Effect of Dietary Vitamin E on Expiration of Pentane and Ethane by the Rat

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ABSTRACT

An analytical method for the measurement of hydrocarbon gases in the breath of rats is described. The method was used to follow the expiration in rat breath of in vivo formed scission products of hydroperoxides. The major products are pentane from the linoleic acid family and ethane from the linolenic acid family. Rats were fed 0, 11 or 40 i.u. vitamin E acetate/kg diet for 7 wk starting at age 21 days. Data obtained by gas chromatographic analysis of breath samples were analyzed by the Mann-Whitney nonparametric *U*-test. This statistical analysis showed that pentane evolved by the group of rats not supplemented with vitamin E was significantly higher during the period 1-7 wk than that evolved by either of the two supplemented groups of rats. Ethane from the nonsupplemented group was significantly higher than that from the group supplemented with 40 i.u. vitamin E/kg of diet by 5 wk, and significantly higher than both supplemented groups by 6 wk. By 7 wk, pentane production was tenfold greater in the nonsupplemented group than in either supplemented group, and ethane was about twofold greater. There was no significant difference between the groups supplemented with 11 and 40 i.u. vitamin E/kg diet for either ethane or pentane. This new technique, which measures scission products from in vivo lipid peroxidation, promises to be useful for application to many experimental areas where lipid peroxidation is expected or known to occur.

INTRODUCTION

There are many reviews of the scientific evidence showing vitamin E to function as a lipid antioxidant (1-6). Some of the evidence is shown by higher levels of malonaldehyde in tissue of rats not supplemented with vitamin E than in tissue of supplemented rats (7); by the presence of fluorescent products of lipid peroxidation that arise in part by malonaldehyde reaction with other biological compounds (8); by muscle degeneration and dystrophy (9); and by red blood cell fragility in vitamin E-insufficient animals as measured by hemolysis in vitro (9). Plaa and Witschi (10) concluded in a recent review that the concept of lipid peroxidation is one of the important concepts of current experimental pathology and toxicology, in spite of the fact that some of the most convincing evidence for the role of lipid peroxidation in vivo is indirect and that many conclusions have been drawn by inference. Recently, Riely et al. (11) showed that ethane production was characteristic of spontaneously peroxidizing mouse tissue in vitro and that carbon tetrachloride provoked formation of ethane in vivo. Abstracts by Hafeman and Hoekstra (12,13) describe the measurement of ethane to show protection by vitamin E and selenium against lipid peroxidation in vivo. Ethane is known to arise from autoxidizing linolenic acid (14), and it is a major thermolysis product of autoxidized linolenic acid (15).

Based upon the report by Riely et al. (11), we projected that not only ethane but also the linoleic acid hydroperoxide product pentane should be a useful index of lipid peroxidation in vivo. This paper describes a study of the effect of dietary vitamin E on the production of the hydrocarbon gases ethane and pentane as measured in breath samples from rats by a new analytical technique.

MATERIALS AND METHODS

Animals and Diets

Specific pathogen-free male rats, descendants of the Sprague-Dawley strain, were obtained at 21 days of age from Hilltop Lab Animals, Inc., Scottdale, PA. The rats were housed two per cage in filter-top plastic hanging cages for the duration of the study. The animals were kept on a 12-hr light and 12-hr dark cycle.

The basal diet used was that described by Draper et al. (16). Essentially, the diet contained in percentage: casein, 20; dextrose, 65.9; vitamin E-stripped corn oil, 10; and mineral and vitamin mix, 4. This basal vitamin E-deficient diet was fed to eight rats; vitamin E acetate was added to the basal diet at 11 i.u./kg and at 40 i.u./kg, and these supplemented diets were fed to groups of six rats each. The powdered

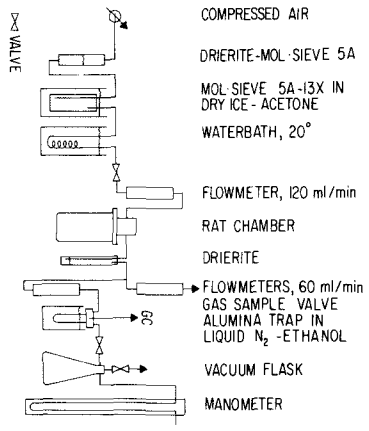

FIG. 1. Schematic diagram of breath sampling system.

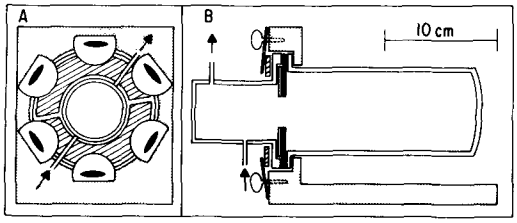

FIG. 2. Diagram of rat chamber drawn to scale. Shaded area is a plastic spacer placed between the wing nuts and head chamber; solid area is the rubber collar placed around the rat's neck; and stippled area is plastic holder for the chamber. (A) Front view; and (B) side view.

diet and tap water were given ad libitum. Food was removed from the cages 18-22 hr before breath samples were collected.

Gas Chromatography

A Varian-Aerograph model 1520 gas chromatograph with a flame ionization detector and fitted with a six-way gas sample valve was used for chromatographic analysis of hydrocarbon gases. A stainless steel column (1/8 in. X 5 ft) filled with activated alumina (80-100 mesh) was used with a nitrogen carrier gas flow rate of 25 ml/min. The detector temperature was 265 C and the injector temperature was 165 C. The column temperature was programmed as follows: 50 C for 1 min, followed by a 20 C rise/min for 7 min to a temperature of 190 C, which was held for 3 min and which was followed by a 20 C rise/min to 250 C. The total program was for 16 min. Between sample applications and after each day's analyses, the column and the alumina trap, to be described below, were held at 250 C with a nitrogen flow of 25 ml/min to remove any high molecular weight hydrocarbons.

Removal of Hydrocarbons from Air and Description of Animal Chamber

Figure 1 shows schematically the apparatus used to produce hydrocarbon scrubbed air and to collect the samples for injection into the gas chromatograph. Compressed air, at a tank pressure of 30 psi, was passed through a gas purifier (120 cc capacity) that was filled with indicating Drierite and molecular sieve 5A. Using 1/8 in. nylon or Teflon tubing and Swagelok fittings for all connections, the air was then passed through another gas purifier (stainless steel, 3.4 X 23 cm) that contained a mixture of molecular sieve 5A and 13X and that was immersed in a dry ice-acetone bath. After leaving the trap, the air passed through a coil of stainless steel tubing immersed in water at 20 C. The air then passed at a flow rate of 120 ml/min through a flowmeter, controlled by a valve, and into the stainless steel head portion of the animal chamber. The air stream, or air-breath stream when an animal was in the chamber, passed on the effluent side of the chamber through a tube that contained indicating Drierite (ca. 5 g) to remove water vapor, and then the air stream was split to obtain a flow rate of 60 ml/min through each of two flowmeters.

The body portion of the animal chamber, shown in Figure 2, was constructed of a glass cylinder that had a ground flanged opening. A rubber gasket with the appropriate size hole for the neck of the animal being tested was placed over the rat's head, followed by a rigid Teflon collar about 1.2 cm smaller in diameter and split on one side to allow its positioning around the rat's neck. It was determined that these two collars prevented the passage of any fecal gases, intestinal gases, or gases from the rest of the rat's body into the head chamber. The animal was placed in the glass chamber, and the stainless steel head chamber, with a glass viewing plate in the end, was placed over the head and held tightly against the rubber collar and the glass chamber flange with wing nuts screwed into a plastic holder. Two semicircular plastic spacers were placed between the head chamber and the wing nuts to apply sufficient pressure to allow complete sealing of the two chambers.

Collection of Breath Samples and Their Injection into the Gas Chromatograph

A length of nylon tubing was attached with Swagelok fittings to the inlet port of a six-way gas sample valve, mounted on the side of the gas chromatograph, and to the end of one of the two flowmeters on the effluent side of the rat chamber. The other flowmeter was always open to prevent a buildup of pressure in the chamber. Attached to the six-way gas sample valve in place of a standard gas sample loop was a loop of 1/8 in. stainless steel tubing, about 8 in. long, that contained activated alumina (80-100 mesh) in the lower 3 in. of the loop. During sample collection, this alumina trap was immersed in a very thick, icy slush of ethanol-liquid nitrogen. The trap was held immersed in the icy slush until the sample was injected into the gas chromatograph. The outlet port of the sample valve was attached to a 1-liter vacuum flask that had been evacuated with a small vacuum pump. The flow of air and breath into the alumina trap was controlled by opening a needle valve placed between the vacuum flask and the outlet side of the gas sample valve. The vacuum flask was attached to a calibrated mercury manometer. With a flow rate of 60 ml/min through the flowmeter and through the attached sample loop, 5 min were required to collect a 300-ml sample. Slight variations in flow rate through the flowmeter and through the attached sample loop were not a problem as 300-ml samples were always collected. After collection of the sample, the ethanol-liquid nitrogen bath was removed from around the sample loop trap, the nitrogen flow to the gas chromatograph was diverted through the trap via the sample valve, and a bath of hot tap water (70 C) was placed around the trap for the 3-min injection period. After 3 min, the nitrogen flow was again diverted from the sample loop trap directly into the chromatographic column.

Starting at 1 wk after initiation of the dietary regimen, each animal was placed in the holding chamber, and after 30 min, a 300-ml sample was collected. This sample represented one-half of the total breath sample in a 5-min interval. After chromatography, the relative peak areas of pentane and ethane were calculated by triangulation, and the picomoles of each gas in the 300-ml breath samples were calculated from standards injected via a 1 cc gas-tight syringe directly into the gas chromatograph. The standards, 0.8 ppm pentane and 1.5 ppm ethane in nitrogen, were obtained from Matheson Gas Products, Newark, CA. One milliliter portions of these standards were measured at an electrometer setting of 2 X 10^{-12} amps/mV. A 30-min time interval before collection of the breath sample was chosen to allow all the room air to be flushed from the tubing and from the head chamber and to accustom the rat to confinement in the chamber. After each day's use, the molecular sieve in the stainless steel trap used to clean hydrocarbons from the air was heated with a heating tape at about 350 C with a slow flow of nitrogen through the molecular sieve. Before animal breath samples were collected each day, a 300-ml sample of the background hydrocarbon-scrubbed air was collected and injected into the

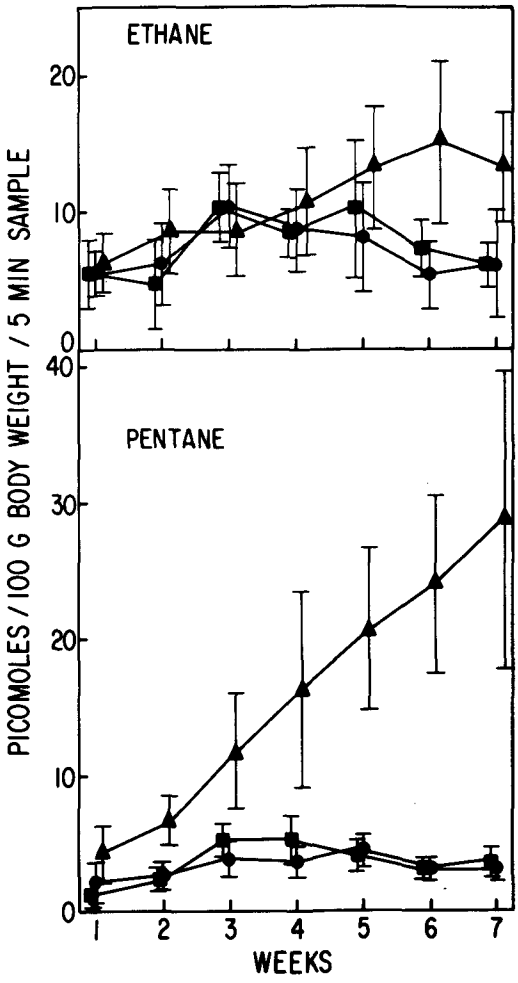

FIG. 3. (A) Ethane and (B) pentane/100 g body weight expired in one-half the total breath sample during a 5-min time interval beginning 30 min after placing a rat in the holding chamber. Diets fed for 7 wk beginning at 21 days of age contained (▲) 0, (■) 11, or (●) 40 i.u. vitamin E acetate/kg. The values plotted are the means ± S.D. for eight animals in the unsupplemented group and six animals in each of the vitamin E-supplemented groups.

TABLE I

Mann-Whitney *U*-Test Values[a] for Significant Differences Among Groups

Hydrocarbon gas	Groups compared[b]	Week of analysis[c]						
		1	2	3	4	5	6	7
Ethane	40 vs. 0	N.S.	N.S.	N.S.	N.S.	0.0294	0.0014	0.0046
	11 vs. 0	N.S.	0.042	N.S.	N.S.	N.S.	0.0046	0.0014
Pentane	40 vs. 0	0.0294	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006
	11 vs. 0	0.0026	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006

[a]$\alpha(=2P) = 0.05$. Comparisons were made of picomoles ethane and pentane/100 g body wt. The sample of breath represented one-half the total expired air over a 5 min period.

[b]The groups were: six rats fed 40 i.u. vitamin E/kg diet; six rats fed 11 i.u. vitamin E/kg diet; and eight rats fed a vitamin E-deficient diet. There were no significant differences between the 40 and 11 groups for ethane or pentane.

[c]Rats were fed their respective diets beginning at 21 days of age and breath samples were analyzed weekly for the following 7 wk.

gas chromatograph to assure that the air the rats were exposed to was free of hydrocarbon gases, thus assuring that all the hydrocarbon gases measured were from the breath of the rat.

RESULTS

Among the hydrocarbon gases that chromatographed from the alumina column during the program described, those that were identified were methane, ethane, ethylene, propane, butane, pentane, and hexane. Of these compounds, only ethane and especially pentane increased dramatically in breath samples in rats fed a vitamin E-deficient diet over a 7-wk period.

The mean values ± S.D. for picomoles of pentane and ethane/100 g body weight are shown in Figure 3. These values represent one-half of the total expired air over a 5-min time interval beginning 30 min after a rat was placed in the holding chamber. The greater than tenfold difference between pentane expired and twofold difference between ethane expired by vitamin E-supplemented rats and the nonsupplemented rats by 7 wk of feeding the diets is striking. There was individual animal variation in pentane and ethane expired, as shown by the standard deviations; however, the α values in Table I for data shown in Figure 3, where $\alpha = 2P$, obtained by the Mann-Whitney *U*-test (17,18), a nonparametric statistical test, show the significant effect that the absence of vitamin E in the diet had on increasing pentane and ethane in the expired breath. The variations in amount of pentane and ethane shown by the standard deviations in Figure 3 are variations among the animals and are not variations in the analytical system. Gas standards collected in the same manner, but with one of the flowmeters on the effluent side of the chamber closed, showed that 100% of the injected standards could be collected consistently. The individual animal variations are not unlike those seen for measurements of hemolysis of red blood cells as a function of dietary vitamin E (9) and the scatter seen for muscle fatty acids as a function of dietary vitamin E (4). Pentane was significantly higher ($2P<0.05$) in the nonsupplemented rats as early as 1 wk after the rats were fed their respective diets. Ethane evolution was significantly higher by the nonsupplemented rats after 5 wk than by the rats supplemented with 40 i.u. vitamin E/kg diet, and by 6 wk it was significantly higher than that of both supplemented groups. There was no significant difference in the amounts of either ethane or pentane between the two groups of rats consuming 40 and 11 i.u. vitamin E/kg diet.

DISCUSSION

This investigation of the use of the hydrocarbon gases pentane and ethane as indices of lipid peroxidation was instigated by the report of Riely et al. (11) that ethane production in vivo was related to treatment of mice with carbon tetrachloride, a rapid inducer of lipid peroxidation in the liver (19). Hafeman and Hoekstra (12,13) reported the measurement of ethane to show protection by vitamin E and selenium against lipid peroxidation in vivo.

A search of the literature for references to the measurement of hydrocarbon gases shows that many volatile compounds have been measured from peroxidizing systems, especially food systems. Pentane evolved from peroxides during their decomposition has been correlated with sensory tests for rancidity (20,21), and flavor studies on pentane formation in foods have shown that it is possible to correlate linoleate content and iodine values with the

induction period for pentane formation (22). Pentane has been measured as a decomposition product of 13-hydroperoxyoctadeca-9,11-dienoic acid, a lipoxidase oxidized product from linoleic acid (15,23,24). In dehydrated food systems, oxidation processes were measured by thermal release of hydrocarbons and the predicted hydrocarbons were found: ethane from linolenic acid; octane from oleic acid; pentane from linoleic acid; pentene and butane from myristoleic acid, and heptene and hexane from vaccenic acid (25). Ethane was shown by Lieberman and Mapson (14) to be produced in nonenzymatic model systems of linolenic acid hydroperoxide where the decomposition was catalyzed by iron, and that the same reaction carried out with a cupric-ascorbate system yielded mainly ethylene. Lieberman and Hochstein (26) also reported enzyme-catalyzed generation of ethylene in rat liver microsomes in the presence of cuprous ions. Horvat et al. (27) also observed hydrocarbon gases during the oxidation of methyl linoleate, and Salke et al. (28) showed that saturated hydrocarbons arose early during autoxidation of soybean oil. Our own unpublished studies have confirmed the iron-catalyzed decomposition of purified linolenic acid and linoleic acid hydroperoxide with the release of ethane and pentane, respectively.

Analytical methods to detect lipid peroxidation in vivo have been quite limited. Other than measurements of red cell hemolysis (9) as a test to establish the state of vitamin E nutrition, there has been no method described to follow the course of vitamin E insufficiency, especially during the early stages of dietary experiments or pro-oxidant stress. This paper has described a technique that offers promise for application to many situations where lipid peroxidation is thought to occur in vivo. This technique is based upon the gas solid chromatography of hydrocarbons on activated alumina. List et al. (29) showed that the technique readily separates C_1 to C_8 paraffins from α-olefins, and that more polar materials such as aldehydes, ketones, and esters are irreversibly adsorbed by the alumina.

The finding that pentane in the breath of rats fed a diet that contained vitamin E-stripped corn oil was significantly higher than in the breath of vitamin E-supplemented rats after only 1 wk is not surprising if one considers that corn oil contains 56-57.5% linoleic acid (9,30). The linoleic acid would be rapidly incorporated into membranes of the growing rat. For example, the total lipids of liver mitochondria from rats that consumed 160 mg of linoleic acid per day from a standard rat diet contained 23.4% linoleic acid, 17% arachidonic acid, and 6.2% docosahexaenoic acid (31). Vitamin E insufficiency would lead to the peroxidation of membrane lipids with the formation of hydroperoxides of the linoleic family, the ω-6-unsaturated fatty acids. In a similar manner, linolenic acid and docosahexaenoic acid, both ω-3-unsaturated fatty acids, would peroxidize to yield ethane as one product. Organic hydroperoxides are known to greatly increase the effectiveness of hemoproteins, cytochromes and hematin in catalyzing lipid peroxidation (32). The order of effectiveness in catalyzing lipid peroxidation was shown to be similar to their order of effectiveness in decomposing hydroperoxides (33). Heme compounds could catalyze the decomposition of hydroperoxides of the linoleic family in vivo to yield among other products, low levels of pentane.

The general route for formation of pentane and ethane from free radical products of hydroperoxide decomposition can be readily visualized. Considering the route to the formation of pentane, some of the ω-6-hydroperoxides can be decomposed by iron:

$$CH_3(CH_2)_4\underset{OOH}{C}\text{-R} + Fe^{++} \rightarrow$$

$$CH_3(CH_2)_4\underset{O\cdot}{C}\text{-R} + Fe^{+++} + OH^-$$

This alkoxy radical would readily undergo β-scission (34) with the production of $CH_3(CH_2)_3\dot{C}H_2$. This pentane radical would form pentane by hydrogen abstraction.

The protection afforded by dietary vitamin E, as shown by lower levels of ethane and pentane in vitamin E-supplemented rats, could be mediated by its chain breaking antioxidant action. The kinetics and mechanisms of chain scission during peroxidation and from hydroperoxide decomposition to yield hydrocarbon gases in vivo are not known. However, studies are underway to determine in vitro decomposition of purified linolenic and linoleic acid hydroperoxides to yield ethane and pentane, respectively.

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