different enzyme systems by the metabolic products. However, no strict correlation has yet been discovered

between carcinogenic activity and enzyme inhibition (4).

Miller and Miller (6) have observed that a firmly bound compound may exist between proteins of the liver and an azo dye in rats fed DAB. The amount of bound dye was reduced when the level of riboflavin, a protective substance, was increased in the diet. No bound dye was found in the neoplasm of the liver. This indicated a qualitative difference in the protein of the liver and the tumor. It was also suggested that DAB might initiate the carcinogenic process through sublethal combinations of this dye or its metabolites with critical proteins in normal liver cells and their descendants. Permanent alteration or loss of proteins essential for the control of growth but not for life may occur.

Silverstone (7) found that tumor incidence increased with the amount of DAB found in the liver. He suggested that variations in the composition of the diet increased or decreased the appearance of hepatomas by increasing or decreasing the level of DAB in the liver. This does not agree with reports of Miller et al. (8).

Several dietary factors have been found to have an inhibiting or accelerating effect on the formation and growth of tumors resulting from ingestion of DAB. Pyridoxine deficiency, for example, inhibits tumor formation (9). Since several studies have indicated that pyridoxine was involved in protein metabolism (10, 11) it was possible that a sufficient lack of pyridoxine inhibits tumor formation by the inability of the animal to utilize sufficient amounts of one or more of the amino acids for protein synthesis. Synthesis of protein in both normal and neoplastic tissue could not proceed. Therefore, a loss in weight and a failure in tumor induction occurred

(9). No effect was observed when the level of pyridoxine was raised above that required for normal growth. The recent work of Ballantyne and McHenry (12) showed that the pyridoxine content of many kinds of human malignant tumors fell in a range similar to that of normal tissue having a low pyridoxine content. The vitamin content of the tumor bore no relationship to that of the surrounding tissue.

Protection against tumor formation was found when dietary sources of riboflavin were raised to a level five times that present in the normal control diet (13). On the other hand, a state of vitamin A deficiency caused a greater susceptibility to carcinogenic agents (14). When casein was replaced by egg white in the diet, tumor formation was retarded (15, 16). One per cent of cystine in the diet also protected against tumor formation (17).

The incidence and time of appearance of tumors was correlated with the degree of calorie consumption (18). This is an important phenomenon which too often has been ignored by investigators of other dietary factors (19).

Since dietary fat as a factor in carcinogenesis is the subject of this study, the general importance of fat may well be discussed. The fatty acids in the body are derived from two sources, the dietary fat and the fat formed in the body from carbohydrate. The dietary importance of fat was first demonstrated by Burr, Burr (20, 21) and Miller (22). They showed that a diet totally devoid of fat caused in rats a deficiency disease characterized by a scaly condition of the skin and tail, swollen red paws, increased water consumption, and renal lesions causing albumin or blood to be present

in the urine. This disease was curable by administration of fats containing highly unsaturated fatty acids or of pure methyl linolate.

Birch and Gyorgy (23) found that rats suffering from a pyridoxine deficiency developed similar dermal symptoms. Hogan and Richardson (24, 25) described a dermatitis induced in rats by feeding low fat diets supplemented by yeast irradiated with ultra violet light. This dermatitis could be cured by certain vegetable oils and by water soluble extracts of yeast.

Birch (26) suggested that at least two factors are concerned in the cure of acrodynia-like dermatitis, water soluble pyridoxine and linoleic acid. Severe acrodynia resulting from a synthetic diet lacking pyridoxine, pantothenic acid, and fat was not improved by the addition of either calcium pantothenate or methyl linolate alone. Addition of pyridoxine produced an increase in weight and appeared to initiate healing of the dermatitis. When both pyridoxine and calcium pantothenate were added, there was a rapid improvement in dermatitis and a gain in weight which became stationary in a few weeks. Addition of all three deficient factors restored the rats to a seemingly normal condition (27).

Quackenbush et al. (28) in an experiment similar to the one just described found that a daily supplement of 10 mg of ethyl linolate cured rat acrodynia. Cures were also obtained with subcurative doses of linolate supplemented with pyridoxine. Pantothenic acid together with pyridoxine improved the dermal condition. Essential fatty acids hold the dominant position though other factors are operative in maintaining the normal dermal condition.

Turpeinen (29) has indicated that arachidonic acid was three times more effective than linoleic in curing unsaturated fatty acid deficiency. He suggested that linoleic was converted to arachidonic acid, and that the latter was the essential acid. Recent work of Kummerow et al. (30) showed that linolenic acid was utilized completely only if pyridoxine was present. The major acid of tung oil, α-eleostearic, a conjugated acid, was ineffective in fat deficiency. Tung oil itself seemed to contain enough linoleic acid to be slowly effective (22).

In the absence of dietary linoleic or arachidonic acid, oleic and stearic acids and their homologs appear to be the only fatty acids the animal can synthesize. The more highly unsaturated acids characteristic of animal fat gradually disappear (31). Experiments with deuterium have established that the conversion of oleic to stearic acid is reversible (32).

Kline et al. (33) have reported that in general DAB is more carcinogenic when the diet contained fat. A greater incidence of tumors was observed with a diet containing 20 per cent hydrogenated fat than with a fat free diet. This tumor incidence increased when 5 per cent corn oil was substituted for Crisco. An even greater incidence of tumors was observed when 20 per cent corn oil was added to the diet. The level of fat in the diet as well as the type of fat is important for the formation of hepatic tumors.

When 0.25 per cent of either of two commercial synthetic detergents was fed with 5 per cent corn oil, no tumors occurred. There were no tumors found when 5 per cent mineral oil replaced corn oil. No large changes occurred in the concentration of dyes

found in the tissue with either stimulatory or protective diets. These data give no support to the possibility that the detergents and mineral oil interfere with the absorption or transport of the dye (8).

The metabolism and carcinogenic activity of DAB has been found to take place within the cells of the tissues. The phospholipid is believed to form an important part of the cell wall, while some phospholipids are found bound to the protein within the cell. Therefore, greater knowledge of the part played by fat in carcinogenic activity may have an important bearing of the mechanism of carcinogenesis of DAB. This work, involving the feeding of fats and of other compounds of either similar structure or of known metabolic relationship under controlled conditions, was undertaken in an attempt to clarify the role of fat in the carcinogenicity of DAB.

MATERIALS AND METHODS

Treatment of Animals

One hundred sixty three weanling female rats of the Sprague-Dawley strain were kept on a fat free diet for two months. This diet (Diet V) consisted of cerelose--84 per cent, purified casein--12 per cent, Wesson's salts--4 per cent, and the required quantities of cystine, inositol, choline chloride, niacin, riboflavin, thiamine hydrochloride, para aminobenzoic acid, pteroyl glutamic acid, biotin, calcium pantothenate, and pyridoxine. Vitamins A and D dissolved in one drop of hydrogenated fat were fed each week.

The rats were then divided into nine groups of 15 and two control groups of 14 rats each. The dietary supplements fed each group are listed in Table 1. The 5 per cent of fat was substitutef for an equal weight of cerelose.

Table 1. Diet supplements

Group	Supplements
I	none
II	5% corn oil
III	.06% DAB
IV	" " 5% tung oil pyridoxine (10 mg/kilo)
V	" " 5% linseed oil " " "
VI	" " 5% hydrogenated fat
VII	" " 5% corn oil
VIII	" " 5% emulsifier*
IX	" " 5% corn oil .5% emulsifier
X	" " 5% corn oil pyridoxine (5 mg/kilo)
XI	" " 5% corn oil .22% choline

* A commercial food emulsifier prepared from corn oil

All rats except the two control groups received the carcinogen para dimethylaminoazobenzene (DAB). The extra pyridoxine given Groups IV and V were at the level of 10 mg/kilogram of ration. Group X received half this amount, or 5 mg/kilogram of ration. The basal diet, containing crude casein, was mixed in 100 lb. quantities, and the supplemented diets were prepared from this in 2 kilo-

gram lots. The DAB was dissolved in the fat which was mixed thoroughly with the basal diet. In some cases the dye was dissolved in ethyl alcohol, mixed with the basal ration, and air dried. The fats were stored at 5°C.

The DAB containing diets were fed ad libitum for fourteen weeks. Throughout this time the animals were weighed and examined approximately once each week. Three unexplained deaths occurred, one each in Groups V, VII, and X.

At the end of this period, one animal from each DAB supplemented group was examined by laparotomy. Tumors were found to be present in Groups V, VII, IX, and XI. Three deaths resulted from this examination. The DAB was removed from the diets, but other supplementation continued.

Three rats from each group (III through XI) were killed with chloroform and examined after five weeks on the DAB free diet. After another period of three weeks all the animals were killed with chloroform. The livers were removed and stored in glass jars at -20°C. until they were analyzed.

Analysis of Tissue

Extraction of Lipid. Since analysis of the phospholipid was desired, extraction was necessary. A simple extraction procedure which had previously given good results in this laboratory was used. The liver tissue was separated into tumor and non-tumor bearing groups, weighed, and placed in a Waring blendor. Anhydrous sodium sulfate in the ratio of 130 g to 100 g tissue and 200 ml Skellysolve F were added. The tissue was extracted for two minutes

with the blendor and allowed to stand several minutes. The solvent was decanted, and the extraction repeated twice. The third extraction was allowed to stand over night before centrifuging and decanting. The extracted material was filtered, the solvent removed on the steam bath, and the extracted residue dried in a vacuum desiccator and weighed as total fat.

Precipitation and Saponification of Phospholipid. The total fat was slowly added to 25 ml acetone in a centrifuge tube with constant stirring. After all fat was added, stirring was continued for several minutes, and the solutions centrifuged. The acetone was decanted and the solvent removed on the steam bath. The acetone soluble residue was dried in the vacuum desiccator and weighed as neutral fat.

Twenty five ml saturated alcoholic potassium hydroxide were added to the acetone insoluble fraction. The mixture was warmed gently or allowed to stand over night until saponification was completed. The solution was neutralized with concentrated hydrochloric acid, cooled, and the fatty acids extracted with Skellysolve F. The solvent and fatty acids were dried over anhydrous sodium sulfate and filtered. The solvent was removed on the steam bath and the fatty acids dried in a vacuum desiccator.

The neutral fat and the fatty acids of the phospholipid fraction were stored in an evacuated desiccator until analyzed.

Determination of Iodine Values. The iodine values were determined on both fractions by the Wijs method as described in Jamieson (34). In some cases the amount of sample was insufficient and a modification of this method had to be used. One hundredth

a gram of fat was weighed accurately into a 50 ml Erlenmeyer flask, 1 ml chloroform and exactly 2 ml Wijs' solution added. The flasks were stoppered and placed in the dark for 1 hour. Two ml 15 per cent potassium iodide were added, the solution mixed well, the sides of the flask washed down with 2 ml distilled water and titrated with .01 N sodium thiosulfate, using starch paste as an indicator.

Determination of Unsaturated Fatty Acids. Linoleic, linolenic and arachidonic acids were determined in each fraction according to the method of Brice et al. (35) as modified by Potter and Kummerow (36). Into a small weighing dish 0.1 g of sample was weighed accurately. The dish and sample were dropped into a large test tube containing 10 ml of a 12½ per cent solution of potassium hydroxide in ethylene glycol which was maintained at a constant temperature of 180°C. in an oil bath for 25 minutes. At the end of the period the tubes were removed from the oil bath and cooled rapidly in water. The solution was removed to a 100 ml volumetric flask and made up to volume with 95 per cent ethyl alcohol. The flask and contents were allowed to stand over night in the refrigerator. The solution was filtered and diluted volumetrically to give readings between 0.2 and 0.8 on the Beckmann ultra violet spectrophotometer at wave lengths of 2320, 2620, 2680, 2740, 3100, 3160, and 3220 Angstrom units. Calculations were made using Potter's constants (36).

Oleic acid was calculated from the Iodine value and the percentages of the other unsaturated acids. Saturated fatty acids were determined by difference.

RESULTS

Growth of Animals and Gross Examination of Carcass

The young rats showed no symptoms of fat deficiency at the beginning of the DAB feeding period. At the end of the feeding period the rats of Control Group I and Group III which had received no fat exhibited only a slight scaliness of paws and tail.

Table 2. Average weight of rats at beginning and end of experimental feeding period. Average weight of livers at end of feeding period. (Tumorous and non-tumorous rats and livers are shown separately.)

Grp.	Tumor rats : Total rats	Rats--av. Weights : initial	final-wo*	final-w*	Livers : normal	tumors
I	0/13	163.5 g	195 g	-	7.0 g	-
II	0/14	162.0	254	-	8.0	-
III	2/15	162.0	185	208	7.5	10.0
IV	1/15	162.5	199	218	7.5	7.5
V	13/14	167.0	225	209	11.0	36.0
VI	5/15	165.0	219	215	7.5	11.5
VII	7/14	162.0	226	239	8.0	13.5
VIII	0/15	158.0	240	-	8.0	-
IX	10/15	163.0	218	219	7.0	26.0
X	7/14	162.0	223	232	8.0	30.0
XI	11/14	158.0	231	222	10.0	24.0

* wo--without tumors
w---with tumors

The average gain of each group throughout the feeding period and the average weights of the tumorous and non-tumorous livers in

each group are given in Table 2.

All the groups except the controls lost weight steadily for several weeks after DAB was added to the ration, but regained their original weight in one or two months. However, these rats ate less than the controls, particularly during the first month when only 2/3 the normal quantity was consumed. Wasted food was not salvaged and weighed, but over the DAB feeding period 17 kilo was prepared for Group V, 18 for Groups IX and X, 19 for Groups VI and VII, 21 for Group XI, 22 for Groups IV and VIII, and 23 for Groups I, II, and III. When DAB was removed from the diet, only Group V ate conspicuously less than the controls. There seems to be no correlation between the quantity of food consumed and incidence of tumors. All the groups made rapid gains when DAB was removed.

The rats receiving tung oil, Group IV, were initially fed extra pyridoxine at the level of 5 mg/kilo of ration. They lost weight so rapidly, however, that this amount was doubled. At the termination of the experiment, only one liver was found to be cancerous. Small transparent "bubbles" appeared in several of the livers, perhaps indicating healing of small tumors. These rats had sightly scaly paws and tails.

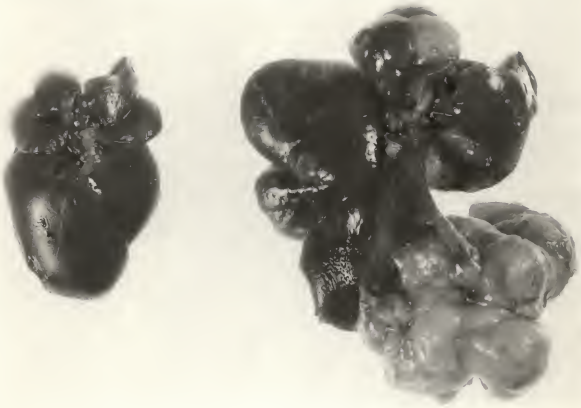
The pyridoxine level of rats receiving linseed oil, Group V, was also increased from 5 to 10 mg/kilo. The linseed oil greatly increased the incidence of tumors and their rate of development. Cirrhosis was marked, 7 in 14 livers weighing from 40 to 75 g. Several of these rats died previous to the end of the experiment, either from tumors or from the effects of the greatly enlarged livers.

Rats which had received hydrogenated fat, Group VI, appeared normal and healthy with only very slight scaliness. Tumors were small and the livers were compact at death.

Rats which had received a food emulsifier, Group VIII, experienced diarrhea the first week on the experimental diet which was probably due to the high concentration of acetic acid in this emulsifier. Five per cent of sodium bicarbonate was added to the diet using ethyl alcohol as a solvent. This had a diuretic effect which was overcome by cutting the sodium bicarbonate to 2 per cent and by using water as a mixing agent. The rats immediately began to gain weight steadily and were normal in appearance. No tumors were observed.

The rats which received corn oil, Group VII, corn oil plus emulsifier, Group IX, and corn oil plus pyridoxine, Group X, grew well after the initial drop and appeared healthy. The rats receiving only corn oil had small tumors and compact livers. The emulsifier, which by itself inhibited tumor formation, when combined with corn oil increased not only the incidence of tumors but their rate of development and the size of the livers, as well. Rats receiving corn oil plus pyridoxine had no greater tumor incidence than those receiving corn oil alone, but cirrhosis was greater. Three of seven livers weighed between 40 and 65 g.

The rats which had received corn oil plus choline, Group XI, grew rather poorly and several of the rats lost a great deal of hair. When the dye was removed from the diet, hair growth resumed and the animals increased in weight rapidly. Incidence of tumors increased greatly over that observed with corn oil alone. Four of



A B

Fig. 1. Comparison of normal and tumor-bearing livers.

A. Normal liver.

B. Cirrhotic tumor-bearing liver.

eleven tumors were greatly enlarged.

Average final weights of the rats in each group do not present a true picture of the individual animals. In early stages of tumor development the liver is small, almost normal, and the rats appear large and healthy. In the latest stages, the liver may be enormous (Fig. 1), while the rest of the body is emaciated. In one case (Group V), a rat weighing 195 g possessed a liver weighing 75 g.

Effect of Diet on Liver Fat

The diet appeared to have little effect upon the percentage of extractable fat (Table 3). The values ranged around 2 or 3 per cent. The slightly higher values associated with the tung oil and linseed oil rats are probably not significant.

Table 3. General fat content of livers.

Gp.	Wt. liver tissue		per cent total fat		Distribution of total fat: % neutral fat		% phospholipid	
	WO	W	WO	W	WO	W	WO	W
I	80	---	2.1	---	75.6	----	24.4	----
II	79.5	---	1.7	---	73.9	----	26.1	----
III	81	20	2.3	2.0	54.3	68.4	45.7	31.6
IV	95	18	3.2	5.3	69.4	88.9	30.6	11.1
V	10	128	3.1	3.2	67.4	83.7	32.6	16.3
VI	60	66	2.4	3.0	70.3	71.6	29.7	28.4
VII	52	75	2.6	2.6	74.8	60.2	25.2	39.8
VIII	97	---	2.4	---	65.1	----	34.9	----
IX	31	118	2.5	2.8	48.0	87.6	52.0	12.4

Table 3. (cont.).

Gp.	Wt. liver tissue		per cent total fat		Distribution of total fat: % neutral fat		% phospholipid	
	wo	w	wo	w	wo	w	wo	w
X	52	114	2.4	2.2	84.8	76.4	15.2	23.6
XI	28	134	2.5	3.1	83.0	80.9	17.0	19.1

wo--without tumors.
w---with tumors.

A large variation in the percentages of phospholipid (Table 3) was noted. It appears that emulsifier increased the percentage of phospholipid, while additional pyridoxine and choline when fed in conjunction with corn oil decrease it. Though these percentages appear low, previous work in this laboratory has shown that the extracted tissue, when further extracted in a Soxhlet apparatus, yielded no more fat.

The iodine values of the control groups of the fatty acids of the phospholipid fraction were higher than those of the neutral fat. This does not hold true for those rats which had received DAB and had remained free from tumors. With the exception of the animals which had received emulsifier the iodine values of the neutral fat are higher than those of the fatty acids of the phospholipids. For the rats which had developed tumors, in the majority of groups the iodine values of the fatty acids of the phospholipid fraction were higher than those of the neutral fats (Table 4).

The lower iodine value of the fatty acids of the phospholipid fraction in rats without tumors was generally associated with a reduction in the percentage of oleic acid (Table 6). With the ex-

ception of Group VIII, there was also a reduction in the percentage of arachidonic acid. Small decreases in the percentage of linoleic acid except in Group IX and Group X also occurred as compared with the control groups (Tables 5 and 6).

Table 4. Iodine values of fat fractions.

Gp.	Tumor incidence per cent	Fat fraction	Iodine values wo	Iodine values w
I	-	Neutral fat	104.9	-
		Fat acid of phos.	120.4	-
II	-	Neutral fat	118.8	-
		Fat acid of phos.	140.4	-
III	13.3	Neutral fat	107.6	101.9
		Fat acid of phos.	105.9	117.0
IV	6.7	Neutral fat	102.3	86.2
		Fat acid of phos.	100.7	122.3
V	92.8	Neutral fat	131.0	113.3
		Fat acid of phos.	102 *	128.0
VI	33.3	Neutral fat	116.8	88.2
		Fat acid of phos.	92.0	94.0
VII	50.0	Neutral fat	126.8	125.1
		Fat acid of phos.	110.0	114.0
VIII	0.0	Neutral fat	116.7	-
		Fat acid of phos.	139.1	-
IX	66.7	Neutral fat	133.7	104.6
		Fat acid of phos.	110.5	106.3
X	50.0	Neutral fat	127.9	118.2
		Fat acid of phos.	100.0	114.8
XI	73.3	Neutral fat	121.8	107.8
		Fat acid of phos.	72. *	129.9

* Estimated values.
w With tumors.
woWithout tumors.

Table 5. Fatty acid distribution in fatty acids of the phospholipid fraction.

Gp.	tumor w or wo	I. V.	lin-oleic acid	lin-olenic acid*	arach-idonic acid	oleic acid	sat. acid
			per cent				
I	wo	120.4	3.03	13.94	9.09	51.7	22.2
	w	-	-	-	-	-	-
II	wo	140.4	9.49	-	28.40	35.3	26.8
	w	-	-	-	-	-	-
III	wo	105.9	.54	11.13	9.18	48.5	9.6
	w	117.0	6.11	13.06	8.04	46.3	26.5
IV	wo	100.7	8.15 9.04**	-	23.98	-	58.8
	w	122.3	9.70 9.00**	-	15.80	39.7	25.8
V	wo	102***	****	****	****	****	****
	w	128	11.77	-	11.63	75.4	1.2
VI	wo	92	4.59	-	17.18	29.4	48.8
	w	94	6.23	-	19.55	19.5	54.7
VII	wo	110	8.74	-	25.61	9.6	56.0
	w	114	15.18	-	39.09	-	45.7
VIII	wo	139	8.43	-	37.51	-	54.1
	w	-	-	-	-	-	-
IX	wo	110.5	12.9	-	24.79	5.0	57.3
	w	106.3	14.89	-	22.28	5.6	57.2
X	wo	100.0	11.88	-	26.20	-	62.9
	w	114.8	13.16	-	29.40	-	57.4
XI	wo	72***	****	****	****	****	****
	w	129.9	13.03	-	12.10	73.3	1.6

* In Groups I and III, the trienoic acid reported as linolenic is probably dihydroarachidonic (37) (38).
** Conjugated dienoic acid present in tung oil fed rat livers.
*** Estimated values.
**** Sample insufficient to permit analysis.

The rats which developed tumors showed without exception an increase in the percentage of linoleic acid in the fatty acid of the phospholipid fraction when compared with either the controls or the group without tumors (Tables 6 and 8).

Since linoleic acid in hydrogenated fat and possibly in tung oil was present in small quantities only these groups were compared with both control groups.

The iodine values of the neutral fat fraction of the non-tumorous livers were in some cases higher and in others lower than those of the controls.

When the percentage values of the unsaturated acids in the neutral fat were compared with the control values, it was observed that the values for linoleic and arachidonic acid in groups receiving corn oil (VII, IX, X, and XI) and hydrogenated fat were higher than the control values. In all other groups the reverse was true.

When the percentages of unsaturated acids in the neutral fat were compared between tumorous and non-tumorous groups on the same diet, a decrease in the percentage of arachidonic acid in the tumorous groups was observed in the majority of cases. There appeared to be no consistent increase or decrease of linoleic acid.

Rats on a diet containing no fat have very little linoleic or arachidonic acid present in their livers (Tables 5 and 7). The percentages of these acids are particularly low in the neutral fat fraction, the phospholipid possessing about a third of that found in rats fed corn oil. The animals which had received DAB and no fat had a particularly low percentage of linoleic acid in the

Table 6. Differences in the percentage of the various fat acids found in fatty acids of the phospholipid fraction compared with control groups.

A. Compared with no fat control

Gp.	per cent tumors	w or wo	Variation from control: lin-oleic	lin-olenic*	arach-idonic	oleic	sat. acids
			per cent				
III	13.3	wo	-2.49	-2.81	+ .1	- 3.0	-12.6
		w	+3.08	- .88	- 1.05	- 5.4	+ 4.3
IV	6.7	wo	+5.12	**	+14.89	-51.7	+36.6
		w	+6.67		+ 6.71	-12.0	+ 3.6
VI	33.3	wo	+1.56		+ 8.09	-22.3	+26.6
		w	+3.20		+10.46	-32.2	+32.5

* Dihydro arachidonic acid
** Values of linolenic not compared
wo Without tumors
w With tumors

B. Compared with corn oil control

Gp.	per cent: tumors	w or wo	linoleic	arach-idonic	oleic	sat. acids
			per cent			
IV	6.7	wo	-1.34	- 4.42	-35.3	+32.0
		w	+ .21	-12.60	+ 4.4	- 1.0
VI	33.3	wo	-4.90	-11.22	- 5.0	+22.0
		w	-3.46	- 8.85	-15.8	+27.9
V	92.8	wo	*	*	*	*
		w	+2.28	-16.77	+40.1	-25.6
VII	50.0	wo	- .75	- 2.79	-25.7	+29.2
		w	+5.69	+10.69	-35.3	+18.9
VIII	00.0	wo	-1.06	- 9.11	-35.3	+27.3
		w	---	---	---	---
IX	66.7	wo	+3.41	- 3.61	-30.3	+30.5
		w	+5.40	- 6.12	-29.7	+30.4
X	50.0	wo	+2.39	- 2.20	-35.3	+36.1
		w	+3.67	+ 1.0	-35.3	+30.6

Table 6. (concl.).

Gp.	per cent tumors	w or wo	linoleic	arachidonic	oleic	sat. acids
			per cent			
XI	73.3	wo	*	*	*	*
		w	+3.54	-16.3	+38.0	-25.2

* Material insufficient for determination
wo Without tumors
w With tumors

phospholipid fraction. This may be due in some way to an effect of the dye upon fat metabolism. A very high percentage of oleic was present, especially in the neutral fat. No saturated fatty acid was present in the neutral fat, but from 10 to 20 per cent was found in phospholipid fraction. The oleic acid was synthesized in the body from carbohydrate. The presence of a trienoic acid has been previously observed in the liver of fat starved rats (37, 38). It has been identified as dihydroarachidonic acid by Munn and Smedley-MacLean who believe that it results from the reduction of arachidonic acid.

The livers of rats receiving hydrogenated fat contained more linoleic and arachidonic acid than those of fat deficient rats, but not as much as those of rats receiving corn oil. This increase in linoleic and arachidonic acid over the fat deficient group occurred principally in the neutral fat fraction. There was a decrease in oleic in both fractions. A small percentage of saturated acid was present in the neutral fat, while 50 per cent of the fatty acids of the phospholipid fraction were saturated.

Livers from rats receiving 5 per cent Emulsifier (Table 10) were found to contain almost as much linoleic acid as those of corn

Table 7. Fatty acid distribution in neutral fat

Gp.	tumors w or wo	I.V.	lin-oleic acid	lin-olenic acid	arach-idonic acid	oleic acid	sat. acid
			per cent				
I	wo	104.9	1.83	3.34*	2.74	92.0	-
	w	-	-	-	-	-	-
II	wo	118.8	13.16	-	15.36	48.6	12.9
	w	-	-	-	-	-	-
III	wo	107.6	1.28	3.82*	1.73	93.2	-
	w	101.9	2.07	4.19	1.51	90.7	1.5
IV	wo	102.3	.39 10.85**	3.71	7.46	52.2	25.4
	w	86.2	5.56 10.80**	1.15	7.64	32.9	41.9
V	wo	131.0	10.28	4.45	11.63	68.2	5.4
	w	113.3	9.76	2.83	8.0	67.9	11.5
VI	wo	116.8	5.65	-	10.74	78.6	5.0
	w	88.2	13.19	.32	4.03	54.0	28.5
VII	wo	126.8	19.07	-	17.15	37.9	25.9
	w	125.1	18.33	-	11.48	58.6	11.6
VIII	wo	116.7	12.07	-	11.15	64.0	12.8
	w	-	-	-	-	-	-
IX	wo	133.7	16.42	-	16.42	54.6	12.6
	w	104.6	20.64	-	5.38	58.1	15.9
X	wo	127.9	21.22	-	17.01	36.3	25.5
	w	118.2	18.56	-	10.86	53.7	16.9
XI	wo	121.8	18.88	-	16.66	35.6	28.9
	w	107.8	14.59	1.0	7.14	60.9	16.4

* Dihydro arachidonic acid
** Conjugated dienoic acid present in tung oil fed rat livers
wo Without tumors
w With tumors

oil rats. There was a difference of 1 per cent in both fractions. The arachidonic acid in the neutral fat was less than that in the

controls, but an unusually large amount was found in the phospholipid fraction. No oleic acid was found in the phospholipid, but a large amount occurred in the neutral fat. The iodine values of both fractions were similar to those observed with the corn oil controls. The saturated fat in phospholipid was about 50 per cent, double the amount in the control group.

Table 8. Comparison of tumorous to nontumorous livers.

Gp.	Fraction	Variation of tumorous livers				
		linoleic	linolenic	arachidonic	oleic	sat. acid
		per cent				
III	Neutral fat	+ .79	+ .37	- .22	+ 1.2	+ 1.5
	Fat acid (phos.)	+5.57	+1.93	- 1.16	- 3.2	+16.9
IV	Neutral fat	+5.17	-2.63	+ .18	-19.3	+16.5
	Fat acid (phos.)	+1.55	-	+ 8.18	+39.7	-33.0
V	Neutral fat	- .52	-1.22	- 3.63	- .3	+ 6.1
	Fat acid (phos.)	- *	-*	- *	- *	- *
VI	Neutral fat	+7.54	+ .32	- 6.71	-24.6	+23.5
	Fat acid (phos.)	+1.64	-	+ 2.37	- 9.9	+ 5.9
VIII	Neutral fat	- .74	-	- 5.67	+20.7	-14.3
	Fat acid (phos.)	+6.74	-	+13.48	- 9.6	-10.3
IX	Neutral fat	+4.22	-	- 1.04	+ 3.5	+ 3.3
	Fat acid (phos.)	+2.00	-	- 2.51	+ .6	-
X	Neutral fat	-2.66	-	- 6.15	+17.4	- 8.6
	Fat acid (phos.)	+1.28	-	+ 3.2	-	- 5.5
XI	Neutral fat	-4.2	-	+ 1.0	- 9.52	+25.3
	Fat acid (phos.)	-*	-*	- *	- *	- *

* Insufficient data

When both DAB and corn oil were fed, the percentage of linoleic acid and arachidonic acid in the neutral fat was greater than

that in the corn oil group without DAB. These acids also decreased in the phospholipid, while the saturated acids had doubled in both fractions. Whether these changes result from the addition of dye is not known.

Table 9. Fatty acid content of dietary supplements

Supplement	linoleic	linolenic	oleic	arachidonic	sat. acid	α-eleostearic
	per cent					
Corn oil (39)	60	10	20	-	10	-
Emulsifier	34.5	.5	14	-	-	-
Linseed oil (39)	20	50	20	-	10	-
Tung oil (39)	0--9	-	1--4	-	5	82--95

When 0.5 per cent emulsifier was fed with 5 per cent corn oil, the increase in linoleic and arachidonic acids in the neutral fat was not as marked as in Group VII (corn oil plus DAB). However, the linoleic acid in the phospholipid increased appreciably over that observed in the control group. The arachidonic acid of the phospholipid decreased. Oleic acid increased in the neutral fat and decreased in the fatty acids of the phospholipid.

The addition of pyridoxine to the corn oil and DAB ration (Group X) increased the linoleic acid in both fractions over that in Group VII (corn oil plus DAB). The arachidonic acid was nearly the same. Oleic acid was similar in the neutral fat, but was absent from the phospholipid. There was an increase of saturated fatty acids in the phospholipid.

Tung oil and pyridoxine (Group IV) caused a great decrease in the linoleic acid of the neutral fat when compared to the corn oil control. This value was even much lower than that obtained in Group I (no fat). The arachidonic acid in the neutral fat was decreased but not as drastically. The values for the phospholipid were lower than those of the corn oil controls but in the same range. Tung oil is mainly composed of α-eleosteric acid (Table 9), a trienoic conjugated acid. It has been found that conjugated fats are not utilized by the animal body (23), and that large amounts may prove fatal. In this experiment in which large amounts of pyridoxine were fed, no fatality occurred. There appeared to be an attempt either to utilize or to destroy the acid. When spectrophotometric analysis was run without previous isomerization, about 10 per cent of a conjugated dienoic acid was found in both the neutral fat and the phospholipid. Only traces of a trienoic acid were present. Evidently, hydrogenation of one bond had taken place. The role of pyridoxine in this process is unknown.

Insufficient material prevented the analysis of the phospholipid of Group XI (corn oil plus choline), but the values obtained for the neutral fat were comparable in every instance to those obtained in the corn oil plus DAB group.

The analysis of phospholipid could not be accomplished for the linseed oil group since insufficient material was available. However, the linoleic and arachidonic acids in the neutral fat were less than that in the corn oil control. About 5 per cent linolenic acid was present. A large value was obtained for oleic acid, but very little saturated acid was present.

The fatty acids of the phospholipids of tumorous rats were distributed in an unusual manner in the choline plus corn oil and the linseed oil groups, both of which showed great tumor incidence (Table 9).

Table 10. Distribution of fatty acids in phospholipid of tumorous livers of Groups V and XI.

Gp.	Supplement	linoleic	arachidonic	oleic	sat. acids
		per cent			
VII	corn oil, DAB	15.18	39.09	-	45.7
V	linseed oil, DAB, pyridoxine	11.77	11.63	75.4	1.2
XI	corn oil, choline, DAB	13.03	12.10	73.3	1.6

In no other group were these values so distributed. These values could not be compared with those for non-tumorous rats, since no analysis could be made.

DISCUSSION

Dietary fats appear to influence the carcinogenecity of DAB in rats by contributing to the nutrition of the cell during a critical period of tumor formation in which either development or regression of the neoplastic tissue may occur depending upon the environment. There seems to be no effect upon the actual mechanism of tumor formation.

Carcinogenesis has been classified into three phases (19):

1) The period of induction during which the neoplasm is formed;

2) The critical period during which an equilibrium exists between the neoplasm and its environment when growth depends upon the proliferative capacity of the cell to withstand the resistance of the normal tissue; 3) The period of progression during which growth is rapid.

The fact that tumors occur at all in any one dietary group would indicate that the carcinogenic agent is still capable of producing tumors. The failure of tumor development appears to take place in the critical period. This is readily seen in the special case of the tung oil group in which tumors were induced but failed to develop and appeared to regress. This is just as clearly illustrated when it is noticed that the probability of tumor formation was increased with the increase of linoleic acid, a growth factor.

In view of this the inhibition of tumors in the tung oil group cannot be explained as a reaction between the azo dye and the more active conjugated double bonds present in the dietary fat. The fact that the α-eleostearic acid had no obviously harmful effect is attributed to the high level of supplementary pyridoxine. It has been reported that the pyridoxine content of human malignant tissue falls in a range similar to that of normal tissue having a low pyridoxine content and is independent of the surrounding tissue (12). Assuming that this applies equally to rats, it is likely that only the normal tissue is capable of counteracting the harmful effect of the α-eleostearic acid. Consequently, the tumor cells are destroyed. Or. it may be that the pyridoxine of the normal tissue provides an advantage in the competition for the essential linoleic acid.

The complete lack of tumor formation or development in the

livers of rats receiving an emulsifier is not so easily explained. Emulsifying agents have been found to increase the absorption of fat and fat soluble substances (40). In that case DAB would have been even more readily absorbed than usual. The distribution of fatty acids in the phospholipid offers no help, since adequate nutritional amounts of linoleic and arachidonic acid were present. In at least one liver evidence of tumor formation and subsequent failure of development was present. Therefore, it appears that the actual mechanism of induction had not been impaired. Perhaps the large concentration of acetic acid present in the emulsifier affects the neoplasm, but not the normal tissue, adversely.

The increased rate of tumors obtained when 0.5 per cent emulsifier was fed with corn oil may be attributed to the increased absorption of both fat and dye, and consequent more ready availability of unsaturated fatty acids. It is not known if the phospholipid turnover can be in any way affected by the emulsifying agent.

Pyridoxine is believed to act as coenzyme in the higher fatty acid dehydrogenase system (41), and to have a role in transamination. The inhibition of tumors in a pyridoxine deficient diet was attributed to the inability of the animal to utilize one or more of the amino acids for protein synthesis. Then protein synthesis in both normal and neoplastic tissue could not proceed (9). An excess of pyridoxine above a good nutritional level has no effect upon tumor formation (Group X).

That an unusual distribution of fatty acids was observed in the phospholipid of tumor-bearing tissue in both the linseed oil and corn oil plus choline groups has been noted. This is in agree-

ment with the results of an investigation by Sueyosi and Miura (42) in which comparison of two kinds of neoplasms with their normal tissue showed a small decrease in the linoleic acid and a large increase in the oleic acid of the phospholipid. In this study this phenomenon was observed only in the two groups in which the greatest incidences of tumors occurred. Since the fatty acids of the phospholipid are normally about 50 per cent saturated, the large amount of oleic acid present may result from an inhibition of the enzyme-controlled reversible desaturation system.

Since chronic choline deficiency alone results in the formation of neoplasms (43, 44), and since choline has been found to inhibit the transaminase system (45), one might expect the addition of extra choline to the diet would inhibit tumor formation. Since this is not the case, other properties of choline must overshadow those above during adequate nutrition. Choline accelerates the synthesis and transfer of phospholipid by the liver. Sinclair (46) has proposed three functions of the phospholipid: 1) They are intermediary metabolites in fat metabolism. 2) They serve as an oxidation-reduction system. 3) They are essential elements in cell structure. Any effect upon the activity of phospholipid would have a bearing upon its functions. It is possible that there is a greater efficiency of phospholipid function which improved conditions of growth and development for both normal and tumor cells. The inhibiting effect upon the desaturation system would then be the result of tumor formation.

Linseed oil cannot be utilized by the animal body in the absence of pyridoxine (30). Since a high level of dietary pyridoxine

was maintained, and since little linolenic acid was found in the tissue fat (none in the phospholipid), the increased tumor formation must result from the large quantity of essential unsaturated fatty acid available.

The fact that large quantities of oleic acid was found in phospholipid of tumor bearing animals only in these two groups may be due to their more advanced stage of tumor development.

SUMMARY

Tumor incidence in rats on a synthetic ration containing DAB and various supplements increased with an increase of linoleic acid in the diet.

Tumor incidence also increased with the percentage of linoleic acid found in the phospholipid fatty acids, though other factors possessed an accelerating or inhibiting effect.

An emulsifier and choline when fed in conjunction with corn oil increased tumor incidence, as does a diet containing linseed oil.

Tung oil inhibits the formation of tumors, possible through the effect of a conjugated dienoic acid present in the tissues. Pyridoxine fed with corn oil has no effect. An emulsifier fed alone inhibits tumor formation completely.

Tumor incidence appears to depend upon the unsaturated acid content and upon the activity of phospholipid.

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