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In vitro Inhibition of Glycogenesis by D-α-Tocopherol.* (26348)

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During a recent study on the glycolytic cycle in Vit. E-deficient rabbits(1,2), it was observed that D-α-tocopherol lowered glycogen levels and lactic acid production in muscle. Zierler *et al.*(3) reported a similar finding for rat diaphragm after *in vivo* administration of α-tocopheryl phosphate. On the basis of data from several pertinent experiments, these authors concluded from indirect evidence that the phosphorylated tocopherol inhibited at the phosphoglucomutase site. It was the purpose of the present investigation to study more directly the action of free tocopherol on glycolysis.

Experimental. Colonies of white, New Zealand rabbits were reared on a Vit. E-deficient diet as previously reported(4). Half the animals (control) received oral supplements of DL-α-tocopheryl acetate and muscle strips were prepared as previously described(1). Oxygen uptake was determined for one hour in presence or absence of D-α-tocopherol with the usual Warburg technics. The tocopherol solution was prepared immediately before use by homogenizing 10 mg of the compound in 0.2 ml of propylene glycol in a 1 ml all glass homogenizer. At termination of the one hour incubation interval, suitable aliquots of tissue were used for estimation of glycogen and lactic acid(1). Isooctane extractions of the medium after incubation were shown to contain 70 to 90% of the added tocopherol as the intact chroman or para quinone by their characteristic ultraviolet absorption.

D-α-tocopherol was also tested on individual purified enzyme systems containing commercially available phosphorylase a, glucose-6-phosphate dehydrogenase and hexokinase. Phosphoglucomutase was also studied and this material was generously supplied by Dr. O. Bodansky.

Phosphorylase a activity was followed by estimating the release of inorganic phosphate while absorption at 340 mμ by reduced triphosphopyridine nucleotide was used to measure the activity of glucose-6-phosphate dehydrogenase(1). The hexokinase reaction was studied by determining the disappearance of glucose according to the method of Nelson(5) as modified by Somogyi(6). The phosphoglucomutase procedure was that of Bodansky(7) in which the disappearance of glucose-l-phosphate (or inorganic phosphate) was followed.

Results. The *in vitro* influence of tocopherol on glycogen levels and lactic acid production in rectus femoris of rabbit is shown in Table I. In the presence of exogenous glucose (0.1 ml of a 10% solution) tocopherol caused a distinct decline (19 to 43%) in glycogen levels in both control and Vit. E-deprived animals. This fall in titer of muscle glycogen was not completely alleviated by increasing the rate of glucose-6-phosphate synthesis from glucose. Addition of 1 mg hexokinase and 1 mg adenosine triphosphate

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TABLE I. *In Vitro* Effect of D-α-Tocopherol on Glycogen and Lactic Acid Levels of Skeletal Muscle.

Condition of animal	No. of animals	Additions	Glycogen, mg/100 mg dry wt* Added tocopherol None	1 mg	% change	Lactic acid, mg/100 mg dry wt* Added tocopherol None	1 mg	% change
Control	6	None	1.44 ±.11	.86 ±.02	−40	.65 ±.08	1.78 ±.16	+174
"	"	1 mg hexokinase + 1 mg ATP	1.95 ±.11	1.32 ±.15	−32	.81 ±.11	2.11 ±.13	+160
Deficient	5	None	.98 ±.10	.56 ±.04	−43	.61 ±.08	1.49 ±.09	+144
"	"	1 mg hexokinase + 1 mg ATP	1.19 ±.10	.97 ±.18	−19	.67 ±.04	1.67 ±.15	+150

* Mean ± S.D.

stimulated glycogen deposition on the average 29% for control and 64% for deficient animals. However those tissues exposed to tocopherol still gave lower glycogen values.

An estimate of lactic acid formation showed an increase in muscle lactate ranging from 144 to 174% (Table I) for all groups. Introduction of hexokinase had a similar slight effect on lactic acid production in both groups of animals. Lactate content was increased by 22 to 24% for control and 10 to 12% for deficient muscle. However the difference between tocopherol-treated and untreated groups remained unchanged.

That a specific concentration of tocopherol was necessary to decrease muscle glycogen levels can be seen in Fig. 1. Glycogen depletion was discernible at tocopherol levels of 0.6 mg for 250 mg wet weight of skeletal muscle. The picture was the same for control and Vit. E-deficient rabbits.

The same figure shows the *in vitro* effect of tocopherol on oxygen uptake by skeletal muscle from control animals. There is a steady and significant increase. Addition of tocopherol to Vit. E-deficient muscle results in a decrease in respiration of the muscle(1). It is to be recalled that oxygen consumption in muscle from Vit. E-deprived animals may be elevated from a slight to a marked degree (reviewed in reference 4).

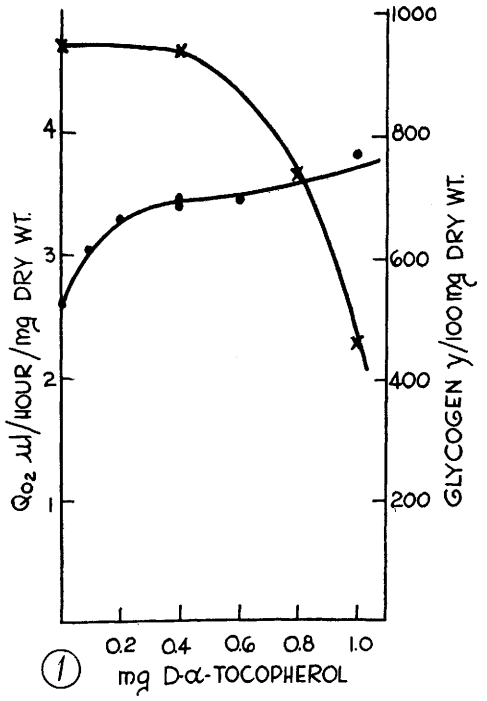
Extension of this investigation to purified enzyme systems demonstrated that tocopherol (0.5 to 1.0 mg) had no influence on the phosphorylase a system, on conversion of glucose-6-phosphate to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase or on the hexokinase system. However in the instance of phosphoglucomutase, conversion of glucose-1-phosphate to glucose-6-phosphate proceeded to approximately 90% in the control incubation and to only 70% in presence of tocopherol (Table II).

The extent of tocopherol interference with the function of phosphoglucomutase became more apparent when the enzyme level was varied. Results of this experiment are depicted in Fig 2. The control system without tocopherol required 3 μg of enzyme per ml of reaction mixture to reach maximum transfer of the phosphate group from position 1 to 6. This level of enzyme was completely inhibited by 1 mg of tocopherol. Doubling the enzyme titer released the inhibition by approximately 70%. No inhibition was ob-

TABLE II. Effect of D-α-Tocopherol on Purified Phosphoglucomutase.*

Enzyme	Tocopherol added, μg	Density	% change
None	0	.38	
"	1000	"	
Phosphoglucomutase	0	.04	
"	500	.08	−12
"	1000	.14	−30

* Glucose-1-phosphate disappearance was estimated in a solution containing 0.1 ml of 0.03 M $MgSO_4$, 1 ml of 0.1 M tris (hydroxymethyl) aminomethane, 0.5 ml of phosphoglucomutase (30 μg/ml in 0.1 M histidine), 0.5 ml 0.025 M glucose-1-phosphate and 0.4 ml of water. 1 ml of reaction mixture was added to cold 5 ml of 5% trichloroacetic acid and after 5 min. a 1 ml aliquot was diluted with 3 ml of water, 1 ml of 5 N H_2SO_4 and heated in a boiling water bath for 5 min. To the cooled solution was added 3.6 ml of water, 1 ml of 2.5% ammonium molybdate and 0.4 ml of the Fiske-SubbaRow reagent.



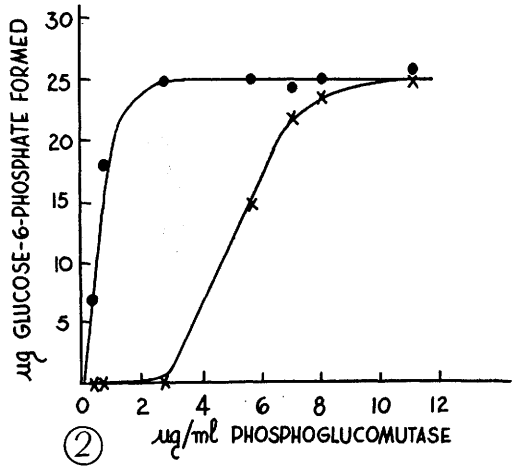


FIG. 1. Effect of varying concentrations of D-α-tocopherol on glycogen (×——×) and oxygen uptake (●——●).

FIG. 2. Formation of glucose-6-phosphate with varying concentrations of phosphoglucomutase in absence (●——●) and presence (×——×) of one mg D-α-tocopherol.

tained at phosphoglucomutase concentrations above 9 μg/ml of the reaction mixture.

Discussion. Zierler *et al.*(3) in an early work had found that tocopheryl phosphate decreased oxygen consumption of rat diaphragm by 25%. This effect on respiration was abolished by addition of glucose or certain members of the Krebs' cycle. Therefore these investigators interpreted the results to mean that tocopheryl phosphate exerted its influence between glycogen and glucose-6-phosphate. Additional evidence from the ability of tocopheryl phosphate-treated diaphragm to dissimilate glycogen and convert glucose-6-phosphate toward lactate led these workers to indicate phosphoglucomutase as the site of tocopheryl phosphate inhibition.

In the present study it was more directly demonstrated that tocopherol could partially inhibit phosphoglucomutase and therefore glycogenesis or glycogenolysis. In the presence of sufficient quantities of glucose-6-phosphate from exogenous glucose or due to added hexokinase, the influence of tocopherol on phosphoglucomutase could be obscured. Some of the excess glucose-6-phosphate can be converted to glycogen while glycolysis leads to an increased production of lactate.

Although it has been reported that activity of phosphoglucomutase declines in human muscular dystrophy(8) and in mice with inherited dystrophy(9), tocopherol levels in blood and tissues were within normal limits. However Carpenter, *et al.*(10), using a Vit. E-deficient diet which permitted rabbits to live 100 days, found no change in level of phosphoglucomutase. The importance of tocopherol in glycogenesis through its influence on phosphoglucomutase remains to be determined.

Summary. The *in vitro* influence of D-α-tocopherol on glycogen levels, lactic acid formation and oxygen uptake has been studied in rectus femoris muscle from control and Vit. E-deficient rabbits. It was shown that less than one mg quantities of tocopherol decrease glycogen stores, increase lactic acid formation in both groups of animals and increase oxygen consumption in control muscle. In purified enzyme systems it was found that tocopherol inhibited phosphoglucomutase and not phosphorylase, hexokinase or glucose-6-phosphate dehydrogenase.

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Effect of Hemorrhagic Shock on Viability of Invading Bacteria.* (26349)

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There is now substantial evidence that bacterial endotoxins play a deciding role in development of irreversibility to transfusion in various types of traumatic shock(1,2). Much of this evidence incriminates the intraintestinal bacteria or their endotoxins, which are absorbed from the intestinal lumen into the circulating blood in the normal as well as the shocked animal(3). In the normal animal the endotoxin is taken up and detoxified by the R.E. system, so that there is no detectable amount in the circulation. The R. E. system of the shocked animal can take up very little, and of that it cannot detoxify more than a small fraction(4). The endotoxin is, therefore, free in the circulation to inflict damage on the peripheral vessels, and so to prevent recovery from shock.

It is of considerable importance to know to what extent the gram-negative bacteria in the tissues, whether they are invaders from the gut or contaminants of wounds, might contribute to the amount of circulating endotoxin. The data to be presented show (1), that bacteria regularly traverse the intestinal barrier; and (2) that even though they multiply when the antibacterial defenses are down, their number in the first filtering depots (liver and mesenteric lymph nodes) is too small to contribute significantly to the endotoxemia.

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Methods. With the use of aseptic technic and sterile equipment, healthy adult white rabbits, with a normal intestinal flora, were given heparin and then bled from a femoral artery, cannulated under local anesthesia, into an elevated reservoir, so as to produce a continuous arterial pressure of 50 mm Hg(5). Two groups (B & C) were maintained at this pressure for 1½ hours, and a third group (D) for 6 hours, after which all the shed blood was returned. Four or 8 hours after this replacement transfusion, a sterile laparotomy was performed under ether anesthesia. The abdomen was prepared for laparotomy by burning the alcohol-soaked skin. Separate sets of instruments were used to incise the skin, to open the peritoneum, to biopsy the ileal lymph nodes, and the liver.

The biopsies were placed in sealed vials of brain heart infusion broth for transfer to the laboratory. Before culturing the samples were removed from the containers, placed in Petri dishes, and exposed to ultraviolet light for 15 minutes to destroy any surface contaminants. They were then ground in a tissue grinder with either brain heart infusion broth or trypticase soy broth. The resultant suspension was inoculated on heart infusion blood agar plates containing 10% defibrinated horse blood, and into brain heart infusion broth and Brewer thioglycollate broth. One set of cultures was incubated aerobically and another anaerobically in Brewer jars under 95% N_2 and 5% CO_2. In most in-