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# ANOMALOUS VITAMIN E EFFECTS IN MITOCHONDRIAL OXIDATIVE METABOLISM

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## SUMMARY

Three different vitamin E effects, suggestive of specific antioxidant effects, were discovered in the protective action of vitamin E against respiratory decline (a decrease in mitochondrial respiration attributed to a "leakage" of electron transport radicals). No correlation was found between respiratory decline and random lipid peroxidation. The mechanisms behind two of the three atypical vitamin E effects were defined. Both involve an artifact in the TBA assay for lipid peroxidation. This artifact occurs when TBA assays are carried out in the presence of sucrose and acetaldehyde; the latter is produced from ethanol, the solvent used to add vitamin E to preparations. The artifact in the TBA assay for peroxidations appears also to be responsible for differing interpretations of the hepatotoxic effect of ethanol.

## INTRODUCTION

A number of different functions, apparently unrelated to its antioxidant properties, have been proposed for vitamin E [1]. These include regulatory effects in the development of rotifers [2], an involvement in the syntheses of heme and creatinine phosphokinase [3, 4], a role in microsomal electron transport [5] and membrane stabilization [6], and control of mitochondrial electron transport by an influence on compartmentalization of NAD [7].

The suggestion of a vitamin E effect on compartmentalization of NAD derives from studies of respiratory decline, a phenomenon which occurs in vitamin E deficient liver homogenates. It consists of an NAD-induced decrease in normal mitochondrial respiration

in the presence of succinate and other citric acid cycle intermediates [7, 8]. Such respiratory decline can be prevented in vitamin E deficient liver homogenates if α-tocopherol is added. But the vitamin under these conditions appears to have no significant effect on random lipid peroxidation [9]. This atypical vitamin E effect has been interpreted by some to signify roles other than antioxidant function [10]. An alternate explanation for this unusual protective action is that the vitamin can exert both general as well as specific antioxidant effects, the latter being the dominant effect at low vitamin concentrations as a result of specific binding interactions.

Our hypothesis of a specific antioxidant effect is largely based on a second atypical vitamin E effect which occurs when isolated mitochondria are used instead of liver homogenates [7]. With isolated mitochondria, it was no longer possible to prevent respiratory decline solely by addition of vitamin E; this was possible only if cytosol was also included in the medium. Cytosol itself has no protective effect. It appears, therefore, as if vitamin E can prevent respiratory decline only after being bound to certain cytosol proteins. This is consistent with the recent discovery of vitamin E-specific binding proteins in cytosol by which the incorporation of the vitamin into mitochondrial membranes is facilitated [11]. In principle, at least, such an interaction can give rise to specifically localized antioxidant effects.

Corwin's observation (that small limiting quantities of vitamin E are able to prevent respiratory decline without affecting lipid peroxidation) may also be explained in terms of specific vitamin E binding to key loci, thereby protecting these but not affecting random lipid peroxidations appreciably.

Earlier work [7] suggests that such a specific vitamin E effect could be directed against sites in the mitochondrial electron transport chain at which changes (induced by NAD) in membrane configuration produce "leaks" of electron transport radicals. Such a leakage of radicals has also been postulated in aging processes [12], and may be involved in the production of $H_2O_2$ during mitochondrial respiration [13]. Other areas of normal metabolism in which specific antioxidant effects could play a regulatory role are those involving such unstable intermediates as superoxides (*e.g.* microsomes, macrophages [14, 15]), singlet oxygen and endo- or hydroperoxide intermediates (*e.g.* prostaglandin synthesis [16, 17]).

The two anomalous vitamin E effects have been further investigated in the present report in the hope of generating direct evidence for the existence of specific antioxidant effects.

## MATERIALS AND METHODS

### *Animals and diets*

Fisher 344 male weanling rats, weighing 25 to 35 grams, were purchased from Charles River Laboratories (Wilmington, Massachusetts). The animals were given tap water and fed *ad libitum* the vitamin E and selenium-deficient Torula yeast diet described below. After 4 weeks on the deficient diet, the rats were supplemented with 0.02 mg%

selenium as sodium selenite. In cases where the rats were given vitamin E at this time, 10 mg% α-tocopherol acetate (Sigma) was added to the diet in addition to the selenium. The deficient Torula yeast diet consisted of the following: 30% Torula yeast (St. Regis, Rhinelander, Wisconsin), 5% Eastman stripped lard, 59% sucrose, 5% high mol. wt. salts (ICN Nutritional Biochemicals Co.), 0.5 ml% of a solution of 0.20 g vitamin A acetate (crystalline, Sigma) and 8000 units vitamin D (Drisdol band) in 100 ml ethanol, and the following vitamins (obtained from Nutritional Biochemicals Co.) per 100 grams of diet: 0.4 mg thiamine–HCl, 0.25 mg riboflavin, 0.20 mg DL-calcium pantothenate, 10 mg niacin, 0.10 mg menadione, 0.20 mg folic acid, 0.10 mg D-biotin, 10 mg of a 0.1% trituration of cyanocobalamin, 212 mg choline dihydrogen citrate, and 765 mg lactose.

*Subcellular fractions*

Except where indicated otherwise, rat liver mitochondria were prepared from rats fed the vitamin E deficient diet. Finely minced liver was homogenized at 0 °C with a Potter–Elvehjem homogenizer (0.004–0.006 in. clearance) in the presence of 0.25 *M* sucrose (Sigma, Grade I) containing 0.01 *M* Tris buffer, pH 7.6. The 10% liver suspension was centrifuged for 12 min at 4 °C and 700 × *g* to remove the nuclear fraction, followed by 15 min centrifugation at 6000 × *g* to obtain the mitochondrial pellet. Mitochondria were washed twice by resuspending in 0.25 *M* sucrose and re-centrifuging at 6000 × *g*. The mitochondria were diluted in 0.25 *M* sucrose so that 0.1 ml of the suspension in 2.9 ml of 0.25 *M* sucrose gave an absorbance value of 0.50 at 600 nm (1 cm lightpath at the standard distance from the phototube of the Gilford spectrophotometer). The contribution to light scattering from glycogen was established by repeating the measurement in 0.2 *N* NaOH and subtracting this reading from the value obtained with the sucrose suspension. Light scatter measurements were found to be a more accurate measure of mitochondrial concentration than protein analysis, since the latter fluctuated considerably as a result of variable amounts of protein removed by washing procedures. This method for mitochondrial quantitation was confirmed by measurements of succinic dehydrogenase activity as described by King [20]. Another important aspect in the preparation of mitochondria is that EDTA (ethylene diamine tetraacetic acid) was not included in the isolation medium, since this prevents respiratory decline; EDTA also causes variability in the quantitation of mitochondria by the light scatter method. Deletion of EDTA had no effect on our experimental results, as shown by experiments in which EDTA (0.2 m*M*) was included in the isolation medium but subsequently removed from the mitochondrial pellet by washing with 0.25 *M* sucrose. Identical results were obtained with both types of mitochondria, provided, of course, that succinic dehydrogenase activity was used as a measure of mitochondrial concentration.

Exogenous vitamin E was added to mitochondria by adding, with rapid stirring, 0.03 ml of an ethanolic solution of DL-α-tocopherol (0.25 g/10 ml) to 3.0 ml of the final mitochondrial suspension (0.5 o.d. at 600 nm per 0.1 ml).

Two types of cytosol were used as vitamin cofactor in the protection against respiratory decline. Both were prepared by centrifuging the mitochondrial supernatant at 100,000 × *g* for 1 h at 4 °C to remove microsomes. The upper portion of the centrifuged

cytosol, containing suspended lipid droplets, was discarded, and the remainder was used in experiments in which native cytosol was employed. Denatured cytosol proteins were prepared by heating cytosol for 5 min at 100 °C and spinning the denatured protein at 35,000 × *g* for 30 min. The pellet, freed from prooxidants such as ascorbic acid, was resuspended in water or isotonic KCl to the original volume of the cytosol.

*Enzyme assays*

Succinic oxidase activity in isolated mitochondria was measured with a Warburg respirometer in the following medium: 90 μmol $PO_4$ (added as sodium phosphate buffer, pH 7.6), 15 μmol $MgCl_2$, 3 μmol ADP, 3 μmol ATP, 0.1 mg cytochrome *C*, 60 μmol sodium succinate, and 0.1 *M* Tris buffer to bring to a final volume of 3.5 ml, including the volumes of 0.5 ml of mitochondria, and 0.2 ml of cytosol. In order to initiate respiratory decline, 6 μmol of NAD were added to the reaction medium. All solutions were prepared in triple-distilled water; respirometer vessels were cleaned in sulfuric acid–dichromate cleaning solution, followed by washing in hot nitric and sulfuric acid, treating with EDTA solution and repeated rinsing with triple-distilled water. Mitochondria and cytosol were added to the sidearm of the reaction vessel, mixed by gentle agitation of the vessel, and tipped into the reaction medium after approximately 10–12 min of temperature equilibration (reaction temperature of 37 °C). To prevent coagulation of mitochondria, Warburg vessels were shaken at a slow rate of 60 cycles/min. When acetaldehyde determinations were performed on the reaction mixture, KOH-moistened filter paper was deleted from the center well of the respirometer vessel, since volatile acetaldehyde is partly lost by condensation on the KOH paper.

The alcohol dehydrogenase activity present in the native cytosol was measured in a reaction medium identical to that used in the Warburg respirometer. The reactions were performed at 37 °C with 87 μmol ethanol, and 6 μmol of NAD. Enzyme activity was calculated from the rate of NADH formation measured at 340 nm with a DW-2 Aminco spectrophotometer.

In the experiment to test for effects of different impurities in NAD, NAD was obtained from Sigma and Calbiochem. Sigma grades III (98%), AA (90–96%) and V (99 + %) were compared.

*Determination of true and false lipid peroxidation*

The studies outlined in the present paper (see Results and Discussion) have shown that acetaldehyde accumulates in our reaction system. When reaction mixtures containing acetaldehyde and sucrose are reacted with thiobarbituric acid (TBA), it produces a chromogen with a 532 mm absorption maximum which is indistinguishable from that formed by the products of lipid peroxidation (*e.g.* malonaldehyde). It was necessary, therefore, to devise analytical procedures by which true lipid peroxidation values could be distinguished from false ones. Since acetaldehyde produces false lipid peroxidation values only when sucrose is present, it was simply a matter of deleting sucrose from the reaction mixture in order to prevent this interference; *i.e.* mitochondria were resuspended in isotonic KCl instead of in isotonic sucrose. The TBA assay was then performed in the

conventional way as follows. The sucrose-free sample of the mitochondrial reaction mixture was heated at 100 °C for 15 min with 0.8 ml of 20% trichloroacetic acid and 1.2 ml 0.5% TBA in a final volume of 3.0 ml. Malonaldehyde standards were prepared by the 0.1 *N* HCl hydrolysis of malonaldehyde bis (dimethyl acetal).

However, in some of our experiments, it was necessary to measure both true as well as false lipid peroxidation. In that case, two different TBA assays were performed: One assay, measuring true lipid peroxidation, was carried out in the absence of sucrose by the method described above; the second assay, measuring both true and false peroxidation, was performed in the same manner except that sucrose (100 μmol) was added to the reaction mixture. By subtracting the first result from the second one, one obtains the value for false lipid peroxidation. A linear relationship was found between acetaldehyde concentration and 532 mm absorption due to false lipid peroxidation, provided that non-limiting quantities of sucrose were used in the assay. We found that sucrose, present in relatively high concentrations, rather than acetaldehyde, was the limiting factor in the TBA assay for false lipid peroxidation. This is due to the fact that it is a minor hydrolysis product of sucrose, and not sucrose itself, which reacts with acetaldehyde and TBA to form the 532 mm chromogen; 100 μmol of sucrose were found to be sufficient to give a linear response in the TBA assay for acetaldehyde up to 2.2 absorbance units. For reasons stated in the text, both lipid peroxidation and acetaldehyde levels were expressed in absorbance units. Under our assay condition, a 1 ml sample of reaction mixture containing 3.5 μmol of acetaldehyde was found to give an absorbance of 1.0. This factor can, therefore, be used to convert our tabulated absorbance values of false lipid peroxidation into molar units of acetaldehyde.

## RESULTS

### *Cytosol – cofactor for vitamin E function*

Typical respiration kinetics (minus endogenous reactions) of isolated vitamin E-deficient mitochondria, with succinate as substrate, are shown in Fig. 1. The effects of NAD, α-tocopherol, and denatured cytosol are also illustrated. The results show clearly that respiratory decline can only be prevented by the combined action of α-tocopherol and cytosol, with no such prevention occurring with either α-tocopherol or cytosol alone. Additional experiments (not shown) indicate that albumin was not effective when substituted for cytosol, and that native and heat-denatured cytosol were equally effective in preventing respiratory decline in the presence of vitamin E. We also found that respiratory decline experiments are best performed with denatured cytosol, since it produces negligible endogenous reactions. In contrast to this, the endogenous reaction with native cytosol is much larger and may, under certain circumstances, be equal to the main reaction with succinate, thereby complicating mechanistic interpretations (see below).

### *Mitochondrial swelling*

Since lipid peroxidation is known to cause mitochondrial swelling, we investigated whether swelling was correlated with the incidence of respiratory decline. Figure 2 shows

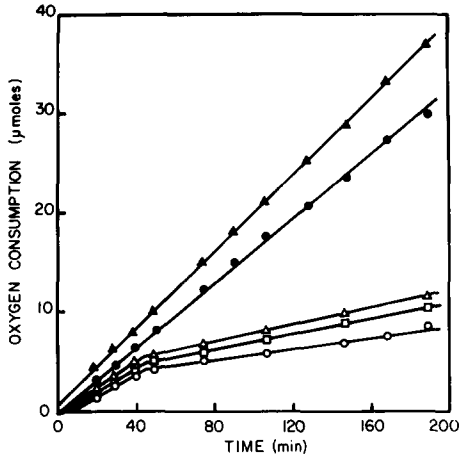


Fig. 1. Typical respiratory decline kinetics for isolated mitochondria (endogenous reactions subtracted) performed with a Warburg respirometer. Method and reaction media are described in experimental procedures. The following reaction systems are shown: □, mitochondria + ethanol + NAD; △, mitochondria + ethanol + NAD + cytosol; ○, mitochondria + NAD + ethanol + α-tocopherol; ●, mitochondria + ethanol; ▲, mitochondria + NAD + cytosol + ethanol + α-tocopherol.

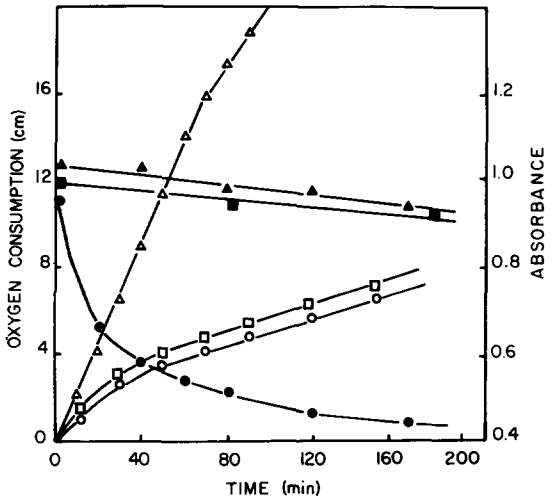


Fig. 2. Relationship between respiratory activity of isolated mitochondria in the presence of NAD (open symbols) and mitochondrial swelling (absorbance values, solid symbols) under the following reaction conditions: respiratory decline caused by the absence of vitamin E and cytosol (○, ●); respiratory decline prevented by the presence of vitamin E and cytosol (△, ▲); respiratory decline occurring in the presence of vitamin E in the absence of cytosol (□, ■).

TABLE I

RELATIONSHIP BETWEEN LIPID PEROXIDATION AND SUCCINIC OXIDASE RESPIRATORY DECLINE

Lipid peroxidation was measured by the TBA assay following 3 h incubation in a Warburg respirometer. Reaction medium and conditions are described in experimental procedures. Results are the averages of three experiments.

| *Expt.* | *Type of cytosol* | *Reaction system*[a] | *TBA assay (532 nm absorbance)* | *Occurrence of respiratory decline* |
|---|---|---|---|---|
| 1 | None | M | 0.03 | – |
| 2 | | M + EtOH | 0.04 | – |
| 3 | | M + NAD | 0.43 | + |
| 4 | | M + NAD + EtOH | 0.46 | + |
| 5 | | M + NAD + EtOH + E | 0.03 | + |
| 6 | Native | M + C | 0.31 | – |
| 7 | | M + C + EtOH | 1.60 | – |
| 8 | | M + C + NAD + EtOH | 2.00 | + |
| 9 | | M + C + EtOH + E | 1.44 | – |
| 10 | | M + C + NAD + EtOH + E | 0.32 | – |
| 11 | Heat | M + C | 0.50 | – |
| 12 | denatured | M + C + EtOH | 0.50 | – |
| 13 | | M + C + NAD + EtOH | 0.88 | + |
| 14 | | M + C + EtOH + E | 0.03 | – |
| 15 | | M + C + NAD + EtOH + E | 0.11 | – |

[a] M, mitochondria; C, cytosol; EtOH, ethanol; E, α-tocopherol.

that drastic ("large amplitude") mitochondrial swelling coincides closely with the onset of respiratory decline. However, since "large amplitude" swelling was prevented by vitamin E alone, *i.e.* without the use of cytosol, it appears that mitochondrial swelling is not directly involved in the decline mechanism.

*Specific and non-specific antioxidant effects*

The possible role of specific lipid peroxidation in the mechanism of respiratory decline was investigated by performing TBA assays at the conclusion of the respiratory-decline experiments outlined in Table I. The following criteria were used to distinguish between random and specific peroxidation processes, and random and specific antioxidant effects: peroxidation, which was inhibited by vitamin E alone, was attributed to random peroxidation processes; the antioxidant effect was, therefore, considered to be non-specific. Any small amounts of peroxidation remaining after vitamin E treatment were assigned to specific peroxidation, provided that these could be inhibited only by the combined action of vitamin E and cytosol, *i.e.*, by the postulated action of vitamin E binding-proteins.

The above experiments were performed both with native and with heat-denatured cytosol in view of their different influences on the endogenous respiration reaction. We

also studied the effect of ethanol – the solvent used conventionally for addition of vitamin E – since this has also been implicated as a possible source of lipid peroxidation [19, 20].

The results in Table I show that random lipid peroxidation is unrelated to the incidence of respiratory decline. This point is well illustrated by experiments 5 and 8, where respiratory decline occurs both at near-zero and at high TBA values, respectively.

Another feature of the data is that TBA values are clearly influenced by the type of cytosol used in these experiments (experiments 7–9 *vs.* 12–14). The reasons for the high TBA values in the case of native cytosol will be discussed in detail below, since they are considerably more complicated than the mere difference in the content of pro-oxidants such as ascorbic acid. For the present, it is sufficient to note that the high TBA values in experiment 9 preclude native cytosol from being used in experiments testing for possible specific antioxidant effects of vitamin E. Consequently, we were able to evaluate this possibility only in experiments in which denatured cytosol was used.

With respect to this latter objective, we see from experiments 1 and 3 that addition of NAD causes respiratory decline with an associated increase in TBA value from 0.03 to 0.43. Addition of vitamin E (experiment 5) depresses this value once more to 0.03 without, however, preventing respiratory decline. When denatured cytosol is now added along with α-tocopherol (experiment 15), respiratory decline is inhibited. However, this is not accompanied by the expected decrease in lipid peroxidation. Instead, a small but significant increase in TBA values, from 0.03 to 0.11, was observed. We attribute this unexpected increase in random lipid peroxidation to an interaction between NAD and a component of the cytosol, since no increase in TBA values was obtained when either one of these agents was added in isolation (experiments 5, 14 and 15). Unfortunately, this increase interferes with our test for specific antioxidant effects, since we cannot now establish whether the small amount of (specific?) residual peroxidation, associated with respiratory decline, was reduced when cytosol was included as co-protective agent. Therefore, the only conclusion that we can reach at this point is that respiratory decline is not caused by random lipid peroxidation reactions.

The other interesting feature of the data in Table I is the aforementioned difference between native and heat-denatured cytosol. This is exemplified by experiments 6 and 7, where addition of ethanol in the presence of native cytosol produces a drastic increase in TBA values. In contrast to this, TBA values do not increase significantly when heated cytosol is used in place of native cytosol (experiments 11 and 12). Surprisingly, this high TBA level, produced by the combined action of alcohol and native cytosol, cannot be decreased to any significant extent by vitamin E (experiments 7 and 9). A decrease occurs only through the combined action of vitamin E and NAD (experiment 10). This result, *i.e.* the apparent cofactor function of NAD in a vitamin E protective effect against what apparently is an alcohol-induced lipid peroxidation, is somewhat paradoxical in view of the obvious pro-oxidant properties of NAD (experiments 1 and 3; 2 and 4; 7 and 8; 12 and 13). The nature of this unexpected (third) anomalous vitamin E effect, and its relationship to the vitamin E effect occurring in homogenate systems [9], was further investigated in the experiments described below.

*False lipid peroxidation*

The fact that ethanol and unheated cytosol are required for the production of high TBA values, along with the failure of vitamin E to affect this increase, suggested to us that the TBA assay may not have detected exclusively lipid peroxidation under our reaction conditions, but that TBA may have reacted with some other substance present in our reaction mixture. One such possibility is a reaction with acetaldehyde, the latter being formed from ethanol, *i.e.*, from the solvent used for addition of vitamin E.

We investigated this possibility by reacting acetaldehyde with TBA reagent. In agreement with previous reports [21], only a weakly absorbing chromogen was obtained with an absorption maximum at 498 nm. A totally different result was, however, obtained when the TBA assay was performed on a mitochondrial reaction mixture which had been spiked with acetaldehyde. Under these conditions, TBA was found to react with acetaldehyde to form a new, and fifty-fold more intensely absorbing chromogen which, by absorbing maximally at 532 nm, was indistinguishable from that formed by lipid peroxidation products (Fig. 3).

From other experiments, which have been described in a preliminary communication [22], it was established that this unexpected interference in the TBA assay for lipid peroxidation required, in addition to acetaldehyde, also the presence of sucrose. The latter is a necessary component of the mitochondrial isolation medium. At the elevated

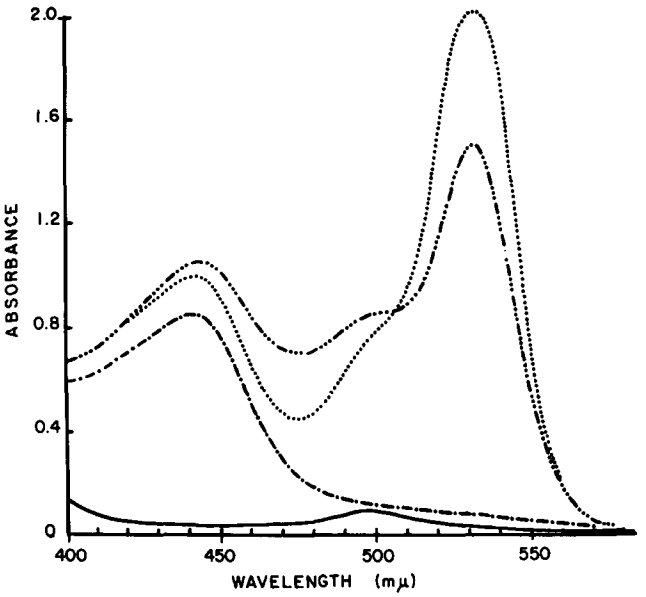


Fig. 3. Absorption spectra of products formed by the reactions between thiobarbituric acid (TBA) reagent, trichloroacetic acid, and the following additives: ———, acetaldehyde (4 μmol); –. –, sucrose (90 μmol); –···–, sucrose (90 μmol) and acetaldehyde (4 μmol); ....., sucrose (90 μmol) and malonaldehyde (0.06 μmol).

temperatures and acidic conditions of the TBA assay, we found sucrose to undergo pyrolysis with the formation of reaction products which, in the presence of acetaldehyde and TBA reagent, form the 532 nm chromogen. It appears, therefore, that the third anomalous vitamin E effect requiring the presence of NAD is in fact due to an influence of vitamin E and NAD on acetaldehyde levels, and not due to an effect on lipid peroxidation. The interference with the TBA assay for lipid peroxidation appears to be specific for acetaldehyde, since it did not occur with a variety of other physiological aldehydes [22].

The interference by acetaldehyde in the TBA assay for lipid peroxidation suggests that it could also have affected the conclusions of several other studies. One possibility is the anomalous vitamin E effect in homogenate systems [9], upon which the concept for specific antioxidant effect is partly based. False lipid peroxidation is also likely to have played a role in the controversy dealing with the involvement of lipid peroxidation in the hepatotoxic mechanism of ethanol [19, 20].

*Effect of cytosol and mitochondria on true and false lipid peroxidation*

In order to clarify the above questions, we investigated the conditions leading to the accumulation of acetaldehyde. In particular, we investigated how this accumulation is prevented by the combined action of vitamin E and NAD, and how acetaldehyde levels are affected by the presence of cytosol, mitochondria, and microsomes, *i.e.*, by the individual components of the homogenate system. To evaluate the role of false lipid peroxidation, we used the TBA assay both for the determination of acetaldehyde levels and lipid peroxidation. The modified TBA assay was used to differentiate between true and false lipid peroxidation (see method section).

Table II shows the effects of cytosol and mitochondria on lipid peroxidation and acetaldehyde accumulation. Little true lipid peroxidation took place, either in the

TABLE II

EFFECT OF CYTOSOL AND MITOCHONDRIA ON FALSE LIPID PEROXIDATION VALUES

TBA values due to acetaldehyde accumulation and lipid peroxidation in indicated reaction systems after 3 h incubation in Warburg respirometer. Reaction conditions and medium are the same as those used in respiratory decline experiments. Results are the averages of 3 experiments.

| *Expt.* | *Reaction system*[a] | *TBA assay (532 nm absorbance)* | |
|---|---|---|---|
| | | *Lipid peroxidation* | *Acetaldehyde* |
| 1 | C + EtOH | 0.02 | 0.03 |
| 2 | C + EtOH + NAD | 0.02 | 0.31 |
| 3 | C + EtOH + E | 0.00 | 0.07 |
| 4 | C + EtOH + E + NAD | 0.02 | 0.32 |
| 5 | C + EtOH + M | 0.07 | 0.91 |
| 6 | C + EtOH + M + NAD | 0.80 | 0.90 |
| 7 | C + EtOH + M + E | 0.02 | 0.83 |
| 8 | C + EtOH + M + NAD + E | 0.04 | 0.20 |

[a] M, mitochondria; C, cytosol; E, α-tocopherol; EtOH, ethanol.

absence or presence of NAD, when only cytosol was present. Also, acetaldehyde levels were found to be generally low (experiments 1–4). When both mitochondria and cytosol were added to the reaction medium, acetaldehyde levels increased significantly, but not genuine lipid peroxidation (experiment 5). Experiment 6 shows that NAD causes an increase in lipid peroxidation in the presence of mitochondria, but that this has no effect on false lipid peroxidation. The remaining experiments (experiments 7 and 8) demonstrate that random lipid peroxidation is decreased by vitamin E alone, but that a decrease in false lipid peroxidation can only be affected by the combined action of NAD and vitamin E.

*Effect of microsomes on true and false lipid peroxidation*

The effect of microsomes on false lipid peroxidation, *i.e.*, acetaldehyde accumulation, was investigated by the experiments described in Table III. Experiments 1 and 2 show that false TBA levels increase significantly when microsomes are added to cytosol. This occurs in spite of the fact that microsomes alone generate no significant quantities of acetaldehyde (experiments 3 and 4). Unlike with mitochondria, acetaldehyde accumulation in the presence of microsomes is not reduced by the synergistic interaction of vitamin E and NAD (experiment 5).

A further increase in acetaldehyde levels occurred when mitochondria were added along with cytosol and microsomes to the reaction medium (experiment 2 *vs.* experiment 6). Once again, this increase could not be prevented by the combined action of vitamin E and NAD (experiments 7 and 8). The failure of vitamin E and NAD in preventing acetaldehyde accumulation was clearly caused by the presence of microsomes,

TABLE III

EFFECT OF MICROSOMES ON FALSE LIPID PEROXIDATION VALUES

TBA values due to acetaldehyde accumulation and lipid peroxidation in indicated reaction systems after 3 h of incubation in Warburg respirometer. Reaction conditions and medium are identical to those used for respiratory decline experiments. Results are the averages of 3 experiments.

| *Expt.* | *Reaction system*[a] | *TBA assay (532 nm absorbance)* | |
|---|---|---|---|
| | | *Lipid peroxidation* | *Acetaldehyde* |
| 1 | C + EtOH | 0.07 | 0.19 |
| 2 | C + MS + EtOH | 0.34 | 0.60 |
| 3 | MS | 0.10 | 0.02 |
| 4 | MS + EtOH | 0.13 | 0.03 |
| 5 | C + MS + EtOH + NAD + E | 1.36 | 0.80 |
| 6 | C + MS + M + EtOH | 0.27 | 1.18 |
| 7 | C + MS + M + EtOH + E | 0.09 | 1.39 |
| 8 | C + MS + M + EtOH + NAD + E | 1.36 | 1.37 |
| 9 | C + MS + M + EtOH + NAD | 2.12 | 1.40 |
| 10 | C + M + EtOH | 0.37 | 0.82 |
| 11 | C + M + EtOH + NAD + E | 0.00 | 0.05 |

[a]C, cytosol; MS, microsomes; M, mitochondria; EtOH, ethanol; E, α-tocopherol.

since the vitamin was effective when added to the same mitochondrial preparation in the absence of microsomes (experiment 11).

The effect of exogenous NAD on peroxidation is also of interest. For example, the inclusion of NAD increased lipid peroxidation drastically in the presence of microsomes (experiment 6 *vs.* experiment 9); presumably this result is due to formation of NADH and the latter's catalytic action on lipid peroxidation. This increase in random lipid peroxidation was only incompletely suppressed by the amount of exogenous vitamin E used in these experiments (experiments 8 and 9), attesting thereby to a marginal vitamin E protection. False lipid peroxidation, on the other hand, was essentially unaffected by vitamin E addition (experiment 8 *vs.* experiment 9).

*Effect of NADH oxidation on acetaldehyde levels*

According to Krebs, the alcohol acetaldehyde couple is at all times in equilibrium with the NADH couple [23]. This observation could explain why addition of mitochondria to cytosol caused the observed increase in acetaldehyde levels, since the increase in the NAD/NADH ratio (brought about by mitochondrial NADH oxidation) would produce a shift in the above equilibrium towards the aldehyde side. According to such a mechanism, the prevention of acetaldehyde accumulation by vitamin E can be attributed to a protective effect on the mitochondrial NADH barrier, *i.e.*, to prevention of swelling, since this would inhibit NADH oxidation through latency effects.

To test this hypothesis, we measured the rate at which NADH, derived from alcohol metabolism, was oxidized by mitochondria under the various experimental conditions described in Table IV. Experiments were carried out with a Warburg respirometer containing, except for the deletion of succinate, the usual reaction medium.

The data in Table IV show that significant respiratory activity due to NADH oxidation occurs in the presence of mitochondria or microsomes, *i.e.*, under the aforementioned conditions of acetaldehyde accumulation. A further increase in oxygen consumption was observed when both microsomes and mitochondria were present. This

TABLE IV

OXYGEN CONSUMPTION IN DIFFERENT REACTION SYSTEMS DUE TO NAD AND ETHANOL

Oxygen consumption was measured manometrically in the absence of succinate using the reaction medium described in experimental procedures for respiratory decline experiments.

| *Substrates* | *Total oxygen consumed (μmol) in 3 h in different reactions systems*[a] | | | |
|---|---|---|---|---|
| | *C* | *C + MS* | *C + M* | *C + MS + M* |
| None (endogenous) | 0 | 4.0 | 4.5 | 5.5 |
| EtOH | 0 | 6.0 | 8.0 | 9.0 |
| NAD | 0 | 10.0 | 10.0 | 12.5 |
| NAD + EtOH | 0 | 12.0 | 16.5 | 30.0 |
| NAD + EtOH + E | – | – | 16.0 | – |

[a] M, mitochondria; MS, microsomes; C, native cytosol; EtOH, ethanol; E, vitamin E.

increase, which is particularly large in the presence of exogenous NAD and ethanol, points to a synergistic interaction between these two organelles resembling that reported by Schenkman for aldehyde catabolism [24]. However, the most significant feature of our data is that the inclusion of exogenous vitamin E has no inhibitory effect on mitochondrial NADH oxidation, indicating thereby that the lower acetaldehyde levels in the presence of vitamin E are not due to a protective effect on the NADH permeability barrier of mitochondria. The results also identify alcohol-associated NADH oxidation as the source for the troublesome endogenous reaction in respiratory decline experiments in which native cytosol is used.

*Additional factors in the control of acetaldehyde levels*

During the course of the study with the cytosol–mitochondria reaction systems, we occasionally encountered reaction conditions where acetaldehyde levels could not even be controlled by the combined action of exogenous vitamin E and NAD. So far, this has only been observed with endogenous reactions, *i.e.*, with reaction mixtures from which succinate had been deleted. Typically, this situation would produce false TBA values of 1.6 absorbance units for the endogenous reaction and 0.22 absorbance units for the main reaction with succinate present.

One possible explanation for this succinate effect assumes that exogenous and/or endogenous vitamin E is not always completely effective in inhibiting deleterious membrane changes. Possible reasons for this failure may be variable peroxidation conditions, accessibility problems, or variations in endogenous vitamin E content. Under such conditions of marginal antioxidant protection, active succinate metabolism can provide additional protection against lipid peroxidation, since the succinic oxidase reaction produces a drastic decrease in oxygen tension in the reaction medium.

We investigated this possibility by using mitochondria containing a higher level of endogenous vitamin E. These were prepared from animals fed a diet containing 4–5 times the normal amounts of vitamin E. With these mitochondria, we found in the majority of cases that it was possible to prevent acetaldehyde accumulation in the mitochondrial–cytosol reaction system merely by the combined action of exogenous and endogenous vitamin E, *i.e.*, under conditions of enhanced endogenous antioxidant protection the assistance of NAD and succinate was found to be no longer necessary.

## DISCUSSION

Earlier studies have shown that respiratory decline of the succinic oxidase system is caused by exogenous NAD. Although exogenous NAD produces also an increase in lipid peroxidation, it became clear from the reported reversibility of respiratory decline (achieved simply by removal of NAD and reintroduction of cytochrome *c* [25]) that succinic oxidase deactivation in respiratory decline is not caused by direct radical attack on the enzyme. If such an attack had taken place, respiratory decline would essentially be irreversible.

The precise mechanism for the deactivation of the succinic oxidase system by exogenous NAD has not been completely clarified to date. Evidence from earlier studies suggests that this may occur either through an NAD-dependent oxaloacetic acid feedback effect and/or a regulatory mechanism involving changes in the membrane-bound NAD/NADH ratio [7, 8, 26]. In either case, this implies that the prevention of respiratory decline by vitamin E is due to the protection of certain membrane properties regulating the compartmentalization or binding of NAD, since both of the above mechanisms for succinic oxidase deactivation are critically dependent on such properties [26, 27].

The observation of two anomalous vitamin E effects, one occurring in isolated mitochondria and the other in homogenates, provided the rationale for the present study, namely that the changes in the compartmentalization or binding of NAD necessary for respiratory decline are caused by specific rather than by random radical processes. It is possible to account for the two anomalous vitamin E effects by such a mechanism if one assumes that the "leakage" of radicals at a specific site can only be inhibited by a correspondingly specific antioxidant effect, the latter being mediated by the action of specific binding proteins from the cytosol.

We used the isolated mitochondrial reaction system to test this hypothesis. As in the case of the homogenate system [9], we found no evidence for a correlation between respiratory decline in isolated mitochondria and the incidence of random lipid peroxidation or gross mitochondrial swelling accompanying such peroxidation. This is evident from the fact that random lipid peroxidation and swelling, but not respiratory decline, could be prevented by vitamin E alone. To prevent respiratory decline, both vitamin E and cytosol had to be present.

Direct evidence for a specific antioxidant effect was sought from experiments in which we investigated whether the inclusion of cytosol could decrease further or abolish completely the already low level of lipid peroxidation remaining when vitamin E alone is added to the mitochondrial reaction medium. This attempt failed because heat-denatured cytosol unexpectedly increased the level of residual lipid peroxidation. Although this increase in random peroxidation was very small in absolute terms, it was nevertheless of sufficient magnitude to interfere with the detection of possible specific antioxidant effects. We attribute the increase in lipid peroxidation to the presence of peroxidation catalyst in cytosol.

We repeated the above experiments with native cytosol in place of heat-denatured cytosol. Totally different results were obtained in these experiments in spite of the fact that both types of cytosol are equally effective in preventing respiratory decline. Here, a third anomalous vitamin E effect was observed which implied that vitamin E could inhibit random lipid peroxidation only with the assistance of exogenous NAD.

This unexpected involvement of NAD in the antioxidant function of vitamin E was further investigated. A detailed study of the TBA assay under our experimental conditions (presence of sucrose and ethanol) revealed that the new anomalous vitamin E effect did not involve lipid peroxidation, *i.e.*, malondialdehyde accumulation, but rather that it was due to an influence of vitamin E and NAD on acetaldehyde levels, the latter being formed from ethanol – the conventional solvent for vitamin E addition. The confusion

between lipid peroxidation and acetaldehyde accumulation arose from a novel reaction between thiobarbituric acid, acetaldehyde, and a hydrolysis product of sucrose, since these react to form a chromogen which is identical to that produced by malondialdehyde.

The mechanism for the control of acetaldehyde levels by the combined action of vitamin E and NAD was further investigated by studying the effect of cytosol, mitochondria, and microsomes on acetaldehyde accumulation. This was done since it appeared likely that false lipid peroxidation could have been responsible for the anomalous vitamin E effect observed in homogenate systems. Another reason for pursuing this question was the possibility that false lipid peroxidation was also responsible for the controversy of whether lipid peroxidation is involved in the hepatotoxic mechanism of ethanol. We pursued this dual objective by expressing acetaldehyde levels in terms of false lipid peroxidation values.

In spite of the fact that cytosol contains active alcohol dehydrogenase activity, as evidenced by the increase in NADH levels, we found that little acetaldehyde accumulated when only cytosol was added to the reaction medium. Since the alcohol/acetaldehyde couple is known to be at all times in equilibrium with the NAD/NADH couple [23], it is possible to explain the absence of acetaldehyde under the above reaction conditions by a shift in the equilibrium towards the acetaldehyde side, *i.e.*, towards the side which is already heavily favored by thermodynamic factors [28].

When mitochondria were added along with cytosol to the reaction medium, acetaldehyde, *i.e.*, false lipid peroxidation, increased dramatically. Since NADH was rapidly oxidized by mitochondria, one can explain the increase in acetaldehyde levels by a shift in the equilibrium towards the acetaldehyde side.

The fact that acetaldehyde accumulation in the presence of mitochondria was prevented by the combined action of vitamin E and NAD was originally attributed to a protective effect of vitamin E on the mitochondrial NADH permeability barrier. Maintenance of this barrier would retard NADH oxidation and thereby prevent a shift in the equilibrium towards the acetaldehyde side. However, on testing this hypothesis, we found that the NADH permeability barrier was not protected by vitamin E in spite of the prevention of gross mitochondrial swelling. This result is consistent with other observations attesting to loss of the NADH permeability barrier in the absence of large amplitude swelling.

An alternate explanation for the regulation of acetaldehyde levels by vitamin E and NAD is a protective effect on the mitochondrial aldehyde oxidase system, since this is one of the main pathways of aldehyde catabolism [29]. Furthermore, since the activity of this enzyme is critically dependent on the integrity of the mitochondrial membrane, in particular on membrane-bound NAD, it is a relatively easy matter to envisage mechanisms for the control of acetaldehyde levels in which synergistic interactions between vitamin E and NAD are involved.

Such a mechanism is supported by work on several other NAD-dependent mitochondrial enzyme systems where loss of activity was found to occur as a result of swelling and loss of membrane-bound NAD [30, 31]. Vitamin E protected these enzymes against deactivation; alternatively their activity could be restored by exogenous NAD. Inter-

estingly, restoration of activity by exogenous NAD did not occur through rebinding of the coenzyme, but instead resulted from the restoration of subtle membrane properties relating to compartmentalization or configurational effects.

It appears likely, therefore, that two independent factors, such as membrane configuration and NAD, are also involved in the control of acetaldehyde levels by the postulated aldehyde oxidase mechanism. This is suggested by our inability to control acetaldehyde levels solely by the action of exogenous NAD, *i.e.*, NAD has so far only been found effective in combination with exogenous vitamin E. This, in turn, suggests that vitamin E preserves certain compartmental and/or configurational properties of the mitochondrial membrane which, in addition to exogenous NAD, are critical for enzyme activity. An interesting corollary to this mechanism is that the NAD involved in aldehyde oxidase activity appears to be lost from the membrane in the absence of gross mitochondrial swelling. This is indicated, since exogenous vitamin E on its own, like NAD, was also found ineffective in controlling acetaldehyde levels under normal experimental conditions in spite of the fact that swelling was prevented by its use (see exceptions below). This situation is somewhat reminiscent of the loss of the NAD barrier in unswollen mitochondria.

Some of the other unusual features observed in the regulation of acetaldehyde levels can also be explained in terms of loss of membrane-bound NAD and compartmentalization effects. One of these is that this control mechanism requires occasionally the presence of succinate. Two possible mechanisms may be involved in this effect; both may be envisaged as supporting a marginal vitamin E protective effect. In one case, reverse electron-transport from succinate to membrane-bound NAD can contribute to the retention of membrane-bound NADH, since the coenzyme can only be lost in the oxidized form [32]. Another possible reason for the succinate effect is that rapid mitochondrial oxidation of succinate decreases the oxygen tension in the reaction medium, thereby potentially reducing lipid peroxidation at critical membrane sites not readily protected by exogenous vitamin E.

Direct evidence for the limited effectiveness of exogenous vitamin E in the mitochondria–cytosol reaction system was obtained when the above experiments were repeated with mitochondria which contained higher levels of endogenous vitamin E. It was found under these conditions that acetaldehyde levels could be controlled solely by the combined action of endogenous and exogenous vitamin E. It appears from this that vitamin E can prevent, under optimum conditions, not only enzyme deactivation due to loss of critical compartmentalization effects, but that it can also protect those membrane properties which are essential for the maintenance of membrane-bound NAD. Furthermore, since endogenous and exogenous vitamin E apparently do not exert the same protective influence, it appears that vitamin E effects are themselves compartmentalized. Most likely this is due to accessibility problems. These occur apparently in spite of the postulated assistance from vitamin E binding proteins.

Finally, we have the observation that acetaldehyde levels, *i.e.*, false lipid peroxidation, cannot be controlled by the combined action of vitamin E and NAD when microsomes are present in the reaction medium. This result is important, since it indicates

that the anomalous vitamin E effect observed by Corwin in homogenate systems is most likely caused by the presence of acetaldehyde and not by lipid peroxidation. Consequently, this particular observation is no longer supportive of a specific antioxidant effect.

The reason why vitamin E and NAD cannot control acetaldehyde levels in the presence of microsomes is not completely clear. Our results suggest that this may be due to a marginal protective effect of vitamin E, since the presence of microsomes and NAD causes excessive lipid peroxidation. Another possible contributing factor is that the increase in alcohol metabolism, caused by the synergistic interaction of mitochondria and microsomes, has enhanced acetaldehyde formation to such an extent that it exceeds the catabolizing capacity of the aldehyde oxidase system.

From our results, it appears also as highly likely that false lipid peroxidation is responsible for the controversy of whether lipid peroxidation plays a role in the hepatotoxic mechanism of ethanol. This is indicated since the controversy has largely revolved around the use of different analytical procedures for the measurement of lipid peroxidation, *i.e.*, diene-conjugation assay *vs.* TBA assay [19, 20].

Still unresolved is the nature of the remaining anomalous vitamin E effect dealing with the need for cytosol in the protection of isolated mitochondria against respiratory decline. The possible involvement of a specific antioxidant effect in this phenomenon is under further study.

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