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THE POSSIBLE ROLE OF α-TOCOPHEROL IN THE RESPIRATORY CHAIN

II. REACTIVATION BY α-TOCOPHEROL

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In the previous paper of this series, it was shown that an active preparation of the respiratory chain, the KEILIN AND HARTREE heart-muscle preparation, as well as liver mitochondria, contained α-tocopherol in amounts of the same order of magnitude as of known components of the respiratory chain[1]. Thus, α-tocopherol fulfills the first of three criteria for a member of the respiratory chain which were listed. The present paper is concerned with the second criterion, namely that removal of a component of the respiratory chain from an enzyme preparation leads to inactivation of the chain, and the activity can be restored by the addition of the proposed component.

The studies of NASON and his co-workers[2-4] appeared to provide good evidence that α-tocopherol satisfied this second criterion. They found that extraction of a preparation of rat skeletal muscle with iso-octane inactivated the reduced diphosphopyridine nucleotide (DPNH) oxidase and DPNH–cytochrome *c* reductase, and that the activity could be specifically restored by the addition of either the lipid extracted from the enzyme preparation by iso-octane or of α-tocopherol.

In similar experiments with the KEILIN AND HARTREE heart-muscle preparation, we have found that the α-tocopherol, isolated from the heart muscle, and pure α-tocopherol were equally effective in restoring the activity of an iso-octane-extracted preparation. On the other hand, an examination of the effects of extraction with iso-octane and of the reactivation have led us to question whether these processes can be simply explained on the basis of removal and restoration of a specific component of the respiratory chain.

A preliminary account of some of these findings has been given[5].

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METHODS

Heart-muscle preparation was prepared in the usual way[6,7].

α-Tocopherol. Except where otherwise stated, DL-α-tocopherol obtained from Hoffman-La Roche was used. In some experiments, D-α-tocopherol kindly provided by Dr. C. BAXTER of Distillation Products and a crude preparation of α-tocopherol isolated from heart-muscle preparation were used. The latter preparation was isolated by following Procedure A of the previous paper[1] up to and including the passage through a column of Floridin Earth.

α-Tocopherylquinone was prepared from DL-α-tocopherol by oxidation with $AuCl_3$[8].

Tocopherol and vitamin K_1 suspensions. The tocopherol was suspended in crystalline bovine serum albumin (*cf.* ref. 3). A weighed amount of α-tocopherol (D- or DL-, as specified in the experiments) was dissolved in 0.3 ml ethanol, and the solution added to 1.7 ml 0.2 % serum albumin in 0.1 *M* Sørensen phosphate buffer, pH 7.4. The suspension was then vigorously shaken by hand. Measurements of the tocopherol content of the suspensions, either by the method of EMMERIE AND ENGEL[9], or by determination of the extinction at 292 mμ of the iso-octane extract, showed that up to 4 mg tocopherol/ml (0.0093 *M*) could be completely suspended in this way. Above this concentration, a large proportion of the tocopherol remained on the glass walls and the concentration in the suspension was not reproducible. The stock suspension of α-tocopherol was usually about 1 mg/ml.

In disagreement with NASON AND LEHMAN[3], it was not possible to calculate the concentration of tocopherol in the suspensions by direct measurement of the extinction at 297 mμ. This extinction is partly due to absorption of light by the α-tocopherol, and partly due to light scatter. No simple relationship was found between the measured extinction and the concentration of α-tocopherol.

Suspensions of vitamin K_1 were made in the same way.

Extraction of heart-muscle preparation with iso-octane

Heart-muscle preparation (about 30 mg protein/ml) was shaken by hand for 2 min at 4° with an equal volume of iso-octane, and the layers were separated by centrifugation at 500 *g* for a few minutes. The iso-octane layer was sucked off, and the aqueous layer either used as such or again extracted with iso-octane a number of times.

Measurement of enzyme activities

Succinic oxidase was measured manometrically at 38° with the following reaction mixture: phosphate, pH 7.3, 0.067 *M*; succinate, 0.04 *M*; ethylenediamine tetraacetate (EDTA), $3.3 \cdot 10^{-4}$ *M*; cytochrome *c*, 8×10^{-6} *M*; heart-muscle preparation, about 0.4 mg protein/ml. After temperature equilibration, the reaction was started by adding the enzyme from the side-arm. (A different reaction mixture was used for the experiment described in Fig. 1.)

Succinic–cytochrome c *reductase* was measured spectrophotometrically at 550 mμ with the following reaction mixture: phosphate, pH 7.3, 0.033 *M*; succinate, 0.0267 *M*; EDTA, 10^{-3} *M*; KCN, 10^{-3} *M*; cytochrome *c*, $1.4 \cdot 10^{-5}$ *M*; heart-muscle preparation, 0.02–0.07 mg protein/ml. The reference cuvette received all additions, including α-tocopherol where used, except the succinate. Activities at a measured temperature were converted to 25°, assuming that $Q_{10} = 2$. The reaction was started by adding the enzyme.

DPNH oxidase was measured spectrophotometrically at 340 mμ using the same reaction mixture as that for succinic–cytochrome *c* reductase, except that succinate was replaced by DPNH ($9.2 \cdot 10^{-5}$ *M*) and cyanide was omitted. Activities were corrected to 20°, as previously described[10]. The reaction was started by adding the enzyme.

DPNH–cytochrome c *reductase* was measured spectrophotometrically at 550 mμ using the same reaction mixture as for the DPNH oxidase with the addition of 0.0013 *M* KCN. The procedure, including correction for temperature, was the same as for the succinic–cytochrome *c* reductase.

Cytochrome c *oxidase* was measured spectrophotometrically at 550 mμ using ferrocytochrome *c* as substrate. The reaction mixture contained phosphate, pH 7.3, 0.033 *M*; ferrocytochrome *c*[11], $2.4 \cdot 10^{-5}$ *M*; heart-muscle preparation, 0.02–0.07 mg protein/ml. Activities at a measured temperature were converted to 25°, assuming $Q_{10} = 2$.

Diaphorase was measured spectrophotometrically at 340 mμ as previously described[10] with the following reaction mixture: phosphate, pH 7.3, 0.034 *M*; EDTA, 0.001 *M*; KCN, 0.003 *M*; DPNH, $9.25 \cdot 10^{-5}$ *M*; methylene blue, $4 \cdot 10^{-4}$ *M*; heart-muscle preparation, 0.02–0.07 mg protein/ml.

RESULTS

Effect of extraction with iso-octane

The effect of extraction of the heart-muscle preparation on six different enzymes or enzyme systems is shown in Table I. One extraction was sufficient largely to inactivate

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the DPNH oxidase or the DPNH–cytochrome *c* reductase systems. The succinic oxidase system was much more stable, four extractions causing a loss of only 25%. The succinic–cytochrome *c* reductase was particularly stable, six extractions causing a negligible loss of activity in most cases. The much greater sensitivity of the DPNH-cytochrome *c* reductase than the succinic–cytochrome *c* reductase is shown in Expt. 5, Table I, where comparative measurements were made on the same extracted suspensions. The cytochrome *c* oxidase activity, and the diaphorase activity were also partially inactivated by the extraction.

TABLE I

EFFECT OF EXTRACTION WITH ISO-OCTANE ON THE ACTIVITIES OF VARIOUS ENZYME SYSTEMS IN HEART MUSCLE PREPARATION AND THE EFFECT OF ADDED TOCOPHEROL

In Expts. 1–6, $4.65 \cdot 10^{-4} M$ α-tocopherol was used (DL in Expts. 1 and 5, D in the other expts.); in Expt. 7, $10^{-4} M$ D-α-tocopherol was used. The concentration of albumin in the last two columns was the same, varying between 0.03 and 0.43 mg/ml.

Expt.	*Activity measured*	*No. of extractions*	*Activity (control = 100)*		
			no addition	*+ albumin*	*+ albumin + α-tocopherol*
1	Succinic oxidase	0	100	90	86
		2	109	95	54
		4	75	70	35
		6	29	25	18
2	Succinic–cyt. *c* reductase	0	100	94	93
		5	79	76	87
		6	93	93	71
3	DPNH oxidase	0	100	61	117
		1	3.7	1.0	77
		2	2.0	0.5	53
		3	1.2	0.6	31
		6	1.0	0.6	9
4	DPNH–cyt. *c* reductase	0	100	142	97
		2	6.1	4.5	146
		5	3.1	2.9	143
		6	3.0	2.5	104
5	DPNH–cyt. *c* reductase	0	100		77
		3	10		70
		5	6.4		45
	Succinic–cyt. *c* reductase	0	100		96
		3	96		92
		5	62		
6	Cyt. *c* oxidase	0	100	105	149
		2	66	66	99
		4	47	38	54
		6	51	51	72
7	Diaphorase	0	100	—	—
		2	48	125	113

Effect of added α-tocopherol

Addition of α-tocopherol suspended in serum albumin markedly activated the DPNH oxidase and the DPNH–cytochrome *c* reductase activities after iso-octane extraction. Expt. 3, Table I, shows that one extraction was sufficient almost completely

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to inactivate the enzyme system, and the addition of α-tocopherol brought the system back to 77% of the control activity. After additional extractions, the degree of reactivation brought about by tocopherol was still great, but the activity in the presence of tocopherol declined with increasing number of extractions. The DPNH–cytochrome *c* reductase was also largely inactivated by one extraction, and full reactivation was obtained in some cases (*e.g.* Expt. 4, Table I) even after six extractions. Serum albumin, on its own, has little effect in the concentrations used in Table I.

The effect of added α-tocopherol on the succinic oxidase system after extraction with iso-octane is most clearly seen in Fig. 1. In this experiment, the chief effect of α-tocopherol was to prevent an initial lag in the rate of oxygen uptake, which was the main result of the iso-octane extraction. In other experiments, the succinic oxidase was definitely inactivated after repeated extractions, and was not reactivated by α-tocopherol (see Table I).

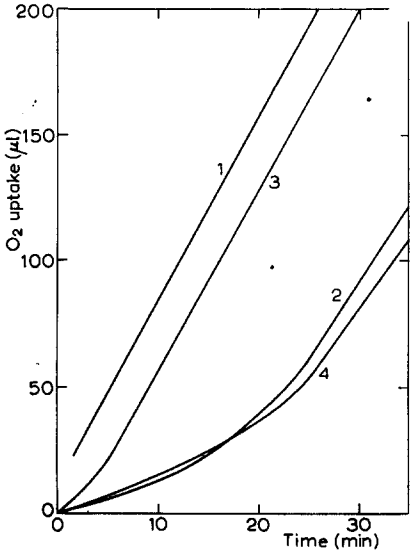

Fig. 1. Effect of extraction with iso-octane (four times) on the succinic oxidase activity of heart-muscle preparation and effect of addition of α-tocopherol ($1.7 \cdot 10^{-3} M$). Concentration of serum albumin, 0.06 mg/ml. Reaction mixture for estimation of activity of succinic oxidase; phosphate, pH 7.3, 0.03 M; succinate, 0.025 M; EDTA, $3 \cdot 10^{-4} M$; cytochrome *c*, $5 \cdot 10^{-5} M$; heart-muscle preparation, 0.3 mg protein/ml. Curve 1, not extracted; Curve 2, extracted; Curve 3, extracted + α-tocopherol; Curve 4, extracted + serum albumin.

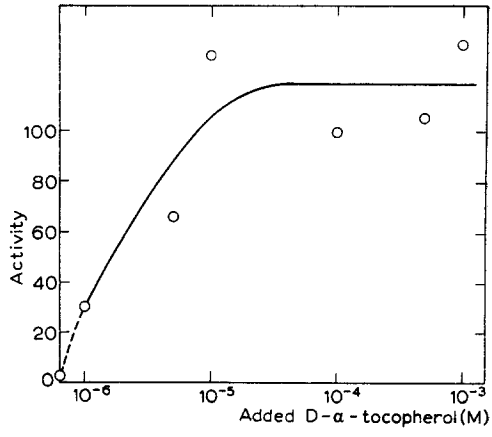

Fig. 2. Effect of different concentrations of D-α-tocopherol on the reactivation of DPNH–cytochrome *c* reductase after two extractions with iso-octane. Activity of unextracted preparation = 100. The concentration of serum albumin added with the α-tocopherol was 0.01 mg/ml with 10^{-5} and $10^{-3} M$ α-tocopherol, 0.06 mg/ml with $5 \cdot 10^{-6}$ and $5 \cdot 10^{-4} M$ α-tocopherol, and 0.12 mg/ml with 10^{-6} and $10^{-4} M$ α-tocopherol.

α-Tocopherol appreciably stimulated the cytochrome oxidase reaction, but in this case the degree of stimulation (about 50%) was not influenced by the iso-octane extraction. The diaphorase activity was restored by the addition of serum albumin without α-tocopherol.

The effect of different concentrations of α-tocopherol is shown in Fig. 2. A large

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reactivation was obtained with 10^{-6} *M* α-tocopherol, while 10^{-5} *M* gave approximately 100% reactivation. The amount required for complete reactivation was, however, found to vary greatly in different experiments (contrast Fig. 2 with Fig. 3).

Comparison between D- *and* DL-α-*tocopherol*

The comparative activity of D- and DL-α-tocopherol in the reactivation is shown in Table II. Each measurement of the activity in the presence of α-tocopherol was done in triplicate in order to reduce the effects of individual fluctuations. It appears that the D-tocopherol is much more effective than the DL-form. NASON AND LEHMAN'S[3] results also show a greater activity of the D-tocopherol.

Table III shows that pure DL-α-tocopherol and the tocopherol isolated from the heart-muscle preparation were about equally effective. The isolation procedure would be expected to lead to considerable racemization.

TABLE II

COMPARISON OF THE EFFECTIVENESS OF D- AND DL-TOCOPHEROL AS REACTIVATING AGENTS FOR THE DPNH–CYTOCHROME *c* REDUCTASE SYSTEM, AFTER ISO-OCTANE EXTRACTION

The heart-muscle preparation was extracted once with iso-octane.

Tocopherol added	*Activity*		*Reactivation by*	
	Unextracted	*Extracted*	D-α-*tocopherol*	DL-α-*tocopherol*
None	100	8.9		
10^{-4} *M* D-α-tocopherol		27.6 33.2 33.2 } 31.3*	22.4	
10^{-4} *M* DL-α-tocopherol		11.0 11.0 15.5 } 12.5*		3.6
$2 \cdot 10^{-4}$ *M* D-α-tocopherol		53.0 48.7 44.3 } 48.7*	39.8	
$2 \cdot 10^{-4}$ *M* DL-α-tocopherol		25.4 19.9 27.6 } 24.3*		15.4

* Means.

TABLE III

COMPARISON OF EFFECTIVENESS OF DL-α-TOCOPHEROL AND α-TOCOPHEROL ISOLATED FROM HEART-MUSCLE PREPARATION AS REACTIVATING AGENTS FOR DPNH-CYTOCHROME *c* REDUCTASE AFTER ISO-OCTANE EXTRACTION

The heart-muscle preparation was extracted twice with iso-octane. The concentration of α-tocopherol in both cases was $1.32 \cdot 10^{-5}$ *M*.

	DPNH-cyt. c reductase (relative activity)
Unextracted	100
Extracted	11
Extracted + serum albumin	27
Extracted + serum albumin + DL-α-tocopherol	85
Extracted + serum albumin + tocopherol from heart-muscle	95

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Effect of vitamin K_1

Since vitamin K_1 and α-tocopherol possess certain resemblances in structure, the effectiveness of these two fat-soluble vitamins in reactivating the DPNH–cytochrome *c* reductase after iso-octane extraction were compared. In disagreement with the results of NASON AND LEHMAN[3], Fig. 3 shows that vitamin K_1 was almost equally effective as α-tocopherol. NASON AND LEHMAN'S[3] results can possibly be explained by the fact that they added it in alcoholic solution instead of as a suspension in albumin. Under these conditions, most of the vitamin would be neither in solution, nor in a fine suspension.

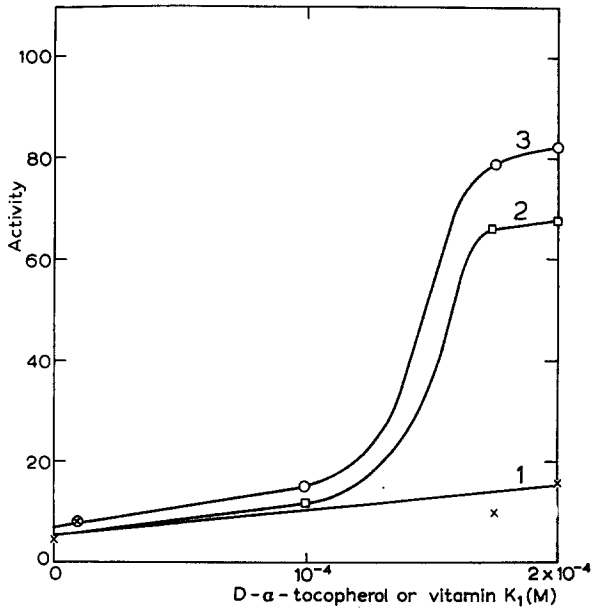

Fig. 3. Comparison of effectiveness of D-α-tocopherol and vitamin K_1 in the reactivation of the DPNH–cytochrome *c* reductase after two extractions with iso-octane. Activity of unextracted preparation = 100. Curve 1 represents the effect of albumin added alone in the same concentrations as used when the suspension of vitamin K_1 (curve 2) or D-α-tocopherol (curve 3) was added.

Effect of α-tocopherol on antimycin-inhibition of DPNH oxidase

NASON AND LEHMAN[2,3] claim that the addition of α-tocopherol partially relieves the inhibition of the DPNH–cytochrome *c* reductase by antimycin. Our experiments with the DPNH oxidase system showed no such effect of α-tocopherol (Table IV).

Experiments to test if α-tocopherol can be oxidized by the heart-muscle preparation

The possible presence of an α-tocopherol oxidase was tested by adding heart-muscle preparation to a suspension of α-tocopherol and determining the change of extinction at 292 mμ, where α-tocopherol has an absorption peak, and at 270 mμ, where α-tocopherylquinone has an absorption peak. No changes of extinction were observed. Under similar circumstances, it was possible to demonstrate the oxidation of reduced vitamin K_3[12], but an important difference is that the latter is water-soluble.

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TABLE IV

EFFECT OF DL-α-TOCOPHEROL ON INHIBITION OF DPNH OXIDASE BY ANTIMYCIN

Antimycin was allowed to react with the heart-muscle preparation for 5 min before adding the DPNH

Antimycin (M)	*% inhibition of DPNH oxidase*	
	without α-tocopherol	*with $8 \cdot 10^{-4}$ M DL-α-tocopherol*
10^{-9}	34	40
$2.5 \cdot 10^{-9}$	32	38
$5 \cdot 10^{-9}$	65	77
10^{-8}	73	73

It was also not possible to demonstrate the reduction of cytochrome *c* by α-tocopherol in the presence of heart-muscle preparation and cyanide.

The same negative results were obtained using a suspension of the crude α-tocopherol preparation isolated from heart-muscle preparation.

NASON AND LEHMAN[3] have also been unable to demonstrate that α-tocopherol can act as a hydrogen-donor.

α-Tocopherylquinone, suspended in a solution of serum albumin, was inactive as a hydrogen-acceptor from DPNH, in the presence of heart-muscle preparation and cyanide. This is also in contrast with the quinone, vitamin K_3[12].

DISCUSSION

NASON AND LEHMAN[2,3] concluded that extraction with iso-octane removed a fat-soluble component of the respiratory chain which acted between cytochromes *b* and *c*. This fat-soluble component of the respiratory chain was replaceable by α-tocopherol but was considered not to be identical with α-tocopherol.

Under our conditions, the damage caused by iso-octane extraction was not confined to a narrow region of the respiratory chain, since both the diaphorase and the cytochrome *c* oxidase activities were affected. The DPNH oxidase and DPNH–cytochrome *c* reductase activities were much more susceptible than the corresponding succinic systems. This would not be expected if iso-octane extraction specifically acted near the antimycin-sensitive factor, as NASON AND LEHMAN[2] believe, since this factor is common to both the succinic and DPNH oxidase systems. However, we were not able to find any competition between α-tocopherol and antimycin, as claimed by NASON AND LEHMAN[2,3].

The much greater damage to the DPNH systems could be caused by the fact that a component of this system is specifically removed, or because the DPNH system is much more susceptible to physical damage to the particle. The much greater reactivation by D-α-tocopherol compared with DL-α-tocopherol suggests at first sight that α-tocopherol is reactivating an enzyme system which has lost its α-tocopherol. Further examination, however, brought out the following facts which are difficult to reconcile with this view:

1. The relative effectiveness of D- and DL-α-tocopherol is much more than the expected ratio of 2.

2. Vitamin K_1 was found to be as effective as D-α-tocopherol in reactivating the system. It is significant that the heart-muscle preparation is devoid of vitamin K_1[13].

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3. Analysis showed that even six extractions with iso-octane removed only about 25% of the total α-tocopherol of the heart-muscle preparation. One extraction was usually sufficient almost completely to inactivate the DPNH–cytochrome *c* reductase.

4. If a suspension of α-tocopherol is capable of reactivating the iso-octane-extracted heart-muscle preparation by virtue of the fact that it restores an essential oxidation–reduction carrier, it would be expected that oxidation of the reduced form of this catalyst or reduction of the oxidized form would also be found. This could not be demonstrated.

Since this work was completed, a paper by DONALDSON AND NASON[14] has appeared in which these authors also show that the reactivation is not specific for α-tocopherol. The reactivating principle present in the iso-octane extract of their enzyme preparation was tentatively identified as a mixed triglyceride with stearate, palmitate and oleate components. A number of other neutral fats (*e.g.* butter and oleomargarine) were also effective. Iso-octane extraction removed only about 10% of the total tocopherol, but inactivated the DPNH–cytochrome *c* reductase by 90%.

DONALDSON AND NASON believe that iso-octane removes α-tocopherol from "active enzyme sites", while reactivating lipides release some of the remaining tocopherol, perhaps from a "bound" form. It appears more probable to us, however, that the reactivation by α-tocopherol is by virtue of some physical property which it possesses in common with vitamin K_1 which allows it to restore the physical integrity of particles damaged by extraction with iso-octane. It is even possible that the inhibition is caused by adsorption of iso-octane on the surface of enzyme (*cf.* WARBURG'S[15] explanation of inhibition by narcotics), and that α-tocopherol and other fat-soluble substances act simply by dissolving and desorbing the iso-octane. These experiments do not provide strong evidence for the participation of α-tocopherol in the respiratory chain. However, they do not disprove this contention. In fact, in the previous paper[1], reasons are given for believing that α-tocopherol might play some part in respiratory enzyme systems, particularly in oxidative phosphorylation.

ACKNOWLEDGEMENTS

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SUMMARY

1. The DPNH oxidase and DPNH–cytochrome *c* reductase systems in the Keilin and Hartree heart-muscle preparation were particularly susceptible to extraction of the preparation by iso-octane. The succinic oxidase, cytochrome *c* oxidase and diaphorase activities were also affected, but to a lesser extent. The succinic–cytochrome *c* reductase was much less sensitive.

2. Suspensions of α-tocopherol in serum albumin reactivated the DPNH oxidase and DPNH-cytochrome *c* reductase after this treatment. Full reactivation of the latter enzyme was often obtained with 10^{-5} *M* α-tocopherol. The effect of α-tocopherol on the other enzyme systems was less clear-cut.

3. D-α-Tocopherol was much more effective than the DL. The tocopherol isolated from the heart-muscle preparation was about equally effective as DL-α-tocopherol.

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4. Vitamin K_1 suspended in the same way as α-tocopherol was almost equally effective as a reactivating agent.

5. The addition of α-tocopherol did not affect the degree of inhibition of the DPNH oxidase system by antimycin.

6. No evidence was obtained that α-tocopherol would be oxidized by oxygen or by cytochrome *c*, or that the quinone could be reduced by DPNH, in the presence of the heart-muscle preparation.

7. It is suggested that the inactivation by iso-octane extraction is caused by adsorption of iso-octane on the surface of the enzyme or by some physical damage of the particle of the enzyme preparation, and that α-tocopherol reactivates by dissolving the iso-octane or by restoring the physical structure of the particle. The experiments do not provide strong evidence for the participation of α-tocopherol in the respiratory chain, nor do they disprove it.

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INCORPORATION OF RADIOACETATE INTO LIPID BY ADIPOSE TISSUE *IN VITRO*: LIPID CHARACTERIZATION

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INTRODUCTION

The ability of adipose tissue to incorporate glucose or acetate into lipid has been established by several investigators[1–5]. This investigation is concerned with characterization of the lipid components synthesized by adipose tissue from ^{14}C-1-acetate in an *in vitro* system.

METHODS

Lipid separation

Lipid separation was accomplished by a modification of the chromatographic and extraction procedure described by BORGSTROM[6]. The column used consisted of silicic acid** (13 g) which had

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** Mallinckrodt Chemical Works, Silicic Acid-100 mesh (suitable for chromatographic analysis by the method of Ramsey and Patterson).

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