Vitamin E: Substrate-Dependent Growth Effect on Cells in Culture (36833)

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The effect of vitamin E in maintaining the viability of tissues in animals is well known. The deficiency of the vitamin when accompanied by a selenium deficiency results in a liver necrosis in rats (1), muscular dystrophy in rabbits (2), and exudative diathesis in chicks (3). A deficiency of vitamin E in monkeys (4) and humans under certain conditions (5) can result in a severe anemia. Explanations for the role of the vitamin in metabolism have been quite varied, ranging from its action as an antioxidant (6), protection of enzyme sulfhydryl groups (7), to a specific role in the synthesis of heme (8). Our purpose in this communication is to present an approach towards the understanding of the different degrees of sensitivity of certain tissues to a vitamin E deficiency in different animals and conditions. To this end we have sought an effect of the vitamin on a variety of cells in culture, a system which is subject to specific controls by the investigator. This paper presents a method for obtaining tocopherol-free media and its usefulness in studying the utilization of the vitamin by the cell and its resultant effect on the rate of growth.

Methods. Tocopherol-free media. There are several commercially available media with no added α-tocopherol. Eagle's minimum essential medium (MEM) with Earle's salts (9) was chosen for this study. The problem, of course, comes with the uncontrolled serum component of the medium. Since tocopherol does not penetrate the placental barrier, fetal calf serum (FCS) was tested for its tocopherol content by Duggan's (10) spectrofluorometric method. No tocopherol was detected. By contrast, human serum contained 12 μg/ml and horse serum 12–24 μg/ml tocopherol. Addition of tocopherol to the medium was accomplished by adding 1.2 mg of *d*-α-tocopheryl acetate, dissolved in 0.1 ml of propylene glycol to 100 ml of FCS. The serum was then filtered through a 0.45 μm Millipore filter. The filtration resulted in about a 20% loss of the vitamin. The serum was added to MEM at a level of 10%. Thus, the final concentration of *d*-α-tocopheryl acetate was about 1.0 μg/ml medium.

Uptake of d-α-tocopheryl-3,4-^{14}C-acetate. The cells used in these experiments were fibroblasts obtained from seminiferous tubules of a Chinese hamster, isolated by Dr. C. Yerganian, and are now a secondary derivative of a primary diploid strain. They are designated STBTL cells. The cells were grown to about three quarter confluency in 5 cm plastic dishes (NUNC, Vanguard International) in MEM (Earle's) with 10% FCS. The medium was removed and replaced with MEM containing 2.5% FCS with 13.7 μg/dish *d*-α-tocopheryl-3,4-^{14}C-acetate obtained through the generosity of W. E. Scott of Hoffman La Roche. At a given time interval two duplicate dishes were taken, the medium removed, the cells washed thrice with phosphate buffered saline and drained. One milliliter of deionized water was added to each dish and allowed to stand for 30 min to cause cell lysis. The plates were scraped with a rubber policeman and the cells uniformly suspended by pipetting up and down several times. Protein was measured on a 0.2 ml aliquot by the Lowry *et al.* (11) method and 0.8 ml counted in Aquasol (New England Nuclear) in a Beckman 200 B scintillation counter.

Growth studies. Cells were grown at 37° in a 5% CO_2 incubator in 5 cm plastic petri

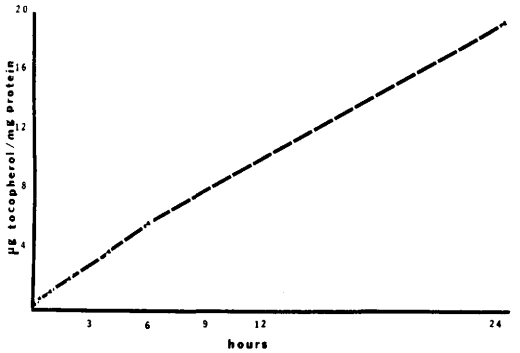

FIG. 1. Uptake of *d*-α-tocopheryl-3,4-^{14}C-acetate by STBTL cells in 5 ml MEM (Earles') with 2.5% fetal calf serum.

dishes (NUNC). The plates were inoculated with 5×10^4 cells in 5 ml of glucose-free MEM (Earle's) supplemented with 10% FCS and an appropriate source of carbon at a level of 0.1%. Growth was followed by protein determinations using the Lowry *et al.* (11) method on aliquots of cells from duplicate plates.

Results. Uptake of d-α-tocopheryl-3,4-^{14}C-acetate. The rate of tocopherol uptake by cells from the blood has never been directly determined. To assess the rate at which cells remove the vitamin from serum proteins, *d*-α-tocopheryl-3,4-^{14}C-acetate was added to serum and its rate of uptake determined as described in Methods. The experiments were done in low level serum (2.5%), so that a minimum amount of growth would occur over the period of the experiment. The results in Fig. 1 indicate that per milligram cell protein, tocopherol uptake is relatively slow and nearly linear over a 24.5 hr period. During the test period, the cell protein increased from 65 to 71 μg/dish in 6 hr and to 120 μg/dish in 24.5 hr. We have obtained almost identical results with the V79 strain of the Chinese hamster lung fibroblast cell line. Thus, it is apparent that cells remove tocopherol from the proteins in the surrounding medium rather slowly. This is, of course, not true transport, since the vitamin is not in solution but adsorbed to serum proteins.

Effect of d-α-tocopheryl actetate on cell growth. Our initial attempts to demonstrate a growth effect of vitamin E in the more usual media, such as MEM containing glucose, were unsuccessful (Fig. 2A). It is well known that cells in culture grow well in media not containing added tocopherol. STBTL cells

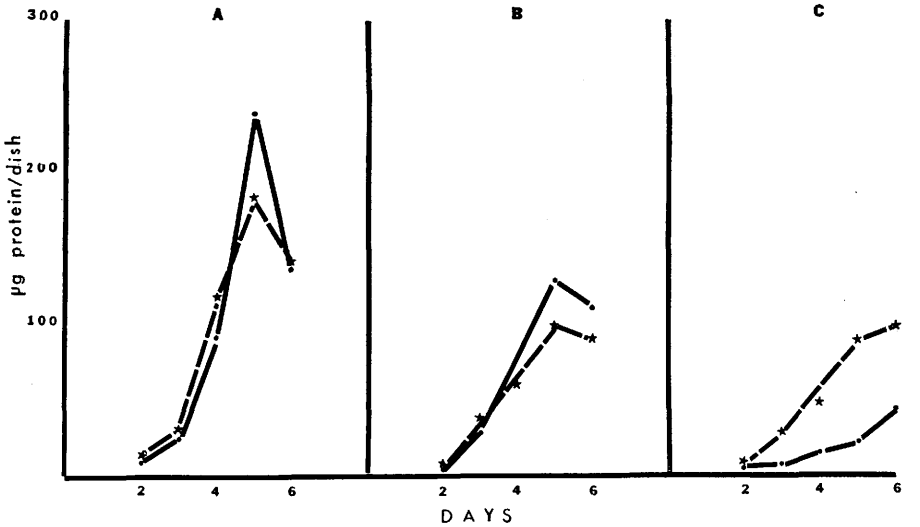

FIG. 2. The effect of *d*-α-tocopherol on the growth of STBTL cells with different sources of carbon. (A) contains 0.1% glucose, (B) contains no added carbon source, and (C) contains 0.1% neutralized sodium pyruvate. All are added to 5 ml MEM (Earles') with 10% fetal calf serum which was made without a carbon source. (— •) Without tocopherol added, and (* - -) contains 1 μg α-tocopherol acetate/ml of medium.

are no exception. Tocopherol has no effect on the STBTL cells grown in glucose-containing medium. In fact, in other cells lines such as the V79 lung fibroblasts, we have noticed a mild inhibitory effect (20–30%).

There is much evidence to support the view that the metabolism of cultured cells differs markedly from the intact tissues from which they are derived (12). In particular, the energy-yielding mechanisms of the Krebs' cycle do not seem important, *i.e.*, succinic dehydrogenase is greatly decreased in cultured cells which are rendered comparatively insensitive to malonate inhibition compared to their parent tissue (13). Since tocopherol has been implicated in heme synthesis (8), we reasoned that the conversion of the cells to glycolytic pathways would decrease the requirements of cells for cytochromes and hence decrease the requirement for tocopherol. Thus, if it were possible to force the cells to utilize the Krebs' cycle, a requirement for tocopherol might be shown. To this end, growth was measured in MEM without glucose (Fig. 2B). Although growth was decreased, the cells grew surprisingly well in the absence of added glucose. Tocopherol again was without effect. When 0.1% sodium pyruvate was substituted for glucose, the growth of the cells was dramatically decreased (Fig. 2c). The cells appear thin in a phase-contrast microscope and multiply slowly over the first few days. When α-tocopheryl acetate is present in the medium, however, the cells look dramatically better and the multiplication rate is enhanced. These results have been obtained repeatedly with this cell strain. On rare occasions, a dish has grown well with pyruvate. Under these conditions, tocopherol is without effect.

To test whether or not the cells had indeed been shifted to a requirement for the Kreb's cycle by utilizing pyruvate as a carbon source, survival of cells in the presence of 10 m*M* malonate was examined. It should be borne in mind that amounts in excess of 100 m*M* malonate have been found necessary to inhibit growth with other cells (13). Dishes were inoculated and allowed to grow for 2 days in the usual MEM–10% FCS medium. These contained either 0.1% glucose or pyruvate as a carbon source, and some were left without an added carbon source. On the third day, half of the dishes in each group had neutralized malonate added to a final concentration of 10 m*M*. After 24 hr, the malonate-treated cells with pyruvate as a carbon source or with no substrate developed vacuoles. By 48 hr, the cells growing in pyruvate were degenerating, and were dead or dying by 72 hr. The cells growing without added substrate had some survivors after 72 hr, but they were vacuolated. The cells growing with glucose had their growth rate decreased by 2–3-fold by malonate, but the cells were still growing, looking healthy, and surviving after 6 days of growth, at which point the experiment was terminated.

Discussion. From these experiments one might conclude that α-tocopherol can be taken up by STBTL cells and used to prevent an inhibition of growth by pyruvate. The mechanism of the pyruvate inhibition and its prevention by tocopherol is not clear, however. One possible explanation is that when pyruvate is used as a substrate, the cell metabolism is converted to an obligate Krebs' cycle requirement and tocopherol is required to allow for the synthesis of cytochromes which are now required for terminal oxidation (8). The malonate experiments indicate that when cells grow with glucose as a carbon source some energy is derived from Krebs' cycle oxidation, but it is not an absolute requirement of the cells. Cells growing on pyruvate, however, are killed by malonate, indicating an absolute requirement for the Krebs' cycle. Apparently, the STBTL cells can grow without any carbon source added to the MEM–10% FCS medium. These cells are much more sensitive to malonate than glucose-grown cells, but there are still some surviving cells which are able to grow even though apparently damaged. This survival might be due to the relatively small amount of glucose in the fetal calf serum.

Various groups have been unable to demonstrate that pyruvate as a carbon source can support growth in any cell line studied (14, 15). To our knowledge, no studies have appeared showing growth without an added

carbon source. Our results with STBTL cells support the finding of poor growth with pyruvate, but we find it to be an inhibitor, rather than merely a nonsupporter of growth. A possible clue as to the nature of pyruvate inhibition which is relieved by tocopherol comes from work on the formation of lipid peroxides in liver homogenates from rats deficient in vitamin E (16). It was shown that when these deficient homogenates were incubated *in vitro* with pyruvate or α-ketoglutarate, an increase in peroxide levels could be demonstrated above that seen without added substrate or with succinate. This increase in peroxide levels could be inhibited by antimycin A and cyanide, inhibitors of cytochrome-mediated oxidation. No peroxides were formed when tocopherol was present. The application of these findings to the present paper require more evidence. Lipid peroxide formation in undamaged tissue, prevented by tocopherol, has not been demonstrated directly (17, 18). In preliminary results, we have not been able to show peroxide formation using the thiobarbituric acid assay in vitamin E-deficient cells, but we may need more refined techniques.

Summary. Chinese hamster fibroblasts derived from seminiferous tubules grow well in Eagle's minimal essential medium containing glucose and 10% fetal calf serum. This medium contains no vitamin E; nevertheless, addition of the vitamin has no effect on the growth of these cells. When pyruvate is substituted for glucose as a carbon source, there is a great decrease in the growth rate which can be alleviated by *d*-α-tocopherol. The growth rate with pyruvate is much less than when no added carbon source is used.

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1. Schwarz, K., and Foltz, C. M., J. Amer. Chem. Soc. **79**, 3293 (1957).

2. MacKenzie, C. G., and McCollum, E. V., J. Nutr. **19**, 345 (1940).

3. Schwarz, K., Bieri, J. G., Briggs, G. M., and Scott, M. C., Proc. Soc. Exp. Biol. Med. **95**, 621 (1957).

4. Porter, F. S., and Fitch, C. J., Scand. J. Haematol. **3**, 175 (1966).

5. Majaj, A. S., Dinning, J. S., Azzam, S. A., and Darby, W. J., Amer. J. Clin. Nutr. **12**, 374 (1963).

6. Dam, H., Pharmacol. Rev. **9**, 1 (1957).

7. Corwin, L. M., and Schwarz, K., Arch. Biochem. Biophys. **100**, 385 (1963).

8. Murty, H. S., Caasi, P. I., Brooks, S. K., and Nair, P. P., J. Biol. Chem. **245**, 5498 (1970).

9. Eagle, H., Science **130**, 432 (1959).

10. Duggan, D. E., Arch. Biochem. Biophys. **84**, 116 (1959).

11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

12. Paul, J., *in* "Cells and Tissues in Culture" (E. N. Willmer, ed.), Vol. 1, Chap. 7, p. 239. Academic Press, New York (1965).

13. Turner, G. S., Nature (London) **193**, 164 (1962).

14. Eagle, H., Barban, S., Levy, M., and Schulze, H. O., J. Biol. Chem. **235**, 551 (1958).

15. Morgan, J. F., and Morton, H. J., Can. J. Biochem. **38**, 69 (1960).

16. Corwin, L. M., Arch. Biochem. Biophys. **97**, 51 (1962).

17. Bieri, J. G., World's Poult. Sci. J. **16**, 245 (1960).

18. Bunyan, J., Murnell, E. A., Green, J., and Diplock, A. T., Brit. J. Nutr. **21**, 475 (1967).

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