Respiration and Lipid Peroxidation in Tocopherol Deficient Rat Hearts* (32764)

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Ever since Evans and Burr (1) first drew attention to a paralysis of young suckling rats with vitamin E-deficient mothers, the relationship of vitamin E deficiency and muscular dystrophy has been under investigation. The hypothesized pathophysiologic links between the two have included a well-defined role for Vitamin E as an antioxidant, a controversial effect of tocopherol deficiency to increase muscle oxygen consumption, and a possible role as a participant in oxidative phosphorylation (2). Since much of the work on oxygen consumption and lipid peroxidation has been derived from *in vitro* preparations such as homogenates and strips (2,3), we chose to study oxygen consumption and lipid peroxidation in a more intact muscle preparation, the isolated perfused rat heart. Vitamin E deficiency is known to cause a myocardial dystrophy (2), but to the authors' knowledge, isolated perfusion studies have only been performed on hearts from animals with a hereditary dystrophy not known to be related to vitamin E deficiency (4). Moreover, we dealt with an earlier phase of deficiency prior to the development of anatomical lesions. This system has the advantage of nutrient supply via a normal vascular system with all muscle cells intact. When adapted for studies of the contribution of "metabolic" factors to myocardial oxygen consumption, it has been found useful to eliminate inotropic and chronotropic contributions by 16 m*M* potassium arrest (5). In addition to the total oxygen consumption, some estimate of the portions related to phosphorylation and nonmitochondrial O_2 fixation can be made using inhibitors such as oligomycin (5) and cyanide.[3]

Materials and Methods. Male Wistar rats between 80–100 gm were placed on the tocopherol deficient or sufficient diet obtained in pellet form from the General Biochemical Company. This diet is identical in composition to that used by Mason and Harris (6). The control diet contains 60 mg of tocopherol acetate per pound. The animals were fed *ad libitum* and the food consumption by the two groups was similar. The animals were on the diet from 3 weeks to 3 months.

Hearts were removed after decapitation and an aortic retrograde perfusion was performed after the technique of Morgan *et al.* (7), as adopted for the measurement of qO_2 (5). Krebs–Ringer–bicarbonate buffer, containing 16 m*M* of potassium was equilibrated with 95% O_2–5% CO_2, 37°C, and perfused at a fixed coronary flow rate of 7.5 ml/min. Arteriovenous pO_2 differences were determined with a Clark microoxygen electrode and converted to qO_2 (ml O_2 per min per gm of dry wt.), using the appropriate O_2 solubility constant.

For determination of lipid peroxidation the modified thiobarbituric acid (TBA) test (8,9) was used as follows: in the intact heart, to 1 ml of perfusate was added 0.5 ml of 35% TCA and 1 ml of 0.75% TBA. The mixture was boiled for 15 min. The chromogen was extracted with an additional 1 ml of 35% TCA and the optical density of the supernate was measured at 530 mμ.

The *in vitro* lipid peroxidation in 10% homogenates of liver or heart in isotonic KCl was measured. A homogenate of each tissue, containing the equivalent to 10 mg of wet weight of tissue, was incubated in phosphate buffer (0.1 *M*, pH 7.35) in total volume of 1 ml in air for 1 hour, at 37°C. The TBA chromogen production was determined as

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TABLE I. TBA Chromogen Production in Liver and Heart Homogenates of Vitamin E-Deficient and Control Rats.[a]

Diet	Organ	TBA index[b]
Deficient	Liver	2.310
Deficient	Heart	1.040
Control	Liver	0.490
Control	Heart	0.300

[a] Three animals in each group. Incubation system: 0.1 *M* phosphate buffer pH 7.35; homogenate equivalent to 10 mg of tissue; final volume 1 ml.

[b] Absorbance at 530 mμ per 100 mg of tissue in 1 hour.

above after the reaction was stopped by the addition of 0.5 ml of 35% TCA to the incubation flask.

Results and Discussion. It is well known that rats on vitamin E-deficient diet develop biochemical lesions much earlier than the anatomical lesions. Such lesions have consisted of: (a) Inhibition of gulonolactone oxidase in liver microsomes (8); (b) Inhibition of steroid synthesis by the adrenal cortex (9); (c) inhibition of succinoxidase in liver mitochondria (10); and (d) Increased production of lipid peroxidation as tested by the thiobarbituric acid production in numerous organs during varied periods of vitamin deprivation (11, 12). The demonstration of high *in vitro* lipid peroxidation in the homogenates of heart and liver of the deficient rats in the present studies (Table I) implies the presence of such biochemical lesions as a result of vitamin E deprivation prior to development of gross anatomical lesions.

Preliminary results indicated no difference in the qO_2 of hearts from animals subjected to tocopherol deficiency for periods varying from 3 weeks to 3 months. Therefore, we analyzed all of the tocopherol-deficient qO_2 data as a single group (Fig. 1).

The oxygen consumption of potassium arrested hearts was identical whether from the control (0.155 ± 0.015) or tocopherol deficient (0.148 ± 0.010) rats. Further support for the lack of effect of tocopherol on heart qO_2 was found when no changes resulted from the addition of 1 mg of tocopherol to an albumin containing buffer perfusing vitamin E-deficient hearts. As previously discussed,

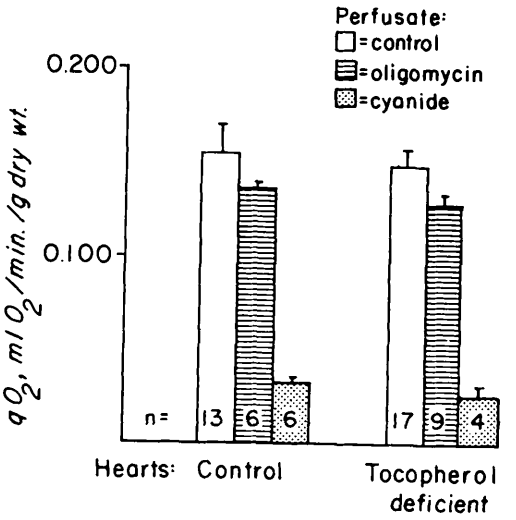

FIG. 1. Oxygen consumption in the isolated perfused hearts of tocopherol-deficient and control rats in the presence of oligomycin, cyanide or Krebs-Ringer-bicarbonate (control). The n indicates number of rats used in each experiment. Bars indicate standard error of the mean.

potassium arrest was chosen to study more directly that part of oxygen consumption related to basal or resting metabolic activities. However, to rule out the possibilities that inotropic or chronotropic activity may be necessary to demonstrate an effect of tocopherol deficiency, a group of three vitamin E-deficient hearts were studied with normal potassium concentration. The qO_2 was found to be 0.494 ± 0.025, a value comparable to that found in many previous control studies.[1] Thus, the total respiratory activity of intact myocardium was not found to be elevated in vitamin E-deficient animals.

In addition, measurement of TBA chromogen production in the perfusate of the two groups was found to be identical and of very small quantity in the beating and arrested control hearts (Table II). This is in contrast to the increased *in vitro* chromogen production of liver and heart in the deficient group (Table I).

To determine if equal O_2 consumption could be due to some defect in oxidative phosphorylation, control and vitamin E-deficient hearts were perfused with oligomycin, as previously described (5). Oligomycin is an antibiotic which inhibits oxidative phosphorylation in

TABLE II. TBA Chromogen Production in Perfusate of Isolated Heart Preparation of Vitamin E-Deficient and Control Rats.[a]

Diet	TBA chromogen[b]	
	Beating heart	Arrested heart
Deficient	.003	.001
Control	.001	.000

[a] Three animals in each group.

[b] Absorbance at 530 mμ in 1 ml of perfusate.

mitochondria; that part of respiration remaining is therefore not linked in an obligatory fashion to phosphorylation (13,14). The analogy of oligomycin effect in intact myocardium in comparison to those described for mitochondria has been established by the demonstration of a decrease in ATP (5), an inhibition of incorporation of ^{32}P into ATP (15), and an increase in DPNH fluorescence (15).

As shown in Fig. 1, a major portion of the resting oxygen consumption is not coupled, in an obligatory fashion, to phosphorylation. This respiration was similar both absolutely [(+E) 0.136±.004 vs (−E) 0.128±0.006] and relatively [(+E) 88% vs (−E) 86%] in tocopherol deficient and control hearts.

The final subdivision of total oxygen uptake to be considered was that possibly related to a role for vitamin E as an antioxidant (2). In this role, vitamin E might control the amount of oxygen utilized in lipid peroxidation (2) or microsomal DPNH or TPNH oxidation (16). Such oxygen fixation would not be blocked by cyanide, an inhibitor of mitochondrial cytochrome oxidase. Hearts were, therefore, perfused with 2.5 m*M* cyanide, a concentration previously found to be maximally effective.[3] Cyanide inhibited oxygen uptake to 0.027 ± 0.005 and 0.032 ± 0.003 for tocopherol-deficient and sufficient hearts, respectively (Fig. 1).

Thus, these studies demonstrate no effect of tocopherol deficiency on the total qO_2 or on those portions related to phosphorylation or nonmitochondrial oxygen fixation, nor demonstrate any significant difference in TBA chromogen production in heart perfusate of the two groups. These results in a relatively intact myocardial preparation stand in contrast to total oxygen consumptions and *in vitro* lipid peroxidation obtained previously in less intact *in vitro* tissue preparations. However, as reviewed by Vasington *et al.* (2), the previous studies have been inconsistent and fraught with technical difficulties and lack of statistical significance. The present studies suggest that the biochemical mechanisms for myocardial disease in vitamin E deficiency are to be sought in areas other than those reflected in the parameters of respiration measured here. An extrapolation of these conclusions to skeletal muscle can be made here only to the extent that such muscle can be considered analagous to myocardium.

Summary. Respiration in the perfused hearts of vitamin E-deficient and -sufficient rats was studied and compared in the control, oligomycin, and cyanide treated hearts. No effect of vitamin E deficiency was noted in the total qO_2, phosphorylated respiration or nonmitochondrial oxygen fixation. Studies of lipid peroxidation, as tested by the thiobarbituric acid (TBA) method, demonstrated high *in vitro* lipid peroxidation in the homogenates of hearts and livers of the vitamin E-deficient rats compared to the control. The TBA test in the heart perfusate of the two groups was the same.

1. Evans, H. M. and Burr. G. O., J. Biol. Chem. **76**, 273 (1928).

2. Vasington, F. D., Reichard, S. M., and Nason, A., in "Vitamins and Hormones," Vol. 18. Academic Press, New York, 1960.

3. Bird, J. W. C., Schottelius, D. D., and Shottelius, B. A., Metabolism **12**, 570 (1963).

4. Opie, L. H., Lochner, A., Brink, A. J., Homburger, F., and Nixon, C. W., Lancet **2**, 1213 (1964).

5. Challoner, D. R. and Steinberg, D., Am. J. Physiol. **211**, 897 (1966).

6. Mason, K. E. and Harris, P. L., Biol. Symp. **12**, 459 (1947).

7. Morgan, H. E., Henderson, M. J., Regen, D. M., and Parker, C. R., J. Biol. Chem. **236**, 253 (1961).

8. Kitabchi, A. E., McCay, P. B., Carpenter, M. P., Trucco, R. E., and Caputto, R. J., J. Biol. Chem. **235**, 1591 (1960).

9. Kitabchi, A. E., Nature **203**, 650 (1964).

10. McCay, P. B., Kitabchi, A. E., Caputto, R., and Trucco, R. E., Biochem. Biophys. Res. Comm. **4**, 469 (1961).

11. Bieri, J. G. and Anderson, A. A., Arch. Biochem. Biophys. **90**, 105 (1960).

12. Carpenter, M. P., Kitabchi, A. E., McCay,

P. B., and Caputto, R., J. Biol. Chem. 234, 2814 (1959).
13. Huijing, F. and Slater, E. C., J. Biochem. Tokyo 49, 493 (1961).
14. Lardy, H. A., Johnson, D., and McMurray, W. C., Biochemistry, 3, 1961 (1964).
15. Challoner, D. R., Am. J. Physiol, in press.
16. Siekevitz, P., Federation Proc. 24, 1153 (1965).

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Endotoxin Tolerance Induced by Detoxified Endotoxin (Endotoxoid) (32765)

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Beeson (1) first described the induction of endotoxin tolerance by pretreatment with extremely low doses of endotoxin. Since then several workers have produced tolerance towards endotoxin by repeated injections of toxic preparations given over several days. However the toxicity of these substances precludes the clinical application of these findings.

Using detoxified endotoxins (endotoxoid), Freedman *et al.* (3) have induced in animals resistance to infection. Further, Noll and Braude (7) have succeeded in inducing pyrogen tolerance in rabbits, and in increasing the resistance of mice to lethal challenge doses of endotoxins, by immunizing the animals with detoxified endotoxins.

Chemically detoxified endotoxins of the type used in the experiments to be described were obtained from *Serratia marcescens* bovine-type endotoxin by Nowotny (8). These endotoxoids were almost nontoxic while retaining several useful properties of the parent endotoxin (4,9).

The question now raised is, whether protection against the lethal action of endotoxins in general can be induced by pretreatment with this endotoxoid in particular. The present paper describes the induction of endotoxin tolerance in guinea pigs (Pirbright) by treatment with endotoxoid-2 (endotoxin partially deacylated with potassium methylate).

Experiments. Section A. Ninety guinea pigs received by the intravenous route 150 μg/100 gm of body weight of endotoxoid-2. Six animals died within 1 day. Twenty-four hours after injection the 84 survivors were divided into three groups (see Table I) each group being then injected with toxic endotoxin from either *E. coli 0111, Proteus vulgaris,* or *Brucella melitensis.* The three control groups of guinea pigs received intravenously only the corresponding toxic endotoxin. The endotoxins were prepared by the trichloroacetic acid method (2) as described by Urbaschek (11).

Section B. The Ouchterlony (10) gel diffusion method, the very sensitive passive hemagglutination technique (6) and the complement fixation technique of Kolmer (5) were employed to study the serological status of the following groups of animals: (i) Ten guinea pigs were injected intravenously with endotoxoid 150 μg/100 gm body weight. These guinea pigs were bled twenty four hours after the endotoxoid injection. (ii) Ten guinea pigs received the same endotoxoid dose and 24 hours later were challenged with a lethal dose of *E. coli* endotoxin. Twenty-four hours after the challenge the animals were bled. 3. Ten guinea pigs and four rabbits were immunized with toxic *E. coli* endotoxin. The immunized animals were bled on the seventh day after the last injection. As antigen *E. coli* endotoxin and endotoxoid were used.

Results. In Section A experiments, endotoxin from *E. coli, P. vulgaris,* and *B. melitensis* caused a high mortality, when injected