Dihydrotachysterol-Induced Changes in Citric Acid Cycle Oxidations*

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ABSTRACT: The *in vitro* and *in vivo* effects of dihydrotachysterol (DHT) on the oxidation and oxidative phosphorylation of various intermediates of the citric acid cycle by rat kidney preparations have been studied. It has been demonstrated that the *in vivo* administration of DHT to rats has a pronounced inhibitory effect on citric acid cycle oxidations measured in kidney mitochondrial preparations. No effect of the sterol was found on oxidative phosphorylation. Although the sterol *in vitro* inhibited endogenous kidney respiration to a significant degree, it was not possible to demonstrate an *in vitro* effect on the oxidation of various substrates using kidney homogenates, mitochondria, and mixed subcellular preparations.

A large body of evidence has been reported within the past 10 years which demonstrates that DHT,[1] PTH, and vitamin D exhibit a number of diverse biochemical effects in addition to the classic rise in serum calcium. Although it is believed that these other effects are associated with it, they have not led to a clearer understanding of the hypercalcemic phenomenon. The effect of the hypercalcemic agents on citric acid metabolism has been of considerable interest in recent years. It is now generally agreed that there exists an intimate relationship between calcium levels of the body and citrate levels in tissues and body fluids (Harrison, 1956).

Steenbock and Bellin (1953) have shown that the citrate content of blood, bone, kidney, and the small intestine of rats on normal or low phosphorous rachitic rations is increased by physiologic doses of vitamin D. DeLuca *et al.* (1957a,b; DeLuca and Steenbock, 1957) have demonstrated that the *in vitro* and *in vivo* administration of vitamin D inhibits the oxidation of citrate and isocitrate in rat kidney homogenates and mitochondria. Oxidative phosphorylation is not affected by the vitamin. Recently, Norman and DeLuca (1964) reported a large accumulation of ^{14}C-labeled citrate after incubation of bone slices with vitamin D. This increase was shown to be due to inhibited metabolism of this intermediate rather than accelerated synthesis from radioactive precursors.

Terepka and Chen (1962) concluded from studies of calcium and phosphorous metabolism in humans that the effects of DHT are virtually identical with those elicited by large doses of vitamin D and only superficially resemble those of PTE.

Both vitamin D and DHT inhibited a partially purified preparation of aconitase (Bruchmann, 1962). The sterol was shown to be about five times more inhibitory than vitamin D on a molar basis. Of particular interest, however, was the fact that at low concentrations DHT inhibited this enzyme-catalyzed reaction by 50%, whereas vitamin D, at the same concentration, had little (less than 10%) effect.

In this report we present evidence that DHT has effects at the subcellular level which are very similar to those reported for vitamin D. The *in vivo* administration of DHT markedly inhibited the oxidation of citrate and isocitrate while it had either a much smaller effect, or none at all, on the oxidation of other substrates by rat kidney mitochondrial preparations.

Materials and Methods

Chemicals. All solvents and inorganic chemicals used were reagent grade. The citric acid cycle intermediates and yeast hexokinase were purchased from the California Corp. for Biochemical Research. Disodium ATP and cytochrome *c* were products of Nutritional Biochemicals Corp. and Sigma Chemical Co., respectively. The crystalline DHT_2, obtained from the Mann Research Laboratories, was used without further purification. The sterol was shown to be homogenous by paper chromatography in several solvent systems and by melting point measurements. Ultraviolet and infrared analyses showed that the crystalline substance contained at least 99% DHT_2.

Animals. Male Sprague–Dawley rats (175–200 g) were used throughout this investigation. They were given a regular laboratory diet and water *ad libitum*. In the *in vivo* studies, the animals were placed under slight ether anesthesia and 2 ml of blood were with-

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[1] Abbreviations used throughout the text: DHT, dihydrotachysterol; PTE, parathyroid extract; PTH, parathyroid hormone; Na_2ATP, disodium adenosine triphosphate.

drawn by cardiac puncture and set aside for calcium analysis (Ashley and Roberts, 1957). They then received an intraperitoneal injection of 1 mg of DHT in 0.5 ml of corn oil. Five days after this initial injection, the rats were given a second dose of the sterol. On the 10th day, *i.e.*, 5 days after the second injection, the animals were anesthetized and blood was taken as before. Approximately 3 hr after this heart puncture, the animals were killed by decapitation, bled as completely as possible, and the subcellular particles were isolated. The control animals were treated identically except that they received injections of corn oil only.

General Experimental Procedures

The Warburg manometric technique was used for the measurement of the citric acid cycle oxidations (Umbreit *et al.*, 1959). The reaction vessels were kept on ice during the addition of the "premix"[2] and other reactants and were then transferred to the manometers and equilibrated for 10 min. The manometers were closed and readings were taken every 10 min for 1 hr. The incubation temperature was 37° in the case of homogenates and 30° when mitochondria or microsomes were used. Air was the gaseous phase. When employed, the hexokinase–glucose trapping system was tipped in after the manometers were closed.

The protein concentration was measured by the biuret reaction. Crystalline bovine serum albumin was used as the standard. The results of the oxidations are reported in terms of microatoms of O_2 consumed per milligram of protein or per milligram of mitochondrial nitrogen (calculated mg of N = mg of protein/6.25). For the determination of the amount of inorganic phosphate liberated, the flask contents prior to and following incubation were deproteinized with 10% perchloric acid. The filtrates were analyzed for inorganic phosphate by the method of Fiske–Subbarow (1925).

Preparation of Kidney Homogenates, Mitochondria, and Microsomes. After the animals were sacrificed by decapitation, the kidneys were rapidly excised and cooled in ice-cold isotonic sucrose. The fascia and capsule were removed with care taken to keep the tissue as cold as possible during this manipulation. The kidneys were then blotted, weighed, and minced. A 10% homogenate was prepared in 0.25 M sucrose by means of a motor-driven Potter–Elvehjem homogenizer fitted with a Teflon pestle. Kidney mitochondria and microsomes were prepared by differential centrifugation according to the method of Schneider (1948).

Results

In Vitro Experiments. The results of the *in vitro* effects of DHT on the oxygen consumption of rat kidney homogenates are presented in Table I. Endogenous respiration amounted to approximately (11 μatoms/mg of protein)/hr and represented 100% activity. It can be seen that the medium used to solubilize the sterol had no appreciable effect on the O_2 uptake of this preparation. However, when 2 μmoles of DHT was added to this medium, it brought about a decrease in O_2 consumption of 4.4 μatoms compared to the control flasks, or almost a 41% decrease. It should be noted that four flasks in each group represent one experimental run so that the data presented here are averages of four separate experiments.

TABLE I: *In Vitro* Effect of DHT on Endogenous Kidney Respiration.

Conditions[a]	O_2 Consumption (μatoms/mg of protein)/hr)	% Control
Homogenate (16)[b]	10.8 ± 0.9[c]	—
Homogenate carrier (16)	10.2 ± 0.5	94
Homogenate + DHT (16)	6.4 ± 0.3	59

[a] Each experimental flask contained, in a final volume of 3.0 ml, the following reactants: 50 μmoles of potassium phosphate buffer, pH 7.3; 300 μmoles of sucrose; 20 μmoles of $MgCl_2$; 6 μmoles of disodium ATP; and 0.5 ml of 10% kidney homogenate (6 mg of protein) in the main compartment. The center well contained 0.20 ml of 2 N NaOH adsorbed on filter paper. DHT (2 μmoles) was placed in the side arm in 0.20 ml of solution with Tween 20–phosphate buffer solution. Control vessels contained the identical reactants with the exception that only the carrier was placed in the side arm. [b] Number of flasks. [c] Mean plus and minus standard deviation.

During the course of preliminary experiments, it became apparent that the solubility of the sterol in the aqueous reaction medium was of particular importance. A number of emulsifying agents and their mixtures which had been reported in the literature as dispersing media for steroids, vitamin D, and similar hydrophobic compounds were tested but met with limited success in our hands. Some of the vehicles used were: proplyene glycol, proplyene glycol in 10% ethanol, sodium cholate, bovine serum albumin, and bovine serum albumin in potassium phosphate buffer. None of these carriers proved entirely satisfactory, *i.e.*, those agents (propylene glycol) which formed uniform suspensions alone reduced the oxidative capacity of the tissue preparations. Agents which did not affect the oxidation of the preparations formed flocculent suspensions and therefore it was impossible to know accurately what amount of sterol was actually being delivered into the flask. The following procedure for suspending crystalline DHT for the *in vitro* studies

[2] The "premix," which was made up on the day of the experiment, consisted of a given volume of potassium phosphate buffer, pH 7.3, which contained specific amounts (see table legends) of $MgCl_2$, ATP, sucrose, etc. After all the additions had been made, the pH of this solution was adjusted to pH 7.3 with a mixture of 0.5 N KOH–0.3 N NaOH.

was developed. This method consisted of adding a given amount of sterol (maximum amount about 18 mg) to a small (2 ml) test tube. Tween 20 (100 μl) was then added and a paste was made by grinding with a small glass rod. To the paste was then added potassium phosphate buffer, pH 7.3, to a final volume of 2 ml. The resulting suspension was very homogeneous. This suspension (200 μl) (containing 10 μl of Tween 20), without the sterol, was added to the incubation flasks and no change in the oxidation rates occurred (Table I). When the amount of Tween 20 was increased to 50 μl/2 ml of buffer–suspension, a decrease of 45% in O_2 consumption was consistently observed in kidney homogenates. Oxygen consumption was completely abolished in these preparations when 200 μl of Tween 20 alone was added to the incubation medium. Accordingly, in the *in vitro* experiments, 200 μl of the Tween 20 phosphate buffer suspension (100 μl of Tween 20 in 1.9 ml of phosphate buffer), containing the desired amount of DHT, was added to the contents of the flasks.

The effect of the sterol on the oxidation of citrate incubated with homogenates was then tested. Using this system, it was not possible to demonstrate any effect on the oxidation of citrate. Succinate, added at the 15-μM level, was also used as an oxidizable source with results similar to those obtained for citrate. It was also demonstrated that DHT had no effect on the oxidation of citrate, succinate, and pyruvate primed with fumarate incubated with kidney mitochondria or mitochondria plus microsomes.

The action of DHT on the efficiency of coupled phosphorylation was studied using various citric acid cycle intermediates (Table II). It can be seen that the addition of succinate gave the most active oxidative preparations. The oxidation of citrate, succinate, α-ketoglutarate, and glutamate was not changed to any degree by the addition of the sterol. It is also apparent that there is no difference in the amount of inorganic phosphate liberated between the control and the sterol-treated preparations. These data are also reflected in the P:O ratios.

In Vivo Experiments. The methods used are described under the sections entitled Animals and General Experimental Procedures. The hypercalcemic effect of *in vivo* administered DHT was observed in each of the injected rats (Ashley and Roberts, 1957). In all instances, the increase in the serum calcium values of the treated animals was at least 1.9 mg % higher than those of the control rats. The intermediates examined in this system were: citrate, 45 μmoles; *d*-isocitrate, 20 μmoles; α-ketoglutarate, 30 μmoles; glutamate, 30 μmoles; succinate, 20 μmoles; and pyruvate, 20 μmoles, primed with 5 μmoles of fumarate.

A summary of the results obtained is recorded in Table III. These results are averages of at least four experiments and in many instances averages of six or more individual experiments. The substrates used as oxidizable source in this *in vivo* system are given in column 1. Columns 2 and 3 present the O_2 uptake of the various substrates oxidized by mitochondria from control animals and animals injected with DHT, respectively. The change in oxidation (% decrease) of the intermediates due to the sterol is given in column 4. The associated P:O ratios of these substances are given in columns 5 and 6. It can be seen that the administration of DHT to intact rats decreased the mitochondrial oxidation of several intermediates to a large extent, *e.g.*, the oxidation of citrate was lowered from 22.2 to 10.4 μatoms (−53%), isocitrate oxidation was inhibited by 45%, being oxidized at the rate of 17.5 μatoms/hr in untreated rats as contrasted to 9.5 μatoms/hr. The oxidations of α-ketoglutarate and glutamate were decreased 30 and 21% by the *in vivo* administration of DHT, respectively. However, DHT reduced the oxidation of succinate and pyruvate to a very limited extent. The P:O ratios obtained with these substrates are essentially identical with the P:O ratios obtained with substrates incubated with kidney mitochondria obtained from DHT-treated animals.

TABLE II: *In Vitro* Effect of DHT on Oxidative Phosphorylation.[a]

Substrate (1)	O_2 Consumption (μatoms/mg of N) (2)	P_i (μmoles) (3)	P:O (4)
Citrate (25)[b]	8.1 ± 0.7[c]	20.3 ± 1.1	2.5
Citrate + DHT	7.4 ± 0.8	19.4 ± 1.0	2.6
Succinate (20)	10.6 ± 1.0	19.1 ± 1.4	1.8
Succinate + DHT	11.7 ± 1.1	19.7 ± 1.1	1.7
α-Ketoglutarate (15)	5.7 ± 0.4	17.7 ± 1.0	3.1
α-Ketoglutarate + DHT	6.1 ± 0.8	18.0 ± 1.6	3.0
Glutamate (20)	6.1 ± 1.0	17.1 ± 1.2	2.8
Glutamate + DHT	6.9 ± 0.9	18.7 ± 1.1	2.7

[a] Flask contents (final volume, 3.0 ml): 50 μmoles of potassium phosphate buffer, pH 7.3; 20 μmoles of $MgCl_2$; 6 μmoles of ATP; 0.08 μmole of cytochrome *c*; 300 μmoles of sucrose; 3 mg of albumin; 1 mg of mitochondrial N; and indicated amount of substrate in the main compartment. The side arm contained 2 μmoles of DHT; 50 μmoles of glucose, and excess hexokinase. The center well contained 0.20 ml of 2 N NaOH adsorbed on filter paper. [b] Figures indicate micromoles of substrate. [c] Mean plus and minus standard deviation.

Discussion

In this report, we have demonstrated that the *in vivo* administration of DHT produces an inhibitory effect on the *in vitro* oxidation of certain tricarboxylic

TABLE III: *In Vivo* Effect of DHT on Oxidation and Oxidative Phosphorylation by Kidney Mitochondria.[a]

Substrate (1)	O_2 Consumption ((μatoms/mg of N)/hr)		%	P:O	
	Control (2)	+ DHT (3)	Decrease (4)	Control (5)	+DHT (6)
Citrate (35),[b] 45 μmoles	22.2 ± 1.7[c]	10.4 ± 0.7[c]	53	2.4	2.4
d-Isocitrate (18), 20 μmoles	17.5 ± 0.5	9.5 ± 0.3	46	3.0	2.9
α-Ketoglutarate (16), 30 μmoles	18.0 ± 0.3	12.5 ± 0.4	30	3.1	2.8
Glutamate (16), 30 μmoles	21.4 ± 0.4	16.5 ± 0.3	21	2.7	2.7
Succinate (18), 20 μmoles	18.8 ± 0.3	17.2 ± 0.2	10	1.7	1.8
Pyruvate–Fumarate (18), 20 μmoles–5 μmoles	15.4 ± 0.4	14.2 ± 0.5	7	—	—

[a] Flask contents: (final volume, 3.0 ml) same as given in legend of Table II except DHT is omitted. [b] Number of flasks. [c] Mean plus and minus standard deviation.

acid cycle intermediates by rat mitochondria. Citrate and isocitrate oxidations were decreased by 53 and 46%, respectively (Table III), by DHT as compared to the control values. The effects of DHT on the oxidations of α-ketoglutarate and glutamate were less pronounced relative to citrate and isocitrate but quite significant. The oxidations of succinate and pyruvate were not affected to any degree by the sterol. The phosphorylations coupled to these oxidations were not affected in any manner by DHT treatment.

These results are similar to those demonstrated with PTE and vitamin D. De Luca *et al.* (1957b) showed that the *in vivo* addition of vitamin D decreased the oxidations of several citric acid cycle intermediates by rat kidney mitochondria. The vitamin did not alter the P:O ratios of the substrates examined. Costello and co-workers (1964a,b) have reported that PTE decreases the capacity of kidney mitochondria to oxidize citrate and isocitrate. Using rabbit liver minces, Cohn *et al.* (1965) demonstrated that *in vivo* administration of PTE or partially purified PTH markedly inhibited the oxidation of citrate, isocitrate, lactate, α-ketoglutarate, and fumarate.

When one considers the *in vivo* effects of PTE, vitamin D, and DHT on the oxidation of citrate and isocitrate, there exists a uniform depression in these oxidations. That this effect results from the hypercalcemia produced by these agents rather than by direct effects on mitochondria is, albeit, subject to discussion but certainly a distinct possibility. Much work is being done concerning calcium ion concentration and mitochondrial function. In connection with the hypercalcemic agents, Engstrom and DeLuca (1964) have shown that kidney mitochondria take up large quantities of calcium and that vitamin D greatly stimulates the release of this "actively bound" calcium. Electron microscopy of rat kidney mitochondria demonstrated that dietary vitamin D has a protective effect on the structural integrity of this organelle (DeLuca *et al.*, 1960).

The *in vitro* effects of PTH, vitamin D, and DHT are not as consistent as their *in vivo* actions. We have shown that the *in vitro* addition of DHT reduced endogenous kidney respiration (Table I) and that it was without effect on the oxidation of added substrates incubated with homogenates or mixed cell preparations. These observations are unlike those reported by DeLuca and Steenbock (1957) but may be partially explained by the fact that the animals used in their studies were vitamin D deficient and received a special dietary regimen. Of the several citric acid cycle intermediates tested in our system, the *in vitro* addition of DHT had no effect on the phosphorylation coupled to these oxidations (Table II). These results parallel those reported for vitamin D (DeLuca *et al.*, 1957b) but are unlike those obtained with high concentrations of highly purified PTH (Fang *et al.*, 1963). From this study, we conclude that the inhibitory actions of DHT on citric acid cycle oxidations are very similar to those reported for vitamin D and PTE.

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Aliphatic Diether Analogs of Glyceride-Derived Lipids. IV. The Occurrence of Di-*O*-dihydrophytylglycerol Ether Containing Lipids in Extremely Halophilic Bacteria*

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ABSTRACT: The lipids of seven extremely halophilic bacteria, which require 4 M NaCl for optimal growth, were compared with those of moderately halophilic bacteria (requiring 1 M NaCl) and related nonhalophilic bacteria. Infrared spectra of the total lipids showed that the extreme halophiles were almost entirely devoid of fatty acid ester groups, in contrast to the moderate and nonhalophiles which had normal fatty acid ester-containing lipids. After saponification, the lipids of the extreme halophiles gave high yields of unsaponifiable material, identified as largely 2,3-di-*O*-dihydrophytylglycerol, and only traces of fatty acids which were probably derived from lipids in the medium; in contrast, the lipids of moderate and nonhalophiles gave no glycerol diethers and large amounts of fatty acids.

The lipids of all the extremely halophilic bacteria examined by silicic acid paper chromatography were found to contain major amounts of the diether analog of phosphatidylglycerophosphate and minor amounts of the diether analog of phosphatidylglycerol and an unidentified ether phosphatide; a diether glycolipid and its sulfate ester were also present. The moderate halophiles were found to contain major amounts of the diester forms of phosphatidylglycerol and its aminoacyl esters, phosphatidylethanolamine, and several minor unidentified components. Two nonhalophilic *Sarcina* species had major amounts of diester phosphatidylglycerol, and traces of its aminoacyl ester, but no phosphatidylethanolamine.

Previous studies on the lipids of the extremely halophilic bacterium, *Halobacterium cutirubrum*, have established that these lipids consist almost entirely of derivatives of a glycerol diether (Sehgal *et al.*, 1962; Kates *et al.*, 1963; Faure *et al.*, 1963, 1964; Kates *et al.*, 1965a,b), identified as 2,3-di-*O*-(3′,7′,11′,15′-tetramethylhexadecyl)-L-glycerol (di-*O*-dihydrophytylglycerol) (Kates *et al.*, 1963, 1965a,b). Two phosphatide components have so far been identified: a major component, phosphatidylglycerophosphate, diether analog (Kates *et al.*, 1963, 1965b; Faure *et al.*, 1963), and a minor component, phosphatidylglycerol, diether analog (Faure *et al.*, 1964). In addition, a new glycolipid, 1-*O*-[glucosylmannosylgalactosyl]-2,3-di-*O*-dihydrophytyl-L-glycerol, and its sulfate ester, have recently been identified (Kates *et al.*, 1966b).

It was of interest to determine whether these glycerol diether derived lipids were uniquely characteristic of *H. cutirubrum* or whether they were also present in other halophiles and in nonhalophiles. We therefore undertook a survey of the lipids of several species of extremely halophilic, moderately halophilic, and related nonhalophilic bacteria. The present report de-

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