THE FACTORS AFFECTING THE METABOLISM OF LINOLENIC ACID

by

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INTRODUCTION AND REVIEW OF LITERATURE

Two types of rancidity are known, hydrolytic and oxidative. The latter, which is largely responsible for food deterioration during cold storage, may be prevented in one or two ways; the deposition of antioxidant materials in the body fats by the living animal or by changing the characteristic unsaturated species fat via dietary supplementation or omission.

Lea (17) was among the first of the early workers to recognize that the stability of animal fat maybe influenced by antioxidants in the diet. Dyme et al. (7) found that the feeding of large amounts of soybeans to hogs increased the iodine value of their fat, but did not decrease its keeping time. This would indicate that the iodine value of a fat is not a criterion of its keeping time in the presence of antioxidant materials.

In feeding the antioxidants, ascorbic acid and hydroquinone, to rats, Overman (21) found no definite changes in the keeping time of the body fats, although there was some indication that ingestion of ascorbic acid improved the stability. Later Barnes et al. (2) reported that the ingestion of certain antioxidant substances such as yeast and hydroquinone did not restore "normal" stability to body fat from vitamin E-deficient rats, but alpha-tocopherol effects such a restoration. Furthermore, Lundberg, Barnes, Clausen, and Burr (19) have shown that the feeding of alpha-tocopherol in various amounts up to 500 milligrams results in the deposition of increasing amounts in

the abdominal fats. The oxidative-induction stability of this extracted fat was directly proportional to its alpha-tocopherol content. Recent work by Burr et al. (6) has shown that the alpha and beta forms of tocopherol give results very much alike when fed to rats, while the gamma form was not more than half as effective as the other two.

Hanson et al. (12) in an effort to show the antioxidant properties of compounds other than the tocopherols, fed rats alpha-naphthol, nordihydroguaiaretic acid and lecithin. Results indicate that none of the above was effective. Van Fleet (27), on the other hand, has shown the vitamins B_2 and B_6 to inhibit the oxidation of fats in living plant tissues.

Recently, Kummerow et al. (15) reported that the supplementation of ethanolamine and choline to the basic diet of turkeys increased the stability of the fat extracted from the skin tissue towards oxidative rancidity taking induction period tests as a criterion. Here, however, it was suggested that the increased ratio of phospholipid to tissue fat in the birds on this dietary regime might have been responsible for the increase in stability.

There is much accumulated evidence that body fats are derived from all of the proximate food principles: fats, carbohydrates, and even proteins. Anderson and Mendel (1) have studied the effect of feeding high concentrations of the above dietary principles to rats. Findings indicated that both protein and carbohydrate diets yielded "hard" fats or fats with

low iodine values. On the other hand, when soybean oil, corn oil, cottonseed oil, or peanut oil was fed in large amounts, the resulting body fats resembled the food fats.

On the surface, then, it would appear that enhanced carcass stability would be achieved if the animals were fed diets high in protein and carbohydrate, with dietary fat present only as saturated compounds. That such a diet is impractical was shown by the work of Evans and Burr (8) by Burr and Burr (4) and Evans and Lipkovsky (9). The above investigators clearly established the fact that the rat does not thrive on diets devoid of fat, but develops a characteristic deficiency disease. The symptoms of this disease are a retardation and gradual complete stoppage of growth, scaly condition of the skin, and abnormally high water consumption.

To date various investigators, Burr and Burr (4), Burr, Burr, and Miller (5) and Turpeinen (26) have claimed curative powers for linoleic, linolenic, and arachidonic acids respectively, whereas oleic (9), alpha-eleaostearic (5), erucic, ricinoleic and chaulmoogric acids (26) have proven ineffective. More recently, Randolph (24) has presented conclusive evidence to show that linolenic acid alone also was ineffective as a curative for rat acrodynia. Kummerow (14), working with chickens, has further demonstrated that the animal fed a diet with hydrogenated vegetable fats as the sole source of dietary fat did very poorly. The young birds on this diet ceased growth, some did not even grow feathers. It is evident, then,

that the need of the animal body is not for fat in general, but for certain unsaturated fatty acids.

Since both the kind and optimum amount of unsaturated fatty acids are not known, it appears that the preparation of a diet containing the exact amount of the necessary unsaturated dietary factor, but with none of these in excess for deposition, is even a laboratory difficulty. Under field conditions, control of the fatty acid intake would be an impossibility. Yet any of these unsaturated fatty acids in excess will find their way into the animal's fat depots. Kummerow et al. (16), for example, have shown that turkeys fed less than one percent linolenic acid in the form of raw linseed oil, deposited linolenic acid in their skin tissues.

Other factors are also involved. Studies via the medium of rat acrodynia have shown that the vitamins B_6 (10, 11) and pantothenic acid (18) are also involved in the metabolism of the unsaturated fatty acids.

The work in this thesis was carried out with acrodynia rats in order to study the effect of the various antioxidants on the metabolism and deposition of linolenic acid. In addition, the effect of the vitamin B_6 analogs and the combined effects of the above antioxidants and the vitamin B_6 were studied.

EXPERIMENTAL

Summary of Experimental Work

Preparation of Diets and Animals. Weanling rats 40-50 g[1] in weight were placed in separate metal cages and started on a fat free basal diet, (Table 4). The animals were weighed once each week and examined for dermal fat deficiency symptoms. When the dermal lesions, which were determined as described by Quackenbush et al. (22) reached a minimum of five, the animals were divided into groups, each group consisting of six rats. Three animals in each group received the dietary supplement plus vitamin B_6, the other three receiving only the supplement. Each animal was fed approximately 0.1 gram of pure ethyl linolenate, which was prepared from hexabromostearic acid by the method described by Rollett (25). The daily feedings of supplements were administered with the aid of calibrated medicine droppers. Where solubility conditions permitted, the supplements were dissolved in the ethyl linolenate.

Three groups were fed the vitamin B_6 analogs, pyridoxamine dihydrochloride, pyridoxal hydrochloride, pyridoxal L tyrosine[2]. Table 2 shows the amounts of the supplements fed daily.

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[1] Purchased from Sprague Dawley Inc., Madison, Wis.
[2] Contributed by Merck and Co., New York, N. Y.

Methods and Procedures

Extraction and Characterization of the Carcass Fat. Each week over the assay period of three weeks the animals were examined for dermal symptoms and the body weights recorded. At the end of this period the animals were killed by anesthetization with ether, saponified, and their total carcass fats extracted by the method of Quackenbush and Steenbock (23). The resulting fatty acids were weighed and the percentage of the total body weight was calculated, (Table 5). The fatty acids were sealed under vacuum immediately after extraction and stored in the cold until analyzed in order to prevent changes in the unsaturated components.

Spectrophotometric (20) and iodine value (28) analyses of the fatty acids were conducted. The calculation of percent increase in body weight eliminated the differences present in the average starting weights of the various groups; similarly, to compensate for the difference in size and amount of fat on the animals receiving B_6 compared with those not receiving that vitamin. The fat content of the carcasses was calculated in milligrams per hundred grams of body weight.

Extraction of Tissue Lipid. Immediately after the animals of a group had been killed, the carcasses of the animals in a dietary group were placed in a one liter beaker, 30 percent potassium hydroxide solution added, and the beaker heated on a steam bath until the carcasses, excluding the bones and teeth, were dissolved (23). Complete disintegration of the carcass,

with subsequent release of all lipid material, was assured by using 30 percent alkali in a ratio of one milliliter to each gram of body weight. The time required to completely dissolve the animal bodies varied according to the size of the animals in a group.

The alkaline solution containing the dissolved rat carcasses was then decanted into a round bottom flask. The bones were washed three times with 95 percent ethyl alcohol. The total volume of alcohol used was equal to one-half the number of milliliters of 30 percent potassium hydroxide used for saponification. The decanted liquid and alcohol washings were combined and refluxed for four hours in order to complete saponification. The mixture was cooled and extracted three times with half-volumes of Skellysolve F to remove the nonsaponifiable fraction. The extracted nonsaponifiable was discarded.

The alkaline saponification mixture was next made slightly acid with concentrated hydrochloric acid. This procedure was carried out in a beaker immersed in an ice bath. The acid was added slowly with constant stirring to prevent localized heating.

The liberated fatty acids were extracted three times with half-volumes of Skellysolve F. Great care was exercised in these extractions and the subsequent water washings to avoid the formation of emulsions. After extraction of the fatty acids from the acidic saponification mixture, the aqueous layer

containing potassium chloride and amino acids was discarded. The Skellysolve layer was washed three times with approximately equal volumes of distilled water to remove excess acid and water soluble materials which may have remained in the Skellysolve phase. The latter was dried with anhydrous sodium sulphate. To avoid any loss of fatty acid in drying, the sodium sulphate medium was washed twice with small amounts of the solvent. The dried Skellysolve extract and washings were combined in a weighed round bottom flask and freed from solvent under vacuum.

To insure complete removal of all solvent, the round bottom flask was placed for 30 minutes in a vacuum oven kept at 100° centigrade and a pressure of 0.1 mm of mercury. The vacuum was applied and removed gradually to prevent any losses due to spattering.

The flask was fitted with a stopper, cooled, and weighed. The difference in weight represented the total fatty acids of the carcass. The percentage of fatty acid in the tissues was calculated from this weight according to the following equation.

$$\text{Percent of fatty acid} = \frac{100 \times \text{weight of fatty acid extracted}}{\text{total carcass weight}}$$

Determination of Iodine Value (23). Approximately 0.1 g of the liquefied fatty acids was pipetted into a glass-stoppered iodine flask, dissolved in 5 milliliters of chloroform, and 15 milliliters of Wijs solution were added. To facilitate solution and thorough mixing, the flask was stoppered and swirled. The stoppered flask was then placed in the dark for exactly one hour.

At the end of this time, 10 milliliters of a 15 percent potassium iodide solution was added. The mixture was thoroughly mixed by shaking, 10 milliliters of distilled water were used to wash the stopper and sides of the flask, and the contents titrated with N/10 sodium thiosulphate. When the titration had proceeded far enough to produce a faint yellow color, a few drops of 2 percent starch indicator were added, and the mixture titrated to its end point. The number of milliliters of N/10 thiosulphate required was recorded. Duplicate blanks were titrated in the same manner.

Wijs solution - Thirteen grams of powdered, crystalline iodine were dissolved in one liter of glacial acetic acid. Ten milliliters of this iodine solution were titrated with standard sodium thiosulphate with the aid of starch as an indicator. About 100 milliliters of the iodine solution were set aside, while dry chlorine gas was passed into the remainder until the solution became straw colored. Ten milliliters of the straw colored solution were then pipetted into 10 milliliters of a 15 percent potassium iodide solution and titrated as before with standard thiosulphate. Sufficient chlorine was added to double the titration of the original iodine solution. A slight excess of iodine was found permissible. The 100 milliliters of original iodine solution held in reserve were used to compensate for any chlorine excess. The prepared Wijs solution was stored in amber-colored bottles for periods not to exceed one month.

Potassium iodide solution - Fifteen grams of potassium iodide were dissolved in 85 milliliters of distilled water.

Thiosulphate solution - Twenty-four and eight tenths grams of sodium thiosulphate were dissolved in one liter of distilled water. The solution was standardized as follows: Exactly 10 milliliters of a N/10 potassium dichromate solution were pipetted into an iodine flask. To this 5 milliliters of concentrated hydrochloric acid and 10 milliliters of a 15 percent potassium iodide solution were added. The mixture was immediately titrated to a blue-green color with the sodium thiosulphate solution to be standardized, starch indicator was added, and the titration continued slowly, with constant agitation. The end point was very sharp, with the solution turning a brilliant green color. The iodine factor of the thiosulphate solution was calculated with the following formula:

$$\text{Iodine factor} = \frac{126.9 \times \text{normality of the } K_2Cr_2O_7 \text{ solution}}{\text{ml thiosulphate required}}$$

The iodine value of the fat sample was determined as follows:

$$\text{Iodine value} = \frac{\text{I. F.} \times (\text{blank titration} - \text{sample titration})}{\text{weight of the sample}}$$

Spectrophotometric Analysis of Fat (20). Four milliliters of ethylene glycol, which contained 7.5 percent potassium hydroxide, were added to the appropriate number of 10 inch ignition tubes. The tubes, plus duplicate blanks, were placed in an oil bath and heated to 180 degrees centigrade. A constant flow of dry nitrogen was passed into the tubes in order to replace the air.

The fat to be analyzed was liquified and thoroughly mixed.

Approximately one tenth of a gram of the fat was accurately weighed, in duplicate, into small weighing dishes. The latter were dropped into the ignition tubes and heated at 180 degrees for 30 minutes. The tubes were then removed from the oil bath, wiped free of oil, and cooled to room temperature in a stream of cold water. The contents of the tubes were quantitatively transferred to hundred milliliter volumetric flasks with the aid of 95 percent ethyl alcohol. The volumetric flasks were made up to volume with ethyl alcohol, thoroughly mixed, and stored at zero degrees centigrade overnight to allow precipitation of silica.

The samples were brought to room temperature and filtered. The first 20 milliliters of filtrate were discarded and the remainder diluted appropriate concentrations. The latter were read on the Beckman spectrophotometer at wavelengths 2320, 2340, 2620, 2680, 2740, 3100, 3160, and 3220 Å against a blank of the same dilution. The concentration of the dilutions depended upon the amount of conjugated material in the isomerized samples. Principal dilutions were 5 milliliters 100 milliliters, and 5 milliliters to 50. Other dilutions were also used. The readings and dilutions were recorded.

Unisomerized samples of the fat were also read in order to correct for errors due to absorption of ultra-violet light by impurities. Samples of approximately 0.1 gram were accurately weighed and dissolved in 95 percent ethyl alcohol to produce a known concentration. These unisomerized samples were read

and recorded in the same manner as the isomerized samples.

The 95 percent ethyl alcohol used was purified for reuse for future determinations by refluxing it over granulated zinc and potassium hydroxide for several hours, followed by distillation. The alcohol reclaimed in this manner was free of materials having absorption in the frequencies mentioned above.

Alkaline ethylene glycol - Fifteen grams of potassium hydroxide were finely ground and dissolved in 180 milliliters of ethylene glycol.

The fatty acid composition was calculated as follows:

Unisomerized samples

$$K_2 = \frac{K_{2320}}{\text{Conc. of sample in gms per liter}} - .07$$

$$K_3 = \frac{2.8}{\text{Conc. of sample in gms per liter}} \left[K_{2680} - \tfrac{1}{2}(K_{2620} + K_{2740})\right]$$

$$K_4 = \frac{2.5}{\text{Conc. of sample in gms per liter}} \left[K_{3160} - \tfrac{1}{2}(K_{3100} + K_{3220})\right]$$

Isomerized samples

$$K'_2 = \frac{K'_{2320}}{\text{Conc. of sample in gm per liter}} + .04$$

$$K'_3 = \frac{4.1}{\text{Conc. of sample in gm per liter}} \left[K'_{2680} - \tfrac{1}{2}(K'_{2620} + K'_{2740})\right]$$

$$K'_4 = \frac{2.5}{\text{Conc. of sample in gm per liter}} \left[K'_{3160} - \tfrac{1}{2}(K'_{3100} + K'_{3220})\right]$$

$$K''_2 = K'_2 - K_2$$

$K''_3 = K'_3 - K_3$

$K''_4 = K'_4 - K_4$

Linoleic acid = $1.125 \times K''_2 - 1.27 \times K''_3 - .04 \times K''_4 = \%$

Linolenic acid = $1.87 \times K''_3 - 4.43 \times K''_4 = \%$

Arachidonic acid = $4.43 \times K''_4 = \%$

Oleic acid = Iodine value $\times$ 100 - [(181.5 $\times$ % linoleic acid + 273.5 $\times$ % linolenic acid + 333.5 $\times$ % arachidonic acid)] /90 = %

Saturated acids = 100% - (% linoleic + % linolenic + % arachidonic + % oleic) = %

Any K value or any part of a K value which has a minus value was considered zero.

Preparation of Ethyl Linolenate (25). Three hundred grams of potassium hydroxide were placed in a five-liter round-bottom flask, 100 milliliters of distilled water were added, and the mixture was carefully agitated to facilitate solution. When solution was complete, the flask and its contents were cooled to room temperature, 1200 milliliters of 95 percent ethyl alcohol were added, followed by one kilogram of raw linseed oil. The mixture was refluxed for 30 minutes.

Upon completion of the reflux period, the hydolysate was cooled to 40 degrees centigrade, diluted with 100 milliliters of cold distilled water, and acidified with 550 milliliters of cold concentrated hydrochloric acid. The acid was added in small portions with constant cooling and agitation.

The cold mixture was then placed in a large separatory

funnel, and shaken vigorously to insure complete decomposition of the soaps. Next, the fatty acids were washed twice with one liter volumes of distilled water. One liter of diethyl ether was added and the fatty acids were washed again with one liter of distilled water. After the last water washing, the fatty acids were drawn off into a clean five-liter round-bottom flask. Fifty grams of anhydrous sodium sulphate were added, and the solution was allowed to stand overnight in a cold room at minus five degrees centigrade.

The following morning, the saturated acids and the sodium sulphate were filtered from the cold solution, and an additional liter of diethyl ether was added to the filtrate. It was then ready for bromination.

The five-liter round-bottom flask containing the still cold filtrate was clamped firmly in an ice-salt bath, and an air driven stirrer was introduced to keep the solution in a constant state of agitation. Bromine was added from a small separatory funnel at such a rate that the temperature of the mixture would not rise above 10 degrees centigrade. Bromination was considered complete when the mixture had acquired a persistent pale pink color due to a slight excess of bromine. Experience has shown a large excess of bromine to be highly undesirable. The flask was corked and again allowed to stand overnight in a cold room at minus five degrees centigrade.

The crude, crystalline hexabromostearic acid was collected on a Buchner funnel, washed to a pure white with diethyl ether,

and recrystallized from hot benzene. The crystals were dried at room temperature. Their melting point, 115 degrees centigrade, indicated a high state of purity.

The dry crystalline hexabromostearic acid and an equal weight of granular zinc were mixed together in a dry two-liter round-bottom flask. One milliliter of absolute ethyl alcohol was added for each gram of hexabromostearic acid and the mixture catalyzed with 10 milliliters of a three normal solution of dry hydrogen chloride in absolute alcohol.

After fitting the flask with a reflux condenser, the mixture was carefully warmed on a steam bath to start the reaction. The violent initial reaction was controlled by immersing the flask and contents in a pail of cold water, after which the reaction mixture was allowed to reflux for two hours with intermittent shaking.

The mixture was then cooled and 200 milliliters of Skellysolve F and sufficient water were added to form a two phase system, one of which was a water-alcohol mixture, the other, ethyl linolenate in Skellysolve F. The aqueous phase was extracted a second time with 200 milliliters of Skellysolve F before it was discarded. The Skellysolve phase was dried by filtering through anhydrous sodium sulphate and freed from solvent under vacuum. The last traces of Skellysolve F were removed in a vacuum oven at 100 degrees centigrade. Duplicate iodine values of the ethyl linolenate indicated a purity of 98.6 percent. The ester was divided into four gram portions,

each of which was sealed under high vacuum until needed.

Estimation of the Dermal Symptoms. The body of the rat contained six areas which were particularly vulnerable to the dermal lesions caused by fat deficiency. The areas involved were the tissues around the eyes, the mouth and throat, fore paws, hind paws, tail, and ears.

During each weekly examination, a rating of from zero to five was given the areas mentioned above according to the method of Quackenbush et al. (22), Table 3.

When the rat had acquired a total deficiency symptom rating of five, it was deemed that it was ready for assay.

RESULTS

The animals which were supplemented with 20 gamma of pyridoxine or its analogs daily developed normally. On the other hand, the animals which were not supplemented with pyridoxine did very poorly, Plates I and II.

The animals which received only ethyl linolenate as a supplement suffered a loss of 14.5 percent in body weight, while those receiving no supplement lost only 6.5 percent. When pyridoxine was added to the above diets, the situation reversed itself. The animals receiving ethyl linolenate displayed a body weight increase of 113.2 percent, while those unsupplemented gained only 104.7 percent. Using the above four groups as a base line, Table 5 clearly shows body weight increases

ranging from 11.5 percent with ascorbic acid and ethyl linolenate to 166.8 percent with butylated hydroxy anisol, pyridoxine, and ethyl linolenate as supplements.

The dermal symptoms as described by Quackenbush et al. (22) increased in severity in all groups not receiving B_6 or its analogs, excluding the groups supplemented with butylated hydroxy anisol and the mixed tocopherols, Table 4. The former group started with an average dermal index of five and completed the assay period with an average index of four, the latter group, starting with an index of six, remained at that point.

Effect on Fat Content. An increase in the production of fatty acids in the animal body was noted when pyridoxine or the analogs of that vitamin were fed. Furthermore, the results (Table 5) indicate that only the tocopherols have an additive effect in fat synthesis, whereas the other antioxidants tend to reduce the amount of fat synthesized.

Effect on the Composition of Mixed Fatty Acids. Despite the fact that all the animals were fed the same basic diet, differences in the unsaturated fatty acids extracted from their carcasses were evident, (Table 6). It is of interest to note that when monoethanol ammonium gallate or 2-methyl naphtha quinone was fed in addition to ethyl linolenate, the percent of linoleic acid in the carcass exceeded the percent of linolenic acid present. When pyridoxine was added in addition to the above dietary components this situation reversed itself-the percent of linolenic acid present exceeded the percent of linoleic acid. On the other hand, in the animals fed butylated

EXPLANATION OF PLATE I

Rats of the Sprague Dawley strain which received various dietary supplements. These animals were the same age and possessed approximately the same dermal lesions at the start of the assay period. The animals were fed the dietary supplements indicated for three weeks.

Fig. 1. Ethyl linolenate only.

Fig. 2. Ethyl linolenate and pyridoxine.

Fig. 3. Ethyl linolenate and 2-methyl naphtha quinone.

Fig. 4. Ethyl linolenate, 2-methyl naphtha quinone, and pyridoxine.

PLATE I

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

EXPLANATION OF PLATE II

Rats of the Sprague Dawley strain which received various dietary supplements. These animals were the same age and possessed approximately the same dermal lesion at the start of the assay period. The animals were fed the dietary supplements indicated for three weeks.

Fig. 1. Ethyl linolenate, butylated hydroxy anisol, and pyridoxine.

Fig. 2. Ethyl linolenate and butylated hydroxy anisol.

PLATE II

Fig. 1.

Fig. 2.

hydroxy anisol, mixed tocopherols, or ascorbic acid the body fats contained nearly the same percent of linoleic acid as those fed the above supplements plus pyridoxine. It is of further interest to note that the variation in the percentage of oleic acid did not exceed 12 percent, irrespective of the dietary supplements. Similarly, the variation in the percentage of saturated acids did not exceed 11 percent.

The three groups which were supplemented with the pyridoxine analogs and ethyl linolenate yielded fats whose unsaturated components were very similar to each other and those of the group supplemented with ethyl linolenate and pyridoxine. However, considerable differences can be noted between the oleic and saturated acid components of the above groups.

Effect on Iodine Value. The mixed fatty acids extracted from the carcasses of the animals supplemented with pyridoxine or its analogs possessed the lowest iodine values, (Table 5). It is interesting to note, however, that the animals supplemented with pyridoxine and ethyl linolenate yielded mixed fatty acids with a lower iodine value than those which received pyridoxine with no fatty acid supplementation.

Effect on Mg of Fatty Acid per 100 g of Body Weight. On the basis of the milligrams per 100 grams of tissue, the nearly constant value for arachadonic acid is of particular interest, (Table 7). Furthermore, the carcasses of the animals supplemented with the various antioxidants, excluding ascorbic acid, contained a greater amount of arachadonic acid per 100 grams

of body weight than the corresponding groups which were fed pyridoxine in addition to the antioxidant. On the other hand, the amount of linoleic, linolenic, oleic, and saturated acid per 100 grams of tissue shows a definite increase when pyridoxine is added to the diet.

Table 1. Composition of basal ration.

Constituent	Amount per 100 lb. of ration
Cerelose	73 lb.
Casein	13 lb.
Cystine	114.66 g
Choline chloride	91.00 g
Inositol	44.31 g
Niacin	4.55 g
Riboflavin	0.40 g
Thiamin	0.20 g
P-amino benzoic acid	0.14 g
Folic acid	0.04 g
Calcium pantothenate	0.05 g
Sodium chloride	300.00 g
Potassium chloride	240.00 g
Potassium dihydrogen phosphate	620.00 g
Calcium phosphate	298.00 g
Calcium carbonate	420.00 g
Magnesium sulphate (anhyd.)	180.00 g
Iron pyrophosphate	119.00 g
Manganese sulphate (anhyd.)	0.40 g
Potassium iodide	0.05 g
$K_2 Al_2(SO_4)\cdot 24 H_2O$	0.09 g
$CuSO_4\cdot H_2O$	0.39 g
Biotin	14 cc
Vitamins A and D	250 and 100 IU weekly

Table 2. Amounts of dietary supplements fed daily.

Supplement	Gamma fed daily
Monoethanol ammonium gallate	200
2-Methyl naphtha quinone	66
Ascorbic acid	200
Butylated hydroxy anisol	200
Mixed tocopherol	200
B_6	20
Pyridoxamine dihydrochloride	20
Pyridoxal hydrochloride	20
Pyridoxyl L tyrosine	20

Table 3. Stages of dermatitis.

	Stage 1	Stage 2	Stage 3	Stage 5
Lips	Hyperemia, usually with small ulcers at corners.	Slight swelling redness along vertical grove.	Medium swelling desquamation to nostrils.	Severe swelling, raw soreness of wide area around mouth and nostrils.
Eyes	Lids wet, slight crusts at corner.	Slight swelling of lids, crusts in corners.	Medium swelling, lids often adherent with gummy exudate.	Severe swelling, lids swollen shut, large crusts on lids.
Fore paws	Slight scaleness redness following desquamation between digits.	Slight swelling digits desquamated, red and sore.	Medium swelling, digits raw and sore, sometimes with puss accumulation.	Severe swelling, desquamated and sore to wrists; gangrenous.
Hind paws	Same as fore paws.	Same as fore paws; also desquamation of large areas on paws.	Same as fore paws; skin cracked and ulcerated at heel and tibia.	Severe swelling, large red areas over paws and inner thigh, paws often gangrenous.
Ears	Hyperemia slight swelling.	Pinnae swollen and thickened due to scale formation.	Medium thickening at tips, large scales sloughing.	Severe thickening necrosis.
Tail	Scaly (ringlets).	Very scaly.	Very scaly; necrosis near tip.	

Table 4. Weight gains and dermal symptoms.

Diet	Start	After 3 wks.	Total	Start	After 3 wks.
1. Without pyridoxine					
Ethyl linolenate	62	53	-9	6	10
" " + ascorbic acid	61	68	7	6	6
" " + gallate[1]	53	65	12	3	8
" " + naphtha quinone[2]	61	70	9	5	11
" " + anisol[3]	62	72	10	5	4
" " + mixed tocopherol	51	88	37	6	6
No supplement whatsoever	81	76	-5	6	9
2. With 20 gamma pyridoxine daily					
Ethyl linolenate	69	147	78	6	1
" " + ascorbic acid	52	133	82	9	1
" " + gallate[1]	55	132	77	7	2
" " + naphtha quinone[2]	58	132	74	5	2
" " + anisol[3]	54	147	93	5	2
" " + mixed tocopherol	60	156	96	6	1
20 gamma pyridoxine only	64	131	67	8	2
3. With 20 gamma pyridoxine analogs					
Ethyl linolenate					
" " + pyridoxamine[4]	55	137	82	5	1
" " + pyridoxal HCl	59	148	89	5	1
" " + pyridoxyl L tyrosine	61	123	62	4	2

[1] Monoethanol ammonium gallate

[2] 2-methyl naphtha quinone

[3] Butylated hydroxy anisol

[4] Pyridoxamine dihydrochloride

Table 5. Percent body weight increases and iodine values.

Diet	Percent fatty acid	Percent increase in body weight	Iodine value
1. Without pyridoxine			
Ethyl linolenate	1.84	-14.5	94.2
" " + ascorbic acid	1.89	11.5	85.3
" " + gallate[1]	2.92	22.6	79.5
" " + naphtha quinone[2]	2.36	14.8	85.8
" " + anisol[3]	3.46	16.1	82.2
" " + mixed tocopherol	2.99	72.5	76.8
No supplement whatsoever	1.84	- 6.17	80.4
2. With 20 gamma pyridoxine daily			
Ethyl linolenate	8.20	113.2	80.1
" " + ascorbic acid	6.20	157.6	70.9
" " + gallate[1]	6.11	140.0	71.5
" " + naphtha quinone[2]	9.76	129.9	69.0
" " + anisol[3]	7.62	166.8	71.3
" " + mixed tocopherol	12.50	163.3	71.5
20 gamma pyridoxine only	9.35	104.7	63.9
3. With 20 gamma pyridoxine analogs			
Ethyl linolenate			
" " + pyridoxamine[4]	7.35	149.2	72.6
" " + pyridoxal HCl	9.02	150.9	70.0
" " + pyridoxyl L tyrosine	7.98	101.5	70.6

[1] Monoethanol ammonium gallate

[2] 2-methyl naphtha quinone

[3] Butylated hydroxy anisol

[4] Pyridoxamine dihydrochloride

Table 6. Spectrophotometric analysis of carcass fat.

Diet	Linoleic	Linolenic	Arach.	Oleic	Sat.
	Percent				
1. Without pyridoxine					
Ethyl linolenate	2.08	0.54	9.20	56.71	31.45
" " + ascorbic acid	1.06	0.26	9.58	56.36	32.74
" " + gallate[1]	0.78	0.88	7.44	56.53	34.37
" " + naphtha quinone[2]	2.68	0.51	9.13	54.51	33.17
" " + anisol[3]	0.98	1.22	7.38	56.42	33.50
" " + mixed tocopherol	0.96	1.16	6.21	56.87	34.80
No supplement whatsoever	1.52	1.56	6.96	55.73	34.23
2. With 20 gamma pyridoxine daily					
Ethyl linolenate	0.87	1.20	2.00	53.97	41.97
" " + ascorbic acid	0.62	0.42	4.12	60.97	33.87
" " + gallate[1]	1.49	1.04	3.21	61.39	32.87
" " + naphtha quinone[2]	1.03	1.93	2.12	60.86	34.06
" " + anisol[3]	1.13	1.63	2.42	63.00	31.82
" " + mixed tocopherol	0.72	1.35	1.89	65.34	30.20
20 gamma pyridoxine only	1.15	0.53	0.77	64.23	33.32
3. With 20 gamma pyridoxine analogs					
Ethyl linolenate					
" " + pyridoxamine[4]	1.09	1.71	2.98	62.22	32.00
" " + pyridoxal HCl	1.13	1.19	2.25	63.63	31.90
" " + pyridoxyl L tyrosine	1.26	1.69	2.79	60.44	33.82

[1] Monoethanol ammonium gallate

[2] 2-methyl naphtha quinone

[3] Butylated hydroxy anisol

[4] Pyridoxamine dihydrochloride

Table 7. Milligrams of fatty acid per 100 grams of body weight.

Diet	Linoleic	Linolenic	Arach.	Oleic	Sat.
	mg				
1. Without pyridoxine					
Ethyl linolenate	38.3	9.95	169	1050	530
" " + ascorbic acid	20.5	4.54	181	1064	600
" " + gallate[1]	22.8	25.7	217	1655	1005
" " + naphtha quinone[2]	63.3	12.1	216	1238	783
" " + anisol[3]	34.0	42.2	270	1950	1160
" " + mixed tocopherol	28.7	34.6	196	1700	1040
No supplement whatsoever	28.0	28.7	128	1025	616
2. With 20 gamma pyridoxine daily					
Ethyl linolenate	71.4	90.5	164	4420	2410
" " + ascorbic acid	38.4	26.1	256	3769	2400
" " + gallate[1]	91.0	63.5	169	3743	2010
" " + naphtha quinone[2]	101.0	188.0	207	5940	3320
" " + anisol[3]	86.2	124.0	194	4800	2432
" " + mixed tocopherol	90.0	231.0	236	8160	4000
20 gamma pyridoxine only	107.5	46.8	72	6000	3110
3. With 20 gamma pyridoxine analogs					
Ethyl linolenate					
" " + pyridoxamine[4]	80.1	126.0	219	4570	2353
" " + pyridoxal HCl	102.0	107.0	203	5810	2980
" " + pyridoxyl L tyrosine	100.0	135.0	223	4820	3050

[1] Monoethanol ammonium gallate

[2] 2-methyl naphtha quinone

[3] Butylated hydroxy anisol

[4] Pyridoxamine dihydrochloride

DISCUSSION

The work of Birch (3), which indicated that pyridoxine is in some way connected with the metabolism of unsaturated fatty acids, has been substantiated. However, the postulation of that author that in the absence of adequate amounts of unsaturated fatty acids the animal is unable to utilize its vitamin B_6 was not confirmed. Animals which received only 20 gamma of B_6 daily supplemental to their fat free basic diet showed definite recovery from the symptoms of acrodynia. However, the animals which received ethyl linolenate and B_6 showed slightly greater recovery over the latter animals. On the other hand, the rats which received ethyl linolenate without B_6 lost a greater percentage of body weight and showed more severe acrodynia symptoms than did those animals which received no fat supplement whatsoever, indicating that linolenic acid without pyridoxine is toxic to the rat.

The feeding of the various antioxidants seems to have a further effect on the dermal symptoms and percentage body weight increases in the animal. In all cases where an antioxidant was ingested without pyridoxine, the animals exhibited percentage weight gains which were greater than those receiving neither pyridoxine nor antioxidant. Similarly, the animals which were fed antioxidant and pyridoxine showed weight gains which were greater than those receiving only pyridoxine. It is therefore thought that these substances in some way reduce the toxicity

of the linolenic acid.

The analogs of pyridoxine seemed to behave in a manner which was similar to the vitamin itself, which is indicative of the possession by the rat of an enzyme system or systems which can convert these analogs of pyridoxine to the vitamin. The data show, however, that the tyrosine analog was not fully as effective as the other analogs tested. This may be due to several considerations: (1) the animal was not able to quantitatively convert this analog to pyridoxine or (2) 20 gamma daily is not adequate supplementation.

The iodine values of the total extracted fatty acids showed that the animals which were supplemented with ethyl linolenate possessed the most highly unsaturated fat, while those animals which received additional supplements of antioxidants possessed a somewhat more saturated fat. On the other hand, supplementation with pyridoxine and ethyl linolenate yielded a fatty acid mixture with a lower iodine value than did additional supplementation with antioxidant substances. Hilditch (13, p. 66) has pointed out that the iodine value does not reflect the mixed fatty acid composition of a fat. Spectrophotometric analysis yields a more complete picture, hence this method of analysis was applied.

From the spectrophotometric data, it can be seen that when ethyl linolenate was fed alone or in combination with ascorbic acid, monoethanol ammonium gallate, or 2-methyl naphtha quinone, the decrease in the percentage of linolenic acid is accompanied

by an increase in linoleic acid. In the supplementation of ethyl linolenate with butylated hydroxy anisol or the mixed tocopherols, linolenic acid was present in greater percentage than linoleic acid. This same general condition was true when pyridoxine was added as a further supplement.

On the basis of the percentage composition of the extracted fatty acids, it appeared that the animals whose fats contained the greatest amounts of linoleic acid possessed the most severe dermal symptoms. This is not in agreement with the accepted theory concerning the curative properties of that unsaturated fatty acid. Further, the great variation in the percentage of arachadonic acid between the groups receiving pyridoxine and those not receiving the vitamin may be misleading as the former weighed two to three times more than the latter. The percentage of fatty acids was therefore converted into milligrams per 100 grams of body tissue.

An explanation for the great variations in arachadonic acid was immediately obvious. Since the complete animals were saponified, and since the liver is the seat of practically all of the arachadonic acid in the body of the rat, it appears that all of the animals possessed livers which contained nearly identical amounts of arachadonic acid per 100 grams of body weight. The sharp increase in the percentage of arachadonic acid in the animals which did not receive pyridoxine is not due then to greater amounts of arachadonic acid, but rather to smaller amounts of the other fatty acids present in the carcass.

The same generalization also accounts for the higher iodine values in the fat of these animals.

The grams of linoleic acid per 100 grams of body tissue (Table 7) was relatively high in all groups of animals which showed signs of recovery from the dermal lesions. This included, in addition to the groups supplemented with pyridoxine or its analogs, the group receiving mixed tocopherol. Since none of the animals had access to any dietary source of linoleic acid, their bodies must have synthesized this acid from some precursor with the aid of pyridoxine. Traces of linoleic acid were found in the carcasses of the animals which did not receive pyridoxine. An explanation of this condition may be made by assuming that all the animals had a certain supply of the vitamin from birth which as the animals grew older was exhausted completely or reduced to suboptimum levels for proper metabolism of the linolenic acid.

The great differences in the number of milligrams of oleic and saturated acids that were found in the rats which were fed pyridoxine or its analogs indicated that this vitamin played some role in the synthesis of these fatty acids from ingested carbohydrate or protein.

SUMMARY

1. The effect of antioxidants on the metabolism of linolenic acid was studied with the use of the medium of rat

acrodynia. Ascorbic acid, monoethanol ammonium gallate, 2-methyl naphtha quinone, butylated hydroxy anisol and mixed tocopherols were supplemented to animals on a pyridoxine adequate and a pyridoxine deficient diet. The pyridoxine analogs, pyridoxamine dihydrochloride, pyridoxal hydrochloride, and pyridoxyl L tyrosine were also supplemented with ethyl linolenate to determine whether these analogs would be functional in the rat.

2. The animals which received pyridoxine or its analogs showed remarkable recovery from the dermal symptoms characteristic of the acrodynia regardless of other dietary supplementation. On the other hand, in the pyridoxine deficient animals, only those which were supplemented with butylated hydroxy anisol showed curative trends.

3. The data show that all the antioxidants have a definite effect on the metabolism of linolenic acid in the rat whether the animals are pyridoxine deficient or not.

4. The iodine values of the fatty acids extracted from the carcasses of the rats which were pyridoxine deficient were considerably higher than the fatty acids of the rats receiving 20 gamma of pyridoxine or its analogs per day. It was pointed out that this effect was due not to an increase of unsaturated fatty acids, but rather to an increase in the saturated fatty acids.

5. Pyridoxine plays a definite role in the synthesis of both saturated and unsaturated fatty acids. However, the amount of oleic and saturated acids synthesized is proportionally far greater than linoleic and linolenic acid.

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