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# The Relationship of Pyridoxine and Riboflavin to the Nutritional Value of Polymerized Fats[1,2]

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THE GROWTH RESPONSE of rats to a fresh, nonoxidized, and unheated fat or an oil has usually served as a criterion of its nutritional value (1). However in the human diet not all edible fats are consumed in the fresh untreated form. For example, in 1953 over 172 million lbs. of oil were heated to 340–400°F. in the production of potato chips (2). Fats used in doughnut fryers and in deep fat fryers in restaurants are also exposed to similar treatment. The temperatures cited are those at which the processes are supposedly conducted. However, because of poor heat transfer and lack of proper agitation, certain zones within a commercial frying unit reach considerably higher temperatures (3).

Prolonged heat treatment of fats and oils promotes thermal polymerization, and exposure of the hot oil to the atmosphere favors autoxidation. If the autoxidation proceeds far enough, oxidative polymerization may possibly occur (4). In commercial frying operation the combination of heat and exposure to oxygen may lead to what we have for convenience termed thermal-oxidative polymerization. No simple quantitative measurement of the degree of polymerization is available for these materials. Therefore they will be described according to the technique used in their production.

Other workers have shown that efforts to alter the properties of fats and oils by drastic thermal and/or oxidative treatments result in the production of minor quantities of toxic materials (5). Thermally polymerized oils seem to involve carbon-to-carbon-linked dimeric acids which appeared to be toxic to rats. Under conditions of economic stress, thermally treated oils were included in commercial products designed for human consumption after World War II. These products have been completely withdrawn from edible use (6).

The biological response to a pure oxidative polymer has not been determined although it is known that the introduction of a single peroxide group into the fatty acid molecule is sufficient to produce toxicity. Holman (7) determined the $LD_{50}$ of linoleate peroxide in mice while Kaneda (8) has stated the $LD_{50}$ of autoxidized oils in terms of milligrams of peroxide oxygen for albino rats. The importance of the peroxide group is clearly demonstrated by the apparent complete elimination of toxicity after treatment of an oxidized oil with reagents which reduce peroxides.

It has been stated (9) that the consumption of polymerized fats does not reach a nutritionally significant level in the human diet. However the production of commercially fried foods in ever-increasing amounts requires re-examination of the problem. Oils which are used in commercial cookery remain organoleptically acceptable since odoriferous degradation products are largely removed by the steam distillation which occurs in the presence of foods containing substantial amounts of water. That the oils have undergone oxidation is apparent however in the relatively short shelf stability of the products fried in such oils. These heated oils are of great interest since they should combine the features of both thermal polymerization and oxidative polymerization.

## Experimental

In the present study the nutritive value of polymerized fats was compared by feeding them, usually at a 10% level, to weanling rats kept on three different basal rations. One was a grain stock ration (10) similar to a diet used in animal colonies for many years (Table I). The second was a fat-free

TABLE I
Modified Stock Ration

| | *lb.* |
|---|---|
| Ground corn | 53.0 |
| Linseed meal | 18.0 |
| Powdered skim milk | 14.0 |
| Alfalfa meal | 3.50 |
| Iod. NaCl | 0.75 |
| Bonemeal | 0.75 |
| Total | 90.00 |

By dropper once a week—vitamin $D_2$ 1.6 mcg.; vitamin A 160 I.U.; mixed tocopherol 295 mcg.

mixture consisting of cerelose, casein, Wesson salts, and all of the vitamins except pyridoxine (Table II) while the third was a fat-free ration consisting of 78% cerelose, 18% casein, 4% Wesson salts, 2 mg. thiamine, and 4 mg. riboflavin per pound of ration. The same fat soluble supplement, as listed in Table II, was provided for the animals on this ration.

A commercially treated fish oil[4] and a corn oil heated at 230°C. for four hours under vacuum (10–30 mm. Hg) were used as examples of thermally polymerized oils. The oxidatively polymerized oils were produced in our laboratories by blowing air through the fresh oils for 72 hrs. at room temperature. The end-products of commercial frying operations were designated as thermally-oxidatively polymerized oils or "used" oils. One sample of corn oil was drawn from the 180 lbs. of residual oil, which

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[4] Fish oil obtained through the courtesy of Archer-Daniels-Midland Company, Minneapolis, Minn.

TABLE II

Modified Fat-Free and Pyridoxine-Free Ration

| | |
|---|---|
| Cerelose | 78 lb. |
| Casein | 18 lb. |
| Wesson salts | 4 lb. |
| Cystine | 114 gr. |
| Glycine | 366 gr. |
| Inositol | 45.5 gr. |
| Choline chloride | 91.0 gr. |
| Niacin | 4.5 gr. |
| Thiamine | 200 mg. |
| Riboflavin | 400 mg. |
| P-amino benzoic acid | 136 mg. |
| Folic acid | 36 mg. |
| Calcium pantothenate | 450 mg. |
| Biotin | 190 mcg. |
| By dropper once a week: | |
| Vitamin $D_2$ | 1.6 mcg. |
| Vitamin A | 160 I.U. |
| Vitamin E | 295 mcg. mixed tocopherols |

remained when 1,191 lbs. of fat had been used in a potato chip fryer of 272-lb. capacity at 340°F. for 51 hrs. over a two-week period. The other corn oil samples were drawn from local continuous potato chip fryers. Used lard was obtained from a 30-lb.-capacity French fryer located in a local drive-in restaurant, and a hydrogenated vegetable oil was obtained from a commercial doughnut fryer. Special treatment was applied, and additional supplements were made to the oil as noted. Laboratory "used" oil was prepared by preparation of potato chips in a small home type of fryer.

## Results

The results indicated that commercially used fats produced poorer growth than did the corresponding fresh fats (Figure 1). At the end of 15 weeks the animals which had been kept on the grain stock ration and supplemented with 10% fresh corn oil weighed 49 g. more than those supplemented with 10% of the commercially used corn oil. Improved growth responses were noted when 10 mg./kg. of pyridoxine were included in the diet, which contained used oil, or when the used oil was washed with alcoholic stannous chloride before addition to the basal ration. The addition of extra pyridoxine did not improve the growth responses of rats given the grain ration containing 10% fresh corn oil. At the end of 34 weeks the animals which had received the pyridoxine supplement weighed 100 g. more, and those which had received the stannous chloride washed oil weighed 52 g. more, than those which had received the untreated used oil.

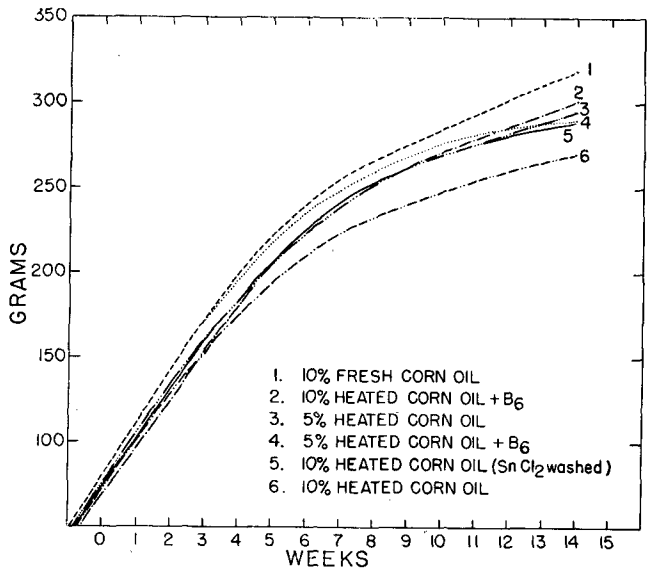


FIG. 1. Growth on stock ration with 58.10% added corn oil.

The growth differences at 14 weeks were statistically significant at the 1% level between either the group receiving 10% fresh corn oil or 10% heated corn oil plus pyridoxine and the group receiving 10% heated corn oil. A difference, statistically significant, at the 5% level was also noted when the group fed 10% fresh corn oil was compared to the group fed 10% heated corn oil washed with alcoholic stannous chloride. No significant difference existed between those receiving 10% heated corn oil compared to those receiving 10% heated corn oil washed with alcoholic stannous chloride.

Feed consumption data indicated that the fat which had been used in commercial potato chip frying operations was quite acceptable to the rats. In six-month-old males, feed consumption averaged 15.8 g. and 15.7 g. daily/rat over a period of 21 days on diets which contained oils from two different types of fryers, as compared with 16.0 g. daily/rat for those which had received fresh oil. During the same period rats which had received a sample of corn oil that had been thermally oxidized in our laboratory consumed 19.8 g. of food per day and made similar weight gains. This finding is consistent with the observations of Lane (11) that rats will consume greater quantities of a ration containing a "heated" oil while making the same or poorer weight gains than a group receiving a similar fresh oil. Since this material had been prepared by blowing air through the oil, the initial content of volatile aldehydes and ketones was low, and the oil was reasonably palatable despite the degree of oxidation. Such an oil will soon develop a rancid flavor through spontaneous breakdown of the oxidative polymers to volatile carbonyl compounds; it is therefore desirable to prepare this type of diet frequently with freshly oxidized oil or with an oil that has been deodorized.

A growth-depressing effect was again noted when used fats were compared with fresh fats on a synthetic diet that was deficient in pyridoxine but complete in other known water-soluble vitamins (Table II). When pyridoxine was added to this ration at the same level as was found in the grain diet by microbiological assay, the results indicated that, while growth on the synthetic ration was slower initially, the animals attained average weights similar to those on the grain diet in 15 weeks (Figure 2). The pyridoxine deficiency apparently served to accentuate the differences in growth response between fresh and used fats. The fresh corn oil, lard, and hydrogenated fat produced growth apparently in proportion to their essential fatty acid content, and the used fats in all three cases produced a depression in these growth responses. As would be expected, the effect of commercial treatment is least pronounced in the hydrogenated shortening, which was lowest of the group in di- and polyunsaturated fatty acid content. In this experiment the growth differences were statistically significant at the 1% level when fresh and used corn oil were compared or when fresh and used lard were compared. There was however no significant difference between the groups receiving fresh or used hydrogenated shortening.

In an effort to elucidate the effect of pyridoxine on the nutritional value of polymerized fats, rats

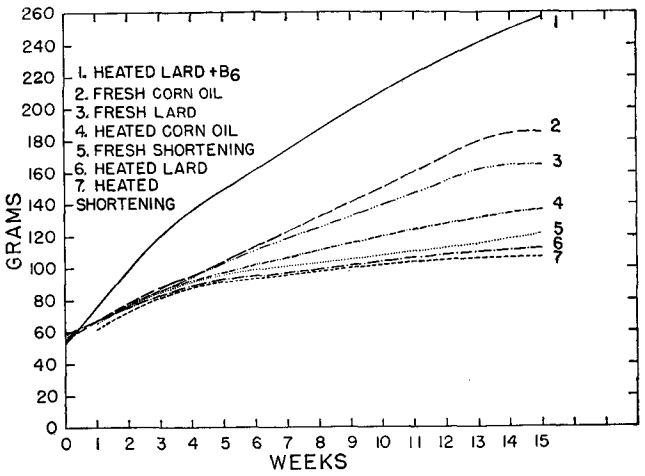


FIG. 2. Growth on fat-free ration + 10% added shortening.

were fed fish oils which had received various treatments. These oils were deliberately chosen to represent far more extreme degrees of oxidative and thermal polymerization than would be found in any oil normally offered for human consumption. Except for a slight bitterness in the thermally polymerized fish oil, these fats were organoleptically acceptable. Autoxidized fish oil should have been the most unpalatable material offered to the experimental animals; however the animals fed this oil plus pyridoxine grew very well, indicating that the oxidized oil must have been acceptable to the rats.

One-half of the rats which had been supplemented with 10% thermally polymerized fish oil died during the first six weeks, and all were dead before the end of the eighth week. Pyridoxine lengthened the life of rats which had received thermally polymerized fish oil, either by removing a simple vitamin deficiency or by improving the ability of the animal to metabolize the polymer. In the group supplemented daily with pyridoxine, only one-third of the rats died during the first eight weeks and two-thirds died in 14 weeks.

No deaths occurred in the groups which had received autoxidized fish oil. Pyridoxine supplementation in this group produced a growth response similar to that obtained by washing the autoxidized oil with alcoholic stannous chloride prior to mixing it with the ration. The animals fed oxidative polymers had matted coats and exhibited slight dermal symptoms while those fed thermal polymers were listless and starved in appearance but showed little change in the character of the fur.

Growth differences, statistically significant at the 1% level, were noted between the groups fed either autoxidized fish oil plus pyridoxine or autoxidized fish oil washed with alcoholic stannous chloride and the group fed autoxidized fish oil. The animals fed the autoxidized fish oil plus pyridoxine, or the autoxidized oil washed with stannous chloride, gained 190 g. and 188 g., respectively, while those on the autoxidized fish oil gained only 97 g. The weight differences between the group receiving fresh fish oil and that receiving autoxidized fish oil was statistically significant at the 5% level.

Further studies were carried out with pyridoxine supplementation at various protein levels. These studies indicated that on synthetic diets which contained more than 20% protein additional supplementation with pyridoxine produced little or no growth response in animals fed used corn oil (Table III). The diet which contained 20% casein did show improvement when pyridoxine was added.

TABLE III

Growth Responses of Rats Fed Diets Containing "Used" Corn Oil and Various Protein Levels[a]

| Casein | I[b] Fresh corn oil | II[b] Used corn oil | III[b] Used corn oil |
|---|---|---|---|
| | Gain | Gain | Gain |
| % | g. | g. | g. |
| 10 | 24.0 | 1.0[c] | 1.3[c] |
| 20 | 33.7 | 19.1 | 24.0 |
| 30 | 47.7 | 29.2 | 28.8 |
| 40 | 49.0 | 30.7 | 29.4 |

[a] Five male weanling rats per group: days on diet, 18. Ten per cent oil added to each diet.
[b] Diet: modified diet (Table II) with 10 mg. pyridoxine/kilo in I and II; and 20 mg./kilo in III. Casein varied at expense of cerelose.
[c] Animals on diet containing 10% casein and used oil could be maintained for only six days.

Riboflavin as well as pyridoxine seemed to be a limiting factor in the growth response to polymerized fats (Table IV). Weanling rats were kept on a fat, and vitamin-deficient ration for three weeks. They were then transferred to a diet which contained 10% used oil and supplemented with ascorbic acid, mixed tocopherols, riboflavin, or pyridoxine. The animals which received these vitamin supplements gained 14, 12, 10, and 30 g. more respectively than those fed only used oil. However neither riboflavin nor mixed tocopherols stimulated growth in the groups which had received 10% thermally polymerized corn oil. When vitamin supplementation was terminated, only the groups which had previously received pyridoxine showed any lasting beneficial effect from the period of supplementation. The addition of 3% rice bran extract (Vitab), 20 mg. of pyridoxine or 60 mg. of riboflavin per kilo of ration improved growth in the animals fed used corn oil. On the other hand, the thermally polymerized oil apparently required increased quantities of tocopherol in its utilization. With the used oil no response was observed after supplementation with 1.0% ascorbic acid or 1% mixed tocopherols. Such statistically significant differences as occurred are noted in Table IV. In the case of both oils, supplementation with tocopherol or ascorbic acid produced rats with very poor coats. The rough, matted appearance of their fur was especially noticeable when compared with the riboflavin-supplemented groups.

## Discussion

The results to date indicate that the dietary level of riboflavin and pyridoxine and the manner in which fats are polymerized have an important bearing on their nutritional value in rat diets. The biological value of pyridoxine appears to be related to the protein content of the diet, especially at lower protein levels. In agreement with Crampton *et al.* (5), who fed thermally polymerized linseed and soybean oil, the thermally polymerized fish oil was more deleterious to rats than the oxidatively polymerized oil. Paschke and Wheeler (12) have shown that, in the thermal polymerization of 9,12 methyl linoleate, the amount of conjugated dienes increases to a maximum of 6 to 8% and then decreases as polymerization becomes more complete. The resulting high ratio of nonconjugated to conjugated linoleate would be fa-

TABLE IV

Comparative Growth Responses to Various Vitamin Supplements of Rats Fed Commercially-Used Corn Oil or Thermally-Polymerized Corn Oil

| | Oil + vitamin supplement for 36 days | | | | Basal only for 29 days | | | | Oil + 3% rice bran extract[a] vitamin supplement for 99 days | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial wt. | | Change in wt. during period | | Initial wt. | | Change in wt. during period | | Initial wt. | | Change in wt. during period | |
| | Used | Thermal | Used | Thermal | Used | Thermal | Used | Thermal | Used | Thermal | Used | Thermal |
| | g. | g. | g. | g. | g. | g. | g. | g. | g. | g. | g. | g. |
| Oil only | 64 | 65 | − 1 | + 8 | 63 | 73 | +14 | − 4 | 77 | 69 | +155 | +149 |
| Oil + 1% ascorbic acid | 65 | 64 | +13 | + 8 | 78 | 72 | −10 | − 5 | 68 | 67 | +153 | +153 |
| Oil + 1% mixed tocopherols[b] | 64 | 65 | +11 | − 1[d] | 75 | 64 | −10 | + 1 | 65 | 65 | +155 | +185 |
| Oil + 60 mg. riboflavin/kilo | 64 | 65 | + 9 | + 4 | 73 | 69 | + 8 | − 2 | 81 | 67 | +199[c] | +143 |
| Oil + 20 mg. pyridoxine/kilo | 66 | 64 | +29[d] | +31[d] | 95 | 96 | + 1 | +10 | 97 | 106 | +205[d] | +145 |

[a] Vitab—Charles Bowman and Company, Holland, Michigan.
[b] Distillation Products Industries, Rochester 3, N. Y.
[c] Significant difference at 5% level when compared to "oil only" group.
[d] Significant difference at 1% level when compared to "oil only" group.

vorable for the formation of co- or cyclic-polymers by a Diels-Alder type of mechanism through a carbon-to-carbon-type linkage. Barbot (13) found that such a dilinoleate could be nitrated, diazotized, and coupled with β-naphthol. It is possible that the intestinal enzymes cannot readily cleave such a benzenoid structure into smaller units.

The present results indicate that the metabolism of such units may depend on the amount of available pyridoxine or tocopherols. The grain diet contained six times more pyridoxine than is normally required by the rat, or 9 γ/g. of ration. Adding 10 γ of pyridoxine per gram of ration however had a beneficial effect. Vilter *et al.* (14) suggested that the requirement of vitamin $B_6$ for man is 2–3 mg. per day. It is possible that for man the normal dietary level of vitamin $B_6$ may be high enough to metabolize the polymerized fats which may be unknowingly consumed.

Oxidative polymers are formed through carbon-to-oxygen linkages and seem to be more readily metabolized than thermally or thermally-oxidatively polymerized fats. The hydroperoxide groups in these oxidative polymers may be responsible for the vitamin destruction which has been associated with rancidity. Washing with stannous chloride removed these hydroperoxides and may therefore have eliminated destruction of the vitamins. Oxidative polymers may have little significance in the diet of man as they break down readily into aldehydes and ketones of objectionable odors and flavors (4). Furthermore a taste panel will reject as unpalatable hydrogenated fats which have a peroxide value of more than 20 and unhydrogenated oils which have a peroxide value of 150 (15). Since ethyl linoleate with a peroxide value of 629 has been shown to be biologically available to rats (16), a large "safety threshold" seems to exist when fats of a peroxide value of 150 are rejected by tasting panels.

Supplementation with riboflavin seemed to compensate for the presence of commercially used oil in the diet but was inactive against thermally polymerized oil. Riboflavin, possibly in the form of the flavin-adenine dinucleotide (FAD), may have entered into the destruction of lipid peroxides and spared pyridoxine. The manner in which pyridoxine facilitates the detoxification of peroxides or promotes growth on deficient diets containing thermal polymers is open to question. Dubolouz (17) has shown that lipid peroxides are not stored in animal tissues. His work indicates that such peroxides are destroyed in the liver by a porphyrin-type pigment which is itself irreversibly destroyed in the reaction. This destruction proceeds along a complicated and poorly understood pathway. Possibly somewhere in this reaction pyridoxine or riboflavin is required in the form of a cofactor. Commercially used oils may contain nutritionally significant quantities of peroxides and thermal polymers. Reduction of the peroxides might be commercially feasible, but the nutritive value of the oil would still be impaired by the thermal polymers. Either a means for removing thermal polymers must be found, or care must be taken to insure that diets containing commercially used oils also contain compensating quantities of the necessary vitamins.

## Summary

The nutritive value of polymerized fats was compared by feeding them at a 10% level to weanling rats kept on three different basal rations and various vitamin supplements. Autoxidatively or thermally autoxidatively polymerized fats depressed growth, and thermally polymerized fats were toxic to rats. The results indicated that the dietary level of riboflavin and pyridoxine and the manner in which the fats were polymerized had an important bearing on their nutritional value. The protein level also influenced the nutritional value of the oils and, at lower levels, appeared to be related to pyridoxine intake as the growth depression and the toxicity were partially overcome by supplementation of the diet with pyridoxine. Riboflavin apparently aided the animals in coping with autoxidized fat but did not contribute any protective action against thermal polymers.

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