UNSATURATED FATTY ACIDS IN THE DIETARY DESTRUCTION OF *N,N*-DIMETHYLAMINOAZOBENZENE (BUTTER YELLOW) AND IN THE PRODUCTION OF ANEMIA IN RATS

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Hepatic injury and the occurrence of malignant hepatoma after ingestion of butter yellow were first seen in rats (1) fed a diet of which 95 per cent was rice and 5 per cent olive oil. To this mixture the butter yellow was added in the proportion of 0.06 gm. per 100 gm. This ration was supplemented daily with a small amount of carrots. In contrast, practically no malignant changes have been seen (2), at least up to 150 days of the experimental period, in the livers of rats fed a diet (called diet C) that contained butter yellow in the same proportion as the original rice diet (0.06 per cent) but that had a different composition, *i.e.*, casein 6 per cent, lard 23, cane sugar 15, cornstarch 50, salt mixture 4, and cod liver oil 2 and was supplemented daily with thiamine, riboflavin, pyridoxine, and pantothenic acid. The lesions in the livers of rats fed this diet were limited to necrosis, cirrhosis, and proliferation of the bile ducts, mostly typical in character, indeed only rarely atypical. Even these pathological changes were prevented to a large extent by the administration of a combination of cystine and choline (2).

Explanation of the difference in the effect of the rice-oil mixture and diet C on the production of malignant hepatoma in rats has been sought by feeding rats a modification of diet C. Crisco or melted butter fat was substituted for lard in one group of experiments and rice (brown unpolished or white) for cornstarch and sugar in another group of experiments. The effects of these substitutions proved in both sets of experiments to be procarcinogenic, especially in those in which brown rice was used. Furthermore, the malignant changes that followed ingestion of the rations containing butter fat or brown rice were not prevented, or were prevented only to a limited degree, by the administration of a combination of cystine and choline.[1]

The problem that arose, therefore, was to learn what principle was operating

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[1] Detailed report of the pathological findings will be given later in cooperation with Dr. Harry Goldblatt of the Institute of Pathology, Western Reserve University.

in the change of the non-carcinogenic diet C into a carcinogenic ration following the incorporation in it of crisco or butter fat in place of lard in one set of experiments and of rice (white and especially brown) instead of cornstarch and sugar in another. The answer has been provided by results of still other experiments in which the source of fat was, first, a crude preparation of linoleic acid[2] and, later, pure specimens of fatty acids and their esters, prepared in the laboratory of one of us.

EXPERIMENTAL

The experimental results, representative examples of which are given in Tables I and II, can be summarized as follows:—

1. Diet C with crude linoleic acid replacing lard (referred to as diet "By" in Tables I and II) proved to be toxic for rats weighing over 130 gm. at the beginning of the experiment. Sixty-seven rats were fed this diet in amounts *ad libitum*, and the results were consistent. The animals lost weight rapidly in spite of satisfactory intake of food. They exhibited marked, progressive anemia, secondary in type, which was accompanied, as a rule, by leucopenia (Table I). They became infested with pediculi, often before the development of anemia when the animals were still very active and not nearly moribund.

The toxic effect on rats of the ration containing linoleic acid was even more pronounced when butter yellow was omitted from diet "By" (referred to as diet "Li" in Table I). In this group comprising 23 rats all the animals died before the 80th experimental day, with an average survival time of 55 days. Four rats showed leucopenia but no anemia before death.

The toxic effect of the rations containing linoleic acid with ("By") or without ("Li") butter yellow could, as a rule, be neutralized preventively and therapeutically by the daily addition of 0.5 to 1 gm. of yeast. Twenty-one rats were fed diet "By" and 11 rats were fed diet "Li." The observation period for these groups was extended to 150 days. In 3 of the 11 rats fed ration "Li" (without butter yellow) the anemia was not prevented by addition of 1 gm. of yeast daily, and the animals died before the end of the experimental period.

In tests involving 49 rats, a diet containing brown rice, linoleic acid, and butter yellow was found to be non-toxic. On the other hand, linoleic acid was almost completely oxidized and destroyed by keeping diet "By," before use, for 3 to 4 weeks at laboratory temperature, and the diet then exerted marked toxic effect on all 23 rats in this group. Diarrhea was a prominent feature, in addition to loss in weight, anemia, leucopenia, and pediculosis.

Postmortem examination revealed that rats fed the modification of diet C with linoleic acid instead of lard and with or without butter yellow were generally free from pathological changes in the liver, even from severe fat infiltration. Animals receiving diet C with linoleic acid and butter yellow never showed malignant hepatoma. The life span of these rats could be prolonged by daily

[2] "Linoleic acid refined light" from the Glyco Products Co., Inc., Brooklyn, N. Y.

TABLE I

Morphological Blood Changes in Rats Fed Rations Containing Crude Linoleic Acid

Rat No. and date when experiment was started	Diet*	Blood examination									
		Date	Hb	Red blood cells $\times 10^6$	White blood cells (corrected) $\times 10^3$	Reticulocytes	Nucleated red cells	Polymorphonuclears	Small lymphocytes	Large lymphocytes	Eosinophils
			per cent			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
7596—12/2	"By" + cystine + choline	2/18	72	5.7	9.4	0.3	1	20	76	4	..
		3/13	46	2.25	7.8	22.5	6	22	78	..	..
		4/3	36	2.6	4.5	5.6	20	12	88	..	..
		4/28	15	1.05	5.4	17.0	32	14	84	2	..
8063— 3/3	"By"	3/25	93	6.1	26.9	3.2	..	16	80	4	..
		4/17	70	6.35	27.0	0.9	6	12	88	..	..
		5/8	59	4.45	9.3	0.4	20	24	76	..	..
		5/19	16	1.1	6.8	—‡	40	20	80	..	..
8132— 3/19	"By" + yeast	4/7	83	7.2	19.2	9.6	2	20	80	..	..
		4/30	88	7.35	12.5	2.6	2	36	64	..	..
		5/21	84	5.5	18.5	4.4	4	28	68	..	4
6995—11/17	"By" + cystine and beginning 1/29 + yeast	1/29	29	3.0	2.3	—‡	2	34	58	8	..
		2/16	63	6.4	3.4	2.1	52	48	52	..	..
		3/31	78	5.7	14.8	7.6	..	28	72	..	..
		5/11	79	6.65	11.2	7.2	..	34	66	..	..
8007— 2/21	"Li"	2/23	94	8.1	15.2	0.3	..	8	88	4	..
		3/17	90	6.6	18.2	0.9	..	10	88	..	2
		4/21	45	4.55	4.5	1.2	2	20	76	4	..
		4/28	13	1.1	0.24	16.0	85	85	15	..	..
8011— 2/21	"Li" + yeast	3/20	92	8.0	12.8	9.6	..	24	76	..	..
		4/13	107	8.25	7.8	2.4	..	20	80	..	..
		5/5	103	8.4	19.0	0.2	..	14	86	..	..

* Diet "By:" casein 6 per cent, cornstarch 50, sucrose 22, cod liver oil 2, salt mixture 4, linoleic acid 16, and butter yellow 0.06. Diet "Li:" same as "By" without butter yellow. Both diet "By" and diet "Li" were supplemented daily with 20 μg. of thiamine, 25 μg. of of riboflavin, 20 μg. of pyridoxine, and 100 μg. of pantothenic acid. Both rations were freshly prepared twice a week.

‡ Not examined.

supplements of cystine (50 mg.) or of cystine (50 mg.) *plus* choline (20 mg.). Only one of the 19 rats that did not receive supplements of cystine or choline survived for 100 days. The average survival time in this group was 61 days.

Again, only one rat in a group of 15 rats that received 20 mg. of choline daily survived for 100 days. The average survival time in this group was 56 days. In contrast, only 6 out of 20 rats receiving daily supplement of cystine (50 mg.) and 4 of 13 rats receiving daily supplement of cystine (50 mg.) plus choline (20 mg.) died before the 100th experimental day. Twelve rats in the first of these two latter groups and 6 in the second were killed at the end of 150 days of the experimental period. The supplements of cystine and of cystine plus choline, however, had no beneficial effect or had only limited beneficial effect on the production of anemia, leucopenia, and pediculosis.

2. The color of the modified diet C containing crude linoleic acid instead of lard, with butter yellow, underwent rapid change even at room temperature and almost completely disappeared after 4 days. In distinct contrast, a mixture containing rice (white or brown), linoleic acid, and butter yellow did not lose its color after 7 days and that with brown rice even after 20 days. Thus, rice appeared to stabilize butter yellow in the presence of linoleic acid.

Quantitative data are given in Table II concerning the iodine number and color of the chloroform extracts of different mixtures which contained, in varying combinations, butter yellow, sand, sucrose, cornstarch, rice starch, white or brown rice, casein, salt mixture, and, as a source of fat apart from crude linoleic acid, pure linoleic acid, arachidonic acid, oleic acid, palmitic acid, methyl palmitate, and methyl oleate.

For the determination of iodine numbers Hanus' (3) method has been used. The concentration of butter yellow in the chloroform extracts was measured by the Klett-Summerson photoelectric colorimeter, using the blue filter No. 42.

DISCUSSION

From the results obtained it is apparent that linoleic acid and, to a less degree, arachidonic acid and even less distinctly oleic acid are connected with the destruction of butter yellow *in vitro*, with a parallel drop in the iodine number of linoleic acid and arachidonic acid. It can be assumed that these unsaturated fatty acids, perhaps through formation of peroxides, decompose butter yellow and at the same time give rise to formation of toxic by-products which in turn produce in rats severe progressive anemia, leucopenia, and loss in weight, together with pediculosis, but leave the liver more or less unharmed. As has been mentioned, these toxic compounds are formed also in the absence of butter yellow.

The decolorization of butter yellow in the presence of linoleic acid is a surface phenomenon; it does not take place in a homogeneous oily mixture. It is accelerated by high temperature (+35°C.) and is retarded by low temperature (−20°C.). It is not observed in the absence of oxygen.

In the original diet C, with lard as the source of fat, no dietary destruction

TABLE II

Changes in Iodine Number and Concentration of Butter Yellow in Different Food Mixtures*

Experiment No.	Food mixtures	5th day		8th day		21st day	
		Iodine No.	Butter yellow* mg./gm. diet	Iodine No.	Butter yellow* mg./gm. diet	Iodine No.	Butter yellow* mg./gm. diet
1	"By" = A‡ + 16 parts linoleic acid "glyco"§	72.0	0.15	42.8	0.09	34.3	0.07
2	"By:" 2 gm. sealed in 5 cc. tube under nitrogen	144.2	...	144.8	0.59	...	...
	"By:" 2 gm. sealed in 5 cc. tube under oxygen	140.2	0.37	129.7	0.33	...	...
	"By:" exposed to air	...	...	56.3	0.09	...	...
3‖	A + 16 parts arachidonic acid	176.8	0.35	104.1	0.16	68.3	0.11
	A + 16 parts linoleic acid	67.4	0.08	59.1	0.07	42.5	0.06
	A + 16 parts oleic acid	101.8	0.44	104.5	0.35	95.1	0.12
	A + 16 parts methyl oleate	98.7	0.66	98.3	0.63	91.5	0.50
	A + 16 parts palmitic acid	15.3	0.55	16.1	0.55	10.1	0.38
	A + 16 parts methyl palmitate	15.3	0.68	14.8	0.64	11.3	0.50
4	B = 16 parts linoleic acid + 0.6 mg./gm. diet of butter yellow	...	0.56	...	...	135.0	0.60
	B + 84 parts brown rice	133.1	0.56	...	...	132.1	0.50
5	B + 84 parts brown rice	134.0	0.69	133.7	0.66	134.7	0.59
	B + 84 parts white rice	123.8	0.66	125.2	0.49	64.7	0.09
6	B + 84 parts brown rice	118.7	0.61	...	...	121.1	0.66
	B + 84 parts quartz sand	86.4	0.22	...	...	52.0	0.07
	B + 84 parts sucrose	81.8	0.22	...	...	40.3	0.06
	B + 84 parts cornstarch	70.8	0.17	...	...	32.9	0.07
	B + 84 parts rice starch	58.0	0.13	...	...	37.3	0.08

* Determined colorimetrically in chloroform extract.

‡ Ration A had the following composition: casein 6 parts, sucrose 22, cornstarch 50, salt mixture 4, cod liver oil 2, supplemented with butter yellow. The concentration of butter yellow was maintained throughout (Experiments 1 to 6) at the level of 0.6 mg. per gm. of the *complete* food mixtures (*i.e.*, per 100 parts of the mixture).

§ The iodine number of different samples before addition to the diet varied from 124.1 to 131.8.

‖ The fatty acids and esters of this experiment were purified samples prepared in the laboratory of one of us.

of butter yellow was visible *in vitro*. It can be assumed that the liberation of unsaturated fatty acids from lard in the process of digestion opens the way to decomposition of butter yellow before it reaches the liver cells. The question

why at the same time a lard diet does not entail toxic manifestations, such as are seen after administration of a diet containing linoleic acid, needs further study for its elucidation.

If these considerations are correct, the procarcinogenic effect of crisco or butter fat as well as that of rice could be explained by the preservation of butter yellow in the diet and the intestine because of a low intake of unsaturated fatty acids, in the case of butter fat or crisco, and by the presence of a stabilizer or antioxidant, in the case of rice.

According to the literature (4, 5) the production of butter yellow cancer is hindered by administration of rice bran oil obtained by extraction from rice bran with ether. This fact puts rice bran oil in the same category as lard. Furthermore it tends to corroborate the assumption that the antioxidant of rice is not fat-soluble and does not pass into the ether extract.

The metabolism of *N*,*N*-dimethylaminoazobenzene has hitherto been regarded as a cellular process (6). The investigations reported here throw light on a reaction which occurs without the participation of living tissue. In the light of these findings it is noteworthy that recently the destruction of hemoglobin and hemin (7) and of carotene (8) by linoleic acid has also been reported. It remains to be shown whether the conservation and destruction of butter yellow in the diet and in the intestine, as illustrated by the investigations here analyzed, exhaust all possibilities of the influence of diet on "butter yellow cancer." No such claim is presented here.

Recently great interest has been aroused by the identification of biotin as a procarcinogenic agent for butter yellow cancer (9). In the relevant experiments of du Vigneaud and his collaborators biotin was given by mouth and thus it may have acted locally in the dietary mixture or in the intestine as stabilizer for butter yellow, an effect similar to that of rice. In preliminary experiments, however, which are being continued, we have been unable to demonstrate such an effect *in vitro*.

The production of progressive anemia and leucopenia when linoleic acid is contained in the diet and their prevention by the administration of yeast recall the old hypothesis concerning the rôle ascribed to unsaturated fatty acids, mainly oleic acid, in the pathogenesis of pernicious anemia and anemia caused by tapeworm (*Bothriocephalus latus*) (10). The fact that in these previous experiments anemia could be produced by parenteral, but not by oral, administration of oleic acid has never been explained. That essential fatty acids or rather, probably, some of their oxidative break-down products, exert an injurious effect on hematopoiesis only if the diet is deficient is a fact not known hitherto and one which has become evident only in the present experiments. Further studies are needed in order to identify this deficiency which, together with the pathogenesis of "butter yellow cancer," is a good illustration of the

often recurring problem whether intoxication or deficiency (11) plays the leading part in a given dietary disturbance.

The special type of anemia and the cancer of the liver found in the present investigations were mutually exclusive. In this connection it is illuminating that in certain races of the Far East primary cancer of the liver is a common disease whereas pernicous anemia occurs very rarely (12). It should be emphasized, however, that the morphologic blood picture of pernicous anemia differs from that seen in rats fed a diet containing linoleic acid, the latter anemia being of the secondary type. This difference may be due either to the differing response of man and rat or to the differing pathogenesis.

SUMMARY

Crude linoleic acid incorporated with or without butter yellow in a synthetic diet proved to be toxic for rats. The toxic effect manifested itself in loss of weight, progressive anemia of the secondary type, leucopenia, and pediculosis. It could be neutralized preventively and therapeutically by administration of yeast. The toxicity of the diet containing linoleic acid appears to be due to oxidative break-down products of the unsaturated fatty acid.

The color of the same diet when it contained crude linoleic acid supplemented with butter yellow faded progressively in the presence of air (O_2), even at room temperature. Purified preparations of linoleic acid and, to a less degree, purified preparations of arachidonic and oleic acids have shown the same destructive effect on butter yellow *in vitro*.

Brown (unpolished) or white rice contains a stabilizer (antioxidant) for the preservation of butter yellow.

In experiments on the production of hepatoma in rats following the ingestion of butter yellow, rice on one hand and crisco or butter fat on the other hand have proved to be procarcinogenic. These results would seem to be correlated with the preservation of butter yellow in the diet and in the intestine, because of the antioxidant in rice and the low supply of unsaturated fatty acids, respectively.

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