Effects of Dietary Vitamin E, Selenium, and Polyunsaturated Fats on In Vivo Lipid Peroxidation in the Rat as Measured by Pentane Production

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ABSTRACT

Starting at 21 days of age, groups of six rats each were fed a basal Torula yeast diet supplemented with 0.4% L-methionine and varying amounts of vitamin E as dl-alpha tocopherol acetate, selenium as sodium selenite, and with either 10% stripped corn oil, stripped lard, or coconut oil. By 7 wk, pentane production by rats fed a corn oil diet deficient in both vitamin E and selenium was twice that by rats fed 0.1 or 1 mg of selenium per kg of the same basal diet. Blood glutathione peroxidase activity after 7 wk was proportional to the logarithm of dietary selenium. Groups of rats fed the vitamin E- and selenium-deficient diets with lard or coconut oil had one-half the pentane production of rats fed the vitamin E- and selenium-deficient corn oil diets. The plasma level of linoleic plus arachidonic acid was 1.8 times greater on a wt % basis in rats fed corn oil than in rats fed lard or coconut oil as the fat source. Pentane production by rats fed 40 i.u. dl-alpha tocopherol acetate per kg of the selenium-deficient corn oil diet was one-sixth of that by rats fed the same diet without vitamin E; the plasma of the rats fed the vitamin E-supplemented corn oil diet had a level of vitamin E that was about six times greater than that of the rats fed the vitamin E-deficient corn oil diet.

INTRODUCTION

Hydrocarbon gases have been related to lipid peroxidation and rancidity in vitro by a number of workers (1-5). Riely et al. (6) were the first to show that ethane, a decomposition product of ω3-fatty acid hydroperoxides, serves as an index of lipid peroxidation in vivo in mice. Hafeman and Hoekstra (7,8) reported that vitamin E and selenium protected rats against lipid peroxidation as determined by measurement of evolved ethane. These investigators (7) showed that ethane evolution was related to the ω3-fatty acid content of the diet. Rats fed a 5% cod liver oil diet evolved about six times more ethane than did rats fed a diet with tocopherol-stripped lard, which contains a lower level of ω3-fatty acids than does cod liver oil.

In this laboratory, in vivo lipid peroxidation in rats fed a vitamin E-deficient diet during a 7-wk period was followed by measurement of expired ethane and pentane (9). Pentane, which derives from ω6-fatty acid hydroperoxides, was shown to be a better index of peroxidative damage in vivo than was ethane when a 10% corn oil diet was fed. Since corn oil contains a relatively high level of linoleic acid, one would expect the rapid incorporation of this fatty acid into membranes of growing rats. The present study was designed to examine the influence of dietary ω6-fatty acids, mainly linoleic acid, on pentane production by rats. A Torula yeast-based diet deficient in both vitamin E and selenium was selected to provide conditions in vivo that would most rapidly result in peroxidation of unsaturated fatty acids. Vitamin E serves as a lipid peroxidation chain-breaker (10), while selenium is required for the function of the lipid hydroperoxide and H_2O_2 decomposing enzyme, glutathione peroxidase (11). The dietary fat sources chosen were tocopherol-stripped lard, tocopherol-stripped corn oil, and coconut oil. In addition to testing the effect of linoleic acid content of the diet on pentane production, the experiments were designed to test (a) the effect of different levels of dietary selenium on pentane production in rats fed a 10% corn oil, Torula yeast-based diet without vitamin E and (b) the effect on pentane production of feeding rats 0.1 ppm selenium together with 40 i.u. dl-alpha tocopherol acetate/kg of a 10% corn oil, Torula yeast-based diet.

MATERIALS AND METHODS

Animals and Diets

The animals used were specific pathogen-free male rats, descendants of the Sprague-Dawley strain. The rats were obtained at 21 days of age from Hilltop Lab Animals, Inc. They were housed in hanging wire cages and were kept on a 14-hr light and 10-hr dark cycle.

The basal diet was a powdered selenium- and vitamin E-deficient diet obtained from Teklad Test Diets. This basal diet contained in percentage: Torula yeast, 30; L-methionine, 0.4;

sucrose, 55.97; fat, 10; mineral mix[1], 3.5; and vitamin mix[2] exclusive of vitamin E, 0.13. The fat source was one dietary variable. The three fats used were tocopherol-stripped corn oil (CO), tocopherol-stripped lard (LD), and coconut oil (CN). The diets were supplemented with vitamin E and selenium as follows (fat source – i.u. dl-alpha tocopherol acetate, mg selenium/kg diet): CO-O E, O Se; CO-O E, 0.01 Se; CO-OE, 0.1 Se; CO-O E, 1.0 Se; CO-40 E, O Se; CO-40 E, 0.1 Se; LD-O E, O Se; and CN-O E, O Se. Selenium was added as sodium selenite. Groups of six rats each were fed the diets and deionized water ad libitum, with fresh diet being provided daily except for Saturdays. The animals were fasted overnight prior to pentane analyses. Weights were recorded at 21 days of age and on each day on which pentane was measured.

Pentane Analysis

Pentane analysis was done on either a Varian 3700 or 1520 gas chromatograph with a flame ionization detector and fitted with a six-way, nut type gas sample valve. Stainless steel columns (1/8 in. x 5 ft for the 1520 model and 1/8 in. x 10 ft for the 3700 model) were filled with 80-100 mesh activated alumina, and a 30 ml/min nitrogen carrier gas flow was used with each column. For the 1520 model, column temperature was programmed as previously described (9). The Varian 3700 was programmed at 20 C/min from 55 to 210 C. The chambers used to hold the rats during sample collection have been described previously (9). The rats were placed in the holding chambers to breathe hydrocarbon-free air (Matheson, ultra pure) for 25 min prior to collection of the samples. One-half of the breath-air stream (120 ml/min) from each rat was collected over a 10-min period to give a 600 ml sample in an activated alumina-filled gas sample loop immersed in liquid nitrogen-ethanol at -130 C. Breath samples were collected from each rat during week 3, 5, 6, and 7 of the dietary feeding program. The relative peak area of pentane on each chromatogram was calculated by triangulation, and the picomoles of the gas were calculated from a standard. One-milliliter portions of the 0.8 ppm pentane standard (Matheson Gas Products) were measured at an electrometer setting of 2×10^{-12} amps/mv on the 1520 model and at a setting of 4×10^{-12} amps/mv on the 3700 model.

Glutathione Peroxidase, Vitamin E, and Fatty Acid Analyses

After 7 wk of feeding the diets, two to four rats in each group were anesthetized with pentobarbital, and blood was removed by heart puncture via a needle and heparinized syringe. One-half ml of fresh blood was used for assay of glutathione peroxidase (12). The remaining blood was centrifuged for 25 min at ca. 2700 rpm in an IEC clinical centrifuge, and the plasma was frozen for later vitamin E and fatty acid analysis. Total vitamin E in plasma pools from each of the eight dietary groups and in samples of the CO-O E, O Se, CO-40 E, O Se, LD-O E, O Se, and CN-O E, O Se diets was measured by the fluorometric assay of Taylor et al. (13). For analysis of diet vitamin E, 1 g of diet was mixed with 5 ml of redistilled absolute ethanol, 7.5 ml of water, and 2.5 ml of 25% ascorbic acid. After heating for 5 min at 70 C, 5 ml of 10 N KOH was added for saponification. Aliquots of 3 ml were used for extraction. Aliquots of each plasma pool were extracted by the Bligh-Dyer (14) procedure, and methyl esters of the fatty acids were prepared by a modification of the procedure of van Wijngarden (15) for analysis on an automated Hewlett-Packard gas chromatograph with a model 3352B data system. Protein analysis was done by an automated Folin technique (16).

RESULTS

Pentane Production

Pentane production by rats during the third, fifth, sixth, and seventh weeks of feeding the eight diets is shown in Figure 1. Table I gives the statistical significance of differences between mean values as determined by Student's *t*-test. There were several findings of interest. At all four weeks of testing, pentane production by the CN-O E, O Se group was significantly (all $P < 0.05$ were considered significant) lower than by the CO-O E, O Se group, whereas, the production of pentane by the LD-O E, O Se group was lower only at week 6. At weeks 5, 6, and 7, there was no significant difference in pentane production by the CO-O E, O Se and CO-O E, 0.01 Se groups. The CO-O E, 0.1 Se and CO-O E, 1.0 Se groups had significantly lower pentane production than the CO-O E, O Se group, yet they were not significantly

[1]Salt mix (% composition): calcium carbonate, 20.71; calcium phosphate, diabasic, 32.29; cupric sulfate, 0.04; ferric citrate, 0.43; magnesium sulfate, 6.57; manganese sulfate, 0.44; potassium chloride, 20.86; potassium iodate, 0.002; sodium phosphate, dibasic, 18.60; and zinc carbonate, 0.06.

[2]Supplying (g/kg diet): thiamin HCl, 0.0004; riboflavin, 0.0025; pyridoxine HCl, 0.002; calcium pantothenate, 0.02; niacin, 0.1; biotin, 0.001; folic acid, 0.002; choline chloride, 1.0; vitamin B_{12} (0.1% trituration in mannitol), 0.1; dry vitamin A palmitate (500,000 u/g), 0.028; dry vitamin D_2 (500,000 u/g), 0.0064; and menadione, 0.001.

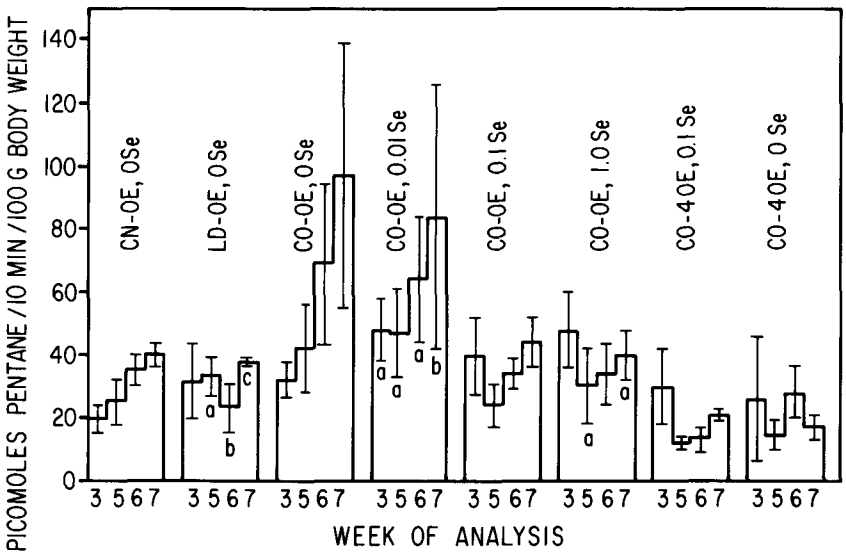

FIG. 1. Pentane production by rats fed diets variable in fat source, selenium and vitamin E. Rats were fed the diets starting at 21 days of age, and breath samples were collected during the following third, fifth, sixth, and seventh weeks. Pentane represents the amount of pentane expired during 10 min of sampling (1200 ml air-breath). The number of rats in each group was six except where indicated: a=5, b=4, and c=2. Bars represent mean ± standard deviation.

different from each other. Only at week 6 was pentane production by the CO-40 E, 0.1 Se group significantly lower than that of the CO-40 E, O Se group. The two groups of rats supplemented with 40 i.u. dl-alpha tocopherol acetate/kg of diet had significantly lower pentane production than all of the rats in the non-supplemented groups.

Glutathione Peroxidase

Table II shows glutathione peroxidase activity in the blood of rats fed the eight diets for 7 wk. The effect on glutathione peroxidase activity of a less than nutritionally required amount of selenium is shown by the absence of activity in the blood of rats fed a 10% corn oil diet with O or 0.01 mg selenium/kg of diet. Glutathione peroxidase activity in the CO-40 E, O Se group was about 16-17% of that in the CO-40 E, 0.1 Se group or the CO-O E, 0.1 Se group, while the activity in the CN-O E, O Se group was about 10% of the levels of these two groups. There was a doubling of specific activity in the blood of rats fed a ten-fold greater level of selenium, that is, in the CO-OE, 1.0 Se group.

Animal Weight Gain and Mortality

Table III shows the effect of the eight diets on final weight attained by the rats. The final weights attained were greatest by the groups of rats supplemented with 0.1 or 1.0 ppm selenium, regardless of dietary vitamin E content. By the seventh week, statistical analysis of the data by Student's *t*-test showed no difference in weights among the CO-O E, O Se, LD-O E, O Se, and CN-O E, O Se groups. The CO-40 E, 0.1 Se group of rats was significantly ($P < 0.01$) heavier than the CO-40 E, O Se group. The mean weight of the CO-O E, O Se group was significantly lower than that of any other corn oil-fed group except for the CO-O E, 0.01 Se group as indicated. CO-40 E, 0.1 Se, $P < 0.001$; CO-40 E, O Se, $P < 0.02$; CO-O E, 1.0 Se, $P < 0.001$; CO-O E, 0.1 Se, $P < 0.001$; and CO-O E, 0.01 Se, N.S.

Deaths resulting from dietary causes occurred mainly in the group fed lard as a fat source; one rat died during week 5, two during week 6, and one during week 7. During week 7, one rat from the CO-O E, 0.01 Se group died. Hematuria and rusty nasal secretions were noted during the sixth and seventh weeks in all groups but the vitamin E-supplemented groups.

Plasma and Diet Vitamin E

Analyses of total tocopherols were done on two different days with duplicate samples of pooled plasma from rats in the eight dietary groups and in triplicate samples of the diets. The average values obtained for the plasma samples were (mg/100 ml): CO-O E, O Se, 0.12; CO-O E, 0.01 Se, 0.22; CN-O E, O Se, 0.18;

TABLE I

Significance of Differences in Pentane Production by Rats Fed Varying Levels of Vitamin E and Selenium and Different Fat Sources[a]

Dietary groups compared[b]	Week 3	5	6	7
CO-O E, Se vs. CN-O E, O Se[c]	<.01[c]	<.05[c]	<.01[c]	<.01[c]
CO-O E, O Se vs. LD-O E, O Se[c]	NS	NS	<.05[c]	NS
CO-O E, O Se[d] vs. CO-O E, 0.01 Se	<.02[d]	NS	NS	NS
CO-O E, O Se[d] vs. CO-O E, 0.1 Se[c]	<.02[d]	<.02[c]	<.01[c]	<.02[c]
CO-O E, O Se[d] vs. CO-O E, 1.0 Se[c]	<.05[d]	NS	<.05[c]	<.02[c]
CO-O E, O Se vs. CO-40 E, O Se[c]	NS	<.001[c]	<.001	<.001[c]
CO-O E, O Se vs. CO-40 E, 0.1 Se[c]	NS	<.001[c]	<.001[c]	<.001[c]
CO-O E, 0.01 Se vs. CO-O E, 0.1 Se[c]	NS	<.01[c]	<.01[c]	<.05[c]
CO-O E, 1.0 Se[c] vs. CO-O E, 0.01 Se	NS	NS	<.02[c]	<.05[c]
CO-O E, 1.0 Se vs. CO-O E, 0.1 Se	NS	NS	NS	NS
CO-40 E, 0.1 Se[c] vs. CO-40 E, O Se	NS	NS	<.01[c]	NS
CN-O E, O Se[c] vs. LD-O E, O Se[d]	<.05[c]	NS	<.05[d]	NS
CO-40 E, O.1 Se[c] vs. CO-O E, 0.1 Se	NS	<.02[c]	<.001[c]	<.001[c]

[a]*P* values determined by *Student's t-test.*
[b]Diets are described in Materials and Methods.
[c,d]*P* value and dietary group with same superscript refers to group with lowest pentane production.

TABLE II

Effect of Dietary Vitamin E and Selenium on Rat Blood Glutathione Peroxidase[a]

Diet[b]	Specific activity[c]
CO-O E, O Se	0.0
CO-O E, 0.01 Se	0.0
LD-O E, O Se	0.0
CN-O E, O Se	2.4 ± 3
CO-O E, 0.1 Se	22.9 ± 3.7
CO-O E, 1.0 Se	41.3 ± 3.3
CO-40 E, O Se	4.0 ± 1.7
CO-40 E, 0.1 Se	25.0 ± 3.7

[a]Rats were fed their respective diets starting at day 21 for 7 wk prior to collection of blood.
[b]Diets are described in the text. Fat source, i.u. dl-alpha tocopherol acetate/kg and mg selenium/kg are indicated.
[c]Nanomoles NADPH oxidized/min/mg protein. The values are the mean ± SD for activity in whole blood from three rats in each dietary group except for the LD-OE, O Se group, from which blood from two rats was analyzed.

TABLE III

Effect of Vitamin E, Selenium, and Fat on Rat Weight[a]

Diet	Body wt[b]
CO-40 E, 0.1 Se	291.8 ± 21.1
CO-40 E, O Se	252.1 ± 21.6
CO-O E, 0.01 Se	212.0 ± 33.6
CO-O E, 0.1 Se	305.0 ± 34.4
CO-O E, 1.0 Se	294.5 ± 16.8
CO-O E, O Se	216.2 ± 21.5
CN-O E, O Se	234.1 ± 32.2
LD-O E, O Se	211.7 ± 27.8

[a]Weights are final weights attained by 7 wk of feeding the diets.
[b]Weight in g ± std. dev.

CO-40 E, O Se, 0.69; CO-O E, 1.0 Se, 0.16; LD-O E, O Se, 0.18; CO-40 E, 0.1 Se, 0.52; and CO-O E, 0.1 Se, 0.14. The average value for the vitamin E in plasma of the rats not supplemented with vitamin E was 0.17 mg/100 ml, and for the vitamin E-supplemented rats, the average value was 0.61 mg/100 ml. The average values of vitamin E obtained for four of the diets were (mg/kg diet); LD-O E, O Se, 2.4; CN-O E, O Se, 5.9; CO-O E, O Se, 3.9; and CO-40 E, O Se, 44.

Plasma Fatty Acid Analysis

Fatty acid analysis on pooled plasma samples from rats fed the eight diets was done primarily to compare the relative amounts of linoleic acid and arachidonic acid. The relative amounts of these two fatty acids were (linoleic acid, arachidonic acid, wt %): CO-O E, O Se, 25.9, 23.3; CO-O E, 0.01 Se, 25.1, 24.6; CO-O E, 0.1 Se, 20.9, 23.5; CO-O E, 1.0 Se, 20.5, 29.6; CO-40 E, O Se, 24.0, 28.0; CO-40 E, 0.1 Se, 23.7, 28.7; CN-O E, O Se, 10.0, 18.9; and LD-O E, O Se 12.3, 18.5.

DISCUSSION

As summarized previously (9), pentane has been shown by other workers to derive from oxidized linoleic acid (4,17) or from lipoxidase oxidized linoleic acid (2,18,19). Dumelin and Tappel (20) showed in vitro that decomposition of preformed linoleate hydroperoxide yielded 1.3 mol % pentane. As one would expect the

peroxidation of linoleic acid in vivo to be dependent upon the amount of linoleic acid provided by the diet and upon the antioxidant status of an animal, the aim of this study with rats was to investigate some of the relationships among linoleic acid, vitamin E, selenium, and pentane production. A diet doubly deficient in vitamin E and selenium was chosen in order to determine whether the effect of selenium, presumably via glutathione peroxidase activity, could be determined in the absence of vitamin E in the diet. A vitamin E-deficient diet is known to increase pentane production in rats (9). Interrelationships between vitamin E and selenium have been studied by many, but it has been difficult to obtain a quantitative approximation of the protective effect of selenium against lipid peroxidation in relationship to the protection afforded by vitamin E. Three fat sources were chosen for use in the hope of providing differing levels of linoleic acid in the diet. Corn oil, lard, and coconut oil have been reported to contain about 34%, 6%, and trace amounts of linoleic acid, respectively (21).

Selenium in the diet had a very definite effect on decreasing the production of pentane by rats fed a vitamin E-deficient 10% corn oil diet. This effect was related to the activity of blood glutathione peroxidase. The blood glutathione peroxidase activity probably can be considered a good index of selenium status in the rat. When fed a selenium-deficient diet, weanling rats lose their glutathione peroxidase activity in 4-5 weeks (22). The doubling of glutathione peroxidase activity when the diet level was increased from 0.1 to 1.0 mg/kg of diet confirms the log relationship of dietary selenium levels to the glutathione peroxidase level in plasma reported by Smith et al. (23). A nutritionally adequate level of selenium decreased the production of pentane by a factor of two. For growth in rats, the nutritional level recommended is 0.04 mg/kg of diet (24). However, increased dietary selenium above the recommended level did not further decrease pentane.

Coconut oil or lard in the vitamin E- and selenium-deficient diet resulted in a level of about one-half of the pentane produced by rats fed a diet with corn oil by 7 wk. The decreased pentane was related to the lower amount of linoleic acid in the diet and linoleic and arachidonic acid in the plasma. Plasma fatty acids can be used as a reflection of the composition of dietary fat in short range experiments (25). It was expected that the coconut oil diet would have a lower level of linoleic acid than the lard diet, but fatty acid analysis of the plasma of rats at the termination of the experiment showed no difference in the amount of linoleic and arachidonic acid in the plasma of rats fed these two diets.

The effect of feeding 40 i.u. dl-alpha tocopherol acetate/kg of diet (CO-40 E, O Se or CO-40 E, 0.1 Se) for 7 wk was to decrease pentane production to one-sixth that produced by rats fed no vitamin E (CO-O E, O Se). The lack of difference between production of pentane by the vitamin E-supplemented rats not fed selenium and those fed 0.1 mg selenium/kg of diet suggests that at this level of vitamin E supplementation no further effect of selenium on pentane production could be seen.

A comparison of pentane production by rats fed a vitamin E- and selenium-deficient Torula yeast-based diet with 10% corn oil and supplemented with 0.4% methionine was made with that produced by rats fed a casein-based, 10% corn oil and tocopherol-deficient diet (9). Pentane production by rats fed these two diets supplemented with 40 i.u. dl-alpha tocopherol acetate/kg of diet was comparable at weeks 5, 6, and 7. Rats fed the Torula yeast-based and methionine-supplemented vitamin E- and selenium-deficient diet in this study produced 16% less pentane by week 7 than did the rats fed a tocopherol-deficient, casein-based diet in the previous study (9). Since the fat source, 10% corn oil, was the same in the diets, one can speculate that L-methionine provided some protection to the rats. Hafeman and Hoekstra reported that methionine was protective as indicated by lower ethane production by methionine-fed rats treated with carbon tetrachloride than by similarly treated rats not fed methionine (7).

The results obtained in this study are in accord with present concepts of in vivo lipid peroxidation and its inhibition by vitamin E and selenium-glutathione peroxidase. Overall lipid peroxidation in bulk lipids is proportional to the content of polyunsaturated fatty acids and inversely proportional to the chain-breaking antioxidant (26), in this case, vitamin E. As an example of a study of the above, Witting (27) studied the relationship of vitamin E to polyunsaturated lipids in the diet and tissues. Results of this study and of others show that the above relationship holds in vivo. In vivo lipid peroxidation is known (28) to be initiated by reactions of the endoplasmic reticulum. Organs that are probable major sources of in vivo lipid peroxidation per unit weight would include liver and testes. Hydroperoxides produced by in vivo lipid peroxidation at a steady state concentration may be reduced by selenium-glutathione peroxidase. The amount of hydroperoxides reduced should be propor-

tional to the amount of enzyme present, although McCay et al. (29) found no hydroxy fatty acids produced in vitro when microsomal phospholipid peroxides were incubated with glutathione peroxidase. Glutathione peroxidase may function to prevent the initiation of peroxidation by H_2O_2 in vivo, since H_2O_2 is an excellent substrate for the enzyme as first reported by Mills (30). Pentane is produced from hydroperoxides by metal-catalyzed decomposition (20). The method described in this paper measures the total body production of pentane as it is expired in the breath. Although it is a minor product (20) of in vivo lipid peroxidation, pentane offers a number of advantages for measurement. Once produced it is an inert product in contrast to most of the products of lipid peroxidation. If there are other sources of pentane, they are small enough not to interfere with the primary measurement. The very low level of pentane produced by the rats fed the CO-40 E, 0.1 Se diet was considered to arise from very low level lipid peroxidation in vivo. Pentane, though soluble in fat, is transported to and volatile enough to be degassed through the lungs. The technique described makes it possible to apply noninvasive measurements to animals.

The lower weight attainment by four groups of rats (CO-O E, O Se, LD-O E, O Se, CN-O E, O Se, and CO-O E, 0.01 Se) was related to a deficiency of dietary vitamin E and selenium, but not necessarily to the fat source. The findings suggest an apparent greater influence of selenium than of vitamin E on the weight attained by the rats during seven weeks of feeding the special diets (see Table II).

Deaths resulting from dietary causes occurred mainly in the group of rats fed lard as the fat source. In a study of essential fatty acid deficiency, Kaunitz (31) compared the mortality of rats. During the first year, the mortality was about the same for rats fed coconut oil, lard, and corn oil, so the deaths were probably unrelated to essential fatty acid deficiency. Hafeman and Hoekstra (8) found deaths with hematuria and lung and liver necrosis 35-105 days after feeding a methionine-supplemented Torula yeast-based diet similar to the diet fed to the rats in this study.

Observations that pentane production is related to dietary linoleic acid, vitamin E, and selenium, via glutathione peroxidase, are of interest in themselves, but the implications of the wider applicability of the technique to areas involving lipid peroxidation is of even greater interest. Evidence for lipid peroxidation being involved in hepatic injury has been shown in rats following treatment with hydrazine (32), acute and chronic doses of ethanol (33), acute and chronic doses of carbon tetrachloride (9,34,35) and orotic acid (36), and during choline deficiency (37). The injury induced by these hepatotoxic agents, as well as lipid peroxidation in the livers is modifiable by antioxidants. Both lipid-soluble and water-soluble antioxidants modify ethanol- and carbon tetrachloride-induced injury, even though the steps in their metabolism to the toxic species is by a different route (38). In 1973, Di Luzio (38) stated that the lipid peroxidation concept had gained increased support, but that the validity of the concept was yet to be unequivocally established. Use of the breath analysis for pentane, which arises in vivo during lipid peroxidation, should prove useful to more firmly establish this process as being basic to many types of liver injury or damage caused by toxic substances.

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