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The Effect of Dihydrotachysterol on the Citric Acid Cycle

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THE EFFECT OF DIHYDROTACHYSTEROL ON THE CITRIC ACID CYCLE

by

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LIFE

Joseph P. Liberti was born in Passaic, New Jersey, on November 2, 1937. He received his high school diploma from Lyndhurst High School, Lyndhurst, New Jersey, in June, 1955.

He graduated from Fairleigh Dickinson University, Rutherford New Jersey, in October, 1959, with the degree of Bachelor of Science in Chemistry.

In September, 1959, he began his graduate work in the Department of Biochemistry and Biophysics at the Stritch School of Medicine and in February, 1962, received the Master of Science degree in Biochemistry. He has held the position of Teaching Assistant in the Department from September, 1959, until June, 1963. Since this time he has held the position of Research Associate under an Institutional Grant.

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He would like to take this opportunity to thank his mother and family for their innumerable sacrifices without which these graduate studies would have been impossible. He will always be grateful for the proper sense of values instilled in him by his mother and father.

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CHAPTER I

A preparation said to contain dihydrotachysterol was introduced commercially in 1934 for the specific treatment of tetany by the German firm of E. Merk, Darmstadt, and called 'antitetanisches Praparat Nr. 10'. As AT-10 in Europe and Hytakerol in this country, it attained wide popularity (47).

Historically, the preparation was an outgrowth of the observation by Holtz in 1930 (34) that, even after the antirachitic properties of crude irradiated ergosterol were eliminated by the destruction of the contained vitamin D, by chemical reduction, heating or aging, hypercalcemic activity remained (61). Initially, it was not known whether this hypercalcemic activity should be attributed to an alteration of the vitamin D itself or to another specific 'calcinofaktor' present in the mixture. After extensive chemical separations and purifications of both crude and reduced irradiation mixtures by Windaus and his associates, it was established that hypercalcemic activity (as opposed to significant antirachitic activity) was present in a number of different irradiation products and their derivatives (34). Among these was tachysterol, a compound normally produced during the irradiation of

ergosterol; but this compound was relatively unstable to air oxidation, expecially when separated and purified. However, if a 'tachysterol-rich' irradiationmixture (one in which vitamin D had been removed or destroyed) was reduced with nascent hydrogen, a stable solution of 'calcinofaktor' was produced (35). Presumably such a product was the one first introduced commercially in 1934, although the exact method of preparation was never disclosed. The active principle in this product has always been stated to be dihydrotachysterol.

It has now been well established that dihydrotachysterol (DHT) is effective in increasing the concentration of calcium in the blood and in relieving the symptoms of parathyroid insufficiency. But in spite of its widespread clinical use, few investigations concerning its biochemical role have been undertaken.

The most satisfactory method found thus far for the preparation of dihydrotachysterol involves the reduction of tachysterol with lithium and liquid ammonia (yield 40%); in this method, dihydrovitamin D_2I is not formed (62, 64). Varying yields may also be produced by reducing calciferol or even crude mixtures of irradiated ergosterol (31, 32, 51). The structural formulas of dihydrotachysterol and related compounds are given in Figure 1. Chemically, dihydrotachysterol is closely related to calciferol (vitamin D_2) differing from it structurally in that the methylene group at C19 is

reduced to $-CH_3$ with the elimination of the double bond. The configuration of the C_3-hydroxyl group is beta in dihydrotachysterol and alpha in vitamin D_2 (1). The characteristic relationship of ring A to the remainder of the molecule determines whether a dihydro- derivative is termed a dihydrotachysterol or a dihydro-vitamin D, since both compounds are formed during the reduction of either calciferol or tachysterol but to varying degrees (22, 56, 62, 63).

Dihydrotachysterol, when administered in appropriate doses, raised the level of total calcium and consequently, the concentration of ionic calcium in the serum (1, 2, 11, 36, 43, 53, 58). Because of this calcemic effect, it is of value in correcting the hypocalcemia of hypoparathyroidism, (idiopathic or postoperative) and pseudohypoparathyroidism, thereby controlling tetany and preventing other manifestations of hypocalcemia. Another important use of Dihydrotachysterol, also dependent upon its calcemic effect, is is the treatment of vitamin D resistant rickets (35). The weight of evidence at the present time is that dihydrotachysterol as well as the vitamins D and parathyroid hormone all have a direct effect on mobilization of mineral from bone and that this effect is partially responsible for the elevation of serum calcium. Dihydrotachysterol also decreases the fecal excretion of calcium. It causes a rise in serum phosphorus, decreases the

I = TACHYSTEROL

II = DIHYDROTACHYSTEROL$_2$

III = DIHYDROVITAMIN D$_2$I

R = C_9H_{17}

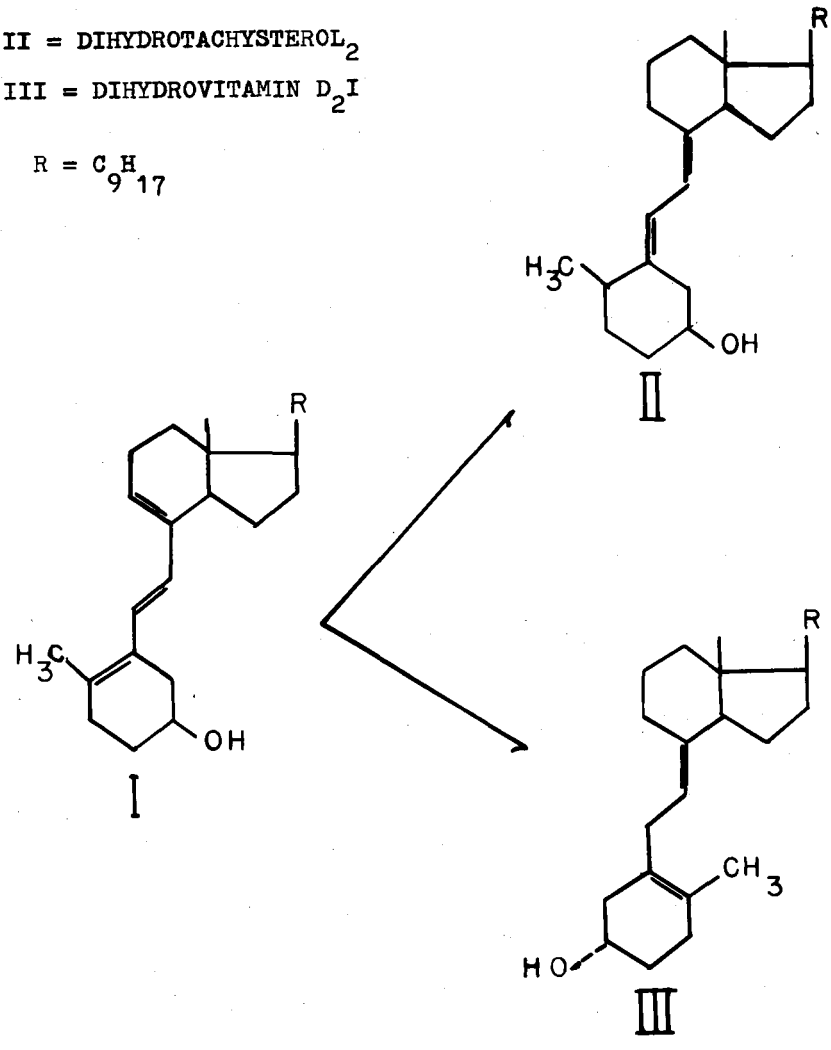


FIGURE 1

DIHYDROTACHYSTEROL$_2$ AND RELATED COMPOUNDS

fecal output and either increases the urinary phosphorus output or maintains it at a constant level in spite of decreased intake.

Hytakerol was used by Albright and co-workers, who, in 1938 published classic metabolic studies comparing the effect of AT-10 and of vitamin D_2 on calcium and phosphorus balances in human hypoparathyroidism (1). They concluded that the action of AT-10 more closely resembled that of parathyroid extract than did that of vitamin D_2 and that "the fundamental action of the parathyroid hormone on phosphorus metabolism is the same as that of AT-10", the latter, only, having an additional action on calcium absorption which was not possessed by parathyroid hormone.

Recent biochemical studies by Terepka et al. (58) have shown that Hytakerol, the presently available commercial preparation of AT-10 (and presumably similar, if not identical, with the preparation previously supplied), does not contain dihydrotachysterol. The major ultraviolet absorbing material in this preparation was found to be an isomer, probably dihydrovitamin D_2II. Experiments in rats and dogs demonstrated that this sterol had effects which were similar to, but not identical with those of crystalline dihydrotachysterol (13). Terepka and Chen (59) re-investigated the comparative effects of dihydrotachysterol and parathyroid extract on calcium and phosphorus balances, in man, using pure dihydrotachysterol.

Also some observations were made on the effects of acute intravenous administration of dihydrotachysterol, vitamin D_2, and parathyroid extract in a patient with hypoparathyroidism. Their results show that oral dihydrotachysterol, like vitamin D, increased the intestinal absorption and urinary excretion of calcium and phosphorus. The phosphaturia induced by intravenous dihydrotachysterol and vitamin D_2, as contrasted with parathyroid extract, appeared more slowly, was not characteristically associated with a fall in serum phosphorus, and, in balance studies, prolonged oral administration of dihydrotachysterol was not followed by post-treatment phosphorus retention. They concluded that dihydrotachysterol had effects on calcium and phosphorus metabolism which are virtually identical with those induced by large doses of vitamin D and only superficially resemble those of parathyroid extract. Both orally and intravenously administered dihydrotachysterol caused a decrease in urinary phosphorus excretion on the first day of treatment, followed by a pronounced phosphaturia (59).

From recent work (43, 53) it appears that dihydrotachysterol$_3$ is the most potent hypercalcemic agent followed in decreasing order by dihydrotachysterol$_2$, vitamin D_3 and vitamin D_2 whether given orally or after administration of one intravenous injection. The maximum calcium levels

appear to be obtained 2-4 days after the administration. This author has obtained similar results with dihydrotachysterol regarding its hypercalcemic effects (42). However, 4 hours after the intraperitoneal administration of dihydrotachysterol, the mean serum calcium concentration of treated rats was 1.5 mg% above the control group. Eight hours after the injection, the serum calcium levels of the experimental animals were essentially the same as those of the control group (+ 0.4 mg%). Twelve hours after the administration of dihydrotachysterol, the serum calcium levels of the treated animals were about 0.6 mg% below those of the control animals. This result was unexpected. However, Carlsson (10) has noted a decrease in the serum calcium levels of rachitic rats 6 hours after the administration of vitamin D.

Dowdle (20) has shown that the transfer of Ca^{45} across everted gut sacs is much greater with those animals which had been treated with either vitamin D or dihydrotachysterol than with the control animals. Dihydrotachysterol has an inhibitory effect upon bone tissue repair in the rat humerus as demonstrated by S^{35} uptake technique (40). Apparently, dihydrotachysterol blocks the synthesis of mucopolysaccharides in the newly formed collagen tissue.

This author has studied the distribution of tritiated dihydrotachysterol$_2$ in various tissues, blood and excreta of

rats at different time intervals after intraperitoneal injection (12). The data obtained revealed that the majority of the dose was located in the liver and to a lesser degree in the spleen and kidney 24 hours after the administration of the labeled sterol. It was also shown that the major portion of the radioactivity of the radioactivity recovered in the urine was found to reside in the aqueous fraction. This may indicate that dihydrotachysterol$_2$ and its breakdown products occur in the urine as water-soluble conjugates such as glucuronides and/or sulfates.

Although many effects of the hypercalcemic agents dihydrotachysterol, vitamin D and parathyroid hormone are well known, the mechanism of action of each is still a matter of conjecture. In this connection, there has been noted during recent years a considerable interest in the production, metabolism and physiological significance of citrate. Investigators agree that factors which influence the calcium economy of the body usually influence the levels of citrate in the tissues and body fluids (29).

Atwell (4, 5) noted a relation between the calcium and citric acid levels in the serum. In dogs injected with parathyroid extract, the serum citrate concentration rose and it followed closely the rise in serum calcium. In animals given calcium salts intravenously, the serum citrate concentration

also rose and the author concluded that the changes in the serum citrate level were secondary to changes in the calcium level.

Further evidence in support of this view was provided by Milne, as reported by Hodgkinson (32), who found that under ordinary physiological conditions any process which altered the calcium level, for example, the injection of calcium gluconate, or parathyroid extract, also caused a parallel change in the urine citrate level. However, factors primarily influencing urinary citrate, as the intravenous infusion of citrate, did not greatly modify the urinary excretion of calcium. He concluded from these and other observations that the changes in the serum and urine citrate levels observed in parathyroid disease are secondary to the variations in concentration of calcium in blood and urine.

Later investigations have given different results. Thus, several authors have observed a significant increase in the urinary excretion of calcium in man and dog following the intravenous infusion of sodium citrate (11, 27, 54). This has led to the suggestion that the small amount of calcium normally excreted in the urine is in the form of a non-ionized complex with citrate which is thought to be less readily reabsorbed than ionized calcium, by the renal tubules (9, 12). Further, it has been suggested that the increased excretion of calcium

observed in idiopathic hypercalciuria may be due in some cases, to a disturbance of citrate metabolism with a resultant increase in the quantity of calcium complexed with citrate (54). In contrast, Karam, Harrison, Hartog and Fraser (37), in studying the effects of growth hormone and sodium fluoroacetate on urinary calcium excretion in the rat, concluded that citrate may facilitate the renal tubular reabsorption of calcium.

A direct effect of parathyroid hormone on citrate metabolism was postulated by Freeman and his colleagues, who observed, in several species of laboratory animals, a transient increase in the serum citrate level following nephrectomy; this effect was not seen in animals deprived of their parathyroid glands (26). The above hypothesis was further elaborated by Neuman (48) who suggested that the effects of parathyroid hormone on calcium metabolism are mediated through their effects on citrate metabolism. In studying the mode of action of parathyroid hormone on bone they suggested that the transfer of labile calcium from bone to blood is under parathyroid control; that a cellular mechanism under the control of the parathyroids regulates the transfer of calcium from bone to blood by the formation of a calcium-citrate complex. Although the originators of this hypothesis have now changed their emphasis on the sequence of events, the postulate is still accepted that the production of acid in bone, including production of citrate

ion, plays a role in the solubilization of bone mineral.

Steenbock and Bellin in 1953 (57) have shown that the citrate content of blood, bone, kidney and the small intestine of rats on normal or low phosphorus rachitic rations was increased by physiological doses of vitamin D.

De Luca and co-workers (15) have shown that the in vivo administration of vitamin D significantly reduced the oxidation of citrate in kidney homogenates prepared from rats which were kept on a nonrachitogenic diet as well as on a rachitogenic diet. They reported that vitamin D, given in doses of 75I.U. at three day intervals, also reduced the oxidation of isocitrate and to a lesser extent, pyruvate-oxalacetate, while it had no apparent effect upon the oxidation of a a-ketoglutarate, succinate, fumarate and pyruvate.

Continuing along this line of research, the Wisconsin group (16) reported that the in vivo administration of vitamin D to rats decreased the oxidation of citrate by kidney mitochondria. They showed that an inhibition of only 15% was obtained without a phosphate acceptor system when the source of the kidney mitochondria was from rats kept on a non-rachitogenic diet. However, the inhibition increased to 57% when the hexokinase-glucose trapping system was added to the incubation medium. When the source of the kidney mitochondria was from rats kept on a rachitogenic diet, the inhibition increased to 29%.

Their data also showed that vitamin D had a pronounced inhibition on the oxidation of isocitrate and lesser inhibitory effects on a-ketoglutarate, glutamate and succinate. No apparent effect of the vitamin was found oxidative phosphorylation with any of the intermediates mentioned. In contrast to these results with kidney mitochondria, similar experiments with liver mitochondria showed that additions of vitamin D to the diet had no effect on the oxidation of citrate and a-ketoglutarate. Also, it had no effect on phosphorylations coupled to these oxidations. They noted the possibility that the resulting accumulation of citrate in kidneys, and possibly other tissues, may be an important factor in calcium transport and deposition (16).

In 1957 (17), the same group of investigators reported that vitamin D_2 and D_3, when added in vitro reduced the oxidationof citrate and isocitrate to a considerable degree (63%) and to a limited extent reduced glutamate oxidation (20%) by rat kidney mitochondria. In vitro addition of the vitamin reduced citrate oxidation and alpha-ketoglutarate production while not affecting coupled phosphorylation efficiency to any degree. Equal quantites of 7-dehydrocholesterol, ergosterol, Δ^7-cholestenol, and cholesterol were inactive in reducing citrate oxidation. The authors conclude with the note " . . . only the triphosphopyridine nucleotide systems are affected if one considers glutamate oxidized by both tri- and diphosphopyridine

nucleotide pathways".

Rasmussen and his co-workers (52) have shown that the addition of parathyroid hormone (100 ug) to kidney and liver mitochondria incubated with glutamate and excess phosphate acceptor induced a 20% increase in oxygen uptake, and a fall in phosphorylation with a consequent change in P/O ratio from 3.0 to as low as 0.7.

The initial impetus for the work reported in this dissertation was a short paper published by Bruchmann (8) in which the author showed that both dihydrotachysterol and vitamin D_2 had a remarkably inhibitory effect on the isolated aconitase enzyme system from pig heart. It was shown that 125 ug of dihydrotachysterol inhibited the conversion of citrate to isocitrate by 48% whereas vitamin D_2, at the same concentration, decreased the conversion of the substrate by less than 10%. At a concentration of 250 ugms., dihydrotachysterol abolished the conversion of citrate to isocitrate. Five hundred ugms. or 20,000 I.U. of vitamin D_2 was required to inhibit this system 93%.

In view of these recent findings, it was decided to investigate the effects of dihydrotachysterol on the oxidation and coupled phosphorylation of various intermediates of the citric acid cycle in rat kidney preparations and to attempt to correlate these findings with the known biochemical effects of this sterol.

CHAPTER II

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURE

For the measurement of the citric acid cycle oxidations, the Warburg manometric technique was used (55). The vessels were kept on ice during the addition of the "premix" and other reactants and were then transferred to the manometers and equilibrated for 10 minutes. The manometers were closed and readings were taken every 10 minutes for one hour. The kidney preparations were incubated for a period of one hour unless otherwise specified. The incubation temperature was 37°C. in the case of homogenates and 30°C. when mitochondria or microsomes were used. Air was the gaseous phase. When employed, the hexokinase-glucose trapping system was tripped in after the manometers were closed.

The protein concentration was measured by the biuret reaction and converted to mg. of nitrogen. Crystalline bovine serum albumin was used as the standard. The results of the oxidations are reported in terms of microatoms of oxygen consumed per mg. of nitrogen. The flask contents prior to and following incubation were deproteinized with 10% perchloric acid. The filtrates, for the calculation of P/O ratios, were analyzed

for inorganic phosphate by the method of Fiske and Subbarrow (24).

PREPARATION OF KIDNEY HOMOGENATES, MITOCHONDRIA AND MICROSOMES

After the animals were sacrificed, 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 Potter-Elvehjem homogenizer fitted with a Teflon pestle. When this type of preparation was used, the protein concentration of the homogenate was adjusted with isotonic sucrose such that approximately 6 mg. of protein was delivered into each flask.

For the preparation of mitochondria, the homogenate was first centrifuged in the International Refrigerated Centrifuge, Model PR-2, at 800 x g for 15 minutes. The resulting supernatant was then subjected to centrifugation at 10,000 x g for 15 minutes. At the end of this operation, the supernatant was discarded, or saved if the microsomal fraction was desired. The mitochondria-containing pellet was disrupted with a glass rod, resuspended in isotonic sucrose solution and again centrifuged at 10,000 x g as before. The concentration of the washed mitochondria was then adjusted so that the final concentration used in the incubations was approximately 1 mg. of mitochondrial nitrogen per flask.

When the microsmal fraction was needed, the supernatant obtained at the end of the 15 minutes centrifugation at 10,000 x g was subjected to further centrifugation at 104,000 x g for 60 minutes in Spinco Model L Preparative Ultracentrifuge at 0°C. The microsomal fraction obtained was then adjusted to the desired protein nitrogen concentration for the incubation.

PREPARATION OF FLASK REACTANTS

Each vessel contained in the main compartment, in a final volume of 3.0 ml., the following reactants: 3 mg. of bovine serum albumin, 6 umoles of disodium ATP, 20 umoles of $MgCl_2$, 350 umoles of sucrose, 0.08 umoles of cytochrome c, 50 umoles of potassium phosphate buffer, pH 7.3, the substrate and the particular tissue preparation. The concentration of the substrates and the changes made in the flask reactants are noted in the text under the section entitled "Experimental Results". When added, fifty umoles of glucose and an excess of yeast hexokinase were placed in the side arm. The center-well contained 0.20 ml. of 2 N NaOH absorbed on filter paper.

It was found that the best method of adding these reactants was to weigh the given amount of each into a volumetric flask and then to dilute to the proper volume with the buffer solution. The pH of this solution was then measured and adjusted to pH 7.3 with a mixture of 5 N KOH and 5 N NaOH such that the ratio of potassium to sodium was 3.5 to 1. Two ml. of this

"premix" was added to each flask at the beginning of the experiment. Those experiments which involved the omission or the addition of various cofactors are noted in the text.

Stock solutions of the various substrates were prepared such that 0.20 ml. of each solution contained the proper concentration to be used in the particular experiment. The given amount of dry substrate was placed in a volumetric flask and diluted almost to the mark with glass-distilled water. The pH of the solution was measured with a pH meter and adjusted to pH 7.3 with the 5 N KOH/NaOH solution. These solutions were kept frozen until the day of the experiment. The preparation of isocitric acid from its lactone required additional treatment. A given amount of the pure lactone was dissolved in 0.1 N NaOH and heated for 10 minutes on a boiling water bath. After cooling, the solution was diluted with water and adjusted to pH 7.3 with the mixed alkali solution.

The dihydrotachysterol which was employed throughout this investigation was used as received from the Mann Research Laboratories without any further purification. The purity of the sterol was assayed as 98.7% dihydrotachysterol$_2$. However, as a check on the stated purity, ultraviolet and infrared analyses were done. The ultraviolet absorption spectrum of the dihydrotachysterol$_2$ was determined at room temperature with a Beckman Model DU Spectrophotometer with 1.00 cm. silica cells. The

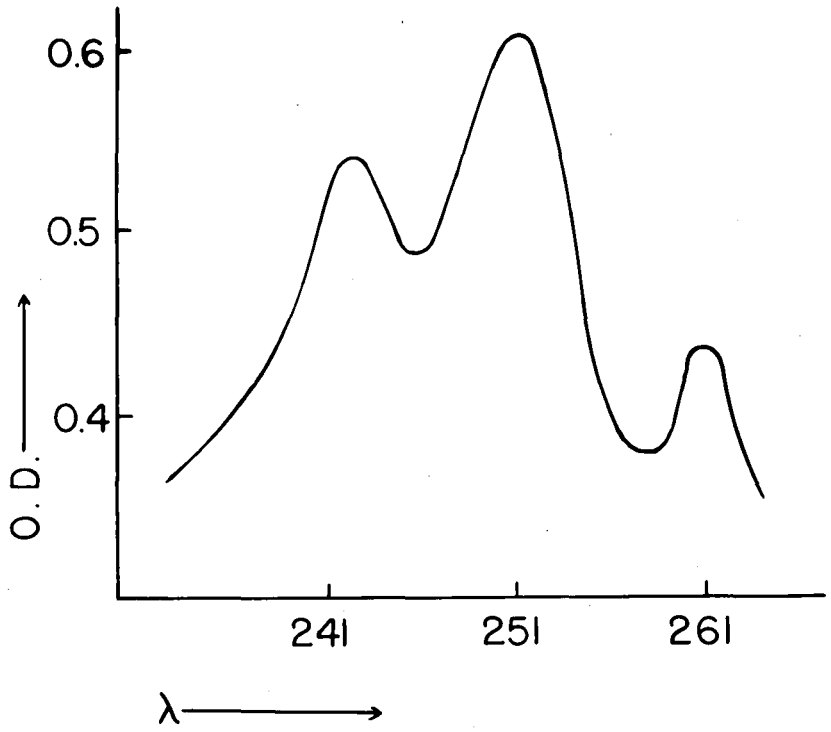
absorption coefficients calculated from the data showed that the steroid is at least 99% dihydrotachysterol$_2$. Figure 2 depicts the ultraviolet absorption spectrum obtained.

Infrared spectroscopy was employed as an additional criterion of purity. The instrument used to produce the spectrum was a Perkin-Elmer Model 21 Infrared Spectrophotmeter. Approximately 0.8 mg. of the steroid was placed in a mull with a small amount of finely ground, highly purified KBr and mixed mechanically for 30 seconds. The sample was then pressed into a pellet under high vacuum and with 12 tons pressure. The spectrum obtained is shown in Figure 3. This spectrum was shown to be superimposable with the infrared spectrum the author obtained with twice recrystallized dihydrotachysterol$_2$ (42) which was shown to be of comparable purity by melting point, ultraviolet absorption and chromatographic analyses.

ANALYTICAL PROCEDURES

Determination of Inorganic Phosphate

Inorganic phosphate was determined by the method of Fiske and Subbarow (24). The color reagent was prepared by mixing 40.0 ml. of 5.0 N H_2SO_4, 40.0 ml. of 2.5% (w/v) ammonium molybdate and 4.0 ml. of the reducing reagent in an Erlynmeyer flask. The reducing reagent was a freshly prepared solution made by adding 10.0 mls. of water to a mixture containing 20 mgs. of a 1-amino-2-naphthol-4-sulfonic acid, 120 mgs. of



$\lambda_{2610} = 655$

$\lambda_{2510} = 1007$

$\lambda_{2425} = 871$

FIGURE 2

ULTRAVIOLET ABSORPTION SPECTRUM OF DIHYDROTACHYSTEROL$_2$

Concentration 6 x 10^{-3} mg/ml in 95% EtOH

sodium bisulfite and 120 mgs. of sodium sulfite. Various aliquots of a standard phosphate solution, prepared with monobasic potassium phosphate were placed in graduated colorimeter tubes. Two ml. of the reducing mixture was pipetted into the tubes and the mixture then diluted to 10.0 ml. with distilled water. The contents were then mixed by inversion and the optical density read after 15 minutes in a Klett-Photoelectric Colorimeter fitted with a No. 66 filter. To measure the color development due to the reagent alone, water was used as the blank in place of the phosphate solution. The data for the calibration curve are given in Table I and the standard curve in represented in Figure 4.

The inorganic phosphate content of the incubation mixture was determined, in triplicate, as follows: 2.0 ml. of the mitochondrial mixture were pipetted into 4.0 ml. of cold 10%. perchloric acid. The sample was then centrifuged for ten minutes in the International Clinical Centrifuge at the highest speed. Two hundred ul. aliquots of the supernatant were pipetted into the colorimeter tubes which contained 5 ml. of water. Two ml. of the reducing reagent were pipetted into the tubes, the mixtures were diluted to 10.0 ml. with water and mixed by inversion. After 20 minutes, the optical density readings were made.

SERUM CALCIUM DETERMINATION

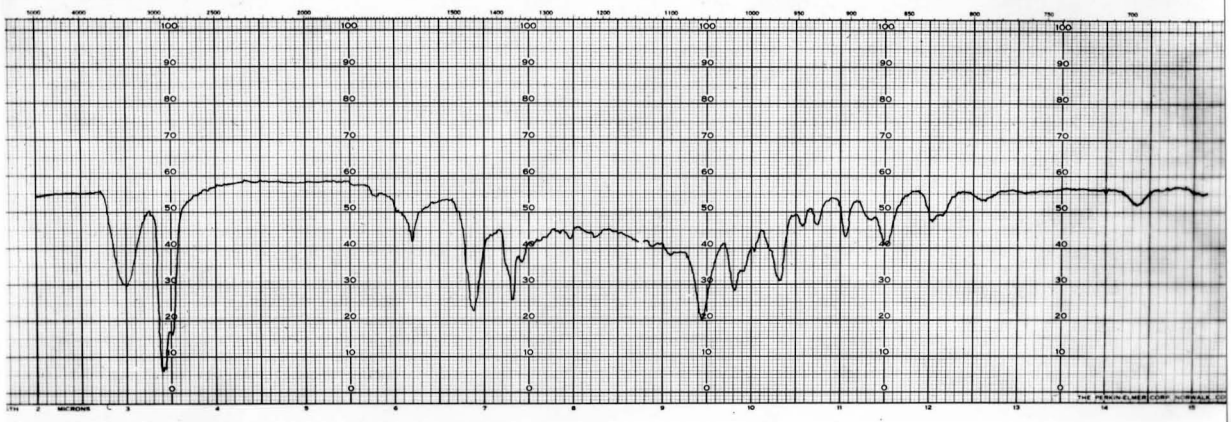


FIGURE 3

INFRARED SPECTRUM OF DIHYDROTACHYSTEROL$_2$

(0.8 mg.)

The procedure described below is a rapid and accurate complexiметric titration according to the method outlined by Ashley and Roberts (3). In this method an amount of the disodium dihydrogen salt of ethylenediamine tetraacetic acid in excess of that necessary to complex all of the available calcium and magnesium is added to the serum sample in the form of a 0.002 molar aqueous solution. Any potential interference arising from the presence of iron or copper in the serum is eliminated by the use of sodium cyanide. The pH value of the solution containing the indicator calcein is adjusted approximately to 12.0 before the titrant is added. Under these conditions, i.e., pH 12 and in the presence ofan excess of EDTA, the calcein indicator is not bound by the calcium ions and the resulting solution is dull brown in color. At pH values less than 12, the indicator assumes a bright yellow-green appearance which persists in the presence of excess calcium. A back titration is then performed by titrating into the calcein-EDTA-serum solution a standard calcium chloride titrant.

The standard calcium titrant complexes with the excess EDTA and because its affinity for EDTA exceeds that of magnesium, displaces the magnesium from this reagent. When all of the EDTA available has reacted with the calcium titrant, additional calcium combines with the calcein indicator producing a striking fluorescence under ultraviolet illumination, thus

TABLE I

STANDARD CURVE DATA FOR INORGANIC PHOSPHORUS ANALYSIS

Number of Determinations	Inorganic Phosphorus umoles	Optical Density
5	0.20	48 ± 2[a]
5	0.40	96 ± 3
5	0.60	147 ± 4
5	0.80	193 ± 4
5	1.00	245 ± 6
5	1.20	275 ± 6

a The last column of figures represents the standard deviation.

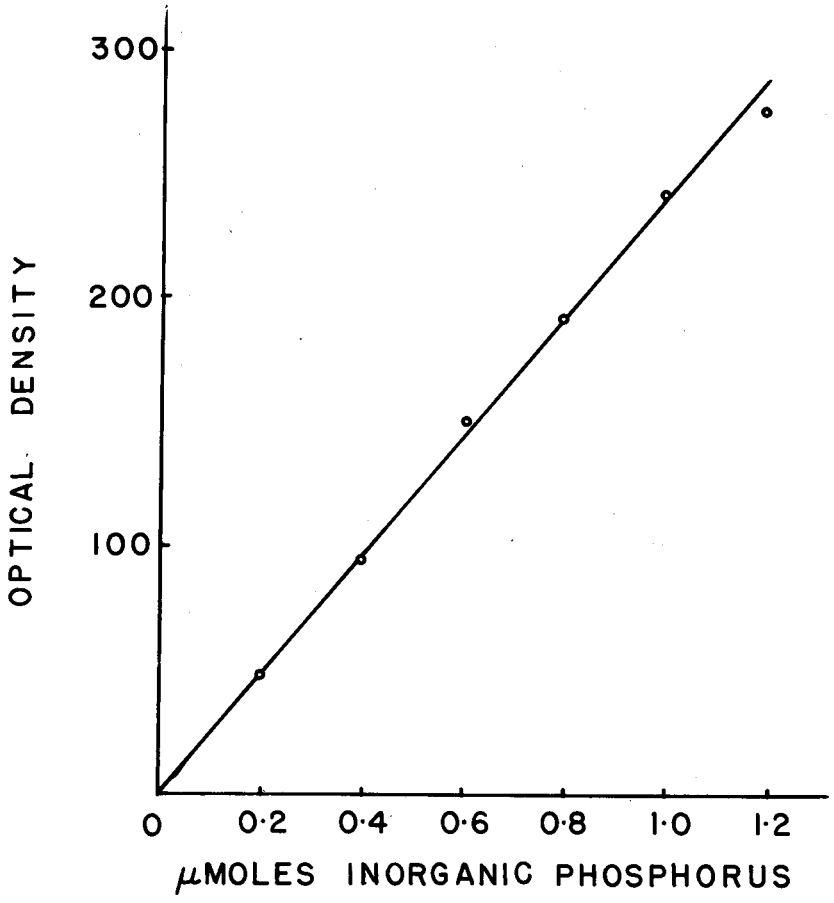


FIGURE 4

STANDARD CURVE FOR INORGANIC PHOSPHORUS ANALYSIS

signalling the end-point of the back titration.

The only special equipment needed in this method was a 5 ml. microburette graduated in 0.01 ml. with a ground glass tip which would accommodate a hypodermic needle. With a 25 gauge needle affixed to the burette tip, volumes of less than 0.01 ml. were delivered. Small magnetic stirrers were easily made by sealing ordinary iron wire of about 1.2 cm. in length inside glass capillary tubes.

The determination of serum calcium was performed as follows: After the blood sample had been withdrawn by cardiac puncture, it was carefully delivered into the bottom of a centrifuge tube. Care was taken during this manipulation to prevent hemolysis. The sample was then centrifuged in the International Clinical Centrifuge for 10 minutes at highest speed. The serum sample obtained was kept cold until analyzed. One of the small magnetic stirring rods was placed in a clean, dry 10 ml. beaker into which was accurately pipetted 0.20 ml. of the serum sample and 1.00 ml. of the 0.002 M EDTA solution. 1.0 ml. of the calcein indicator solution was added, followed by the addition of three drops each of a 1% solution of NaCN and a 1N solution of NaOH. The beaker and its contents was placed on a magnetic stirrer and under the tip of the microburette. The solution was then illuminated with a long-wave ultravioletlight (Mineralight, Model SL 3660) which was placed

about 10 cm. above the solution. The excess EDTA was back titrated while the solution was vigorously stirred. The solution, which orignally is a dull-brown in color, develops a bright green fluorescence at the end point.

The equivalence of the standard calcium solution and the 0.002 M EDTA solution was measured by titrating 1.00 ml. of the EDTA solution with the standard calcium solution. The technique and all the reagents employed in this standardization were the same as for the actual serum samples with the exception that 0.02 ml. of double distilled water was used in place of the serum. The concentration of the calcium was then calculated according to the following relationship:

$$\text{Calcium (mg\%)} = \frac{\text{Net ml. of standard calcium solution required to titrate 1.00 ml. of 0.002 M EDTA.}}{\text{Net ml. of standard calcium solution required to titrate serum sample}} \times 50$$

Figure 5 indicates the linear relationship between the ml. of titrant and the concentration of serum calcium. In Table II appear the data used to plot this standard curve.

TABLE II

STANDARD CURVE DATA FOR CALCIUM DETERMINATION

Number of Determinations	Calcium mg%	Ml. of Titrant
7	6	0.123 ± 0.004[a]
7	8	0.164 ± 0.003
8	10	0.202 ± 0.003
8	12	0.242 ± 0.004
8	14	0.286 ± 0.003
8	16	0.324 ± 0.005

a The last column of figures represents the standard deviation.

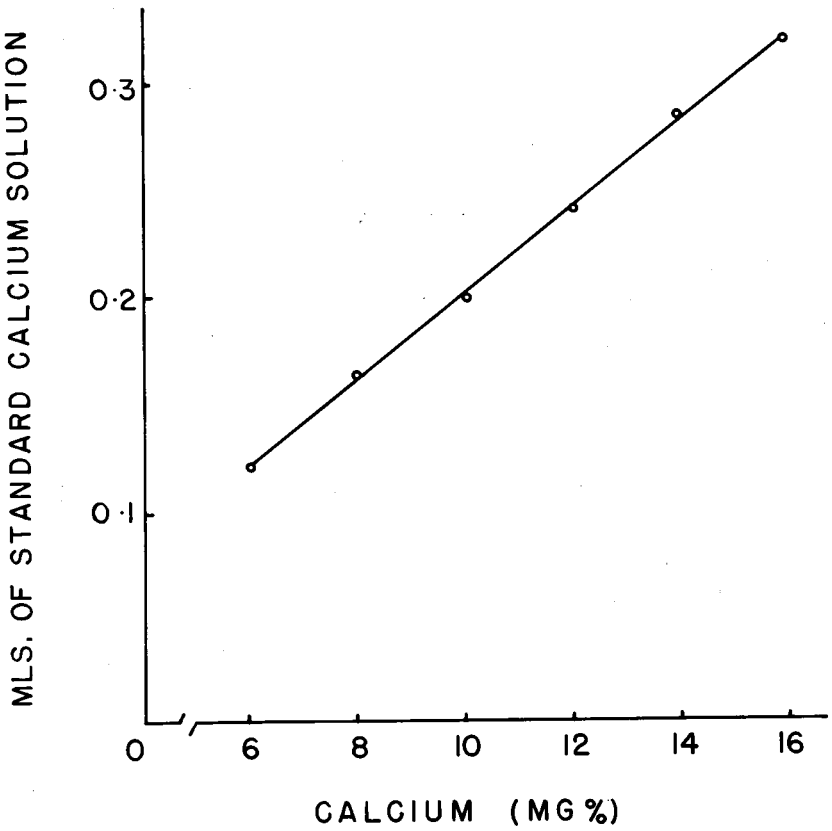


FIGURE 5

STANDARD CURVE FOR SERUM CALCIUM ANALYSIS

Protein Determination

Protein was determined by the biuret reaction. The reagents and conditions described below are those of Garnall at al (28). The procedure for the determination of the standard curve was as follows: 4.0 ml. of biuret reagent was added to 1.0 ml. of a solution containing 1 to 10 mg. of crystalline bovine serum albumin. The solution was mixed by inversion and allowed to stand for 10 minutes at room temperature. The optical density was measured with a Klett Photoelectric Colorimeter fitted with a No. 54 filter. The amount of color development produced by the reagents alone was determined by using 1.0 ml. of water in place of the albumin solution. The data for the calibration curve are given in Table III and the standard curve is represented in Figure 6.

To determine the amount of protein in a particular tissue preparation, 4.0 ml. of the biuret reagent was added to 0.20 ml. of the preparation and 0.8 ml. of distilled water. The contents in the tubes were then mixed by inversion, allowed to stand for 10 minutes at room temperature, and read as described. above.

TABLE III

STANDARD CURVE DATA FOR PROTEIN DETERMINATION

Number of Determinations	Protein mg.	Optical Density
5	0.50	11 ± 2[a]
5	1.0	24 ± 2
5	1.5	36 ± 3
5	2.0	51 ± 3
5	2.5	67 ± 4
5	3.0	81 ± 4
5	4.0	107 ± 6
5	5.0	130 ± 6

a The last column of figures represents the standard deviation.

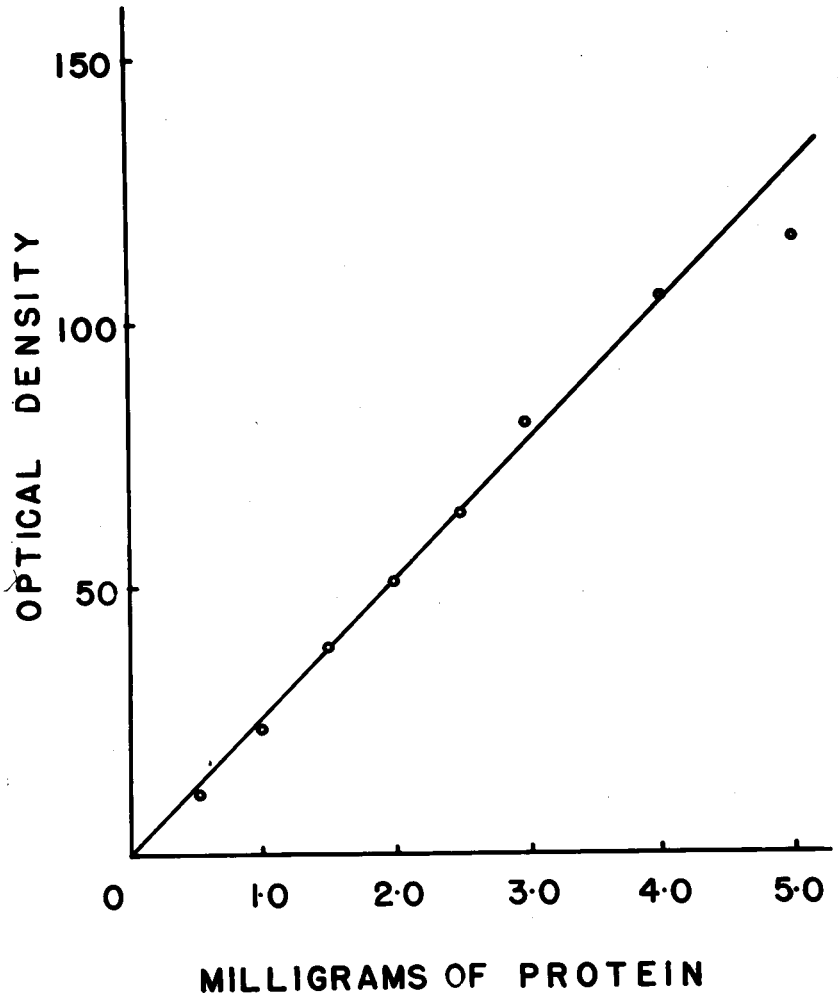


FIGURE 6

STANDARD CURVE FOR PROTEIN ANALYSIS

CHAPTER III

EXPERIMENTAL RESULTS

When undertaking experimentation with any *in vitro* system, the problem of preparing a viable tissue preparation which will give satisfactory oxidation rates and which can be prepared without too many manipulations so that the preparations will be relatively constant on a day to day basis is of prime consideration. The problem of maintaining as many variables as possible relatively constant warrants serious consideration also. Every attempt should be made in order that the preparation is as nearly close to physiological conditions as the experimental design permits.

With regard to the first mentioned consideration, various concentrations of kidney homogenates were prepared as described in the section entitled "Materials and Methods", which ranged from 10% to 30% in isotonic sucrose. It was found that a concentration of 10% was quite satisfactory for our purposes, i.e., the rate of oxidation was not too sluggish without the addition of exogenous substrate or too rapid when oxidizable intermediates were added to the reaction medium.

Various manipulations were employed in order to rid the kidney homogenate of cell debris. The homogenate preparation was either filtered through several layers of cheesecloth or centrifuged in a Clinical Centrifuge for 5 minutes at top speed. It was found that filtration of the homogenate through cheesecloth yielded satisfactory preparations.

A major problem which has confronted investigators who have undertaken similar experimentations was the insolubility of the steroid in the aqueous reaction medium. The carrier systems employed to date are usually emulsions or suspensions, with the result that the amount of steroid which actually is involved in bringing about the effect is not known. Various emulsifying agents and their mixtures were investigated but met with limited success. A few of those which were attempted here may be mentioned: propylene glycol in 10% ethyl alcohol, 3% sodium cholate in 10% propylene glycol, 10% ethyl alcohol-serum albumin-phosphate buffer suspension, 5 and 10% bovine serum and also bovine serum albumin in phosphate buffer solution. None of these carriers proved to be entirely satisfactory. Those agents which formed uniform suspensions, e.g., propylene glycol, reduced the oxidation of the tissue preparation markedly. Agents such as albumin, bovine serum and their mixtures with sodium cholate formed heterogeneous suspensions. However, one

emulsifying agent, Tween 20 was found to be satisfactory as a carrier. Tween 20, which is made up of a variety of long chain fatty acids, had the property of solubilizing as much as 8 umoles of the steroid in 0.20 ml. carrier solution, and when added in a small enough quantity, did not affect the respiration of the tissue preperation. After several trial and error experimentations, a successful method of adding the steroid to the aqueous reaction mixture was developed. A given amount of the steroid, the maxium amount which could be suspended uniformly being approximately 38mg., was made into a paste by adding 100 ul. of Tween 20 and grinding with a glass stirring rod. To the paste was then added potassium phosphate buffer solution, at pH 7.3, until 2.0 ml. of solution was attained. This resulting suspension was quite homogeneous and when 0.2 ml. (10 ul. of Tween 20) of it was added to the incubation flask, no measurable effect on the oxygen consumption of the kidney preparation was observed. 50 ul. of the emulsifier in the buffered solution inhibited the respiration about 45 per cent. 200 ul. of Tween 20 alone completely abolished the oxygen consumption of the kidney homogenate. Accordingly, in the experiments 0.2 ml. of the Tween 20/phosphate buffer solution containing the desired amount of dihydrotachysterol was added to the contents of the flask. Control flasks contained an

equivalent amount of carrier alone.

IN VITRO EXPERIMENTS WITH KIDNEY HOMOGENATES

Kidney homogenates were prepared, as previously described under 'Materials and Methods', from rats weighing between 150-200 gm. 0.5 ml. of this preparation was pipetted into the previously prepared vessels which were kept on ice. Each flask contained, in a final volume of 3.0 ml., the following reactants: 50 umoles of potassium phosphate buffer at pH 7.3, 300 umoles of sucrose, 20 umoles of $MgCl_2$, 6 umoles of disodium ATP, approximately 6 mg. of protein, and 25 umoles of citrate or 15 umoles of succinate in the main compartment. The center-well contained 0.20 ml. of 2 N NaOH adsorbed on filter paper. Between 1 and 8 umoles of dihydrotachysterol was placed in the side arm in solution with Tween 20/phosphate buffer solution. Flasks which served as controls contained 0.20 ml. of the carrier solution only in the side arm. Immediately after the homogenate was pipetted into the ice-cold flasks, the vessels and their contents were transferred to the manometer and equilibrated at 37°C. for 15 minutes in the Warburg apparatus. After that time, the manometers were closed and the contents of the side arm tipped in. The results of these experiments are shown in Table IV. These data show that dihydrotachysterol did not affect the oxidation of the flasks which contained

citrate. In a few instances the oxidation was decreased slightly while in other experiments the respiration was either increased by a small amount or not at all. However, in those flasks which contained just the endogenous preparation and the steroid, it was found that the dihydrotachysterol decreased the respiration about 40% on the average.

TABLE IV

THE IN VITRO EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION OF CITRATE BY RAT KIDNEY HOMOGENATES

Experimental Condition	O_2 Consumption ustoms/mg. protein
Homogenate (16)[a]	10.8 ± 1.8[b]
Homogenate + Carrier (16)	10.2 ± 1.5
Homogenate + DHT (16)	6.42 ± 0.93
Homogenate + Citrate (16)	17.3 ± 1.6
Homogenate + Carrier + Citrate (16)	18.5 ± 2.3
Homogenate + Citrate + DHT (16)	15.8 ± 2.2

a The figures in the parentheses represent the number of flasks used.

b The values in the last column represent the standard deviation.

The data recorded in this table are the averages taken from four individual experiments.

Experiments which were carried out in the same manner with succinate (15 umoles) as the oxidizable substrate yielded results almost identical with those just presented. In subsequent experiments, varying the concentrations of the substrates and dihydrotachysterol as well as the protein content did not bring about any measurable differences from the results just presented. It was also shown that the inhibition of oxidation did not vary proportionately to the amount of steroid present. This no doubt is due to the fact that not all of the steroid is being used to elicit the effects recorded. Most likely, less than 1 umole of the dihydrotachysterol is actually being utilized by the preparation. It was decided to isolate the kidney mitochondria and investigate the effect of dihydrotachysterol on this cell fraction employing the same type of experimental approach as with the homogenates.

It could be possible that the inhibition of endogenous respiration by dihydrotachysterol takes place in the mitochondria which is the site of the citric acid cycle oxidations or, then again, it might have its effect on some other cell constituent such as the microsomes or even on a mixed preparation of these two fractions. In these following experiments, the osmolarity was kept as close to 0.31 as possible by the use of

sucrose or neutral salts. Another parameter which had to be considered was that of the monovalent ions, K^+ and Na^+. Although measurements of these constituents are very difficult and subject to great variations and must be interpreted with great care, investigators agree that this ratio ranges from 3 to 7 to 1 (K:Na). In order to maintain the ratio of these ions close to 3:1, all the substrates were neutralized with the 5 N NaOH-KOH mixture. In addition to this, the premix solution was neutralized with this mixture of bases. Experiments with mitochondria were done with these changes in the procedure.

IN VITRO EXPERIMENTS WITH KIDNEY MITOCHONDRIA

Male Sprague-Dawley rats ranging in weight from 170-200 gm. were used in these experiments. The animals were offered Purina rat food and water ad libitum until 24 hours prior to the day of the experiment. During this period, they were given about 5 gm. of food and water ad libitum and placed in individual metabolism cages. The animals were then killed by decapitation and the kidney mitochondria prepared as described in the section entitled "Materials and Methods". The mitochondrial suspension was adjusted so that 1 mg. of protein nitrogen was added to each flask. This suspension was pipetted into chilled vessels which contained, in a final volume of 3.0 ml., the following constituents: potassium phosphate buffer, pH 7.3, 50 umoles, albumin, 3 mg., $MgCl_2$, 20 umoles, sucrose, 350 umoles,

cytochrome c, 0.08 umoles, citrate, 25 umoles or succinate, 15 umoles and the mitochondria in the main compartment. The center well contained 0.20 ml. of 20 N NaOH adsorbed on filter paper. 4 umoles of the steroid was added in a volume of 0.20 ml. of Tween 20/buffer solution to the side arm. The results of these experiments are presented in Table V.

TABLE V

THE IN VITRO EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION OF CITRATE AND SUCCINATE BY RAT KIDNEY MITOCHONDRIA

Experiment Number	Conditions	O_2 Consumption uatoms/mg. N
1 (a,b,c)	Mitochondria +[a] Citrate (9)	18.4 ± 1.7[b]
2 (a,b,c)	Mitochondria + Citrate + Carrier (9)	19.2 ± 1.8
3 (a,b,c)	Mitochondria + Citrate + DHT (9)	18.1 ± 1.4
4 (a,b,c)	Mitochondria + Succinate (9)	19.9 ± 2.5
5 (a,b,c)	Mitochondria + Succinate + Carrier (9)	18 .9 ± 2.1
6 (a,b,c)	Mitochondria + Succinate + DHT (9)	19.9 ± 2.0

a The number in parentheses indicates the number of flasks used.

b The values in the last column represent the standard deviation.

The data recorded here are the averages of three individual experiments. The data from these experiments show that the steroid had no significant effect on the oxidation of either succinate or citrate. In some experiments the steroid decreased the oxidation of citrate to a slight degree whereas in other instances the reverse was true. In subsequent experiments, 50 umoles of glucose and an excess amount of yeast hexokinase were added to the reaction medium to prevent limitation of oxidation due to lack of phosphate acceptor. However, no difference could be detected between either of the two experimental reaction mixtures with regard to the rate or amount of oxidation.

Lardy (41) in a classical paper showed that the uncoupling of oxidative phosphorylation by thyroxine was masked only until malonate was added to the reaction medium. Malonate has the ability to specifically inhibit the conversion of succinate to fumarate (21). Therefore, if citrate were added as the substrate, the cycle would start at this intermediate and halt at succinate. From the experimental data presented in the literature, it was further reasoned that if the steroid did indeed inhibit the citric acid cycle, the effect would probably take place between citrate and succinate. In the following experiments each flask contained 50 umoles of the phosphate buffer solution at pH 7.3, 20 umoles of $MgCl_2$, 6 umoles ATP,

0.08 umoles cytochrome c, 300 umoles sucrose, 10 umoles malonate, 3 mg. albumin and mitochondria in the main compartment. The center well contained 2.0 N NaOH adsorbed on filter paper. Four umoles of the steroid was added in a volume of 0.20 ml. of Tween 20/buffer solution to the side arm. The hexokinase-glucose trapping system was employed as before. Two substrates were used. One was citrate (25 umoles) and the second a mixture of pyruvate (20 umoles) and fumarate (5 umoles). The pyruvate-fumarate mixture was employed so that the cycle would be proceeding from a point immediately after succinate and therefore would present a complete picture of the events taking place in the cycle. The data obtained from these experiments are given in Table VI.

The data recorded in this table is the average of two individual experiments. These data show that dihydrotachysterol did not affect the oxidation of either citrate or the pyruvate-fumarate mixture to any degree. The small amount of oxygen utilization, as compared with the data given in the previous tables is due to the inhibitory action of the added malonate.

Experiments on the *in vitro* effect of the steroid on the oxidation of various intermediates of the citric acid cycle were not pursued further at this time. Attention was turned to the possibility of investigating the effect of the steroid on oxidative phosphorylation. Although DeLuca and his co-

TABLE VI

THE IN VITRO EFFECT OF DIHYDROTACHYSTEROL ON RAT KIDNEY MITOCHONDRIA OXIDATION WITH ADDED MALONATE

Experiment Number	Experimental Conditions	O_2 Consumption uatoms/mg. N
1 (a and b)	Mitochondria +[a] Citrate (8)	10.4 ± 1.4[b]
2 (a and b)	Mitochondria + Citrate + DHT (8)	9.15 ± 1.2
3 (a and b)	Mitochondria + Pyruvate - Fumarate (8)	8.27 ± 1.0
4 (a and b)	Mitochondria + Pyruvate - Fumarate + DHT (8)	9.49 ± 1.3

a The numbers in the parentheses indicate the number of flasks used.

b The last column of figures represents the standard deviation.

workers (17) have reported that vitamin D is without effect on the uncoupling of oxidative phosphorylation, Rasmussen et al. (52) have been able to demonstrate that parathyroid hormone, as prepared by the method of gel filtration, does affect oxidative phosphorylation when glutamate was employed as the exogenous source of oxidizable substrate.

THE EFFECT OF DIHYDROTACHYSTEROL ON OXIDATIVE PHOSPHORYLATION WITH RAT KIDNEY MITOCHONDRIA

For the measurement of oxidative phosphorylation, the oxygen uptake was determined manometrically and the inorganic phosphate colorimetrically by the method of Fiske-Subbarow (24). The flask contents, prior to and following incubation, were deproteinized with cold 10% perchloric acid and analyzed for inorganic phosphate.

In this series of experiments, each flask contained, in a final volume of 3.0 ml., the following reactants: 50 umoles of the phosphate buffer solution at pH 7.3, 50 umoles of glucose and excess hexokinase, 20 umoles $MgCl_2$, 6 umoles of ATP, 0.08 umoles cytochrome c, 300 umoles of sucrose, 3 mg. albumin, substrate and 1 mg. of mitochondrial N in the main compartment. The center-well contained 0.2 ml. of 2 N NaOH adsorbed on filter paper. 4 umoles of dihydrotachysterol in 0.2 ml. of Tween 20/ phosphate buffer solution was placed in the side-arm. The substrates employed in the study were citrate, 25 umoles, alpha-ketoglutarate, 20 umoles, succinate, 15 umoles and glutamate, 20 umoles. The results of these experiments are presented in Table VII.

TABLE VII

THE EFFECT OF DIHYDROTACHYSTEROL ON OXIDATIVE PHOSPHORYLATION

Experimental Condition	O_2 Consumption uatoms/mg. N	P_i umoles	P/O
Citrate (4)[a]	8.1 ± 1.1[b]	20.3 ± 2.1[b]	2.5
Citrate + DHT (4)	7.4 ± 0.9	19.4 ± 1.6	2.6
Succinate (4)	10.6 ± 1.3	19.1 ± 2.0	1.8
Succinate + DHT (4)	11.7 ± 1.5	19.7 ± 1.8	1.7
Alpha-Ketoglutarate (4)	5.7 ± 0.8	17.7 ± 1.9	3.1
Alpha-Ketoglutarate + DHT (4)	6.1 ± 1.0	18.0 ± 2.1	3.0
Glutamate (4)	6.11 ± 1.2	17.1 ± 1.5	2.8
Glutamate + DHT (4)	6.93 ± 1.3	18.7 ± 1.8	2.7

a The figures in the parentheses indicate the number of flasks.

b The last column of figures represents the standard deviation.

The data show that the P/O ratios obtained for the oxidizable substrates only agree well with those values reported in the literature. It can be seen that dihydrotachysterol has no effect on the oxidative phosphorylation.

Even though these experiments presented demonstrate that the steroid has no effect upon either oxidation of various substrates or on oxidative phosphorylation, the fact remained that

dihydrotachysterol did inhibit endogenous respiration of the kidney homogenates repeatedly and to a significant amount. Since this inhibition was demonstrated with the whole cell preparation and not with mitochondria, it seemed worthwhile to investigate the effect of the steroid on substrate oxidation by kidney mitochondria, together with the microsome fraction.

The preparation of the microsomes is described under the section entitled 'Materials and Methods'. The kidneys were removed from normal male Sprague-Dawley rats. Each flask contained, in a final volume of 3.0 ml., the following reactants: 50 umoles of the phosphate buffer solution at pH 7.3, 20 umoles of $MgCl_2$, 6 umoles of disodium ATP, 0.08 umoles of cytochrome c, 300 umoles of sucrose, 3 mg. of albumin, substrate and 1 mg. each of mitochondrial nitrogen and microsomal nitrogen in the main compartment; 0.2 ml. of 2 N NaOH adsorbed on filter paper in the center-well and 4 umoles of dihydrotachysterol in 0.2 ml. of the Tween 20/phosphate buffer solution in the side arm. The substrates used in the series of experiments were the pre-neutralized citrate, 25 umoles, and succinate, 15 umoles. The data obtained is the average of two experiments and are given in Table VIII.

TABLE VIII

THE EFFECT OF DIHYDROTACHYSTEROL

ON THE OXIDATION OF CITRATE AND SUCCINATE

BY MIXED MITOCHONDRIA--MICROSOME FRACTIONS

Experimental Conditions	O_2 Consumption uatoms/mg. N
Mixed preparation +[a] Citrate (6)	20.3 ± 2.3[b]
Mixed preparation + Citrate + DHT (6)	18.1 ± 1.9
Mixed preparation + Succinate (6)	23.7 ± 2.2
Mixed preparation + Succinate + DHT (6)	22.5 ± 2.5

a The figures in parentheses indicate the number of flasks.

b The last column of figures represents the standard deviation.

The data show that no effect of dihydrotachysterol on the oxidation of these intermediates was obtained. It can be seen that the amount of oxidation of the substrates is greater than those obtained with the mitochondria only. The data show that succinate is metabolized to a slightly greater extent than is citrate. This has been reported in the literature also.

Since the in vitro approach of experimentation failed to yield any significant effects of dihydrotachysterol on the oxidation of various metabolites of the citric acid cycle and

on oxidative phosphorylation, with mitochondria and mixed cell fractions, attention was directed to an *in vivo* approach.

IN VIVO EXPERIMENTS WITH RAT KIDNEY MITOCHONDRIA

Although significant effects were obtained by employing an *in vivo* system, it seems worthwhile to discuss the procedures used and the data obtained from them which led to the final experimental design adopted and the rationale behind them.

In the initial experiments, male Sprague-Dawley rats ranging in weight from 175-200 gm. were used exclusively. In the first of these experiments six rats were given an intraperitoneal injection of 1 mg. of dihydrotachysterol dissolved in corn oil. The animals were then placed in metabolism cages and given Purina rat food and water *ad libitum*. The control animals were treated in the same manner with the exception that they received corn oil only. Twenty-nine hours after the injection, the animals were killed by decapitation. The kidneys were excised and the mitochondria prepared as described previously. Each incubation flask contained, in a final volume of 3.0 ml., the following reactants: 50 umoles potassium phosphate buffer, pH 7.3, 350 umoles sucrose, 6 umoles disodium ATP, 20 umoles $MgCl_2$, 0.08 umoles cytochrome c, 3 mg. albumin, 25 umoles citrate and 0.5 ml. of the mitochondrial suspension in the main compartment. The center well contained 0.20 ml.

of 2 N NaOH adsorbed on filter paper. The mitochondria preparation was adjusted with isotonic sucrose solution such that 1 mg. of mitochondrial nitrogen was pipetted into each vessel.

Table IX presents the data obtained for the control and experimental animals.

TABLE IX

CITRATE OXIDATION BY KIDNEY MITOCHONDRIA FROM RATS TREATED WITH DIHYDROTACHYSTEROL

Treatment	O_2 Consumption uatoms/mg. N	Decrease %
Control (8)[a]	20.3 ± 1.1[b]	
Experimental (8)	16.3 ± 1.6	20

a The figures in the parentheses indicate the number of flasks.

b The last column of figures represents the standard deviation.

The data show that there is a significant effect of the steroid upon the oxidation of citrate. It was of interest to study the effect of the steroid on the oxidation of citrate by increasing the dose of administered dihydrotachysterol and by increasing the length of time before sacrificing the animals.

The experiments were carried out as follows: six rats ranging in weight from 175-200 gm. were used. Three were given an intraperitoneal injection of 1 mg. of dihydrotachysterol in corn oil at 24 hour intervals for three consecutive days. The animals were then placed in individual metabolism cages and were offered about 5 gm. of Purina rat food with water ad libitum. Four days after the initial injection of the steroid, the group of animals was killed by decapitation. The kidneys were quickly removed, pooled and the mitochondria prepared as before. Three control animals were treated in the same manner with the exception that they received injections of corn oil only. The contents of the flasks and their preparation were identical with those of the previous experiment. Citrate, at a level of 25 umoles, was the oxidizable substrate. The results of this experiment are presented in Table X.

TABLE X

CITRATE OXIDATION BY KIDNEY MITOCHONDRIA FROM RATS TREATED WITH DIHYDROTACHYSTEROL

Treatment	O_2 Consumption uatoms/mg. N	Decrease %
Control (10)[a]	20.0 ± 1.5[b]	
Experimental (10)	16.2 ± 1.5	19

a The figures in the parentheses indicate the number of flasks.

b The last column of figures represents the standard deviation.

The results show that the *in vivo* administration of dihydrotachysterol decreased the oxidation of citrate. Modifying the amount of steroid injected from 1 to 3 mg. and permitting it to act for three days, did not increase the effect of that obtained in the preceding experiment.

In order to ascertain if this effect were a reflection of the substrate employed, succinate (15 umoles) was used as the oxidizable substrate. The administration of the steroid and the preparation of the animals were identical to those already described. The results of this experiment are recorded in Table XI.

TABLE XI

SUCCINATE OXIDATION BY KIDNEY MITOCHONDRIA FROM RATS TREATED WITH DIHYDROTACHYSTEROL

Treatment	O_2 Consumption uatoms/mg N
Control (12)[a]	20.8 ± 2.2
Experimental (12)	19.1 ± 1.9

a The figures in parentheses indicate the number of flasks.

b The last column of figures represents the standard deviation.

It can be seen that the steroid in no way affected the metabolism of succinate. It was then decided to employ a combination of *in vitro* and *in vivo* approaches.

Accordingly, six Sprague-Dawley male rats ranging in weight from 175-200 gm. were each injected intraperitoneally with 1 mg. of dihydrotachysterol in corn oil daily for three days. The animals were placed in metabolism cages and offered Purina rat food and water *ad libitum*. Twenty-four hours after the last injection, the animals were killed and the kidney mitochondria prepared. Control animals were treated in an identical manner with the exception that they received corn oil only. The contents of the vessels, in a final volume of 3.0 ml., were the following: potassium phosphate buffer, pH 7.3, 50 umoles, sucrose, 350 umoles, $MgCl_2$, 20 umoles, disodium ATP, 6 umoles, cytochrome c, 0.08 umoles, 3 mg. albumin, citrate, 25 umoles, or succinate, 15 umoles, and approximately 1 mg. mitochondrial protein nitrogen in the main compartment. The center well contained 0.20 ml. of 2 N NaOH adsorbed on filter paper. 0.2 ml. solution of Tween 20/phosphate buffer which contained 4 umoles of dihydrotachysterol were placed in the side arm. In those flasks which contained no steroid, only 0.20 ml. of the carrier solution was added.

The results of these experiments with citrate as the oxidizable source are given in Table XII.

TABLE XII

THE EFFECT OF DIHYDROTACHYSTEROL ON CITRATE OXIDATION BY RAT KIDNEY MITOCHONDRIA

Treatment	Flask Additions	O_2 Consumption uatoms/mg. N	Decrease %
Controls (14)[a]	Carrier	18.3 ± 2.1[b]	
Controls (14)	Carrier + DHT	19.4 ± 2.3	
Experimental (14)	Carrier	15.4 ± 1.8	16
Experimental (14)	Carrier + DHT	15.0 ± 1.6	18

a The figures in parentheses indicate the number of flasks used.

b The last column of figures represents the standard deviation.

The data presented is the average of three separate experiments. The results show that the oxidation of citrate by kidney mitochondria prepared from the treated animals is less than that by mitochondria from untreated animals. They also show that the oxidation of citrate by the kidney mitochondria which were prepared from the treated animals was not affected by the *in vitro* addition of dihydrotachysterol. Similar experiments with mitochondria prepared from treated and untreated rats in

which 15 umoles of succinate was used as the exogenous source of oxidation showed that neither the addition of the sterol directly to the reaction vessels nor the administration of the sterol to the rats had any effect on the oxygen consumption.

It was clearly evident at this time that the *in vitro*, *in vivo* and the mixed preparations did not yield conclusive enough evidence to support the preliminary thesis. The fact that the inhibition of the aconitase enzyme system by DHT reported by Bruchmann (8) and that vitamin D inhibited various substrates of the citric acid cycle both *in vitro* and *in vivo* (15, 16, 17) were facts that had to be taken into account. Two very important innovations were introduced into the experimental design at this time: 1) a phosphate acceptor system was placed into the flasks so as not to limit oxidation by lack of phosphate acceptor and 2) the animals were sacrificed ten days after the initial injection of the steroid. More specifically, 50 umoles of glucose and an excess amount of yeast hexokinase were added to the reaction medium in the side arm. Second, the animals were given intraperitoneal injections of the steroid at five day intervals. At the end of ten days, i.e., after two injections of 1 mg. each, the animals were sacrificed and the kidney mitochondria prepared as before.

Sprague-Dawley male rats ranging in weight from 175-200 gm. were used in the series of experiments. They were offered

regular diet food and water ad libitum. The animals were placed under slight anesthesia with ether, approximately 2 ml. of blood were removed by cardiac puncture and the serum was set aside for calcium analysis. They then received an intraperitoneal injection of 1 mg. of dihydrotachysterol in 0.5 ml. of corn oil. Five days after this initial injection, the rats were given a second dose of the steroid. Five days after the second injection, the animals were anesthetized and blood was withdrawn as before, and set aside for serum calcium analysis. The control animals were treated in the same manner with the exception that they received injections of corn oil only. Approximately three hours after the heart puncture, the animals were killed by decapitation and bled as completely as possible. The kidneys were excised and the mitochondria prepared as described previously. Each incubation flask contained, in a final volume of 3.0 ml., the following reactants: potassium phosphate buffer, pH 7.3, 50 umoles, sucrose, 300 umoles, $MgCl_2$, 20 umoles, disodium ATP, 6 umoles, cytochrome c, 0.08 umoles, albumin, 3 mg., 50 umoles of glucose and excess hexokinase, oxidizable substrate and 1 mg. of mitochondrial nitrogen in the main compartment. The center well contained 0.20 ml. of 2 N NaOH adsorbed on filter paper. The concentration of the particular substrate used as the oxidizable source of metabolism is noted in the text.

The hypercalcemic effect of dihydrotachysterol was observed in each of the injected rats. In all instances, the increase in the serum calcium values of the treated animals was at least 1.9 mg.% higher than those of control rats.

EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION AND OXIDATIVE PHOSPHORYLATION OF CITRATE

The first substrate to be investigated using this system was citric acid. 45 umoles of the pre-neutralized citrate was used per flask. The oxidations were allowed to continue for either 40 or 60 minutes. The results of these experiments are given in Table XIII.

TABLE XIII

IN VIVO INHIBITION OF DIHYDROTACHYSTEROL ON THE OXIDATION OF CITRATE BY RAT KIDNEY MITOCHONDRIA

Experiment[a]	O_2 Consumption without DHT uatoms O_2/mg. N	O_2 Consumption with DHT uatoms O_2/mg. N	Decrease %
I (12)[b]	17.2 ± 1.37[c]	7.8 ± 0.5	54
II (8)	24.4 ± 1.73	10.8 ± 0.8	55
III (10)	20.1 ± 1.84	10.0 ± 0.5	50

a The kidney preparations were incubated for a period of 40 minutes in Experiment I and for a period of 60 minutes in Experiments II and III.

b The figures in the parentheses indicate the number of flasks used.

c The last column of figures represents the standard deviation.

The data show that there is a marked inhibition in the oxidation of this intermediate by dihydrotachysterol amounting to approximately 53%. Bruchmann (8), who investigated the effect of dihydrotachysterol on the isolated aconitase enzyme system, has shown that this sterol, when supplied at a level of 125 ugm. or about 0.4 umoles, had a marked inhibition on the oxidation of citric acid. It is therefore interesting to note that the sterol exhibits a similar effect in vivo.

The effect of dihydrotachysterol on coupled phosphorylation using citrate as the exogenous source of oxidation is given in Table XIV. The data indicate that the sterol has no apparent effect on this metabolic process. The P/O ratios recorded therein are well within the accepted experimental range.

EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION AND OXIDATIVE PHOSPHORYLATION OF ISOCITRATE

Isocitric acid, the isomer of citric acid, is intimately concerned with the further metabolism of citric acid. These

intermediates, along with cis-aconitic acid, are in equilibrium mediated by the aconitase enzyme system. At equilibrium the relative amounts of these acids are 90%, 4% and 6% for citric, cis-aconitic and isocitric acid respectively. In one experiment 15 umoles of isocitrate was added to the incubation flask and in a second experiment, 20 umoles were added. The effect of the sterol on the oxidation of isocitrate is shown in Table XV.

TABLE XIV

THE EFFECT OF DIHYDROTACHYSTEROL

ON OXIDATIVE PHOSPHORYLATION WITH CITRATE

BY RAT KIDNEY MITOCHONDRIA

Conditions	O_2 Utilized uatoms	P_i Uptake umoles	P/O
Complete System	6.9 ± 0.6[a]	16.6 ± 0.9[a]	2.4
Complete System + DHT	7.1 ± 0.4	16.6 ± 0.6	2.4

a The last column represents the standard deviation.

TABLE XV

IN VIVO INHIBITION OF DIHYDROTACHYSTEROL

ON THE OXIDATION OF ISOCITRATE

BY RAT KIDNEY MITOCHONDRIA

Experiment	O_2 Consumption Without DHT uatoms O_2	With DHT uatoms O_2	Decrease %
I (8)[a]	8.5 ± 0.40[b]	3.7 ± 0.29[b]	56
II (10)	17.5 ± 0.52	9.5 ± 0.28	46

a The figures in the parentheses indicate the number of flasks used.

b The figures in the last column represents the standard deviation.

NOTE: Experiment I was allowed to incubate for a period of 40 minutes. Experiment II was allowed to incubate for a period of 60 minutes.

The inhibition which amounted to about 50% is approximately the same as found with citrate as the oxidizable substrate.

The data representing the effect of dihydrotachysterol on the efficiency of coupled phosphorylation is given in Table XVI. The data show that the sterol has no apparent effect in this regard.

TABLE XVI

THE EFFECT OF DIHYDROTACHYSTEROL ON OXIDATIVE PHOSPHORYLATION WITH ISOCITRATE BY RAT KIDNEY MITOCHONDRIA

Conditions	O_2 Utilized uatoms	P_i Uptake umoles	P/O
Complete System	6.03 ± 0.2[a]	18.3 ± 1.1[a]	3.0
Complete System + DHT	6.3 ± 0.3	18.4 ± 0.3	2.9

a The last column of figures represents the standard deviation.

EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION AND OXIDATIVE PHOSPHORYLATION OF a-KETOGLUTARATE

Alpha-ketoglutarate was the next intermediate to be investigated. Each vessel contained 30 umoles of the pre-neutralized substrate. The effect of dihydrotachysterol on the oxidation

of this acid is given in Table XVII. The inhibition obtained with this substrate is not as great as those encountered with citrate and isocitrate. De Luca and Steenbock (16) have reported that vitamin D also caused an inhibition in the oxidation of this substrate. However, the magnitude of this decrease was not as large, being in the order of 20%.

TABLE XVII

IN VIVO INHIBITION OF DIHYDROTACHYSTEROL ON THE OXIDATION OF α-KETOGLUTARATE BY RAT KIDNEY MITOCHONDRIA

Experiment	O_2 Consumption: Without DHT uatoms O_2	O_2 Consumption: With DHT uatoms O_2	Decrease %
I (8)[a]	18.6 ± 0.30[b]	12.8 ± 0.28[b]	31
II (8)	17.3 ± 0.26	12.3 ± 0.54	29

a The figures in the parentheses indicate the number of flasks used.

b The figures in the last column represent the standard deviation.

Table XVIII shows that dihydrotachysterol has no effect on oxidative phosphorylation when alpha-ketoglutarate was used as the exogenous source of oxidation.

TABLE XVIII

THE EFFECT OF DIHYDROTACHYSTEROL ON OXIDATIVE PHOSPHORYLATION OF a-KETOGLUTARATE BY RAT KIDNEY MITOCHONDRIA

Conditions	O_2 Utilized uatoms	P_i Uptake umoles	P/O
Complete System	6.0 ± 0.3[a]	16.7 ± 1.0	2.8
Complete System + DHT	5.3 ± 0.2	16.2 ± 0.7	3.1

a The last column of figures represents the standard deviation.

EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION AND OXIDATIVE PHOSPHORYLATION OF GLUTAMATE

Glutamic acid, can give rise to citric acid cycle intermediates by enzymatic transamination with pyruvate, for example, to yield a-ketoglutarate and L-alanine. The effect of vitamin D on the oxidation of this metabolite both *in vivo* and *in vitro* has been reported by De Luca (15, 17). It was shown that the vitamin did have a slight but significant inhibitory effect on the oxidation of this substrate. Recent investigations by Rasmussen and his co-workers (52) have shown that parathyroid hormone, as prepared by gel filtration, does not only stimulate the oxidation of this intermediate but also uncouples oxidative

phosphorylation to a remarkable degree. They reported a decrease in the P/O ratio from 3.0 to as low as 0.7. They conclude that the uncoupling superficially resembles that of 2,4-dinitrophenol but differs in one striking respect in that the hormonal response requires the presence of inorganic phosphate whereas that due to 2, 4-dinitrophenol does not. It therefore seemed worthwhile to test the effect of dihydrotachysterol on these metabolic processes. The results obtained with glutamate, which was supplied at the 30 umolar level, are given in Table XIX. These data indicate that the effect on the oxidation of this substrate is very similar ot that obtained with alpha-ketoglutarate as the substrate. The decrease in oxidation was, on the average, about 21%. Again, the inhibition of oxidation was significant but not as great as that recorded with citrate or isocitrate.

TABLE XIX

IN VIVO INHIBITION OF DIHYDROTACHYSTEROL

ON THE OXIDATION OF GLUTAMATE

BY RAT KIDNEY MITOCHONDRIA

O_2 Consumption

Experiment	Without DHT uatoms O_2	With DHT uatoms O_2	Decrease %
I (8)[a]	22.4 ± 0.41[b]	17.2 ± 0.45[b]	23
II (8)	19.6 ± 0.49	15.8 ± 0.22	19

a The figures in the parentheses indicate the number of flasks used.

b The last column of figures represents the standard deviation.

Table XX, which contains the data obtained on the effect of dihydrotachysterol on oxidative phosphorylation using glutamate as the oxidizable source, shows that the sterol has no effect on this process.

TABLE XX

THE EFFECT OF DIHYDROTACHYSTEROL ON OXIDATIVE PHOSPHORYLATION WITH GLUTAMATE BY RAT KIDNEY MITOCHONDRIA

Conditions	O_2 Utilized uatoms	P_i Uptake umoles	P/O
Complete System	8.9 ± 0.2[a]	24.5 ± 1.3[a]	2.7
Complete System + DHT	8.5 ± 0.33	23.6 ± 1.5	2.7

a The last column of figures represents the standard deviation.

EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION AND OXIDATIVE PHOSPHORYLATION OF SUCCINATE

The oxidation of succinate to fumarate, mediated by succinic dehydrogenase, is the only dehydrogenation step in the citric acid cycle in which the pyridine nucleotides do not participate. This intermediate was added at the 15 umolar

level. The data in Table XXI show that there is a very slight decrease in the oxidation of this substrate with the kidney mitochondria from dihydrotachysterol treated animals.

TABLE XXI

IN VIVO INHIBITION OF DIHYDROTACHYSTEROL

ON THE OXIDATION OF SUCCINATE BY RAT KIDNEY MITOCHONDRIA

	O_2 Consumption		
Experiment	Without DHT uatoms O_2	With DHT uatoms O_2	Decrease %
I (8)[a]	17.3 ± 0.32[b]	15.5 ± 0.14[b]	10
II (10)	20.3 ± 0.28	18.0 ± 0.29	11

a The figures in the parentheses indicate the number of flasks used.

b The last column of figures represents the standard deviation.

The data showing the effect of DHT on oxidative phosphorylation with succinate is given in Table XXII. It can be seen that there is no evident effect in this regard. The values obtained are quite close to the theoretical value of 2.0.

TABLE XXII

THE EFFECT OF DIHYDROTACHYSTEROL ON OXIDATIVE PHOSPHORYLATION WITH SUCCINATE BY RAT KIDNEY MITOCHONDRIA

Conditions	O_2 Utilized uatoms	P_i Uptake umoles	P/O
Complete System	9.8 ± 0.2[a]	17.1 ± 1.7[a]	1.7
Complete System + DHT	8.6 ± 0.22	15.6 ± 0.72	1.8

a The last column of figures represents the standard deviation.

EFFECT OF DIHYDROTACHYSTEROL ON THE OXIDATION OF PYRUVATE

The last intermediate to be considered in this investigation was pyruvic acid which can enter the citric acid cycle as acetate and condense with oxalacetate to form citric acid. Pyruvic acid, being structurally similar to a-ketoglutarate, can undergo analogous reactions such as transamination and condensation with acetyl-CoA. A pyruvate-fumarate mixture was provided at a ratio of 4:1. The actual concentration of the mixture was 20 umoles of pyruvate and 5 umoles of fumarate. In this system, the fumarate acted as a 'primer' for the oxidation of the main substrate. The data obtained with this system is given in Table XXIII. It can be seen that there is no effect of dihydrotachysterol on the oxidation of pyruvate.

TABLE XXIII

IN VIVO INHIBITION OF DIHYDROTACHYSTEROL ON THE OXIDATION OF PYRUVATE BY RAT KIDNEY MITOCHONDRIA

	O_2 Consumption		
Experiment[a]	Without DHT uatoms O_2	With DHT uatoms O_2	Decrease %
I (10)[b]	15.6 ± 0.41[c]	14.1 ± 0.65[c]	9
II (8)	15.1 ± 0.45	14.3 ± 0.37	5

a The kidney preparations were incubated for a period of 50 minutes in Experiment I and 60 minutes in Experiment II.

b The figures in the parentheses indicate the number of flasks.

c The last column of figures represents the standard deviation.

CHAPTER IV

SUMMARY AND DISCUSSION

The *in vivo* and *in vitro* effects of dihydrotachysterol on the oxidation and oxidative phosphorylation of various intermediates of the citric acid cycle by rat kidney preparations have been investigated.

Kidney homogenates, mitochondria and microsomes were prepared essentially by the method of Schneider (55). Incubations of these preparations were carried out at 37°C in the case of the homogenates and at 30°C in the case of the mitochondria and the mixed cell preparations. Oxidations of the substrates were measured manometrically. The filtrates, for the calculation of the P/O ratios, were analyzed for inorganic phosphate by the method of Fiske and Subbarow (24).

A method was developed for dispersing the sterol in the aqueous reaction medium. This method consisted of making a paste of the sterol with Tween 20, an emulsifying agent. Phosphate buffer solution was then added until the desired volume of the suspension was obtained. Incubations with the kidney preparations showed that this carrier did not affect the respiration to any extent.

The initial *in vitro* incubations employed rat kidney homogenates. The data obtained with these preparations showed

that dihydrotachysterol, when added directly to the reaction medium, did not affect the oxidation of exogenous citrate or succinate. However, when the endogenous preparations alone were incubated with varying amounts of the sterol, an inhibition of oxidation amounting to 40% was observed.

In order to localize the effect of this inhibition, rat kidney mitochondria were prepared and the action of dihydrotachysterol on the oxidation of various substrates of the citric acid cycle was studied. In these experiments close attention was given to variables such as osmolarity, which was adjusted to 0.30, and the ratio of K^+/Na^+, which was kept close to 3:1. The data obtained showed that addition of dihydrotachysterol to mitochondria containing either citrate or succinate as the oxidizable substrate did not affect the oxidation to any degree. Experiments in which a hexokinase-glucose trapping system was added to the medium showed similar results.

At that time it was decided to add malonate to the incubation medium so that the cycle would proceed from the point of the particular substrate added and halt at succinate. When citrate was used as the exogenous source of oxidation in the mitochondrial preparations, in the presence of malonate, the oxidation of citrate was reduced by 50%. However, the amount of oxygen consumption was not depressed by the addition of the sterol. Similar results were obtained when a mixture of pyru-

vate and fumarate was incubated in this system.

Interest was then turned to the possible effect of dihydrotachysterol on oxidative phosphorylation of various substrates by kidney mitochondria. The substrates employed included citrate, a-ketoglutarate, succinate, and glutamate. The data obtained showed that the P/O ratios for these intermediates agree well with those values reported in the literature. It was also shown that the sterol had no effect on the efficiency of coupled phosphorylation.

The fact still remained that dihydrotachysterol did inhibit the respiration of the kidney homogenates. Since this effect was demonstrated with the whole cell preparation and not with mitochondria, the effect of the sterol on substrate oxidation by kidney mitochondria with added microsomes was investigated. The substrates used in this series of experiments were citrate and succinate. The data obtained showed that the sterol did not affect the oxidation of these intermediates. It was also shown that succinate was metabolized to a greater extent than citrate.

Since the <u>in vitro</u> approaches failed to yield any significant effects of the sterol on the oxidation of the metabolites used and on oxidative phosphorylation with mitochondria and mixed cell fractions, attention was turned to an <u>in vivo</u> approach.

Male Sprague-Dawley rats were given a single intraperitoneal injection of 1 mg. of dihydrotachysterol in corn oil. The animals were placed in metabolism cages and given food and water *ad libitum*. The control animals were treated in the same manner with the exception that they received corn oil only. Twenty-nine hours after the injection, the animals were sacrificed and the mitochondria were prepared.

The data showed that there was a small but significant decrease (20%) on the mitochondrial oxidation of citrate by the sterol. This effect is similar to that reported by DeLuca and co-workers (15) who found that the *in vivo* administration of vitamin D to rachitic rats reduced the oxidation of citrate by kidney mitochondria by 27%.

In subsequent experiments 1 mg. of the sterol was injected daily for three days. The rat kidney mitochondria were prepared twenty-four hours after the third injection. Again, the oxidation of citrate was inhibited by 20 per cent. When succinate was used as the substrate, no inhibitory effect upon the oxidation was obtained.

It was then decided to employ a combination of an *in vitro* and an *in vivo* approach. Accordingly, kidney mitochondria were prepared from animals injected as just described. The oxidation of citrate by kidney mitochondria prepared from the treated animals was 19% less than that by mitochondria which had been

prepared from untreated animals. It was also found that the oxidation of citrate by the mitochondria which were prepared from the treated animals was not affected by the direct addition of dihydrotachysterol to the reaction medium. Similar experiments with succinate as the oxidizable source showed that neither the *in vivo* administration of the sterol nor its *in vitro* addition had any effect on the oxidation of this metabolite.

An important change was introduced into the experimental design at this time. A hexokinase-glucose trapping system was added to the flasks so as not to limit the oxidation by lack of phosphate acceptor. Rats were given two injections of the sterol, one mg. each, five days apart. On the tenth day, the animals were sacrificed and the mitochondria prepared.

A summary of the effects of dihydrotachysterol on the oxidation and oxidative phosphorylation of various intermediates of the citric acid cycle by kidney mitochondria prepared from these treated animals is presented in Table XXIV.

The comparative effect of dihydrotachysterol on the oxidation of various substrates shows that the sterol had a pronounced effect on citrate and isocitrate oxidation. Its effect on glutamate and alpha-ketoglutarate oxidation was less marked but significant while on the oxidation of succinate and pyruvate in the presence of fumarate it had little effect.

These results extend the findings reported by Bruchmann

TABLE XXIV

OXIDATION OF VARIOUS SUBSTRATES AND COUPLED PHOSPHORYLATION BY KIDNEY MITOCHONDRIA FROM RATS INJECTED WITH DIHYDROTACHYSTEROL

Substrate	O_2 Consumption			P/O	
	Without DHT	With DHT	Per Cent Decrease	Without DHT	With DHT
Citrate	22.2 ± 1.7[a]	10.4 ± 0.7[a]	53	2.4	2.4
d-Isocitrate	17.5 ± 0.5	9.5 ± 0.3	46	3.0	2.9
alpha-Ketoglutarate	18.0 ± 0.3	12.5 ± 0.4	30	3.1	2.8
Glutamate	21.4 ± 0.4	16.5 ± 0.3	21	2.7	2.7
Succinate	18.8 ± 0.3	17.2 ± 0.2	10	1.7	1.8
Pyruvate-Fumarate	15.4 ± 0.4	14.2 ± 0.5	7	---	---

a The last column of figures represents the standard deviation.

(8) and also reveal the similarity of effects of vitamin D and dihydrotachysterol. Bruchmann has reported that the effect of dihydrotachysterol$_2$ on incubations of the isolated heart aconitase system is almost six times as potent as vitamin D in inhibiting the conversion of citrate to cis-aconitate. It is interesting to note that both dihydrotachysterol$_3$ and dihydrotachysterol$_2$ are more active hypercalcemic agents than either vitamin D_3 or vitamin D_2 when given orally or intravenously.

The investigations of De Luca et al. (16) have shown that the <u>in vivo</u> administration of vitamin D_2 to rachitogenic and non-rachitogenic diets inhibited the oxidation of citrate (57%), isocitrate (28%), alpha-ketoglutarate (17%) and glutamate (11%) by rat kidney mitochondria but had essentially no effect on the oxidation of succinate, pyruvate plus oxalacetate and beta-hydroxybutyrate. When comparing the <u>in vivo</u> effects of dihydrotachysterol$_2$ and vitamin D_2 on the inhibition of oxidation of these intermediates, one notes that dihydrotachysterol and vitamin D inhibit the oxidation of the same intermediates and to the same order of magnitude except in the case of isocitrate.

In a more recent study, De Luca and Steenbock (17) demonstrated an <u>in vitro</u> effect of vitamin D on the oxidation of citrate, isocitrate and glutamate by kidney mitochondria prepared from vitamin D deficient rats.. In contrast, in the present study, no in vitro effect of dihydrotachysterol on the oxida-

tion of citrate was observed.

It has been demonstrated here that dihydrotachysterol did not affect oxidative phosphorylation of various intermediates of the citric acid cycle by rat kidney mitochondria. The same observation has been reported in the case of vitamin D (16).

The results of the experiments described in this dissertation demonstrate a similarity of action of vitamin D and dihydrotachysterol in reducing citrate oxidation by kidney mitochondria while not affecting the efficiency of coupled phosphorylation to any degree.

Calcium may be mobilized by an accumulation of citrate and the possibility that the resulting accumulation of citrate in kidney, due to reduced citrate oxidation, and possibly in other tissues may be an important factor in calcium transport and deposition is of considerable interest.

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APPENDIX

ULTRAVIOLET ABSORPTION DATA

Dihydrotachysterol$_2$; Concentration, 6.0×10^{-3} mg/ml in 95% EtOH; Blank, 95% EtOH.

Wavelength	Optical Density	Wavelength	Optical Density
2800	-0.003	2480	0.512
2760	-0.002	2460	0.460
2720	0.005	2440	0.507
2680	0.049	2430	0.520
2640	0.245	2425	0.523
2620	0.390	2420	0.497
2610	0.393	2410	0.458
2600	0.346	2400	0.410
2580	0.360	2380	0.320
2560	0.391	2360	0.267
2540	0.530	2320	0.147
2520	0.598	2300	0.063
2510	0.604	2280	0.007
2500	0.572	2240	0.001

EXPERIMENTAL DATA: TABLE XIII

Substrate; Citrate, 45 umoles

Experiment and Number of Flasks Used	Oxygen Consumption uatoms/mg. N					
	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Expt. I Controls (6)	2.20	7.21	12.5	16.9		
	2.30	7.42	12.7	17.3		
	2.15	7.01	11.9	16.7		
	2.17	7.63	13.0	17.8		
	2.22	7.39	12.8	17.2		
	2.25	7.27	12.6	17.2		
Average	2.38	7.32	12.6	17.2		
Expt. I Treated (6)	2.08	3.17	5.89	7.18		
	1.97	3.21	6.12	8.12		
	2.11	3.09	5.78	7.26		
	1.99	3.22	6.10	6.09		
	2.20	3.11	6.02	7.98		
	2.02	3.14	6.15	8.19		
Average	2.06	3.15	6.01	7.80		
Expt. II Controls (4)	2.41	7.19	12.2	16.7	20.3	24.2
	2.01	6.81	12.5	17.2	19.6	23.6
	2.21	6.95	11.9	16.4	21.4	24.8
	2.36	7.28	12.7	16.9	20.7	25.2
Average	2.25	7.06	12.3	16.8	20.5	24.4
Expt. II Treated (4)	1.77	3.21	5.72	7.21	8.72	9.82
	2.09	2.89	5.89	6.92	9.27	10.2
	1.70	3.10	5.67	7.39	9.02	10.4
	1.82	2.98	5.79	7.14	8.96	10.3
Average	1.84	3.04	5.77	7.16	8.99	10.2
Expt. III Controls (5)	2.38	6.22	10.0	12.8	17.3	20.2
	2.43	6.51	10.3	13.2	17.7	21.4
	2.35	6.19	9.79	12.6	16.8	19.3

Experiment and Number of Flasks Used	Oxygen Consumption uatoms/mg. N					
	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Expt. III Controls (5)	2.50	6.41	10.1	13.1	17.5	20.1
	2.44	6.37	9.89	12.7	17.0	19.4
Average	2.42	6.34	10.0	12.9	17.3	20.1
Expt. III Treated (5)	1.80	3.79	6.29	8.11	9.82	10.2
	1.63	3.22	5.85	7.59	9.10	9.89
	1.81	3.75	6.19	7.70	9.66	9.88
	1.72	3.60	5.94	7.64	9.54	10.5
	1.75	3.58	5.87	7.83	9.72	10.7
Average	1.74	3.59	6.03	7.77	9.57	10.0

EXPERIMENTAL DATA: TABLE XV

Substrate: Isocitrate, 15 umoles (Expt. I) and 20 umoles (Expt. II)

Experiment and Number of Flasks Used	Oxygen Consumption uatoms/mg. N					
	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Expt. I Controls (4)	1.09	1.96	3.07	4.87	5.91	8.01
	1.48	2.78	3.98	5.52	6.80	8.93
	1.25	2.09	3.51	4.98	6.33	8.46
	1.33	2.41	3.66	5.21	6.51	8.63
Average	1.29	2.31	3.55	5.19	6.39	8.51
Expt. I Treated (4)	0.72	1.50	2.06	2.71	3.33	4.07
	0.58	1.19	1.60	2.39	2.64	3.39
	0.61	1.26	1.77	2.46	2.89	3.54
	0.63	1.38	1.66	2.52	3.12	3.78
Average	0.63	1.33	1.82	2.52	2.99	3.69
Expt. II Controls (5)	2.56	5.20	7.81	10.3	13.1	16.9
	3.01	5.92	8.73	11.5	14.8	18.1
	2.73	5.69	8.21	11.2	14.3	17.4
	2.44	5.36	7.97	10.7	13.7	17.0
	2.66	5.48	8.09	10.8	14.0	17.2
Average	2.68	5.53	7.96	10.9	14.4	17.5
Expt. II Treated (5)	1.91	3.82	5.20	7.12	8.25	10.1
	1.56	3.31	4.59	6.31	7.52	9.43
	1.78	3.46	4.72	6.49	7.68	9.57
	1.22	3.11	4.50	6.01	7.03	9.29
	1.37	3.29	4.72	6.29	7.11	9.33
Average	1.57	3.39	4.74	6.42	7.56	9.54

EXPERIMENTAL DATA: TABLE XVII

Substrate; alpha-Ketoglutarate, 30 umoles

Experiment and Number of Flasks Used	Oxygen Consumption uatoms/mg. N					
	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Expt. I	2.59	6.41	8.92	12.1	15.2	18.5
Controls	2.41	5.93	8.21	13.0	14.8	19.0
(4)	2.38	5.64	8.67	12.5	15.5	18.7
	2.52	6.09	8.54	11.8	15.3	18.3
Average	2.47	6.02	8.58	12.3	15.2	18.6
Expt. I	2.09	4.43	7.21	8.80	10.7	13.1
Treated	1.91	3.88	6.83	8.43	10.3	12.7
(4)	2.19	4.11	6.74	7.92	10.4	12.5
	1.81	3.92	6.29	8.27	10.1	13.0
Average	2.00	4.08	6.52	8.35	10.4	12.8
Expt. II	2.63	4.86	8.11	11.2	13.4	17.0
Controls	2.81	5.04	8.59	12.4	14.3	17.6
(4)	2.77	4.93	8.24	12.0	13.9	17.4
	2.93	4.79	8.37	11.7	13.8	17.2
Average	2.78	4.40	8.33	11.8	13.8	17.3
Expt. II	1.64	3.51	4.90	7.01	9.11	11.6
Treated	1.77	3.24	5.83	7.90	10.3	12.9
(4)	1.00	3.69	5.42	7.58	9.76	12.2
	1.61	3.03	5.25	7.37	9.92	12.4
Average	1.70	3.37	5.35	7.46	9.77	12.3

EXPERIMENTAL DATA: TABLE XIX

Substrate: Glutamate, 30 umoles

Experiment and Number of Flasks Used	Oxygen Consumption uatoms/mg. N					
	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Expt. I Controls (4)	3.61	8.29	11.2	14.4	18.0	23.1
	3.29	8.01	10.6	14.9	18.6	22.5
	3.42	7.97	10.3	14.5	17.9	21.8
	3.36	8.18	11.1	14.4	18.8	22.2
Average	3.42	8.11	10.8	14.5	18.1	22.4
Expt. I Treated (4)	3.01	7.71	8.41	11.3	13.7	17.9
	2.67	7.39	8.09	9.90	14.1	16.8
	2.81	7.52	7.92	10.5	13.8	17.2
	3.19	7.40	8.19	10.2	14.0	17.1
Average	2.92	7.50	8.15	10.5	14.4	17.2
Expt. II Controls (4)	3.09	6.11	8.98	11.8	15.7	19.0
	2.87	5.89	9.33	12.2	16.2	20.2
	2.93	5.95	9.26	11.6	15.7	19.5
	2.76	6.06	9.41	12.7	16.4	19.6
Average	2.91	6.00	9.24	12.1	16.0	19.6
Expt. II Treated (4)	2.33	4.71	7.39	10.1	12.4	16.1
	1.98	4.02	6.81	9.87	11.8	15.6
	1.91	3.98	6.93	9.28	12.1	15.9
	2.19	4.26	7.08	9.76	11.6	15.7
Average	2.10	4.19	7.05	9.75	11.9	15.8

EXPERIMENTAL DATA: TABLE XXI

Substrate: Succinate, 15 umoles

Experiment and Number of Flasks Used	Oxygen Consumption uatoms/mg. N					
	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Expt. I Controls (5)	3.49	6.52	9.44	12.7	16.3	20.5
	3.20	6.79	10.0	12.4	15.8	20.1
	3.37	6.79	9.22	12.9	16.7	21.0
	3.42	6.82	8.97	13.1	16.2	19.8
	3.19	6.91	9.72	12.3	15.9	20.3
Average	3.33	6.69	9.47	12.7	16.2	20.3
Expt. I Treated (5)	2.88	5.81	8.62	11.0	14.8	18.2
	2.71	5.45	9.01	11.4	15.1	17.8
	3.10	6.01	8.29	10.9	14.6	18.3
	2.66	5.67	8.57	10.9	14.9	18.1
	2.92	5.72	8.96	11.6	14.7	17.6
Average	2.85	5.73	8.69	11.3	14.8	18.0
Expt. II Controls (4)	2.12	5.48	8.19	10.8	13.8	17.2
	2.09	5.36	8.01	9.91	14.1	17.4
	2.41	5.51	7.92	11.3	13.9	17.3
	2.28	5.62	8.31	10.4	13.7	17.4
Average	2.22	5.49	8.11	10.6	13.6	17.3
Expt. II Treated (4)	1.82	4.81	7.22	9.77	12.3	15.7
	1.79	4.62	7.41	9.92	12.5	15.3
	1.86	4.91	7.09	9.19	12.0	15.5
	1.74	4.76	7.37	9.54	12.4	15.6
Average	1.55	4.77	7.27	9.60	12.3	15.5

EXPERIMENTAL DATA: TABLE XXIII

Substrate: Pyruvate, 20 umoles and Fumarate, 5 umoles

Experiment and Number Of Flasks Used	Oxygen Consumption umtoms/mg. N					
	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Expt. I Controls (5)	3.29	6.11	9.22	12.7	16.2	
	2.88	5.57	8.49	11.2	15.1	
	3.10	5.83	8.76	11.5	15.7	
	2.81	5.70	8.66	11.3	15.4	
	3.12	5.88	8.59	11.6	15.8	
Average	3.04	5.82	8.74	11.6	15.6	
Expt. I Treated (5)	2.41	5.12	8.47	11.4	14.9	
	2.55	4.93	8.06	10.3	13.3	
	2.68	4.81	8.01	10.4	13.7	
	2.38	4.87	8.13	10.8	14.2	
	2.49	5.09	7.91	10.3	14.4	
Average	2.50	4.96	8.11	10.6	14.1	
Expt. II Controls (4)	2.66	4.93	7.44	9.49	11.4	14.6
	2.53	5.03	7.62	9.90	11.8	15.4
	2.61	4.88	6.89	10.3	12.8	15.6
	2.77	5.29	7.71	10.6	13.2	15.0
Average	2.64	5.03	7.41	10.1	12.3	15.1
Expt. II Treated (4)	2.08	4.22	5.93	8.70	10.6	13.9
	2.41	4.68	6.91	9.22	11.7	14.8
	2.22	4.36	6.66	8.83	11.2	14.3
	2.36	4.53	6.71	8.97	11.5	14.4
Average	2.28	4.45	6.06	8.93	11.2	14.3

Abstract of the dissertation entitled, "The Effect of Dihydrotachysterol on the Citric Acid Cycle", submitted by Joseph P. Liberti in partial fulfillment of the requirements for the degree of Doctor of Philosophy, February 1964.

The *in vitro* and *in vivo* effects of dihydrotachysterol on the oxidation and oxidative phosphorylation of various intermediates of the citric acid cycle by rat kidney preparations have been studied.

Kidney homogenates, mitochondria and microsomes were prepared by differential centrifugation. Incubations of these preparations were carried out at 37°C in the case of homogenates and at 30°C in the case of mitochondria and the mixed cell preparations. Oxidations of the substrates were measured manometrically. The flask contents, prior to and following incubation, were deproteinized with perchloric acid. The filtrates, for the calculation of P/O ratios, were analyzed for inorganic phosphate.

Incubations employing kidney homogenates showed that dihydrotachysterol, when added directly to the reaction medium, did not affect the oxidation of exogenous citrate or succinate. However, the *in vitro* addition of the sterol to endogenous preparations alone, inhibited respiration by 40%.

When the action of dihydrotachysterol on the oxidation of citrate and succinate by kidney mitochondria was studied, it was shown that the sterol had no effect on the oxidation of these intermediates. Similar results were obtained when citrate,

succinate or pyruvate in the presence of fumarate were incubated with kidney mitochondria in the presence of added malonate.

Dihydrotachysterol had no effect upon the efficiency of coupled phosphorylation of citrate, succinate, alpha-ketoglutarate and glutamate by kidney mitochondria *in vitro*.

The effect of the sterol on substrate oxidation by mitochondria with added microsomes was investigated. The data obtained showed that dihydrotachysterol did not affect the oxidation of citrate or succinate in this system.

Attention was turned to the *in vivo* effects of dihydrotachysterol. Male rats were given a single intraperitoneal injection of 1 mg. of dihydrotachysterol in corn oil. Twenty-nine hours after the injection, the animals were killed and the mitochondria were prepared. The data showed that there was a small but significant decrease on the mitochondrial oxidation of citrate by sterol. This inhibitory effect was not enhanced by mitochondria prepared from rats treated with 1 mg. of dihydrotachysterol daily for three days. It was found that the oxidation of citrate by the mitochondria which were prepared from the treated animals was not affected by the direct addition of sterol to the reaction medium.

Subsequent *in vivo* experiments included the addition of a hexokinase trapping system to the reaction medium.

Rats were given two injections of the sterol, one mg. each, five days apart. On the tenth day, the animals were sacrificed

and the mitochondria prepared. It was shown that the sterol had a pronounced effect upon citrate and isocitrate oxidation. Its effect upon glutamate and alpha-ketogluterate oxidation was less marked but significant while on the oxidation of succinate and pyruvate in the presence of fumarate it had little effect. Dihydrotachysterol did not effect oxidative phosphorylation of these intermediates of the citric acid cycle.

The evidence presented demonstrates a similarity of action of dihydrotachysterol and Vitamin D in reducing the oxidation of citrate and isocitrate while not affecting the efficiency of coupled phosphorylation to any degree. Calcium may be mobilized by complex formation with citrate. The increased accumulation of citrate in kidney, and possibly in other tissues, due to these hypercalcemic agents, may be an important factor in calcium transport and deposition.

APPROVAL SHEET

The dissertation submitted by Joseph P. Liberti has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

January 15, 1964
Date

Maurice V. P. Heureux
Signature of Adviser