Failure of the Vitamin E-Deficient Rat to Show Decreased Liver Codeine-O-Demethylase Activity (34578)

MARY L. QUAIFE AND LEONARD FRIEDMAN
(Introduced by E. L. Hove)

Division of Pharmacology and Toxicology and Division of Nutrition, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C. 20204

How nutritional status of man may affect his ability to metabolize a foreign compound (drug, pesticide) is of both scientific and practical nutritional interest. Increasing attention to the role of vitamin E in human nutrition led us to study the effect of its deficiency on the activity of a liver enzyme which metabolizes codeine to morphine, codeine-*O*-demethylase (CDM). Our findings on CDM activity in the E-deficient rat were negative. This was unexpected since Carpenter found pronounced loss in activity of two liver demethylating enzymes in such animal (1). We also found only a slight effect of E-deficiency on the hexobarbital sleeping time of rats.

Materials. Glucose-6-phosphate (G-6-P) dehydrogenase, 100 units/ml (per 0.34 mg of protein), was obtained from Sigma Chemical Co. and 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (DDT), 98.2% pure, from Geigy Chemical Corp. All other chemicals were of reagent, analytical or USP grade.

Rats and Diets. Male weanling rats were purchased from the Holtzman Co. One set was used for studies on homogenates and supernatants and another separately purchased set was used for microsome and hexobarbital tests. The diet fed from weaning until time of killing contained (as %): vitamin-free casein, 18; sucrose, 64; stripped lard (Eastman Kodak), 9; cod liver oil USP, 1; salt mix (2), 4; and vitamins (including A but not E) with cerelose (3), 4. Half the rats were fed this vitamin E-deficient (—E) diet; the other half received an (otherwise identical) vitamin E-supplemented (+E) diet which contained 0.02 % DL-α-tocopheryl acetate. Rats were caged separately and food and water were given *ad libitum*.

Assay of blood vitamin E. Blood tocopherol content was measured on the hexane portion of a 1:1 ethanol-*n*-hexane extract of plasma, either directly by a modified Emmerie–Engel assay (4) or by gas-liquid chromatography (GLC) (5) after its passage through a Celite-digitonin column (6) to remove interfering cholesterol. By the latter technique, suggested by Dr. A. Sheppard, we recovered quantitatively pure α-tocopherol (1 mg) from excess cholesterol; previously, we recovered microgram amounts from cholesterol digitonide precipitates (7).

Blood hemolysis tests. Extent of hemolysis of red blood cells by dialuric acid *in vitro* was measured according to the method of Friedman *et al.* (8).

Liver fractions. At a given time, equal numbers of —E and +E rats were decapitated and a portion of the bifurcated liver lobe of each was rapidly excised and homogenized separately as described (9). For some experiments, homogenates were centrifuged in a Sorvall centrifuge (Model RC2-B) at 15,000*g* for 30 min at 2° to give supernatants. On occasion, separated microsomes were prepared from the latter by centrifuging in a Beckman centrifuge (Model L2-65B) at 95,000*g* for 1 hr at 2°. The pellet was twice suspended in, and sedimented from, fresh homogenizing medium and finally resuspended in the latter at a concentration of microsomes equivalent to that in the original homogenate.

CDM assay. CDM activity was measured on liver homogenate or microsomal fraction derived from 0.25 g of liver (wet wt) as described by Gilbert and Golberg (9) with exceptions as noted here. A single incubation time, 15 min, was used. In morphine assay,

TABLE I. Liver Codeine-O-Demethylase (CDM) Activity, Body and Testis Weights, and Tooth Scores of Rats Fed Vitamin E-Deficient (—E) and Vitamin E-Supplemented (+E) Diets.

Vitamin E status	No. of rats	Range of time on diet (months)	Body wt[a] (g)	Wt of testes[a] (% body wt)	Tooth score[a,b]	CDM activity of liver preparation[a] (μmoles/hr /g of liver)	CDM activity of liver preparation[a] (mμmoles/min /mg of microsomal protein)
						Whole homogenates	
—E	11	2–9	331 ± 13	0.57 ± 0.06[c]	1.8 ± 0.2[c]	3.78 ± 0.28	—
+E	11	2–9	379 ± 19	0.90 ± 0.02	4.9 ± 0.1	4.13 ± 0.31	—
						15,000g supernatants[d]	
—E	4	9.5–10	334 ± 32[c]	0.55 ± 0.05[c]	1.7 ± 0.3[c]	2.37 ± 0.30	—
+E	4	9.5–10	444 ± 26	0.86 ± 0.04	4.8 ± 0.1	3.09 ± 0.24	—
						Microsomes[d]	
—E	4	5	341 ± 6[c]	0.48 ± 0.04[c]	1.0 ± 0.0[c]	2.83 ± 0.27	1.9 ± 0.2
+E	4	5	376 ± 10	0.93 ± 0.04	4.5 ± 0.3	3.32 ± 0.30	2.3 ± 0.2

[a] Values are mean ± SE.

[b] Scored arbitrarily on scale of 1 to 5: nearly white, 1; brownish-yellow, 5.

[c] $p < 0.05$.

[d] Rats were fasted overnight before being killed.

volumes of each color reagent were tripled to enable use of a Zeiss spectrophotometer (Model PM QII); this gave optical density at 650 mμ (with micro cells of 1-cm light path) of 0.261 for 100 μg of morphine–HCl in the incubation mixture. With separated microsomes only, concentration of nicotinamide adenine dinucleotide phosphate was 10 times that described (9), that of G-6-P, 4 times, and that of G-6-P dehydrogenase, twice. CDM assays were run in triplicate; maximum deviation from the mean was ±10 %.

Protein assay. Microsomal protein was determined with the biuret reagent (10).

Hexobarbital tests. Sodium hexobarbital in physiological saline was given ip to rats at a dosage of 100 mg/kg of body wt. The time between loss and recovery of the righting reflex was determined.

Trials of enzyme induction with DDT. In attempt to induce CDM, a +E and a —E test rat each received a daily ip injection of DDT dissolved in medium-chain-length triglycerides (MCT) at 50 mg/kg of body wt for 3 consecutive days. A corresponding control pair received MCT only. CDM in liver homogenates was measured on the fourth day.

Test for significance. Results are considered significant when $p \leq 0.05$ by two-tailed t test.

Results. That classic vitamin E deficiency was attained in these —E rats is shown by the degrees of testicular atrophy and incisor depigmentation (each significant at $p<0.01$) and by their lower body weights (Table I). (Liver weights did not differ significantly from those of the +E rats.) After 2 months on diet and before any enzyme or hexobarbital tests were made, —E rats showed complete (>90 %) red cell hemolysis and +E rats, none (<5 %). Blood tocopherol content at this time (measured only in the first set of rats) was 0.3–0.4 mg/100 ml in —E rats vs. 1.1.–1.4 mg/100 ml in +E animals, by Emmerie–Engel assay. At the end of the test series and after 10 months' feeding, —E and +E rats showed 0.0 and 0.6 mg/100 ml of blood tocopherol, respectively, as measured by GLC.

Table I shows that *in vitro* CDM activity of liver in —E rats, after 2 to 10 months on test diet, did not differ significantly from that of their +E counterparts, whether measured in homogenates, 15,000g supernatants, or separated microsomes. Nor was there any trend in degree of difference in CDM of

homogenates between —E and +E rats with length of time they were fed the diet.

Because (ethylenedinitrilo)tetraacetic acid, (EDTA) added *in vitro* restores impaired synthesis of ascorbic acid by liver microsomal preparations of —E rats (11), the effect of its omission on CDM activity in liver homogenates was tested. No significant effect was observed. For example, with EDTA present, activity was 3.51 ± 0.60 and 3.50 ± 0.36 μmoles/hr/g for 4 —E and 4 +E rats, respectively; without EDTA, activity was 3.75 ± 0.08 for 2 —E rats and 4.06 ± 0.10 for 2 +E rats. Therefore, these values are included in Table I.

In three replicate trials, we gave DDT on the same dosage schedule (except for solvent change) with which Hart and Fouts (12) induced several liver microsomal enzymes in the rat; however, we could not induce CDM in either —E or +E rats. Values (not tabulated separately) ranged from 3.86 to 6.31 μmoles/hr/g. Analysis of variance showed no significant effect due to DDT, vitamin E, "replication," or their various interactions. Therefore, these values are also included in Table I.

Length of sleeping time induced by hexobarbital, a test frequently used as an indicator for degree of activity of hepatic microsomal enzymes, was measured on —E and +E rats after 6.5 weeks and, again in the same animals, after 14.5 weeks on experimental diet. Results (Table II) show a significant increase for the —E rats only in the second trial. The increase, however, is only about 20 % in magnitude. Repeat trials with a third set of rats (values not listed here) gave similar results.

Discussion. Our values for CDM in rat liver homogenates agree well with those obtained with the same assay method by Gilbert and Goldberg (9). Possibly indicating unique status for CDM among liver microsomal enzymes is these authors' finding of only slightly reduced CDM activity in rats after prolonged dosage with 3, 5-di-*tert*-butyl-4-hydroxytoluene (BHT), in contrast to doubled or tripled activities of hexobarbital oxidase and *o*-nitroanisole and aminopyrine demethylases in response to this agent (9). We could not induce CDM with DDT; nor are we aware of any other attempts to do so.

TABLE II. Hexobarbital Sleeping Time of Male Rats Fed Vitamin E-Deficient (—E) or Vitamin E-Supplemented (+E) Diets.

Vitamin E status	No. of rats	Weeks on diet	Sleeping time mean ± SE (min)
—E	9[a]	6.5	23 ± 1.3
+E	12[b]	6.5	23 ± 1.4
—E	9[a]	14.5	34 ± 1.7[c]
+E	13[b]	14.5	28 ± 0.7

[a,b] Same rats, tested twice. (For unknown reasons, one +E rat failed to sleep in the first trial.)
[c] $p < 0.01$.

We found no significant decrease in liver CDM in homogenates, supernatants, or separated microsomes from —E rats; however, Carpenter found sharp decreases in both codeine-(*O* + *N*)- and aminopyrine demethylases in such animals (1). The findings could differ because *O*- and *N*-codeine demethylases are probably two different enzymes (13, 14) or because of some difference in age or strain of rat or in the experimental technique used. We cannot explain the difference at present.

Our findings that vitamin E deficiency does not significantly alter liver CDM activity and only slightly affects hexobarbital sleeping time would not support a concept of a general effect of vitamin E on an animal's capacity to metabolize foreign compounds.

Summary. Classic vitamin E deficiency in the male Holtzman rat over a span of 2 to 10 months on test diet resulted in no significant difference in activity of liver codeine-*O*-demethylase, whether measured on whole homogenates, 15,000*g* supernatants, or separated microsomes. It did significantly, but to relatively slight extent, increase hexobarbital sleeping time.

Appreciation is expressed to our colleagues, Dr. A. Sheppard and Dr. L. Weiss, for help with the gas chromatography of blood tocopherol and the determination of hexobarbital sleeping times, respectively.

1. Carpenter, M. P., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **26**, 475 (1967).

2. Jones, J. H., and Foster, C., J. Nutr. **24**, 245 (1942).

3. Seward, C. R., Vaughan, G., and Hove, E. L., Proc. Soc. Exp. Biol. Med. **121**, 850 (1966).

4. Emmerie, A., and Engel, C., Nature (London) **142**, 873 (1938).

5. Sheppard, A. J., Prosser, A. R., and Hubbard W. D., *in* "Methods in Enzymology" (D. B. McCormick and L. D. Wright, eds.), Vol. 18, Chap. on "Gas Chromatography of Vitamin E." Academic Press, New York (1970), in press.

6. Schwartz, D. P., Brewington, C. R., and Burgwald, L. H., J. Lipid Res. **8**, 54 (1967).

7. Bolliger, H. R., and Quaife, M. L., "Proc. 3rd International Congress on Vitamin E., Venice, 1955," p. 30. Valdonega, Verona (1956).

8. Friedman, L., Weiss, W., Wherry, F., and Kline, O. L., J. Nutr. **65**, 143 (1958).

9. Gilbert, G., and Golberg, L., Food Cosmet. Toxicol. **3**, 417 (1965).

10. Layne, E., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, p. 450. Academic Press, New York (1957).

11. Caputto, R., McCay, P. B., and Carpenter, M. P., J. Biol. Chem. **233**, 1025 (1958).

12. Hart, L. G., and Fouts, J. R., Proc. Soc. Exp. Biol. Med. **114**, 388 (1963).

13. Leadbeater, L., and Davies, D. R., Biochem. Pharmacol. **13**, 1607 (1964).

14. Takemori, A. E., and Mannering, G. J., J. Pharmacol. Exp. Ther. **123**, 171 (1958).

Received Sept. 19, 1969. P.S.E.B.M., 1970, Vol. 133.