Observations on the reactivation of isooctane-extracted DPNH-cytochrome *c* reductase with D-[^{14}C]-α-tocopherol

Previous experiments[1,2] have demonstrated that after administration of [5-Me-^{14}C]-D-α-tocopheryl succinate to rats, two radioactive metabolites, in addition to α-tocopherol, are extractable from the liver. One of these ("Compound F") exhibits an absorption maximum at 257 mμ and is reducible to α-tocopherol with $SnCl_2$ in HCl. The other ("Compound O") has an absorption maximum at 255 mμ and is associated predominantly with the microsomes. Evidence that the DPNH-cytochrome *c* reductases of rat skeletal muscle and bovine heart muscle can be inactivated by isooctane extraction or aging, and restored specifically by α-tocopherol[3,4], prompted a study of the metabolism of [^{14}C]-α-tocopherol by similar preparations of the electron-transport system.

Keilin-Hartree heart-muscle preparations containing about 15 mg protein/ml were used[5]. Extraction was carried out by shaking 5 times for 15 min with 5 vol. isooctane at 4°. After each extraction the preparation was centrifuged for 2 min at 500 × *g* and the isooctane layer was removed. The resulting fine emulsion, which contained an active DPNH-cytochrome *c* reductase, could be partially inactivated by centrifuging for 2 h at 15,000 × *g* in the refrigerated centrifuge, and further inactivated by another centrifugation at 30,000 × *g* for 2 h, the clear isooctane layer being removed each time and the remainder resuspended in a hand homogenizer.

DPNH-cytochrome *c* reductase was measured spectrophotometrically at 550 mμ using a 3.5-ml reaction mixture similar to that of DEUL *et al.*[6]: heart-muscle preparation, 0.1 mg protein/ml; cytochrome *c*, $2.8 \cdot 10^{-5}$ *M*; DPNH, $1.8 \cdot 10^{-4}$ *M*; KCN, 10^{-3} *M*; EDTA, 10^{-3} *M*; phosphate buffer, pH 7.3, 0.033 *M*. DPNH oxidase was measured at 340 mμ with KCN omitted. *d*-α-tocopherol was added at 10^{-4} *M* as a suspension in 0.2 % serum albumin[6].

After testing for reactivation with unlabelled tocopherol, the enzyme preparation was regenerated with D-[5-Me-^{14}C]-α-tocopherol (10^{-4} *M*, 8,100 disintegrations/min/7 ml reaction mixture). A parallel experiment was carried out using an unextracted heart-muscle preparation. Following a 30-min reaction period the mixture was extracted 3 times with peroxide-free ether which, after backwashing with water, was concentrated *in vacuo* under N_2 at 50°. The residue was applied in ethanol to three different paper chromatographic systems which are suitable for the separation of metabolites of α-tocopherol formed *in vivo*[1,2]. Scanograms were prepared from the developed chromatograms for the determination of R_F values.

The radioactivity in the ether extract of both the reactivated DPNH-cytochrome *c* reductase and the unextracted enzyme was due entirely to unchanged D-[^{14}C]-α-tocopherol. Isooctane extraction partially inactivated DPNH oxidase, but no appreciable reactivation was achieved with α-tocopherol. Incubation of labelled tocopherol with unextracted heart-muscle preparation in the DPNH oxidase system also failed to yield a labelled metabolite.

The results demonstrate that the reactivation of isooctane-extracted DPNH-cytochrome *c* reductase is not accompanied by enzymic oxidation of α-tocopherol and is probably due to a physical effect upon residual isooctane in the system, as

Abbreviations: DPNH, reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; DPPD, N,N'-diphenyl-*p*-phenylenediamine.

suggested elsewhere[6]. The effectiveness of various isoprenoid compounds in restoring isooctane-extracted systems has been demonstrated by other workers[6–8].

Experiments with rabbits and rats in this laboratory have provided strong evidence for the direct substitution of α-tocopherol by the synthetic antioxidant DPPD in functions relating to the two classical symptoms of vitamin E deficiency, namely, muscular dystrophy and resorption gestation[9,10]. Accordingly, it was of interest to determine whether this compound could replace α-tocopherol in the regeneration of the isooctane-extracted DPNH-cytochrome *c* reductase. Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline), another synthetic antioxidant which has been shown to be effective in preventing vitamin E deficiency in animals, was also tested. The results, shown in Table I, demonstrate that both antioxidants are effective in regenerating the extracted enzyme in molar concentrations comparable to, or less than, that required for α-tocopherol. Similar observations have been made by other workers[11]. In agreement with previous reports[6–8], reactivation was also obtained with ubiquinone (10^{-4} *M*) and vitamin K_1 (10^{-3} *M*).

TABLE I

INFLUENCE OF ANTIOXIDANTS ON ISOOCTANE-EXTRACTED DPNH-CYTOCHROME *c* REDUCTASE

	Relative activity
Unextracted heart-muscle preparation	100
Extracted heart-muscle preparation	25
Extracted heart-muscle preparation + DPPD ($5.5 \cdot 10^{-6}$ *M*)	32
Extracted heart-muscle preparation + DPPD ($1.1 \cdot 10^{-5}$ *M*)	33
Extracted heart-muscle preparation + DPPD ($5.5 \cdot 10^{-5}$ *M*)	89
Extracted heart-muscle preparation + Santoquin ($6.2 \cdot 10^{-5}$ *M*)	71
Extracted heart-muscle preparation + D-α-tocopherol (10^{-4} *M*)	75

See text for composition of reaction mixture. Antioxidants and α-tocopherol added in 10 μl ethanol.

These findings do not support the suggestion[7,8] that a necessary criterion for regeneration of the isooctane-treated enzyme is a long isoprenoid side chain. The possibility that reactivation is dependent upon antioxidant activity, on the other hand, is also remote, as compounds without such activity are effective and since [^{14}C]-α-tocopherol undergoes no oxidative change coincident with reactivation of the system. A purely physical mechanism, such as isooctane dispersion on the enzyme[6], is therefore indicated.

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