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IN VITRO EFFECT OF TOCOPHEROL METABOLITES ON RESPIRATORY DECLINE IN DIETARY NECROTIC LIVER DEGENERATION*

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SUMMARY

The latent phase of necrotic liver degeneration in the rat, produced by dietary deficiency of vitamin E and Factor 3, is characterized by respiratory decline, *i.e.*, a failure of respiration of liver slices in the Warburg. Tocopherol readily reverses this defect when injected intravenously but not when added to the Warburg medium.

The *in vitro* effect of various tocopherol derivatives on respiratory decline was investigated. DL-α-tocopherol, *d*-α-tocopheryl polyethylene glycol-1000 succinate, DL-α-tocopherylhydroquinone, DL-α-tocopherylquinone, and DL-"α-tocopheroxide" (acetal of α-tocopherylquinone) did not influence the breakdown of respiration. Two tocopherol metabolites, isolated from the urines of rabbits and humans, 2-(3-hydroxy-3-methyl-5-carboxy)-pentyl-3,5,6-trimethylbenzoquinone and its γ-lactone, were found to prevent respiratory decline when added to the Warburg medium. A dose response curve was obtained. The 50% effective dose was at approx. 6.25 μg. The diacetate of the hydroquinone of the lactone was active at higher dose levels. The significance of the findings is discussed.

In addition to the effect on respiratory decline, the γ-lactone at levels of 100 μg per flask produced a stimulation of the initial O_2 consumption. The stimulatory effect was less pronounced, but present, with liver slices from normal controls.

* An account of this work has been presented Nov. 2, 1956, at the Symposium on Approaches to the Quantitative Description of Liver Function at San Francisco, Calif.

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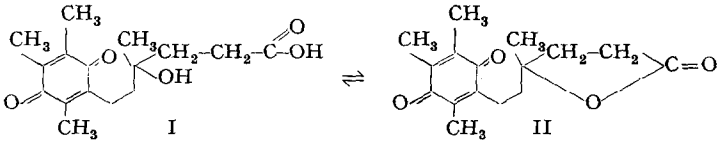
INTRODUCTION

Fatal necrotic liver degeneration can be produced in the rat and in other species by dietary deficiency[1]. The disease results from the simultaneous lack of two different factors, namely vitamin E[2] and Factor 3[3*]. The latter has recently been identified as an organic selenium compound[4]. L-Cystine, previously also considered a protective agent[5], has now been shown to owe its activity to a trace contamination by Factor 3-active selenium[31]. Each of the two protective factors prevents liver necrosis independently. For 50 % protection against death 0.7 mg of DL-α-tocopheryl acetate are necessary in 100 g of diet[6].

Under the standardized conditions in use at the National Institutes of Health, death from liver necrosis in weanling rats occurs on the average after 21 days on the deficient diet. The sudden appearance of liver necrosis shortly before death is preceded by a *latent* period which lasts 8–12 days[7]. The existence of this phase is evident (a) from a profound disturbance of the ultrastructure of the cell, involving a cystic degeneration of the endoplasmic reticulum (microsomes) and swelling and disappearance of mitochondria, as seen by electron microscopy[8]; and (b) from respiratory decline, a metabolic disturbance demonstrable in liver slices of such animals in the Warburg apparatus[9]. Respiratory decline, described in detail elsewhere, consists of a failure of O_2 consumption after 30–60 min of initially normal respiration. The lesion is associated with the breakdown of [^{14}C]acetate utilization for CO_2 production, fatty acid synthesis, and ketonbody formation[10]. The present communication deals with an attempt to identify the form of tocopherol which *in vitro* prevents respiratory decline.

The concept arose that tocopherol may be converted into an "active form" before exerting its metabolic effect in the organism, since direct addition of the vitamin to the Warburg medium is completely ineffective against the respiratory lesion, as shown below, whereas injection of emulsified vitamin E into the *portal* vein immediately before extirpation of the liver will reverse the condition completely. This has been shown by two-step Warburg experiments[11]. Doses of 200 μg of DL-α-tocopherol suffice to reconstitute normal maintenance of O_2 consumption over extended periods of observation. A comparison of graded dose levels of various forms of DL-α-tocopherol, and also of the β-, γ- and δ-tocopherols showed in general good agreement with the results of the classical dietary vitamin E bioassay against resorption sterility. Injection of the tocopherol into *peripheral* veins produced exactly the same dose response curve as that obtained by direct application to the liver through the portal route[12].

A number of well-known derivatives of α-tocopherol, some of which showed activity when injected intraportally, were ineffective when added to the Warburg medium. In 1956 the isolation of two metabolites of α-tocopherol from the urines of rabbits and humans was reported[13]. On the basis of chemical and spectroscopic evidence the following structures were assigned to them[14]:



* Since various compounds of selenium show greatly different biological activity, the term "Factor 3" is used to designate the biologically active, selenium-containing component, or components, present in nutrients and other materials of natural origin.

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2-(3-hydroxy-3-methyl-5-carboxy)-pentyl-3,5,6-trimethylbenzoquinone (Compound I) and its γ-lactone (Compound II).

A crystalline derivative was made by the reductive acetylation of the lactone and was shown to be the hydroquinone diacetate. Testing of these metabolites of α-tocopherol for *in vitro* effectiveness showed a high degree of activity in the prevention of the respiratory lesion.

EXPERIMENTAL

For the present investigations inbred rats of the Fischer 344 strain were used which routinely demonstrate well-developed respiratory decline after 13 to 15 days on the liver necrosis-producing diet. Experimental details concerning the production of the disease[15], the composition of the diet[16] and the Warburg procedure[11] have been published elsewhere. Solutions of the substances to be tested were prepared in minimal amounts of ethanol; these never exceeded 0.001 ml per flask and were without effect on O_2 consumption. The solutions were added to the Warburg medium immediately before addition of the liver slices.

The results were evaluated as follows: The O_2 consumption ($Q_{O_2(F100)}$ = O_2 consumption, in microliters, per hour per 100 mg of liver slices) for the first 30-minute interval (a) was compared to that of the fourth 30-minute interval (b). The degree of respiratory decline was expressed in per cent of the initial respiration ($d = (a - b)/a \times 100$). By comparison of respiratory decline of unsupplemented slices (dA) with that of supplemented slices from the same animal (dB), the effect of the supplement can be expressed as per cent prevention (% prevention = $(dA - dB)/dA \times 100$).

RESULTS

It is readily seen from Table I that DL-α-tocopherol, d-α-tocopheryl polyethylene glycol 1000 succinate, DL-α-tocopherylhydroquinone, DL-α-tocopherylquinone, and DL-"α-tocopheroxide" (acetal of α-tocopherylquinone)[17]* at 200 μg or above did not influence respiratory decline to any significant degree when added to 3 ml of medium.

The metabolites, on the other hand, were highly active. The oxidized (quinone) forms of the metabolite are quite stable. When the free γ-hydroxy acid (Compd. I) was added at a level of 1.56 μg/3 ml of medium and approx. 100 mg of liver slices, a significant effect was produced. The 50 % effective dose for protection against the breakdown of respiration was found to be approx. 6.25 μg. Higher doses prevented respiratory decline practically completely. Similar results were obtained with the lactone (Compd. II). At the 6.25 μg and the 25 μg levels the lactone and the hydroxy acid were about equally effective in preventing respiratory decline. However, the lactone did not seem significantly active at the 1.56 μg level.

With the lactone, the evaluation of the results is complicated by an additional effect of the substance on O_2 consumption (Table II): There is a considerable stimulation of the initial O_2 consumption by liver slices. With 100 μg of lactone the initial 30 minute O_2 consumption is increased by approximately 70 %; smaller doses produce much less, if any, stimulatory effect. The experiments with liver slices from normal

* Compounds obtained from the Distillation Products Industries, Eastman Kodak Company, Rochester, New York.

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TABLE I

In vitro EFFECT OF TOCOPHEROL DERIVATIVES ON RESPIRATORY DECLINE

Supplement	*Dose in µg/flask*	*No. of expts.*	Q_{O_2} *(F100), average*				*% Resp. decline*		*Prevention in %*
			A Control		*B With supplement*		*d A*	*d B*	
			0–30 min	*90–120 min*	*0–30 min*	*90–120 min*			
DL-α-Tocopherol	200	5	332	94	347	116	72	66	(8 ± 3.6)
d-α-Tocopheryl polyethylene glycol-1000 succinate	1000	5	274	66	269	72	75	72	(5 ± 4.2)
DL-α-Tocopherylhydroquinone	200	5	308	91	310	70	71	77	(—12 ± 12.7)
DL-α-Tocopherylquinone	200	4	322	94	316	83	72	74	(—3 ± 6.4)
DL-α-"Tocopheroxide" (acetal of quinone)	200	4	295	101	286	65	67	77	(—19 ± 11.7)
Compound I:	1.56	5	251	87	290	145	66	51	27 ± 8.6
Tocopherol-metabolite	6.25	5	228	60	258	161	73	39	50 ± 20.6
(free acid, quinone form)	25	4	243	69	294	256	71	13	83 ± 4.4
	100	5	252	77	58	247	70	4	92 ± 5.3
Di-acetate:	6.25	5	269	56	293	70	79	77	(3 ± 5.3)
Di-acetyl derivative of	25	5	269	56	299	105	79	65	16 ± 9.3
lactone (hydroquinone form)	100	5	280	84	246	218	70	11	83 ± 3.2

TABLE II

In vitro EFFECT OF LACTONE ON RESPIRATORY DECLINE

Supplement	*Dose in µg/flask*	*No. of expts.*	$Q_{O_2(F100)}$, *average*				*% Increase of initial O_2 consumption by lactone**	*% Resp. decline*		*% Prevention*
			A Control		*B With addition*			*d A*	*d B*	
			0–30 min	*90–120 min*	*0–30 min*	*90–120 min*				
Necrosis-producing diet										
Compound II: Lactone of metabolite (quinone form)	1.56	5	262	95	*291*	78	(11)	75	74	(−12 ± 13.8)
	6.25	5	258	72	*279*	198	(8)	72	29	64 ± 12.4
	25	6	246	62	*292*	238	19	75	21	74 ± 16.4
	100	6	262	63	*442*	335	69	76	24	67 ± 0.26
Complete diet**										
Compound II: Lactone of metabolite (quinone form)	100	5	262	257	334	274	27	—	—	

* Increase $= \dfrac{(B_{0-30} - A_{0-30})}{A_{0-30}} \times 100$.

** Hunt Club Dog Food.

control animals maintained on an optimal, natural diet show that the stimulatory effect is also present in this instance. However, it seems to be much less pronounced.

The reduced (hydroquinone) forms of the metabolites could not be assayed directly because of their marked lability with respect to oxidation. The diacetate of the hydroquinone form of the lactone, however, was tested (Table I). It was found to be active, but higher doses were required for prevention of the metabolic lesion. The observed activity may be due to enzymic hydrolysis of the diacetate in the Warburg vessel.

DISCUSSION

The exact metabolic function of tocopherol, as well as the site and nature of the primary metabolic defect in livers undergoing necrotic degeneration, are presently not known. Results published earlier from this group[9,10,11] point to the breakdown of electron transfer mechanisms and the (subsequent?) failure of ATP formation in livers undergoing respiratory decline. With succinate as substrate, a significant difference has also been detected between liver mitochondria from vitamin E supplemented rats and those from vitamin E deficient animals during the latent phase of necrotic degeneration[18]. The observed phenomenon resembles the respiratory decline in liver slices. Thus, it seems feasible that the present results are related to an active role of tocopherol, or of a metabolic derivative of tocopherol, in enzymic oxidation-reduction reactions, as postulated previously[6].

From most other metabolic lesions which have been attributed to vitamin E deficiency, respiratory decline, of course, differs fundamentally in that it requires the concomitant absence of Factor 3-selenium, besides that of tocopherol. The dietary condition which produces necrotic liver degeneration in the rat and in other species will produce widespread damages in other animals, such as multiple necrotic degeneration (heart, liver, kidney and muscle necrosis) in the mouse[19] and exudative diathesis in chicks[20]. With the latter test animal, the nature of Factor 3 as a selenium compound was independently confirmed[21,22]. The fact that tocopherol and Factor 3-active selenium compounds have to be absent before liver necrosis and other profound degenerative lesions can occur must mean that the functions of these two essential dietary agents have an important connection in intermediary metabolism*. Taking the results accumulated thus far into account, a possible working hypothesis for the fact that vitamin E and Factor 3-selenium are independently protective has been developed. It is based on the assumption that these two factors participate in alternate pathways of electron transfer, both of which have to be impaired before necrosis ensues[7].

It is conceivable that the organism compensates for the lack of one or the other essential agent by shifting to alternate pathways of electron transfer, thereby obscuring the primary metabolic defects. Thus, in uncomplicated tocopherol deficiency for example muscular dystrophy of the rabbit, an increase of O_2 consumption is frequently but not consistently observed[23,24]. In view of this consideration it is likely that respiratory decline, during the latent phase of necrotic liver degeneration (and also during the latent phase of the necrotic degeneration of the heart muscle, of

* The relation of Factor 3-active selenium compounds to respiratory decline will be the subject of a separate report.

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pheripheral muscle, of kidney, etc. in other species) offers a system which is well suitable for the clarification of the function of vitamin E. Here is a reproducible, serious metabolic defect involving oxidative functions which is promptly reversible by application of physiological doses of tocopherol *in vivo*, as well as by the presence of the tocopherol metabolites *in vitro*. It is interesting to note that no other presently known derivative of vitamin E shows this capacity*.

The possibility that the metabolites reach the site of action in the liver slice more readily than α-tocopherol since they are less hydrophobic than the latter must at present be left open. However, this interpretation is contradicted by the fact that the water-soluble DL-α-tocopheryl polyethylene succinate, which when injected into the portal vein is far superior to D-α-tocopherol[11], is *in vitro* as ineffective as the emulsified α-tocopherol. The *in vitro* superiority of the metabolite may rather be related to the fact that not α-tocopherol *per se*, but a metabolic conversion product of the latter is the form which is catalytically active.

Participation of vitamin E in electron transfer systems has recently been investigated by several groups: The possible role of tocopherol in the chain of respiratory enzymes has been studied with particulate preparations from heart muscle by NASON and collaborators[25,26] as well as by SLATER *et al.*[27,28]. From these studies it is clear that certain preparations of DPNH-cytochrome *c* reductase (among others) contain α-tocopherol in more or less bound form. These preparations can be inactivated by isooctane extraction and also by repeated freezing and thawing. Such inactivated systems are reactivated by suspensions of α-tocopherol. As yet, however, no evidence seems to be available for the direct participation of α-tocopherol as such in the electron transfer chain: the vitamin does not seem to be oxidized by O_2 or cytochrome *c*, nor can its quinone, in turn, be reduced by DPNH in these isolated systems. Therefore, the alternative that α-tocopherol acts as a link between electron transfer and oxidative phosphorylation, originally suggested by WEIL-MALHERBE[29] and formulated in more detail by MARTIUS[30], may also have to be considered in the current context.

The pronounced stimulation of the initial O_2 consumption produced by the lactone (Compd. II) cannot readily be explained as a direct substrate effect, since the quinone does not lend itself to oxidation. It is unlikely that the liver cell is capable of oxidizing the sidechain of the molecule. It may be possible that Compd. II uncouples oxidative phosphorylation, or that it assists in the more effective utilization of some other intermediate. The phenomenon is under further investigation.

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* A study of similar effects obtained with synthetic antioxidants will be published elsewhere.

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