

DOCTORAL DISSERTATION SERIES

TITLE *The Effects Of Various Sources Of
Vitamin D And Dihydrocholesterol On
The Blood Calcium And Phosphorus*

Levels In

Cows, Dogs, Chickens, And Rats

AUTHOR *Benjamin Hartley Pringle*

UNIVERSITY *Michigan State College* DATE *1947*

DEGREE *Ph. D.* PUBLICATION NO. *917*



UNIVERSITY MICROFILMS
ANN ARBOR - MICHIGAN

THE EFFECTS OF VARIOUS SOURCES
OF VITAMIN D AND DIHYDROTACHYSTEROL ON THE
BLOOD CALCIUM AND PHOSPHORUS LEVELS IN
COWS, DOGS, CHICKENS, AND RATS

by

Benjamin Hartley Pringle

A THESIS

Submitted to the School of Graduate Studies
of Michigan State College of Agriculture
and Applied Science in partial
fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1947

ACKNOWLEDGEMENT

The author wishes to express his appreciation to Doctor C. A. Hoppert, for his enlightening understanding of the difficulties encountered in working with biological material, and for his interest and kindly criticism throughout these studies and in the preparation of this manuscript.

*

TABLE OF CONTENTS

	<u>Page</u>
Introduction.....	1
Historical.....	3
A. General.....	3
B. Antirachitic Compounds and Side Products.....	18
C. Effects of Antirachitic Compounds and Side Products on Mineral Metabolism.....	20
D. Effects of Antirachitic Compounds and Side Products on Blood Constituents.....	22
E. Toxicity of Antirachitic Compounds and Side Products.....	25
Chemistry of the Provitamins D and of the Irradiation Process.....	30
Physiology of the Antirachitic Compounds.....	44
Significance.....	48
A. Calcium levels.....	48
B. Phosphorus levels.....	50
C. Phosphatase levels.....	51
Description of Methods.....	53
A. Calcium.....	53
B. Phosphorus.....	54
C. Phosphatase.....	57
Description of Rations, Products, and Animals Used.....	66
A. Rats.....	66
B. Dogs.....	66
C. Chickens.....	66
D. Cows.....	67
Experimental Procedures.....	69
A. Rats.....	69
B. Dogs.....	69
C. Chickens.....	69
D. Cows.....	70
Data.....	71
A. Calcium.....	71
1. Rats.....	71
2. Dogs.....	78
3. Chickens.....	80
4. Cows.....	82
B. Phosphorus.....	83
1. Rats.....	83
2. Dogs.....	85
3. Chickens.....	88
4. Cows.....	91
C. Phosphatase.....	93
1. Rats.....	93
2. Cows.....	95

(Cont'd next page)

TABLE OF CONTENTS (Cont'd.)

	<u>Page</u>
Discussion.....	96
A. Calcium.....	96
1. Rats.....	96
2. Dogs.....	98
3. Chickens.....	99
4. Cows.....	100
B. Phosphorus.....	101
1. Rats.....	101
2. Dogs.....	102
3. Chickens.....	103
4. Cows.....	103
C. Phosphatase.....	104
1. Rats.....	104
c. Cows.....	105
Conclusions.....	106
Bibliography.....	109

INTRODUCTION

Since its discovery and isolation, vitamin D has been studied from numerous viewpoints without regard for the side-products produced in the activation process.

The activation of ergosterol involves the formation of certain intermediates such as lumisterol and tachysterol and upon continued irradiation some of the vitamin D is converted to toxic compounds such as toxisterol and the suprasterols. The presence of these toxic compounds in the early vitamin D preparations, led to the development of pure vitamin D products which have been in use for a number of years. There has however been recently developed an idea that the natural sources of vitamin D might contain intermediates which might have physiological value.

From tachysterol there has been produced a compound called dihydrotachysterol, known in the early history of the compound as A.T. 10 which has found considerable use in raising the blood calcium level in deficiencies of the parathyroid. The physiological effects of the side-products have not been studied to any extent, except that in the case of some of them, their anti-rachitic values have been determined. Certain studies have also been made on their toxicity and on the effect of the mode of administration on their physiological activity.

Because of this lack of information it was considered that a comparison between pure vitamin D and certain natural

vitamin D products such as fish oils and irradiated yeast might yield some interesting results. It was of chief concern to determine whether there is a difference in physiological behavior between the natural and pure products.

Although most of the work on vitamin D products and on the various side-products has been performed on rats and chickens, dogs and cows were also used in these studies.

The blood picture was studied to determine the effects of the various products used. Because of the fact that vitamin D is chiefly concerned with calcium and phosphorus metabolism, a detailed study was made of the calcium and phosphorus changes of the blood accompanying the administration of the various products. In addition some of the experiments involved the determination of the acid and alkaline phosphatase level in the blood.

HISTORICAL

A. General

In 1906 Hopkins suggested that rickets was due to some deficiency of nutrition. Available evidence at that time pointed to the conclusion that the supposedly adequate diet needed some supplementary constituent in order to prevent rickets. Exhaustive investigations have confirmed the views that rickets has to be considered as a deficiency disease. The names of Mellanby, McCallum, Sherman, and Pappenheimer are prominently connected with this work.

It was found that experimental rickets could be induced in dogs by giving them a diet from which the antirachitic principle was missing, and on the other hand that experimental rickets as well as the rickets occurring spontaneously could be cured by supplementing the diet with certain oils containing the antirachitic substance. Of these, cod liver oil proved to be the most effective.

The symptoms of rickets consist mainly in changes in the development of the skeleton and are usually accompanied by a general lowering of the vitality and by a retardation of growth.

The development of methods for producing experimental rickets made it possible to test different food stuffs for their antirachitic properties. As experimental rickets can be cured by supplying the antirachitic substances it is possible

to work out the doses necessary to cure rickets. This is called the "curative test". The second method of testing consists in determining the amount of an antirachitic substance necessary to prevent the development of rickets. This is called the "preventive test". It has been found that doses necessary to cure rickets are always greater than the doses preventing rickets. More recently chemical methods have been used for the determination of vitamin D.

The discovery of methods of producing experimental rickets followed by the development of tests for measuring the antirachitic value was the first great advance in elucidating the nature of rickets.

The second great discovery was due to a German physician Huldschinsky, who in 1919 found that cod liver oil was not the only curative agent for rickets, and proved by a large number of clinical observations that light, and especially ultra-violet light, had excellent curative action for human rickets. Later, in 1922, Hess and Lindhagen, observed that the blood content of inorganic phosphate decreases and increases with the seasonal changes of ultra-violet light from the sun. Rats placed on a rickets-producing diet could be protected against rickets by sunlight. Ultra-violet light therefore had the same effect as the substance vitamin D.

The connecting link between these apparently unrelated observations was the third great discovery in this field.

In 1924, Hess and Steenbock found simultaneously that it was by no means necessary to expose the rachitic animal to ultra-violet light provided the food was irradiated. The interpretation of this discovery was soon found. The foodstuffs as well as the skin contain substances which on irradiation with ultra-violet light produce vitamin D. These substances are called pro-vitamins D. Their presence is easily demonstrated. Vegetable oils inactive before irradiation become active after irradiation.

The naturally-occurring vitamin D seems to be formed by photochemical processes from a pro-vitamin D. Summer milk is richer in vitamin D than winter milk. This is due largely to the production of vitamin D in the skin of the animals.

The origin of vitamin D in fishes is not yet clearly understood. The cod fish lives at a depth where the necessary amount of ultra-violet light is not considered sufficient for the formation of vitamin D. Drummond (1) assumes that the cod possesses an exceptional power of storing vitamin D. Coppens and Metz (2) found that the tissues of the lungs of rats destroys vitamin D. The absence of lungs in fishes might account for their large storing capacity. Another possibility would be a biochemical synthesis of vitamin D in fishes.

The investigation of irradiated vegetable oils gave evidence that the active principle is to be found in the unsaponifiable part of the oil. The phytosterols, therefore, were examined. After irradiation they became active; the same

activation was observed after the irradiation of crude cholesterol. Thus in 1925 it was first believed that the pro-vitamin D was a sterol.

In 1927 Pohl (1)* investigated the ultra-violet absorption of cholesterol. The maximum was found at 280 mu. During irradiation the maximum is shifted to the ultra-violet. After a short irradiation this shift was so considerable that a large quantity of cholesterol was supposed to have undergone a photochemical transformation. The analysis of the irradiated material indicated it to be mostly cholesterol, and also some antirachitic substance. This activity was lost by recrystallization from alcohol. *Rosenheim and Webster (3) obtained the same result, by purifying irradiated cholesterol by formation of the dibromide or dichloride. In so doing all of the original activity was lost. Consequently the vitamin D must be associated with cholesterol or the phytosterols. The study of the absorption spectra of pure and crude cholesterol indicated that the crude cholesterol had a maximum at 280 mu whereas the purified cholesterol had a maximum at 240 mu.

✓ In 1927 Windeus (4) compared the absorption spectra of all the known sterols with that of pro-vitamin D. They discovered that the sterol of ergot, ergosterol, had the same absorption spectrum as provitamin D. They irradiated ergosterol and got a product 1000 times more active than irradiated cholesterol. This proved ergosterol to be pro-vitamin D.

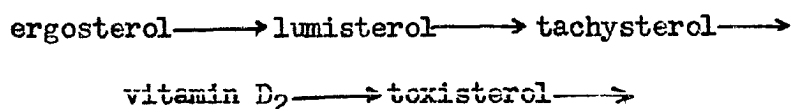
It was now assumed that ergosterol is associated with the known sterols, especially those of the skin. Its presence in skin was supposed to account for the formation of vitamin D upon exposure to some source of ultra violet light. The reason for the long delay in isolating the naturally-occurring pro-vitamin D is easily seen. Its solubility is so much like that of cholesterol that it was difficult to separate it from cholesterol.

After the discovery that ergosterol could be easily changed into a strongly antirachitic substance the isolation of pro-vitamin D and of vitamin D from natural sources was postponed, and research was concentrated on the active substances formed by irradiation of ergosterol. This work was troublesome and slow. Little progress was made until 1931 when Windaus in Germany, and Askew, Bourdillon and their collaborators in England reported the isolation of pure crystalline vitamin D from irradiated ergosterol.

Windaus and his colleagues realized from the beginning that the irradiation of ergosterol produced a mixture of compounds. The first antirachitic product isolated from irradiated ergosterol was a mixture of equal molecular amounts of vitamin D with a second irradiation compound called lumisterol. This mixture was designated as Vitamin D₁. A year later Windaus, Linfert (5) and their collaborators succeeded in isolating pure crystalline vitamin D. This was now

designated as Vitamin D₂. The name calciferol, used in the English literature, was originally applied to Vitamin D₁ but was later transferred to Vitamin D₂. Vitamin D₂ and calciferol are used synonymously.

In the following years other products of irradiation of ergosterol were found by Windaus. They are arranged in the following photo-chemical series:



Suprasterols

An elementary analysis of these products gave the striking result that all have the same formula as ergosterol; namely, C₂₈H₄₄O. Like ergosterol, they all are unsaturated sterols. The first assumption was that the action of light on ergosterol produced mainly steric rearrangements and shifting of the double bonds. Further investigations by Lettrec*(6) showed that the process was far more complicated than had been thought in the beginning.

All proof to date has shown that the antirachitic property depends on the presence of a free hydroxyl group present in the 3 ring skeleton, provided with three conjugated double bonds in closely limited positions.

The toxic effect is produced by substances which contain two or three conjugated double bonds in the 3 ring skeleton.

This is summed up by the following table taken from Friedmann's book, "Sterols and Related Compounds", pg. 60 (7).

	<u>Rings</u>	<u>Double Bonds</u>	<u>Conjugated Double Bonds</u>	<u>Isolated Double Bonds</u>	<u>Anti-rachitic</u>	<u>Toxic</u>
Ergosterol	4	3	2	1	—	—
Lumisterol	4	3	2	1	—	—
Tachysterol	3	4	3	1	—	+
Vitamin D ₂	3	4	3	1	+	+
Toxisterol	3 (?)	4 (?)	2 (?)	2 (?)	—	+
Suprasterol I	4	3	—	—	—	—
Suprasterol II	4 (3)	3 (4)	—	3 (4)	—	—

The successful isolation of a substance with highly anti-rachitic properties from irradiated ergosterol brings the research back to its starting point--the isolation of vitamin D₂ from natural sources. Was the vitamin D in cod liver oil similar to or identical with the vitamin D₂ from irradiation of ergosterol? Much evidence showed this to be true. The chemical composition, solubility and reactions towards different reagents was similar; it was toxic in high doses, and offered the same ratio between toxic and antirachitic doses as vitamin D₂. However, its physiological activity indicated that there was a difference. A comparison of their antirachitic activity showed no difference in rats with vitamin D₂, but in the case of chicks the vitamin D from fish oil sources was much more powerful.

Windaus subsequently discovered the existence of another pro-vitamin D; namely, 7-dehydrocholesterol. This compound bears the same relationship to the vitamin D in fish liver oils

as ergosterol does to Vitamin D₂.

In 1936 Werder and Windaus (8) isolated vitamin D₃ from the irradiation products of 7-dehydrocholesterol.

A.T. 10 or dihydrotachysterol a derivative of an irradiation product of ergosterol, is known to have important effects upon calcium and phosphorous metabolism. This has been amply shown not only by Holtz (9) and others, its discoverers, who showed its ability to raise serum calcium concentration after removal of the parathyroids, but also by the studies of Albright (10,11) and collaborators on the metabolism of calcium and phosphorus in idiopathic hypoparathyroidism and in rickets. They compared the effects of A.T. 10 with those of vitamin D₂ and parathyroid extract. It is their thesis that vitamin D has two primary actions; to increase phosphate excretion in the urine, and to increase calcium absorption from the gastrointestinal tract. Parathyroid hormone does not increase absorption of calcium but does increase phosphate in the urine markedly. According to these authors A.T. 10 has the same two actions as vitamin D, but increases calcium absorption less and phosphate excretion more. In confirmation of this hypothesis it has been shown that A.T. 10 will prevent or cure rickets in rats produced by a low calcium-low phosphorus diet in the same dosage that is without effect on the rickets produced by the classical high calcium-low phosphorous diets by Shohl, Farr and Forber(12).

It has been reported by Hornapp (13), that A.T. 10 has 1/400 to 1/600 the antirachetic potency of vitamin D₂. Shohl and Forber (14) have shown that A.T. 10 in amounts which approximate toxic dosage, is effective in preventing experimental rickets in rats fed a high-calcium-low phosphorus rachitogenic diet.

It was noted by Hess and Supplee (15) that irradiated ergosterol is much less effective than vitamin D from natural sources in promoting normal calcification in the chick. These observations have been amply confirmed, but no satisfactory explanation of the phenomenon has been found. Recently, Correll and Wise (16) have shown that one antirachitic rat unit of dehydrotachysterol is equivalent in the chick to between 4 and 5 units of the U.S.P. Reference Oil. A similar relationship of dose level to ratio of effectiveness for the chick has been shown to exist between vitamin D₂ and natural vitamin D by Massengale and Bells (17).

Summarizing there exists this peculiar situation: Vitamin D₂ is equivalent in the rat to 40,000 U.S.P. Units per milligram (Cawood 18) but in the chick to only about 1000 U.S.P. Units, Remp and Marshall (19). Vitamin D₃ is equivalent to 40,000 U.S.P. Units in both rat and chicken and crystalline dihydrotachysterol is equivalent to 80 U.S.P. Units per milligram but to about 360 units in the chicken. Most recent work shows vitamin D₂ and D₃ equivalent to about 50,000 units per milligram.

Russell and co-workers (20,21) have attempted to explain the low effectiveness in the chick by showing that the amounts of vitamin D₂ and natural vitamin D recoverable from the feces of the chick are quite comparable (26.5 and 34.1% of the dose administered respectively). It was not definitely settled whether or not any of the remainder of the dose was destroyed in the digestive tract. They did show that the vitamin D₃ was effective in the chick when given either by mouth or intraperitoneally, whereas six times as many units of vitamin D₂ given intraperitoneally in oil failed to promote equivalent calcification. From these studies they concluded that the low effectiveness of vitamin D₂ in the chicken could not be attributed to failure of absorption from or destruction in the digestive tract.

McChesney and workers (22,23,24), found that the ratios of oral effectiveness in the chick of D₃ to D₂ was 35:1 and of dihydrostachysterol to D₃ = 4.5:1 confirming the work of other workers. Most workers have found vitamin D₃, D₂, and DHT most effective when given orally. These authors (25) have also found in studying of the oral and parenteral utilization of vitamins D₂ and D₃ that the latter (D₃) is somewhat better absorbed from the digestive tract.

Correll and Wise (26) found A.T. 10 had an antirachitic potency of 300 units per mgm by rat technique. They also found that the action of A.T. 10 resembles that of vitamin D

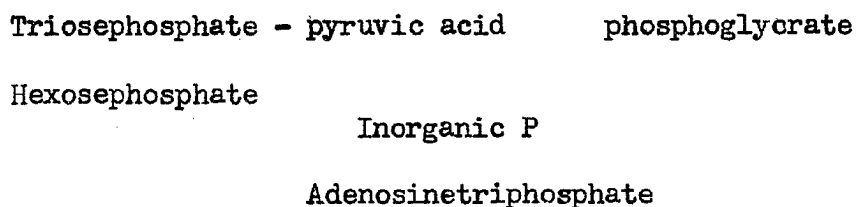
rather than the parathyroid hormone. Rat unit for rat unit they found A.T. 10 four times more effective as an antirachitic than vitamin D from cod liver oil. On a rat unit basis they also found A.T. 10 to be nearly 200 times more effective on the chick than D₂.

Jones and Rapaport (27) found that irradiated ergosterol fed to dogs on basis of 30,000 units of vitamin D per kilo of body weight produced a hypercalcemia. Schneider and Steenback (28) found that a synthetic diet low in phosphorous and free of vitamin D produced rickets, but when supplemented with sufficient phosphorous and vitamin D, it sufficed as the sole source of nutrients for three successive generation of rats. They found that vitamin D induced the utilization of P by bone, thereby depriving the soft tissues of their supply of P, which in turn inhibited growth.

Correll and Wise (29) found that the antirachitic vitamin from several sources exerted varying degrees of influence on the phosphatase values in chicken serum. Cod liver oil was more effective than irradiated ergosterol and the tuna liver oils in reducing serum phosphatase activity in the growing chick.

In 1938 Rapaport and Guest (30), reported that in rats given a high Ca and low P diet (rachitogenic) the development of rickets was associated with decreases first of inorganic P and of adenosinetriphosphate (A.T.P.) and then of diphosphoglyceric acid in the blood cells. In the healing of rickets,

the reverse of this process was found to take place. They suggest the following scheme:



Jones (31), in 1944, found small doses of vitamin D given to rats kept on a low phosphorus-high calcium diet produce a hypercalcemia. Irradiated ergosterol, pure calciferol, or irradiated 7-dehydrocholesterol were equally effective, whereas A.T. 10 was much less effective. The degree of hypercalcemia produced was dependent on the amount of vitamin D administered. The degree of growth inhibition was directly related to the degree of hypercalcemia. Calcification as judged by femur ash was greater in those cases in which there was a definitive increase in calcium. Thus this data gave additional support in favor of the view that vitamin D increases the absorption of calcium from the intestines and serves to indicate the importance of the calcium-phosphorus product of the serum in calcification.

Waddell (32) has shown that irradiated crude cholesterol is equivalent to cod liver oil and superior to irradiated ergosterol in preventing the development of leg weakness in chicks. Bethke (33) has suggested that perhaps two or more factors may be present in irradiated ergosterol, one of which

is effective in both the chicken and the rat and the other only in the rat. This doesn't seem to hold because it has been shown that crystalline vitamin D₂ and Viosterol were found to have relatively the same potency when tested on rats as well as on chicks. Thus in the chick it seems to be a matter of the degree of utilization of the several forms.

McChesney (34) found that differences of absorption and or destruction can not alone account for the very high doses of vitamin D₂ needed to produce the hypercalcemic effect in chicks. It required 125 mgms per kg of vitamin D₂ to raise the serum calcium to a level of 12 mgm %. This level was also reached in the chick with a dose of 10 mgm per kg of vitamin D₃ but not after a dose of 5 mgm per kg. Caward (35) in a group of separate researchers established that vitamins D₂ and D₃ have the same antirachitic potency in rats; i.e., 40,000 I.U. per mgm. Russell (36) in a review concluded that the vitamin D₃ was 30 times as effective as D₂ and 6 times as effective as A.T. 10 in the chick. Toxic effects were observed when cod liver oil was fed at about 5000 times, the calciferol at about 100 times, and A.T. 10 at about 10 times the levels necessary for normal mineral metabolism. Morgan and Shimatori (37) in their work on dogs fed various sources of vitamin D₂ and D₃ that there was produced a prolonged blood calcium rise, which was greatest in the case of irradiated ergosterol followed by Delsterol and Tuna liver oil.

Sherman and Booker (38) found that when the diet was composed of natural food stuffs arranged so as to make calcium the sole significant variable, that at each of the ages studied, the amount of calcium in the body of the growing animal varied in accordance with the calcium content of the diet. Lanford and Sherman (39) found on diets of different calcium levels that although the percentage of body calcium finally reached on the two higher calcium rations were the same, the rate of calcification was somewhat greater on the diet providing .8 percent than on that with .64 percent of calcium. Jones, Rapaport and Hades (40), in working with dogs, report that the source of the calcium in the hypercalcemia produced by irradiated ergosterol is the food and not the body tissue. Greenwald (41) observed that it was impossible to establish a definite relationship between the concentrations of calcium, phosphate, and protein in the sera of dogs.

Klein and Russell (42) found that of the irradiated ergosterol fed to chicks during the first 4 weeks of life, 26.5 percent was recovered in the droppings. When cod liver oil was used 43.1 percent was recovered.

Outhouse, Smith, and Twomey (43) found that rations containing lactose and cod liver oil caused greater retention of calcium, phosphorus, and magnesium than rations containing starch or sucrose.

McLean (44) found that dihydrotachysterol and calciferol are highly effective in increasing the concentration of calcium in the blood and in relieving the symptoms resulting from hypocalcemia in cases of insufficiency of the parathyroids. He found no toxic effects when administered over considerable periods of time. Albright and co-workers (45) working with human subjects established the following results with vitamin D, A.T. 10, and parathyroid hormone:

	Calcium Absorption	Phosphorus excretion in the urine
Vitamin D	+ + +	+
A.T. 10	+	+ + +
Parathyroid extract	0	+ + + +

Cohn and Greenberg (46) in their work with radiophosphorus and vitamin D concluded that the influence of vitamin D on the deposition of inorganic phosphorus in the bone of rachitic rats must be ascribed to a more direct effect than one merely resulting from an increasing absorption. They thought that vitamin D must have a specific effect on organic bone phosphorus and a specific role of this fraction in bone formation is indicated.

McDonald and Massengole (47) found that massive doses of irradiated ergosterol administered to pullets gave only 185 times more vitamin D potency in the eggs than did one ten thousandth as much vitamin D₃ (in the form of cod liver oil).

Russell, Taylor and Wilcox, in work on chicks (48) obtained essentially the same bone ash percentages when vitamin D was administered by injection or by capsule.

B. Antirachitic Compounds and Side Products

Vitamin D is shown to be a generic name for a class of substances possessing antirachitic properties. Certain members of the group have been purified, and their structures elucidated. The commonest procedure for the formation of an antirachitic substance is irradiation with ultra-violet in which ergosterol and 7 dehydro cholesterol are the usual starting compounds. Antirachitically acting products may also be produced from cholesterol by a variety of chemical procedures. These products have however not attained commercial importance. (49).

The more common antirachitic compounds are listed below:

- (1) Vitamin D₂, Califerol. The product of irradiation of ergosterol. This occurs in many irradiated foods of plant origin.
- (2) Vitamin D₄, 22-dihydrocalciferol. The product of irradiation of 22-dihydroergosterol.
- (3) Ertron. This is electrically activated ergosterol and may possibly be a distinct entity.
- (4) The product from the action of nitrites on ergosterol. This substance is feebly active but is certainly different from other forms of vitamin D which are destroyed by nitrites.
- (5) The product of irradiation of 22, 23-oxido-ergosterol. This substance is also feebly antirachitic.

- (6) Vitamin D₃, irradiated 7-dehydrocholesterol. This has been shown to be the chief form of the vitamin found in certain fish oils.
- (7) The product of irradiation of 7-dehydrositosterol. This probably occurs to a small extent in nature, although it is only feebly antirachitic.

It has been shown, that in addition to these compounds, there is a possibility that still other forms of vitamin D may exist in cod liver oil or in other natural products.

It has been shown that a real and significant difference, both quantitative and qualitative, exists between the chick and the rat in the reaction of the various forms of vitamin D, and it is concluded that recent studies of the action of several forms of vitamin D show that the human reacts in general in the same manner as the rat to the various vitamins D.

In the irradiation of ergosterol there are formed as previously mentioned several side or related products such as lumisterol, tachysterol, toxisterol, suprasterol I, and Suprasterol II. Their antirachitic activities are relatively slight although certain workers have shown some of their derivatives such as dihydrotachysterol, to have appreciable antirachitic effect and a particularly marked influence on the blood calcium level.

C. Effects of Anti-rachitic Compounds and Side-products on Mineral Metabolism

The study of the influence of vitamin D on mineral metabolism has occupied the attention of many investigators, and it should be appreciated at once that such investigations offer many difficulties. As a consequence, the observations have not been uniform, and the conclusions have led, therefore, to much confusion.

Eddy and Dalldorf (50) in their work on the calcium intake of rats found that normally the rat loses 20 to 40 per cent of the calcium intake and 15 percent of the phosphorus in the feces, whereas in rachitic animals the calcium loss amounts to 90-100 percent and phosphorus 60-70 percent. When vitamin D is administered to rachitic rats restores the proportionate absorption. This makes it appear that the function of vitamin D is to promote absorption of calcium and phosphorus. The process is somewhat more complicated however. Kern and Montgomery (51) found that on high doses of vitamin D, there was an increased intestinal absorption of calcium and to a less extent phosphorous.

Taylor and Weld (52) in their work on rats found that vitamin D hypercalcemia was due to mobilization of calcium from the bones and that the vitamin depressed the power of the intestine to excrete calcium. Bauer, Marble, and Claffin (53) found no effect on nitrogen metabolism from the ingestion of vitamin D over periods up to a month or more. Blood calcium

and phosphorus levels were likewise unaffected. They found the mineral balances not appreciably affected.

In an interesting study of the effects of vitamin D on calves, Duncan and Huffman (54) found that there was but little alteration of blood calcium, whereas inorganic phosphorus was increased definitely and returned to normal levels after fourteen days. There was decreased fecal and increased renal excretion of both elements.

Wallis, Palmer, and Gullickson (55) reported that a calcium-phosphorus retention ratio of 2 was present in both normal and rachitic calves and that vitamin D increased retention without altering the ratio. Patroharden and Chitre (56) claimed that 4500 units of vitamin D fed daily to adult rats increased renal excretion of calcium, phosphorus and nitrogen.

Reed, Struck, and Steck (57) in their mineral balance study on humans with arthritis, could reach no definite conclusions because of such variations.

Nicolaysen (58, 59, 60, 61, 62, and 63) in a series of studies on rachitic rats has shown that the rat may adapt itself to excess vitamin D which would cause inconsistent results as in the case of the humans with arthritis. He also found that with varying levels of calcium added to this diet, the output of endogenous phosphorus in the feces was progressively increased, but the vitamin generally inhibited the increase to some extent. With varying levels of phosphorus in the diet, the fecal output

of calcium was likewise progressively increased and again the increase was less when vitamin D was given.

It is apparent that there is no conventional picture of mineral metabolism in arthritis and that there is no characteristic response to vitamin D that can be considered specific in this relation. Also it is apparent that the vitamin is capable of producing opposite results under different conditions. What factors are responsible for establishing the point of reversal is not entirely clear, but certainly the size of the dose is not the only factor involved. The absolute amounts of calcium as well as the calcium-phosphorus ratio, the state of the digestive tract as well as of the liver, are all concerned. There may be many other important influences.

D. Effects of Antirachitic Compounds and Side-products on Blood Constituents

Since the blood stands as an intermediary between the alimentary canal, the peripheral tissues, and the kidneys, it is where one would expect to look for some clue as to the nature of the metabolic responses to vitamin D. Alterations in blood composition are not always specific and often cannot be interpreted alone, yet certain trends are recognizable as more or less indicative of changes in the tissues. Although it is probable that too much stress has been laid on certain blood changes in response to vitamin D, yet in the absence of better criteria it has been necessary to make use of such

data in seeking information of its action.

Schmidt and Greenberg (64) in their studies on some of the electrolytes in body fluids found the physiological range for calcium in the blood of humans to range between 9 and 11.5. The normal blood calcium level in most common laboratory animals is of about the same order as for man except in case of birds during the laying season. Species differences are not readily explained.

Quite commonly one finds statements to the effect that the action of vitamin D is to produce hypercalcemia or hyperphosphatemia or both, yet there is abundant evidence that either one may occur independently or that neither may follow the administration of large amounts of vitamin D. Very often the experimental conditions are not sufficiently standardized to enable one to determine just why a given variation occurs; in fact, even when all conditions are as well standardized as possible, it may still be difficult to account for these variations.

There is now incontrovertible evidence that a high degree of hypercalcemia of long duration is compatible with life. Sustained hyperphosphatemia is much more likely to disturb body functions. Some years ago Reed and Leed suggested that the primary gross effect of vitamin D is one of stabilizing calcium-phosphorus metabolism, regardless of direction.

Not many observations have been reported on the effects of vitamin D on the formed elements of the blood. Phillips and

colloborators (65) have shown vitamin D to increase the thrombocytes of the blood and to decrease coagulation time in rats. Sure and Kik (66) in their study of hematopoisis in rats found that anemia of rickets is not due to a vitamin D deficiency.

Taylor, Weld, and Branion and Kay (67) reported that vitamin D greatly reduced the phosphatase content in the rat, rabbit, fowl, and dog. Leenhardt and co-workers (68) in their studies of richitic children found that the acid soluble phosphorous was increased markedly whereas the phosphatase activity was decreased.

Dreyer and Reed (69) worked with a group of arthritic patients fed massive doses of vitamin D and found that the serum phosphatase activity was apparently of little use as an objective diagnostic test in arthritis, because of the wide variation in values found.

In summary, studies on blood showed that the only constituents affected in any consistent manner by massive doses of vitamin D were total calcium (upward), cholesterol, glucose, phosphatase (general trend downward), and phosphorus (upward).

Dihydrotachysterol has been shown to cause increases in both phosphorus and calcium and a downward trend in phosphatase activity.

E. Toxicity of Antirachitic Compounds and Side-products

The voluminous discussion of the toxic effects of vitamin D has been complicated by a number of confusing factors. In the first place standard terminology is still lacking. "Hypervitaminosis D", and "Vitamin D intoxication", have all been used loosely and interchangeably to designate the same physiologic state. Evidence has shown that there is a definite distinction to be made among these terms. There has also been a general failure to record quantitatively the dosage of vitamin D in relation to body weight and to indicate the duration of administration. Moreover the species variation in susceptibility has very often not been taken into account. Then too, the purity of the vitamin D preparations used was unquestionably a factor of importance in all the work on this problem up to the period when calciferol or vitamin D₂ was made available. Most work thereafter did not differentiate between the effects produced by the early impure preparations and those of pure calciferol. Finally the symptoms of intoxication have not always been as closely observed as would be desirable, nor has due account been taken of the influence of diet and of other physiological factors.

Both toxisterol and tachysterol are capable of producing severe symptoms of intoxication. Furthermore both produce histological and chemical changes in the body tissues generally that are definitely pathologic.

Calciferol also is able to produce toxic effects. Herein lies the justification for the assumption that hypervitaminosis D and vitamin D intoxication are one and the same state. The common occurrence of hypercalcemia in toxic conditions at first led to the assumption that this phenomenon was responsible for the toxic condition. Reed and Thacker (70) have called attention to the frequent occurrence of hypercalcemia of long duration without any symptoms of toxicity. From their studies they concluded, therefore, that vitamin D intoxication and hypercalcemia are not synonymous, although the two conditions may exist simultaneously.

The identity of vitamin D intoxication and hyperparathyroidism are not thought to be identical in as much as not all of the objective and subjective evidences of intoxication by one or the other can be proved by an excess of the other. Hyperavitaminosis D and the vitamin D intoxication can not be used synonymously inasmuch as the toxic threshold varies among individuals and thus hypervitaminosis D does not inevitably lead to intoxication. Obviously, a subject receiving 30,000 units of vitamin D per kilogram per day is in a state of hypervitaminosis D, even though no toxication occurs. Likewise the subject receiving 2,000 units per kilogram per day, may under some circumstances show a toxic condition but scarcely can be said to be in a condition of hypervitaminosis D, since an increase of vitamin D concentration in the blood stream may not be demonstrable.

It is suggested, therefore, that "hypervitaminosis D" be employed to designate only those states in which the concentration of the vitamin in the blood stream can be demonstrated to be definitely increased. Regardless of the amount ingested, if it is not circulating it must have been metabolized, stored, excreted, or unabsorbed, and there can be no hypervitaminosis. If under these conditions a toxic state develops, it is obviously not hypervitaminosis per se although probably a secondary effect thereof.

It is apparent that in some respects the toxic action of vitamin D is not unlike the dehydrating effect of parathyroid extract Basset (71). It is apparent also that the toxic action may affect any and all tissues but the kidney is most commonly involved. Moreover, the effects seem to be exerted on tissue cells, and within physiological ranges of application there is improvement in cellular function. However this process appears to be reversible by either a deficiency or an excess as pointed out by Freudenberg (72).

Widenbauer (73) claimed that vitamin A or B complex protected mice against toxication with either vitamin D or dihydrotachysterol. Numerous reports have indicated that activated ergosterol is more toxic than cod liver oil with comparable vitamin D unitage.

Investigations with mammals have generally indicated that massive doses of vitamin D₂ are toxic, demonstrating increased

calcium levels accompanied by bone dissolution. The less frequently studied by-product of ergosterol irradiation dihydro-tachysterol has been shown by Correll and Wise (74) to have a low antirachitic effect and for mammals, it is hypercalcemic and toxic.

In summing up the toxic effects of these compounds, we find that severe and fatal toxication may be produced by excessive amounts of vitamin D. The incidence and severity may be conditioned by:

- (1) the dose per unit of body weight
- (2) the duration of administration
- (3) the composition of the diet, in both mineral and organic content
- (4) the state of the alimentary tract
- (5) the species susceptibility
- (6) individual susceptibility
- (7) age
- (8) pre-existing pathology
- (9) the vehicle in which the vitamin is administered
- (10) the route of administration
- (11) the functional state of the endocrine system
- (12) the purity of the vitamin preparation
- (13) the source of the vitamin
- (14) probably other factors not yet recognized

There is a tendency to deposition of calcium and, to a lesser extent, of phosphorus in soft tissues with approach to a toxic stage. This process is reversible to a considerable extent and apparently leaves no chronic injury.

The incidence of toxication in human subjects is relatively low. The threshold of toxication for humans and dogs appears to be 20,000 U.S.P. units of vitamin D per kilogram of body weight per day for most preparations used.

Of all tissues studied the kidney appears to be the most vulnerable, and the aorta next. Nevertheless hypertension has not been encountered in human subjects.

The extreme pathological changes described in animals have, with few exceptions, been produced by doses of vitamin D greatly in excess of the highest doses recommended for human therapy. If the doses are calculated on the basis of unit weight.

Goormaghtigh and Handowsky (75) have recently shown with dogs doses of vitamin D (pure calciferol) ranging from 1600 to 2800 units per kilogram for 37 to 102 days, the blood pressure was increased from an average of 134 mm before treatment to 149 mm after treatment. They also found that renal damage was produced by doses in the range of 20,000 units per kilogram per day or above. Doses of 4,000 units were harmless and even beneficial. Even on the large doses they found that the arteriolar musculature could recover with no permanent damage. In young animals however regression of the thymus might occur resulting in cessation of growth.

Changes described were found only in the kidney and to a lesser extent in the spleen. Larger doses produced similar changes in the neurohypophysis, thyroid, gonads, adrenal and pancreas. The aorta was not affected except by still larger doses.

CHEMISTRY OF THE PROVITAMINS D AND OF THE IRRADIATION PROCESS

An understanding of the structure of the molecule of vitamin D and of the chemical reactions leading to its formation as well as of the changes brought about by various chemical and physical agents can best be gained by a discussion of the chemistry of sterols. This is due, of course, to the fact that vitamin D is derived from sterols, both in nature and in technical manufacture.

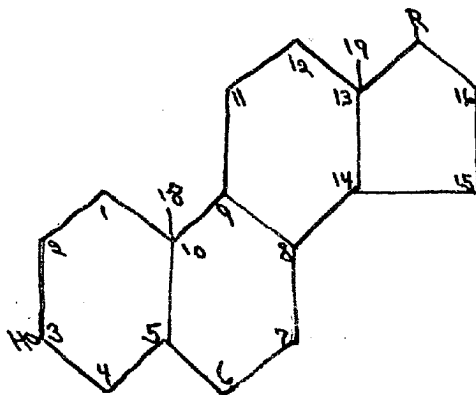
The chemistry of sterols since the isolation of cholesterol from gall-stones by de la Salle about 1769 has been reviewed by Fieser (76) and Bills (77). Sterols have been found in practically all animal and plant tissues—a fact which has led biologists to the conclusion that they have some important functions in cellular metabolism. Another indication of this importance is the fact that it has been amply proved that synthesis may take place in the animal organism.

The elementary formulas of most of the sterols which have been isolated in pure form have been derived, and a few of these are included in the following table, which lists also the melting point, optical rotation, and chief source of certain of the more common sterols.

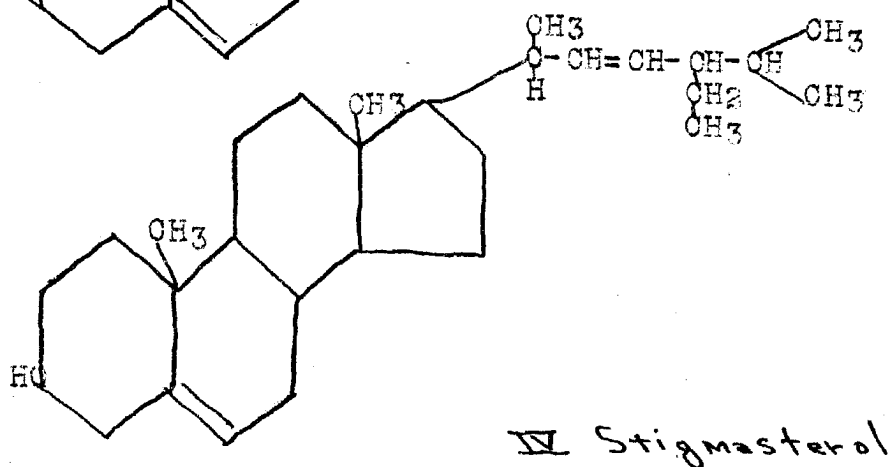
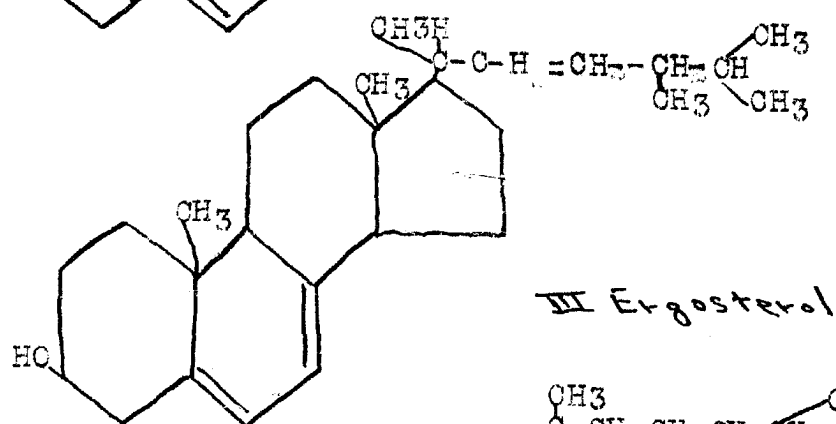
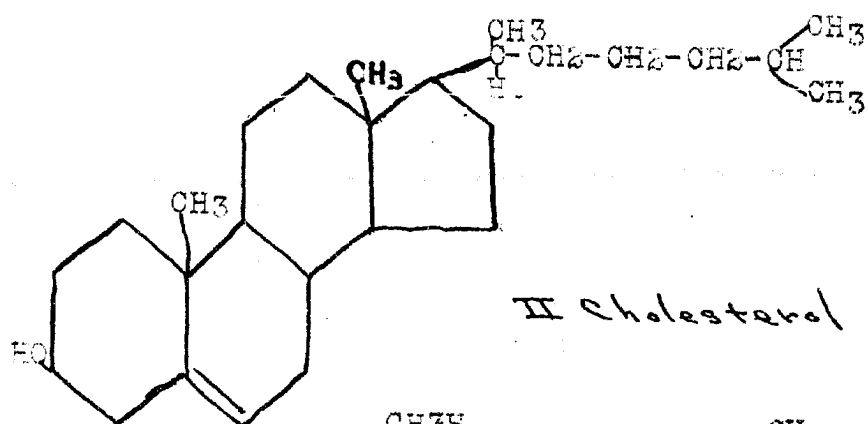
Name	Formula	M.P.	α D	Occurrence
Cholesterol	C ₂₇ H ₄₆ O	150°	-38.8°	All animal cells
Dihydrocholesterol	C ₂₇ H ₄₈ O	142	+28.8	Companion of cholesterol
Coprosterol	C ₂₇ H ₄₈ O	102	+23.5	Feces
Ostreasterol	C ₂₉ H ₄₈ O	143	-43.9	Oysters, gastropods
Lanosterol	C ₃₀ H ₅₀ O	141	+58.0	Wool fat
Agnosterol	C ₃₀ H ₄₈ O	162	+70.6	Wool fat
Ergosterol	C ₂₈ H ₄₄ O	163	-133.0	Ergot, yeast
Sitosterol	C ₂₉ H ₅₀ O	146	-42.4	Fats of higher plants
Stigmasterol	C ₂₉ H ₄₈ O	170	-45.0	Calabor beans, soy beans
Chincol	C ₂₉ H ₅₀ O	140	-24.0	Chinchona bark
Fucosterol	C ₂₉ H ₄₈ O	124	-38.4	Algae
Tymosterol	C ₂₉ H ₄₄ O	110	+47.3	Yeast

The study of the chemistry of sterols has assumed a greater importance in recent years since it has been found that in addition to vitamin D certain other biologically important substances are also steroid derivatives. For example certain of the sex hormones are modified sterols. The bile acids are sterols, as are certain cardiac poisons such as strophanthin and the toad poisons such as bufotoxin. Certain of the carcinogenic hydrocarbons contain the phenanthrene nucleus found in all sterols.

So far as is known at present, all sterols are modifications of the general structure shown in formula I.



The rings are not aromatic in nature but are not necessarily completely saturated. The side group, indicated by R in Formula I, varies with the sterol and may be saturated or contain one or two double bonds. The accepted formulas of cholesterol and ergosterol are given in Formulas II and III as examples and for comparison. A typical plant sterol, stigmasterol, is shown in Formula IV.



It is noticeable that these three formulas have a remarkable similarity in spite of the wide diversity of the sources of the sterols they represent. It is to be noted further that the greatest differences lie in the side chain, and indeed the variations in chemical and physiological behavior are associated more with changes in the side chain than with any deep seated rearrangements or substitutions in the ring system itself. The nucleus is remarkably stable, and only strong methods of treatment will cause rupture.

Other properties of the sterols may be explained by the formulas given. They are all secondary alcohols, since the ring to which the hydroxyl group is attached is saturated. Thus they are neutral rather than phenolic. They are readily esterified. A large proportion of the cholesterol in mammalian tissues exists as esters of various fatty acids. As would be expected from steric considerations as well as from the general properties of all secondary alcohols, the esters are readily saponified.

The similarity of structures II, III and IV suggests that the physical and chemical properties would be expected to vary little among the various sterols. This is the case. Sterols can be prepared from their various natural sources without great difficulty, but even when such preparations yield crystalline products they are almost invariably mixtures of two or more sterols. The separation and purification of a single

sterol is a process often requiring great ingenuity and frequently taxes the resources of the chemist to the utmost, since solubilities are almost identical, and distillation is difficult. In addition sterols form molecular compounds or complexes with each other which are exceedingly difficult to separate.

Formulas II, III, and IV indicate also the vast possibilities for formation of stereoisomers. There are eight asymmetric carbon atoms in cholesterol as well as in ergosterol. On this basis alone, two hundred and fifty six isomers of each substance are possible. The ring structures present opportunities for cis and trans isomerism. These possibilities are important, since many of the various naturally occurring sterols differ from one another only by spatial configuration of the groups around certain of the asymmetric carbon atoms. When it is remembered that no chemical or physical differences exist between optical antipodes except the rotation of polarized light and that the ✓ differences between cis and trans isomers and between epimers are often not very great, the difficulties of separation and identification of the various sterols are readily understood.

Altho there has been accumulated a rather large amount of information regarding reactions of sterols, most of this has not been of particular interest for presentation here. The reactions which are useful for analytical purposes may be described briefly here, since they are similar to reactions which

have been studied in attempts to develop a chemical procedure for the assay of vitamin D. Certain sterols when treated with strong acids under dehydrating conditions give rise to colored compost.

These reactions form the basis for several color tests for sterols, although these colors are not specific for any particular sterol nor are they quantitative except under rigidly controlled conditions.

If the tests are not as reliable as is desirable for distinguishing between various unsaturated sterols they do serve to distinguish sharply between saturated and unsaturated sterols. Only unsaturated sterols give colors under the conditions of the experiment. The colored compounds are probably halochromic salts formed by attachment of the acid (or, when used, a metal salt) to an unsaturated center of the molecule. The reaction appears to involve a ring system of the molecule, since many other polynuclear hydroaromatic compounds which are saturated or potentially saturated behave likewise.

Another reaction of importance is one discovered by Windaus. He showed that cholesterol (and since many other sterols) forms a molecular compound with digetonin. This latter substance is a rare glucosidic saponin found in *digestilis* seeds and has the formula $C_{56}H_{92}O_{29}$. The compounds formed are called digitonides and are formed from one molecule each of the sterol and digitonin. They are usually exceedingly insoluble in

certain solvents and this has been used to separate the sterol forming insoluble digitonides from those which form soluble ones. In order to form digitonides the hydroxyl group must be free and must have the same steric arrangement as in cholesterol.

Steenback, Hess and others soon after their discovery that rachitogenic diets could be rendered protective against rickets, discovered that sterols could be activated by ultra-violet light. This made it clear then that the sterol fraction of foods was influenced by the activating rays.

It was next discovered thru the study of the ultra violet absorption spectrum that cholesterol was not the provitamin. It was soon shown that it was a contaminant. When crude cholesterol was purified by the dibromide treatment, it was no longer activatable and no longer showed the characteristic absorption spectrum of the original product.

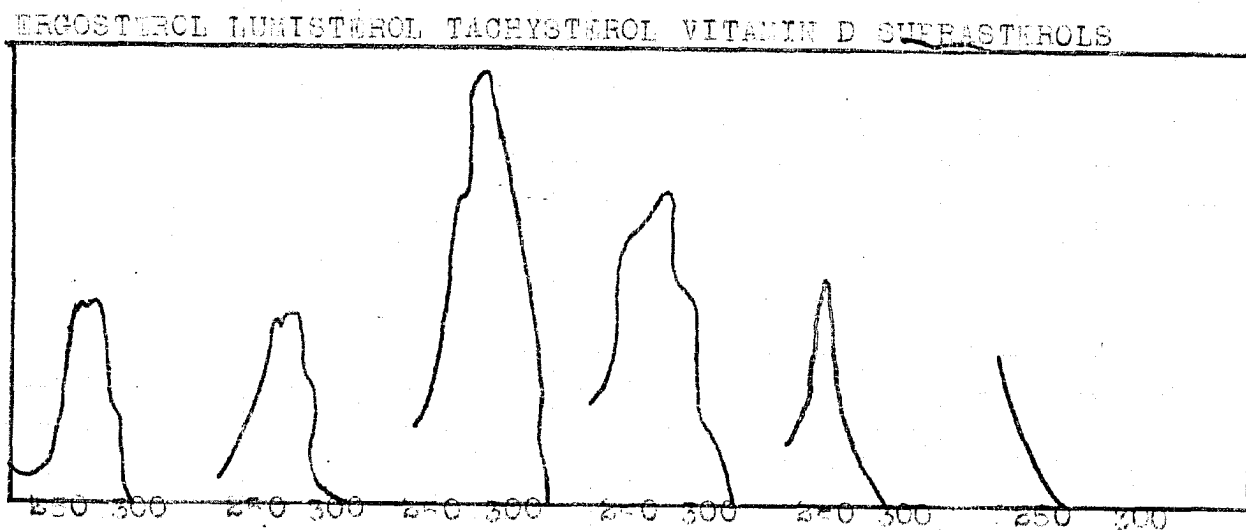
Heilbron, Kanam, and Morton (79) that the repeated fractional crystallization of cholesterol led to the accumulation in the least soluble fraction of the substance responsible for the characteristic absorption spectrum of crude cholesterol. After further study this was identified as ergosterol.

It was at first thought that (ergosterol) was the only provitamin D but this was shown to be doubtful. Convincing evidence was temporarily not forthcoming because intensive studies were then being made on the physics and chemistry of the irradiation of ergosterol.

It was found that sources of radiant energy other than ultra-violet were effective in forming vitamin D from ergosterol. Among those found effective were cathode rays which gave little vitamin D and much decomposition and radium emanation which was moderately effective. X-Rays were shown not to activate ergosterol, as were high intensity radio ways.

Intensive studies on the portion of the spectrum chiefly responsible for the activation showed that ergosterol absorbs strongly in the range 305-230 mμ and that there is some absorption on either side of this range. Some work has shown the longer waves up to 313 mμ seem to be of some importance in the decomposition of ergosterol and possibly of the primary irradiation products.

FROM FREIDMANN'S " STEROLS AND RELATED COMPOUNDS "



ABSORPTION SPECTRA OF ERGOSTEROL AND ITS PHOTO-DERIVATIVES
Absorption Spectra of Ergosterol and its
Photo-derivatives (.02 percent in ether)

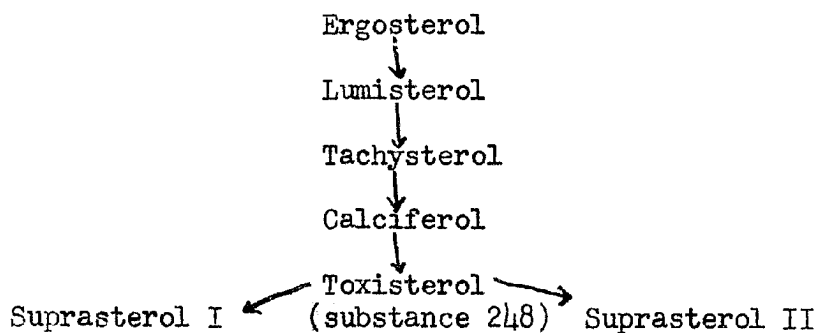
Difficulties in determining the activation energy of ergosterol are contained in the fact that there is a series of by-products formed during its activation. Quantity and sequence vary with the condition of irradiation. It has been concluded that one high velocity electron produces, under the conditions of Hoffman and Daniels, less than one molecule of vitamin D, but decomposes about eighty ergosterol molecules. Owen and Sherman have shown that the activation energy for the conversion of ergosterol to calciferol lies between 72 and 92 kilogram calories. Webster and Boundillon have concluded on the basis of a low coefficient of activation and low temperature that the formation of vitamin D from provitamin D is a monomolecular reaction.

The conditions under which ergosterol is irradiated influence greatly the character and amounts of other products obtained. Irradiation of dry ergosterol yields poor products because the products first formed the surface act as filters and prevent light from reaching the lower layers. Dissolved oxygen affects the results of activation, this effect being mostly on the by-products. Vitamin D has been shown by many workers to be stable towards oxygen.

Other factors being constant, the use of various solvents had a marked effect on the vitamin D potency formed in the irradiation. Ether solutions required a longer time of irradiation to achieve the maximum potency than was required for

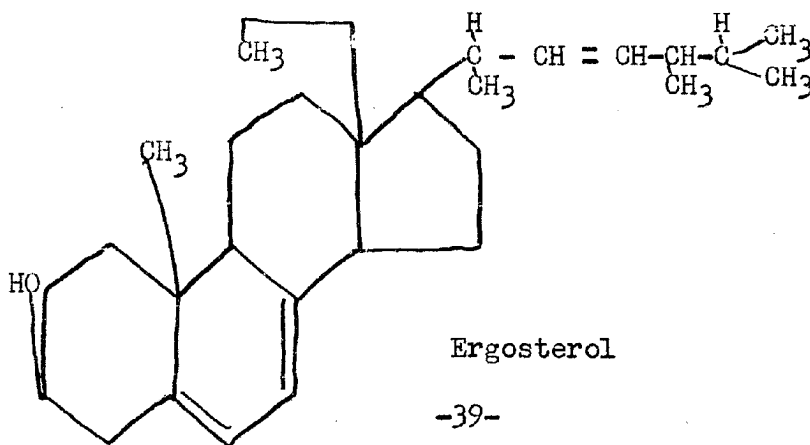
the entire sequence of activation and destruction in alcohol. The maximum potency obtainable was much greater in ether than in alcohol or cyclohexane, but the spectral changes were most conspicuous in alcohol.

Altho the activation of ergosterol to vitamin D is very probably a monomolecular reaction and consists of the isomerization of the ergosterol molecule, the process is not a direct one. There are several intermediate steps as given below:



Ergosterol

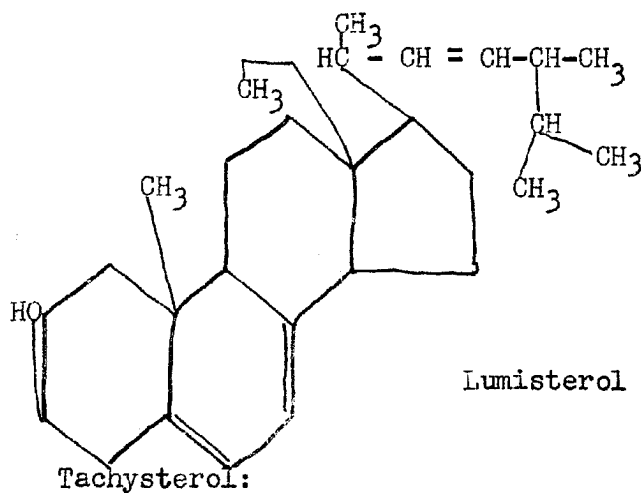
1. White crystalline solid
2. M.P. = 163
3. $[\alpha]_D = -133^\circ$ in CHCl_3
4. Not antirachitic



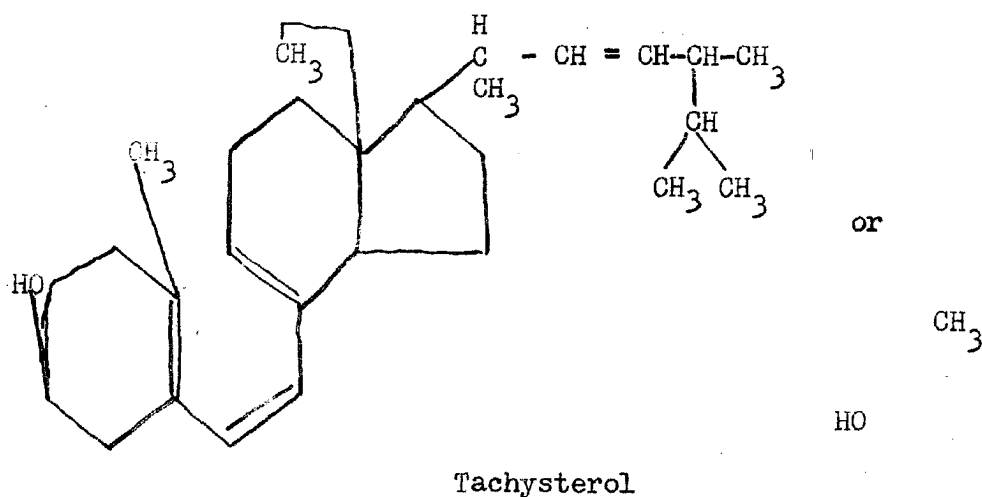
Of these intermediates all except toxisterol have been isolated either in pure, crystalline form or in solutions of high concentration.

Lumisterol:

1. a stereoisomer of ergosterol
2. has no antirachitic value
3. transformed to vitamin D by irradiation
4. gives the various color reactions
5. forms a 1:1 addition compound with calciferol
which is difficult to separate (D₁ of German workers)

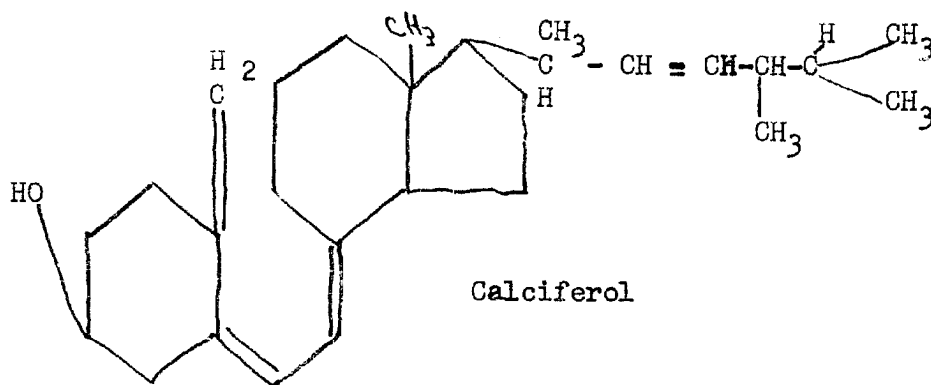


1. does not crystallize readily
2. has great affinity for oxygen
3. questionable antirachitic action
4. toxic



Calciferol = Vitamin D₂

1. isomeric with ergosterol
2. M.P. = 115° - 117° C.
3. White
4. $\alpha_{5461}^{20} = +123.25$ to $+125.75$
5. Sp. Absorption $\begin{matrix} 1\% \\ 1\text{cm} \end{matrix}$ 265 mu = 460-500
6. antirachitic potency 40,000 Int. units per mgm.
7. toxic



Toxisterol

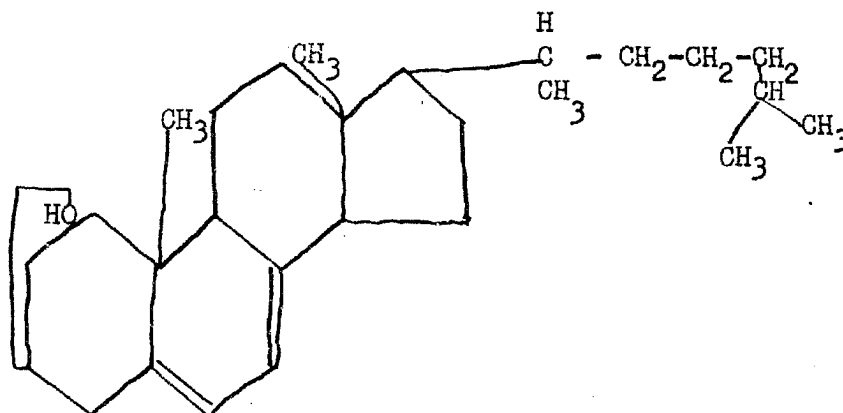
1. formula unknown - probably contains 3 rings
and perhaps 4 double bonds two being conjugated
and the other two isolated.
2. toxic
3. not antirachitic

Suprosterols I and II

1. isolated but formulas not yet determined
2. not toxic
3. not antirachitic
4. give modified color reactions
5. isomeric with ergosterol

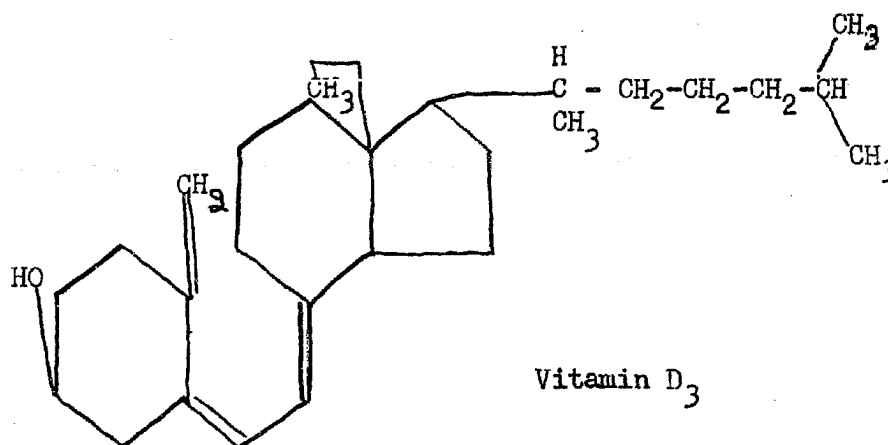
7-dehydrocholesterol

1. not antirachitic
2. provitamin D₃



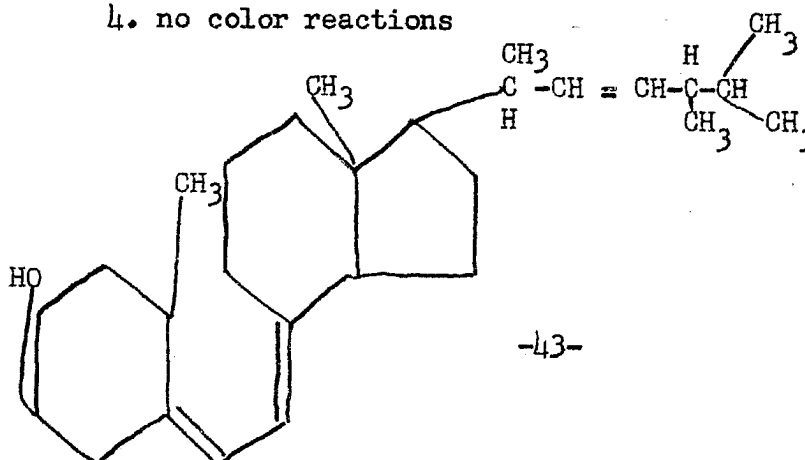
Vitamin D₃

1. M.P. 82-84
2. colorless crystals
3. $\alpha_D^{20} = +83.3^\circ$ (in acetone)
4. absorption maximum 265 mu
5. 40,000 units per mgm
6. more effective in poultry



Dihydrotachysterol

1. toxic
2. slightly antirachitic
3. prepared by reducing tachysterol with sodium in alcohol
4. no color reactions



PHYSIOLOGY OF THE ANTIRACHITIC COMPOUNDS

Bills (79) made a review of the literature concerning the physiology of vitamin D and concluded his reviews with the following statement: "Confusion in this field is understandable when one contemplates the permutations and combinations which are possible in a system comprising several forms of calcium and phosphorus in the blood, a reservoir and depository of these elements in bone, a fluctuating intake and output of them in the diet and excretions, and dumping place for them in the soft tissues—all under the influence of many factors besides the parathyroid glands and the several forms of vitamin D. As for the hormone and the vitamin, which seem to be the major forces in the system, it is most satisfactory to regard them as acting together—complementing, supplementing or opposing each other as the occasion demands".

Altho it cannot be said that any clear explanation of the physiological action of vitamin D has crystallized out of the mass of investigation since Bill's review, many new facts are available which have brought final understanding perhaps a little nearer.

It is remarkable, when one considers that the experimental investigations have been so generally carried out on the rat and that this animal is so widely used as a test subject in the assays of vitamin D, that no one has made comprehensive study of the comparative physiology of the rat. To this fact one may

attribute a large part of the responsibility for the lack of comprehensive knowledge of the physiological relations of vitamin D (48). That this point has received attention is evidenced by the increased tendency to attack various aspects of the general problem from this point of view.

Evidence is accumulating that the responses to vitamin D which have occupied most attention, such as hypercalcemia, phosphatemia, modification of rates of absorption and excretion, ossification--are not primary changes.

Smith and McLean (80) have observed that there was no evidence from chemical analyses of bones of rats fed thyroid to the state of severe hyperthyroidism that there was any disturbance of calcium metabolism in either growing animals or adults. These observations call into question the relationship of the thyroid to calcium metabolism indicated in the earlier reports.

The investigations of Haase (81) on the role of the thyroid would raise a question about this point.

However, it must be admitted that most of the evidence on the relation to endocrine function has to do with effects of amounts of vitamin D which are probably larger than those ever involved in physiological ranges. The only point demonstrated, therefore, is that if sufficient amounts of the vitamin are present it can exert an influence through the particular endocrines. It must be conceded that there is a possibility of

effects on these organs mediated by smaller amounts of the vitamin that are not demonstrable because of the crudeness of available tests. The failure of correlation of blood changes with symptoms is a good example of this.

It is clear that the action of the vitamin whether direct or indirect, affects a variety of tissues since any tissue may show a higher calcium content even though there may be no histological evidence of cell injury. It is speculated that the vitamin may have both a general and a localized action. Evidence for the general action is supported by such work as that of Gelfan, Presnall, Vernor and Todd, and Fischmann (82). In support of localized action the influence on the parathyroid, thyroid, and anterior pituitary may be mentioned as well as the influence on calcium absorption which appears to be localized in the intestinal mucosa.

That there is some definite effect on resistance to infection seems probable.

Deficiency effects are clearly due in part to deficient absorption of calcium.

That the vitamin remains in the mammalian blood stream for months after the administration of excess amounts suggests that it is readily stored in the tissues or else that the storage turnover is very active. It also appears that the body does not possess an efficient mechanism for destruction or inactivation of the vitamin D when supplied in excess.

Altho the antirachitic effects of the various forms of vitamin D appear to be identical in the human, so far as they have been investigated, it seems unlikely that differences do not exist in some of the other effects that have been described for calciferol, cod liver oil, and ertron.

That the vitamin has some influence on muscle function seems inevitable from both direct and indirect evidence. That it may influence the nervous system is suggested by the work of many investigators.

That the vitamin directly influences absorption of calcium and indirectly of phosphorus seems fairly well established. That its main function is exerted elsewhere seems even more certain from the invitro experiments cited on periphenal tissues, bone, endocrines, or central nervous system.

Capillary resistance and periphenal vasomotor reactions appear to be influenced to some extent by the vitamin.

Lastly the influence on skeletal growth must not be disregarded, although this is probably secondary to the general influence on bone.

SIGNIFICANCE

A. Calcium levels

Since calcium is contained entirely in the plasma it is obvious that the calcium content of the whole blood will vary inversely as the corpuscular volume. Because of the great variability of the latter factor, determinations of calcium are usually not made on whole blood. The calcium content of human serum normally ranges from 9-11 mgm. per 100 cc. The plasma content is from .5 to 1 mgm lower although some observers believe it to be higher. The serum calcium is quite constant under normal conditions. Serum calcium consists of two physiologically distinct fractions which have been termed diffusible and non-diffusible. The diffusible probably contain the physiologically active calcium and represents 40 to 60 percent of the total serum calcium.

The quantitative relationship between the various calcium states in the blood are:

Total serum calcium =	Diffusible Ca +	non diffusible Ca
9-11.5 mg	4.5-5.5 mg	4.5-6 mg.
	(1) ionized 2 mg	non-ionizable
	(2) nonionized	

Benjamin and Hess (72) have postulated four physico-chemical states of calcium in normal blood serum:

1. an adsorbable calcium-phosphorus complex constituting two-thirds of the diffusible calcium.
2. Calcium ion (remaining diffusible calcium).

3. adsorbable, nondiffusible calcium-phosphorus complex
(1/4 of nondiffusible)
4. protein bound calcium (3/4 of nondiffusible).

Conditions causing or in which a hypercalcemia coexists
are:

1. Hyperparathyroidism
2. Excess vitamin D (hypervitaminosis)
3. Nephritis (rare cases with uremia)
4. Polycythemia
5. Multiple myeloma
6. Increased CO₂ tension
7. Neoplastic disease of bone
8. Ovulation in birds

Conditions causing or associated with a hypocalcemia are:

1. Hypoparathyroidism
2. Vitamin D deficiency
3. Calcium deficiency
4. Osteomalocia
5. Hunger Osteopathy
6. Celiac disease
7. Sprue
8. Nephrosis
9. Nephrites (occasionally)

10. Kala-azar

11. Milk fever of cattle

Given below are a few normal serum calcium values for the animals used in these studies:

Cow.....	9-12 mgms/100 cc serum
Dog.....	9-11.5 mgms/100 cc serum
Rat.....	9-11.5 mgms/100 cc serum
Chicken (nonlaying).....	9-12.5 mgms/100 cc serum
Chicken (laying).....	17-39 mgms/100 cc serum

B. Phosphorus levels

Absorption--Approximately two thirds of the P present in average normal diets is absorbed from the intestinal tract.

Most of the food phosphorus is present in nucleoproteins, phosphoproteins, and phospholipids. Excessive amounts of calcium in the diet inhibit phosphate absorption. Vitamin D is necessary for optimal absorption during childhood.

Distribution--Most of the phosphorus of the body is in the bones and teeth. However, small amounts are present in all nucleated cells, since phosphoric acid is a constituent of nucleoproteins. The total phosphorus of plasma varies from 8 to 18 mgms per 100 cc. Red blood cells contain from 47 to 114 mgm in each 100 cc. Most of the blood phosphorus is present in organic form.

Normal values for inorganic phosphorus are given below:

Cow.....	2.25-9.63 mgms./100 ccs
Dog.....	3-6 mgms./100 ccs
Chicken (laying).....	5.9-10-26 mgms./100 ccs
Chicken (nonlaying).....	7.1-7.98 mgms./100 ccs
Rat.....	3.5-6.5

Functions of Phosphorus:

1. Phosphorus is required for the synthesis of bones and teeth.
2. It is necessary for the formation of nucleo-proteins phospholipids, sugar esters, and other organic phosphorus compounds in the tissues.
3. Phosphate salts act as buffers in blood.
4. Phosphoric acid and organic phosphorus compounds are necessary for normal muscle contraction.
5. It is necessary for the formation of milk. Casein, one of the principal milk proteins, is a phosphoprotein.
6. It may be necessary for the absorption of sugars from the digestive tract and the kidney tubules.
7. It is required for normal growth.

Excretion:

About two thirds of the phosphorus eliminated from the body is excreted in the urine. The remainder is present in the stools.

C. Phosphatase - General Statement

They are present in practically all living cells. Certain tissues and fluids have been investigated especially; these are bone, blood plasma, erythrocytes, leucocytes, liver, kidney, intestinal epithelium, milk, urine, rice, bran, moulds.

Phosphatase activity assumed significance as a laboratory diagnostic aid when Kay found that values in plasma were increased in various conditions, but particularly in bone diseases.

A large body of clinical data has since accumulated, defining the particular levels in these diseases more closely. There has also been considerable investigation concerning the nature of the enzymes in the various tissues and the source of the serum phosphatase.

The significance of the increase in serum phosphatase in bone disease has been variously interpreted. Kay offered the explanation that the increase is either the result of an overproduction in the bone in attempting to compensate for the lesion or of forced exit from the injured bone due to mechanical stress.

Interesting changes have been demonstrated to take place in the phosphatase activity of the blood in disease, particularly in generalized bone disease. There is almost invariably a marked increase in plasma phosphatase, the value rising in some cases to twenty or more times the normal average value. The abnormality is confined almost exclusively to cases of bone disease, and in a general way varies in extent with the severity of the disease. It suggests a definite correlation between bone disease and abnormal phosphatase distribution in the body, a finding which is of interest in connection with the suggested role of phosphatase in bone formation and maintenance.

DESCRIPTION OF METHODS

A. Calcium --

The Clark-Collip modification of the Kramer-Tisdall Method, was used in these studies. The calcium is precipitated directly from the serum as oxalate and the latter is titrated with potassium permanganate.

Procedure --

Introduce into a graduated 15 ml centrifuge tube 2 cc of clear serum, 2 ml of distilled water, and 1 ml of 4 percent ammonium oxalate solution. Mix thoroughly. The centrifuge tube should have an inside diameter of 6-7 mm at the .1 ml mark. Mixing is aided by holding the tube at the mouth and giving it a circular motion by tapping the lower end. Let stand 30 minutes or longer. Again mix the contents. Centrifuge for about 5 minutes at 2500 revolutions per minute. Carefully pour off the supernatant liquid and while the tube is still inverted let it drain in a rack for 5 minutes, resting the mouth of the tube on a piece of filter paper. Wipe the mouth of the tube dry with a soft cloth. Stir up the precipitate and wash the sides of the tube with 3 ml of dilute ammonia (2 ml of concentrated ammonia to 98 ml water) directed in a very fine stream, from a wash bottle. Centrifuge the suspension and drain again as before. Add 2 ml of approximately normal sulfuric acid (28 ml of concentrated per liter) by blowing it from a pipette directly upon

the precipitate so as to break up the mat and facilitate solution. Place tube in a boiling water bath for a minute. Titrate with .01N potassium permanganate to a definite pink color which persists for at least one minute. If necessary during the course of titration warm the tube by placing in a water bath kept at 70-75°. A micro-burette graduated in .01 ml should be used.

Calculation:

$$(X-b) \times .2 \times \frac{100}{2} = \text{mgms Ca per 100 ml of serum.}$$

Where X = number cc's of permanganate required

b = blank (reagents)

B. Phosphorus --

Method of Youngburg's:

Transfer 4 ml of 10% trichloroacetic acid to a test tube and add 1 ml of plasma or serum while mixing. Shake well and filter thru ashless filter paper. Transfer 2 cc of the filtrate to a test tube and 2 cc of standard phosphate solution (0.02 mg P) to a similar tube. Then to each tube add 5 ml of water, 2 ml of molybdic-sulfuric acid reagents, and finally, without delay, blow in from a pipette exactly 1 ml of dilute stannous chloride solution. Mix after 1 minute determine the color with a photoelectric colorimeter (Lumetron).

Reagents for Phosphorus Determination:

Ten per-cent Trichloroacetic Acid:

The C.P. acid is purified by distilling under reduced pressure. Dilute to make 10%

10 N. Sulfuric:

450 ml of concentrated sulfuric C.P. are added to 1200 ml of water. This solution is titrated and diluted to make it 10N.

Molybdate-Sulfuric Acid Mixtures:

Solution A. 50 ml of 7.5% sodium molybdate (P free).

50 ml of 10 N sulfuric acid.

Stannous Chloride Solution:

Dissolve 10 grams of C.P. Stannous chloride in 25 ml of concentrated C.P. Hydrochloric acid. Store in a brown glass stoppered bottle. Prepare fresh after four weeks. Dilute 1 ml of the above stock solution to 200 ml with water. Discard unused portions.

Data for Phosphorus Curve:

The stock standard for this curve was prepared in the following manner--

Dissolve .4389 grams of pure dry monopotassium phosphate in water enough to make 1000 ml.

10 ml = 1 mgm P Dilute 10 ml to 100 (1 ml = .01mgm P).

This stock was diluted to give the following curve points.

(Refer to above).

A. Stock (1 cc equals .1mgm).

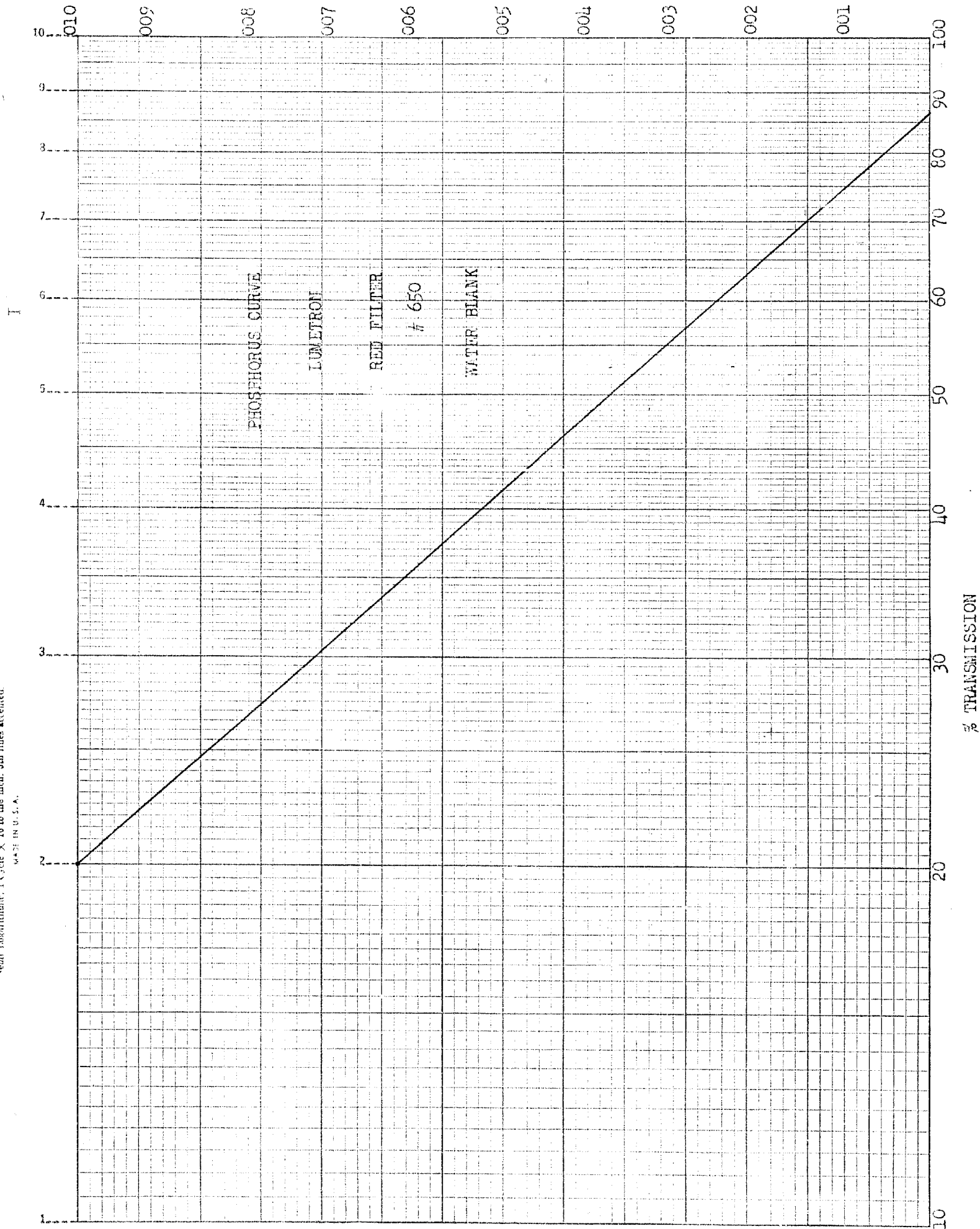
B. 10 ml A diluted to 100 ml (1 ml equals .01 mgms).

C. 10 ml B diluted to 100 ml (1 ml equals .001 mgms).

D. 10 ml C diluted to 100 ml (1 ml equals .0001 mgms).

DILUTIONS FOR STANDARD P CURVE

NO.	CONC.	HOW MADE	AMT. H ₂ O	AMT OF MO.A	SnCl ₂	TOTAL
I	.01	1 ml B	6	2	1	10
II	.007	7 ml C	0	2	1	10
III	.005	5 ml C	2	2	1	10
IV	.003	3 ml C	4	2	1	10
V	.001	1 ml C	6	2	1	10
VI	.0007	7 ml D	0	2	1	10



C. Phosphatase Activity

Modified King and Armstrong Method:

Approximately 12 to 15 ml of blood are withdrawn from a vein and allowed to clot in a test tube or centrifuge tube. Precautions against hemolysis are observed by using a dry syringe and tube and by avoiding fragmentation of the erythrocytes through too vigorous expression of the blood from the syringe into the tube. After the blood has clotted, the outer surface of the clot is separated from the inner surface of the tube by rimming it with a wooden applicator stick. The blood is then centrifuged for 5 to 10 minutes, after which the serum is pipetted off. Three ml of serum are needed for the test. If it is necessary to ship serum samples to a distant point for analysis, the enzyme activity can be preserved in transit by adding 2 to 4 drops of toluol to 4 or 5 ml of serum and stoppering tightly.

Solutions Required: Note: All chemicals should be of analytical reagent quality.

(1) pH 5 buffer substrate:

Disodium-monophenyl-phosphate- 1.09 grams.

0.2N sodium acetate- 700 ml (27.22 gm. sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) per liter of distilled water).

0.2N acetic acid-300 ml (11.3 ml glacial acetic acid per liter of distilled water).

The disodium-monophenyl-phosphate is dissolved

in 300 ml of 0.2N acetic acid, then 0.2N sodium acetate is added to dilute to 1000 ml. This constitutes a 0.005M solution of disodium-monophenyl-phosphate.

Add 5 ml of chloroform. Keep on ice.

(2) pH 9.3 buffer substrate:

Disodium-monophenyl-phosphate- 1.09 grams.

Barbital sodium - 10.3 grams.

Dissolve in distilled water and dilute to 1 liter.

Add 5 ml. of chloroform. Keep on ice.

(3) Phenol reagent of Folin and Ciocalten

The reagent should have no greenish tint. It should be kept well protected from dist, because organic materials will gradually produce slight reductions. Dilute this reagent one in three (250 ml reagent to 750 ml of water).

Into a 1500 ml Florence flask introduce 100 gm sodium tungstate, $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 25 gm. sodium molybdate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 700 ml water, 50 cc. 85 percent phosphoric acid and 100 cc. concentrated hydrochloric acid, and reflux gently for 10 hours. Add 150 gm. lithium sulfate, 50 ml of water and a few drops of bromine. Boil mixture for fifteen minutes without condenser, to remove excess bromine. Cool, dilute to 1 liter, and filter.

(4) 20% sodium carbonate:

Dissolve 200 gm. of anhydrous sodium carbonate (Na_2CO_3) in one liter of distilled water.

(5) Stock phenol solution:

Dissolve 1 gm. crystalline phenol in 0.1N HCl and make up to 1 liter with 0.1N HCl.

Transfer 25 ml of this solution to a 250 ml flask, add 50 ml of 0.1N sodium hydroxide and heat to 65° centigrade.

To the hot solution add 25 ml of 0.1N iodine solution; stopper the flask and let it stand at room temperature for thirty to forty minutes.

Add 5 ml of concentrated hydrochloric acid and titrate the excess of iodine with 0.1N thiosulfate solution. Each cubic centimeter of 0.1N iodine solution corresponds to 1.567 mg. of phenol. On the basis of the result dilute the phenol solution so that 10 ml contain 1 mg. of phenol. Five ml of this solution (equivalent to 0.5 mg. of phenol), when 10 ml of the phospho-tungstic phosphomolybdic reagent and 25 ml of the saturated sodium carbonate solution are added, and whole made up with water at about 30° C. to 100 ml gives a convenient standard.

Preparation of a Standard Curve:

Prepare a series of solutions, diluting the dilute stock standard phenol solution (10 mg. phenol per 100 ml of solution) with distilled water in appropriate amounts of phenol ranging from 0.005 mg. to 0.1 mg. Take 5.0 ml portions of each dilution in separate 25 ml volumetric flasks. Add 4.5 ml diluted phenol reagent (Folin-Ciocalten) to each. Add 2.5 ml 20% Na_2CO_3 to each. (The time should be noted when Na_2CO_3 is added to the first flask and a convenient time interval, 30 seconds or 1 minute observed between additions to subsequent flasks, so that the same time sequence may be obtained in taking colorimeter readings). Dilute to 25 ml with distilled water. Pour 10 to 15 ml from each volumetric flask into separate colorimeter tubes. Read in exactly 20 minutes from the time Na_2CO_3 was added to each solution. Readings are taken on an Lumetron colorimeter, model No. 400, with a 650 m μ filter, and a low setting on the colorimeter. From these readings a standard curve is constructed on semi-logarithmic graph paper; ordinates are mg. of phenol per 25 ml volumetric flask, abscissa are readings on the colorimeter scale. The standard curve should be checked periodically. (The dilute stock standard phenol solution will keep at least 3 months in a refrigerator.

DILUTIONS OF STOCK STANDARD PHENOL
FOR STANDARD CURVE

STOCK		DILUTIONS /5 ml
5 ml. K	25 ml. (L)	.005 mg./5 ml.
2.5 ml. A	25 ml. (J)	.010 mg./5 ml.
5 ml. A	25 ml. (B)	.020 mg./5 ml.
7.5 ml. A	25 ml. (H)	.030 mg./5 ml.
10.0 ml. A	25 ml. (C)	.040 mg./5 ml.
12.5 ml. A	25 ml. (G)	.050 mg./5 ml.
15.0 ml. A	25 ml. (D)	.060 mg./5 ml.
17.5 ml. A	25 ml. (M)	.070 mg./5 ml.
20.0 ml. A	25 ml. (E)	.080 mg./5 ml.
22.5 ml. A	25 ml. (F)	.090 mg./5 ml.
5 ml. stock	25 ml. (A)	.100 mg./5 ml.
K = 1 ml. A dil 4 ml.		
1 ml. K = .005 mg.		

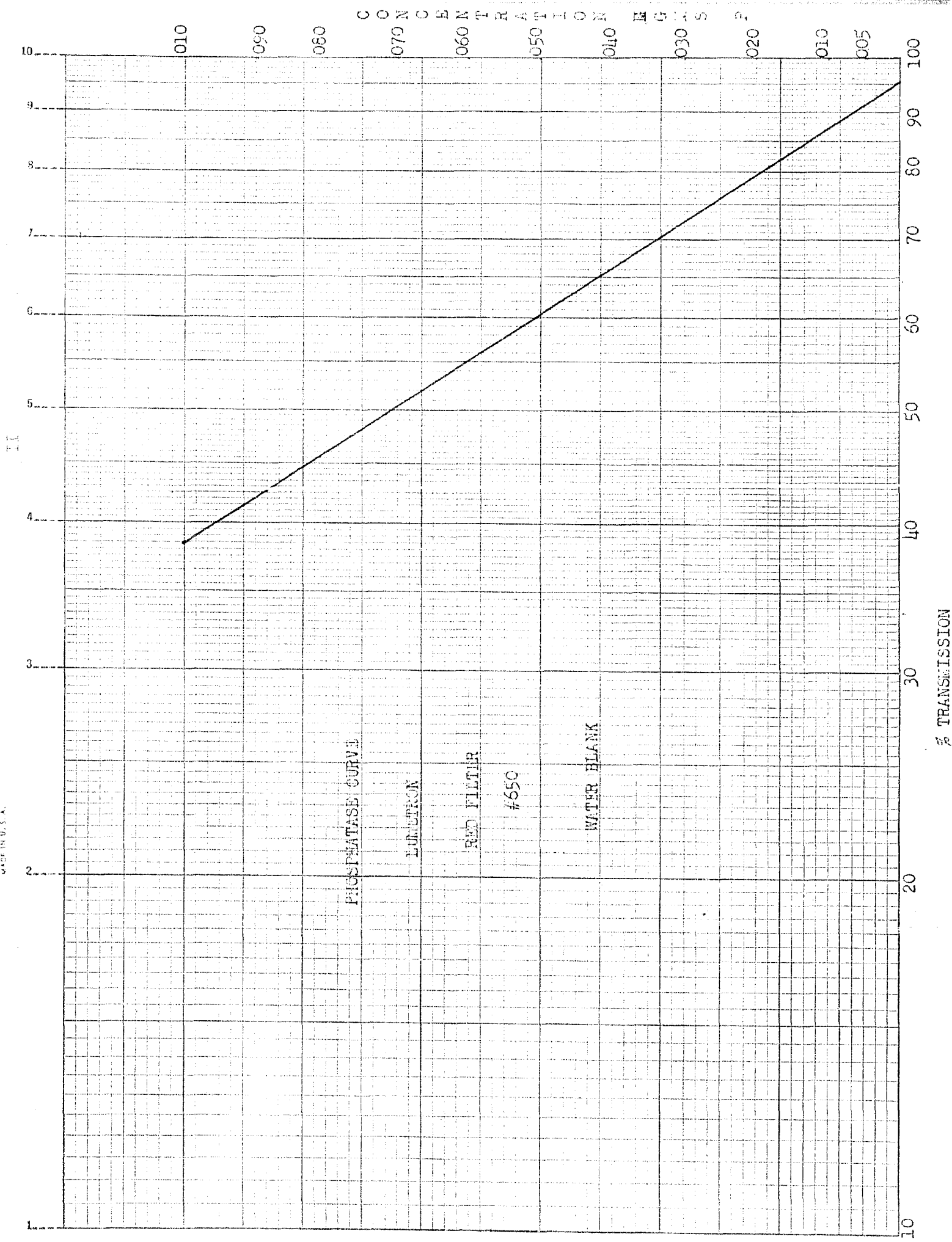
1 ml. stock phenol = 1.1232 mg. phenol

100 ml. stock phenol diluted to 1123.2 ml. with .1N HCl

1 ml. diluted phenol = 0.1 mg. phenol

10 ml. diluted phenol = 1 mg. phenol

KEUFFEL & ESSER CO., N. Y. NO. 355-51
Scale: Logarithmic, 1 Cycle X 10 to the Inch. 5th lines omitted
MADE IN U.S.A.



POINTS USED FOR PLOTTING STANDARD PHENOL CURVE

<u>DILUTIONS</u>	<u>READINGS</u>
.100 mg./5 ml.	38.7
.090 mg./5 ml.	42.4
.080 mg./5 ml.	46.2
.070 mg./5 ml.	50.5
.060 mg./5 ml.	55.0
.050 mg./5 ml.	60.0
.040 mg./5 ml.	65.5
.030 mg./5 ml.	72.7
.020 mg./5 ml.	79.0
.010 mg./5 ml.	86.7
.005 mg./5 ml.	90.7

Readings on the standard curve were taken with the orange filter also but were not plotted, because of the similarity to the red. These readings may be found in the data. Readings were also made with the orange filter on all the unknowns but were not carried over into the report.

Procedure:

All tests are made in duplicate. Buffer-substrate solutions should be brought to room temperature before starting test. In 4 test tubes are placed two 10 ml portions each of pH 5 and of pH 9.3 buffer substrate solution. Allow tubes to remain in the oven at 37.5°C. until solutions have reached this

same temperature, remaining at least five minutes. Add 0.5 ml serum to each, stopper with rubber stoppers, invert twice, and allow to remain in the oven for exactly 30 minutes. At the end of this time add 4.5 ml of dilute Folin-Ciocalten reagent, invert several times, and centrifuge for 2 to 3 minutes. It is important that the Folin-Ciocalten reagent be added to each tube exactly 30 minutes after the serum was added to it, so that a precise period of incubation is observed for each sample.

Control:

In 2 test tubes place 10 ml portions each of pH 5 and of pH 9.3 buffer substrate solution. Add 4.5 ml dilute Folin-Ciocalten reagent, 0.5 ml serum, invert twice, and centrifuge. (It is important that the Folin-Ciocalten reagent be added before the serum).

Test:

Pipette 2 ml filtrate from test and control solutions into 25 ml volumetric flasks. Add 2.5 ml of 20% sodium carbonate solution to each; this should be carried out with convenient time intervals, 30 seconds or 1 minute, between additions to successive flasks, so that colorimeter readings may be made in the same manner. Dilute with distilled water to 25 ml., stopper, invert several times, and pour 10 to 15 ml of each solution into colorimeter tubes. Read each solution in colorimeter exactly 20 minutes after the sodium carbonate was added.

Calculation:

The equivalent mg. of phenol per 25 ml of solution for each colorimeter reading is obtained from the standard curve. Then, mg. of phenol per 25 ml of solution $\times 15/2 \times 100/0.5$ (which cancels out to read: mg. of phenol per 25 ml of solution $\times 1500$) equals mg. of phenol per 100 ml serum in test or control. The King and Armstrong unit is defined thus: one unit of phosphatase activity is that amount of enzyme which, when allowed to act upon excess disodium-monophenylphosphate at proper pH for 30 minutes at 37.5°C., will liberate 1 mg. of phenol. Therefore, mg. of phenol per 100 ml. of control (unincubated) serum equals units of phosphatase per 100 ml of serum.

Many laboratories use the older Hodansky method for determining serum alkaline phosphatase. Sodiumbetaglycero-phosphate is used as a substrate and monosodium-diethylbarbiturate to buffer to proper pH. One ml of serum to be tested is incubated in the buffer substrate solution at 37 degrees Centigrade for exactly one hour. The enzyme activity is halted by adding 5% trichloroacetic acid. The filtrate is then analyzed for inorganic phosphorous. One unit of alkaline phosphatase activity is defined as that amount of enzyme which when allowed to act upon a given excess of sodiumbetaglycero-phosphate at proper pH (8.6) for one hour at 37 degrees Centigrade, will liberate 1.0 mg. of phosphorous.

Units are expressed per 100 ml of serum. The alkaline phosphatase activity of the serum of normal adult ranges from 1 to 4 Bodansky units per 100 ml. Unpublished data indicate that, allowing for differences in hydrolysis of substrates and in time of incubation, one Bodansky unit is approximately equal to 1.8 King and Armstrong units.

Note: All the calculations contained in the data from these experiments are calculated on the basis of King and Armstrong units. In order to secure Bodansky units, it is necessary to divide the King and Armstrong units used herein by 1.8.

One Bodansky unit is equal to 1.8 King and Armstrong units.

DESCRIPTION OF RATIONS, PRODUCTS, AND ANIMALS USED

A. Rats

Both young and mature albino rats were used in these studies.

Rations

Low P diet--

Yellow cornmeal....68%
Wheat gluten.....25%
Brewers yeast..... 3%
CaCO₃..... 3%
Table salt..... 1%

Stock diet--

Cornmeal.....40%
Oatmeal.....20%
Milk powder....20%
Oil meal.....10%
Alfalfa..... 6%
Brewers yeast.. 3%
Salt..... 1%

Special low P diet--

Yellow cornmeal....71%
Wheat gluten.....25%
Brewers yeast..... 3%
Table salt..... 1%

Low Calcium diet--

Cornmeal.....49%
Oatmeal.....30%
Wheat gluten...20%
NaCl..... 1%

B. Dogs

A total of thirty three dogs were used, most of them being of a Spaniel or Shepard type. They were fed a complete commercial dog ration.

C. Chickens

The birds were secured from the poultry department and consisted of groups of young and old healthy birds of Rhode Island Red and Barred Rock breed. They were fed a complete growing mash.

D. Cows

The cows used were from the experimental dairy herd of M. S. C. They were healthy cows of the Holstein type weighing between 1100 and 1300 pounds. They were maintained on a ration of hay and grain.

Products used:

1. Irradiated Ergosterol (viosterol) containing 400,000 units per gram of vitamin D in vegetable oil.
2. Irradiated Ergosterol (viosterol) containing 400,000 units per gram of vitamin D (Standard Brands) in sesame oil.
3. Irradiated Ergosterol in neutral oil, 500,000 units vitamin D per gram.
4. Tuna Liver oil, 20,000 units Vitamin D per gram.
5. Crystalline Calciferol
6. Hydee Yeast (Standard Brands), 24,000 units vitamin D per gram.
7. Brewers Yeast (Irradiated) Fleishmanns, 9,000 units vitamin D per gram.
8. Dihydrrotachysterol (Hytakerol) 1 cc = 1.25 mgm of dihydrrotachysterol in neutral vegetable oil.
9. Cod Liver oil, 180 units vitamin D per gram.
10. Irradiated Ergosterol - (Viosterol)
Sample I 300,000 units Vitamin D/gram
Sample II 500,000 units Vitamin D/gram

11. Tuna liver oil, 17,000 units Vitamin D per gram
12. Hydee yeast extract, 24,000 units Vitamin D per gram
(as bioassayed three different times).

EXPERIMENTAL PROCEDURES

A. Rats

The animals were fed ad libitum of the ration used. The supplements except the yeasts which were mixed with the rations, were fed in Hendryx cups being mixed with a small amount of sucrose to induce ready consumption.

At the end of the experimental period the animals were sacrificed and the blood from each group pooled for analysis.

B. Dogs

The dogs were kept in quarters which had separate pens for isolation if desired. In most cases they were allowed to run in a large enclosed area. They were fed twice a day. The supplements were administered orally by syringe except in certain cases in which the supplement was fed in ground meat. They were bled from the heart on a specially constructed table, at the beginning of the experiment and usually at intervals of three or four days during and after the administration of the supplements.

C. Chickens

The birds were maintained in regular poultry racks. They were allowed to eat all of the stock ration they desired and the supplements were fed daily orally by syringe. At the end of the experimental period the birds were sacrificed by decapitation and the blood collected.

D. Cows

The cows used were those from the experimental dairy barn. They were fed hay and grain as their regular stock feed. The oil supplements were administered daily by number 10 veterinary capsule using a Bolling gum. They were bled, initially and on every third day of the experimental period, from the jugular vein.

The yeast (Hydee) was fed by mixing with the grain ration.

DATA

A. Calcium

I Rats--

TABLE I

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Controls Kg. or o days	mgm Ca/100 ml Days	serum		
		Rats						3	5	7	10
1		3	Std. Brands IE D ₂ in V0	8,000,000	Stock	Young Rats	.05 8.25	9.27			
2	"	3	"	8,000,000	"	"	.05 8.25		8.66		
3	"	3	"	8,000,000	"	"	.05 8.25			9.51	
4	"	3	in SO	8,000,000	"	"	.05 8.25	9.03			
5	"	3	"	8,000,000	"	"	.05 8.25		9.52		
6	"	3	"	8,000,000	"	"	.05 8.25			9.88	
7	"	3	Std. Brands IE D ₂ in V0	4,000,000	"	"	.05 8.25	8.91			
8	"	3	"	"	"	"	.05 8.25		9.52		
9	"	3	"	"	"	"	.05 8.25			9.76	
10	"	3	in SO	"	"	"	.05 8.25	8.52			
11	"	3	"	"	"	"	.05 8.25		8.30		
12	"	3	"	"	"	"	.05 8.25			9.03	
13	"	4	Std. Brands IE D ₂ in V0	"	"	"	.06 11.97				11.61
14	"	"	"	2,000,000	"	"	.06 11.97				12.06
15	"	"	"	1,600,000	"	"	.06 11.97				13.05

(Table I cont'd next page)

TABLE I CONT'D

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Controls		mgms Ca/100 ml			
							Kg.	or	serum			
		Rats						o days		Days		
									3	5	7	10
16		4	Std Brands IE D ₂ in VO	1,200,000	Stock	Young rats	.06	11.97				12.33
17	"	4	"	800,000	"	"	.06	11.97				12.42
18	"	5	"	800,000	"	"	.05	10.25			10.74	
19	"	5	in SO Std.	800,000	"	"	.05	10.25			11.20	
20	"	5	Brands IE D ₂ in VO	400,000	"	"	.05	10.25			10.37	
21	"	5	in SO Std.	400,000	"	"	.05	10.25			11.35	
22	"	4	Brands IE D ₂ in VO	400,000	"	"	.06	11.97			12.33	
23	"	4	"	200,000	"	"	.06	11.97			11.70	

IE = Irradiated ergosterol
VO = Vegetable oil
SO = Sesame oil

TABLE II

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Controls Kg. or days	mgm Ca/100 ml serum Days	7	15	20	30
		Rats				Lo Mature						
1		4	DHT	1 mgm	P	Rats	.165 13.81				16.21	
2	"	4	"	"	SpLo	"	.165 12.80				14.91	
3	"	5	"	"	Lo	Young	P Rats .05 13.84					19.81
4	"	5	"	"	SpLo	"	P .05 12.32					16.72
5	"	3	"	"	Stock	"	.05 11.23		14.76			
6	"	3	"	"	"	"	.05 11.23					14.92
7	"	2	"	"	"	Mature Rats	.18 12.80					15.71
8	"	4	"	.5 mgm	"	Young Rats	.05 8.46	8.84				
9	"	4	"	.5 mgm	"	"	.05 8.57	9.07				
10	"	4	"	.5 mgm	Lo	Mature P Rats	.165 13.81				14.98	
11	"	4	"	.5 mgm	SpLo	"	P .165 12.80				13.96	
12	"	5	"	.5 mgm	Lo	Young P Rats	.05 13.84					17.92
13	"	5	"	.5 mgm	SpLo	"	P .05 12.32					15.36
14	"	3	"	.5 mgm	Stock	"	.05 11.23				12.38	
15	"	3	"	.5 mgm	"	"	.05 11.23					12.38
16	"	4	"	.25 mgm	"	"	.05 8.46	8.57				
17	"	4	"	.25 mgm	"	"	.05 8.57	8.04				
18	"	3	"	.25 mgm	"	"	.05 11.23		11.72			
19	"	3	"	.25 mgm	"	"	.05 11.23					11.56
20	"	3	"	.75 mgm	"	"	.05 11.23		12.96			
21	"	3	"	.75 mgm	"	"	.05 11.23					13.20

DHT = Dihydrotachysterol
 Lo P = Low phosphorus
 Sp Lo P = Special Low Phosphorous

TABLE III

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Controls Kg. or o days	mgms Ca/100 ml Days	serum
								10 15 20 25	
1		5	Irrad. Brewers Yeast 9000u/ gm	2.5% or 22,500 units/feed 100 gms	LoP (basal)	Young Rats	.055 9.52		10.86
2	"	4	"	"	Stock Lo P (basal)	Mature Rats	.17 7.44	8.78	
3	"	3	"	"	"	"	.17 11.10	12.81	
4	"	3	"	"	"	"	.17 12.57	12.20	
5	"	3	"	"	"	"	.17 13.42		14.57
6	"	3	"	45,000	"	"	.17 11.10	11.60	
7	"	3	"	"	"	"	.17 12.57	12.01	
8	"	3	"	"	"	"	.17 13.42		13.91
9	"	4	"	"	Stock Lo P (basal)	" Young Rats	.17 7.44	7.44	
10	"	5	"	"	"	"	.055 9.52		10.74
11	"	5	"	90,000	"	"	.055 9.52		9.76
12	"	4	"	"	Stock Lo P (basal)	Mature Rats	.17 7.44	8.42	
13	"	3	"	"	"	"	.17 11.10	11.07	
14	"	3	"	"	"	"	.17 12.57	12.20	
15	"	3	"	"	"	"	.17 13.42		13.54

Lo P = Low Phosphorous

TABLE IV

Twenty rats (160-170 grams) were placed on a stock diet and fed Jamieson's C.L.O. (Vitamin D₃) conc. 1000 u/ml as follows:

	No. of Animals	mgms Ca/100 ml Serum 10 Days
Fed 50,000 u/10 kg 1 day/10 days	5	8.78
Fed 100,000 u/10 kg 1 day/10 days	5	10.49
Fed 150,000 u/10 kg 1 day/10 days	5	10.86
Controls	5	10.52

C.L.O. = Cod Liver Oil

TABLE V

The compounds used in the following experiments were:

1. Pure calciferol in oil D₂ (Dr. Ewing) 200,000 u/gm
2. Natural fish oils D₃ (Dr. Ewing) 1500-1700 u/gm
3. Hydee Yeast Std. Brands 24,000/gm

One hundred and twelve animals about 100 gms each
Two rations (low Ca and stock ration)

Group	Rats	Vit. D Source	No. of Animals	Supplements	mgms Ca/100 ml serum	
					Exp. 99 15 days	Exp. 100 15 days
A	Stock ration	Pure Calciferol	4 & 4	2,000u/10kg	9.08	7.82
B	" "	Vit. D ₂	4 & 4	40,000u/10kg	10.3	8.57
C	Low Ca	"	4 & 4	2,000u/10kg	9.31	8.51
D	" "	"	4 & 4	40,000u/10kg	10.21	10.39
E	Stock ration	Yeast Hydee	4 & 4	2,000u/10kg	10.05	7.69
F	" "	"	4 & 4	40,000u/10kg	10.01	8.70
G	Low Ca	"	4 & 4	2,000u/10kg	11.45	11.06
H	" "	"	4 & 4	40,000u/10kg	9.81	8.60
J	Stock ration	D ₃ Fish Oils	4 & 4	2,000u/10kg	10.18	8.96
K	" "	"	4 & 4	40,000u/10kg	9.45	8.51
L	Low Ca	"	4 & 4	2,000u/10kg	9.96	8.84
M	" "	"	4 & 4	40,000u/10kg	10.44	8.79
N	Stock ration	Control	4 & 4	Stock only	9.70	9.78
P	Low Ca	"	4 & 4	Low Ca only	9.06	9.13

TABLE VI

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Wt. Kg.	Controls or o days	mgms serum 4	Ca/100 ml Days 7
Dogs									
1		1	DHT	1 mgm	K	10	11.03	16.20	19.40
2		1	"	1 mgm	K	10	10.10	15.92	18.93
3		1	"	.5 mgm	"	10	13.02	19.15	19.89
4		1	"	.5 mgm	"	10	15.10	19.35	19.53
5		1	"	.5 mgm	"	10	9.50	13.78	13.93
6		1	"	.5 mgm	"	10	13.50	19.05	20.16
7		1	"	.5 mgm	"	10	13.30	16.00	17.60
8		1	"	.5 mgm	"	10	13.24	17.50	18.06
9		1	"	.5 mgm	"	10	12.00	14.10	17.20
10		1	"	.5 mgm	"	10	10.61	13.71	15.58
11		1	"	.5 mgm	"	10	12.33	15.06	17.21
12		1	"	.5 mgm	"	10	10.81	12.30	15.30
13		1	"	.25 mgm	"	10	10.29	11.08	11.70
14		1	"	.25 mgm	"	10	12.01	12.01	12.90
15		1	"	.25 mgm	"	10	13.80	14.20	14.24
16		1	"	.25 mgm	"	10	14.60	14.89	14.90
17		1	"	.25 mgm	"	10	12.01	12.19	13.69
18		1	"	.25 mgm	"	10	12.21	12.95	15.08

DHT = Dihydrotachysterol

II Dogs--

TABLE VII

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Kg.	Controls or o days	mgms serum	Ca/100 ml
Dogs									Days 4 7 15 30	
1		1	Std Brands IE D ₂ in VO	500,000	K	Supple- ments for 10 days	10	12.31	23.41	Tissue Calci- fied died
2		1	"	500,000	"	"	10	13.62	19.65	22.65 15.0
3		1	Pure Calci- ferol D ₂ in WO	400,000	"	"	10	12.81	14.76	19.21
4		1	"	400,000	"		10	12.20	14.34	18.70
5		1	"	200,000	"		10	12.04	13.20	14.83
6		1	"	200,000	"		10	12.61	13.68	14.69
7		1	"	200,000	"		10	13.20	15.04	16.24
8		1	"	200,000	"		10	11.08	12.90	14.21
9		1	"	200,000	"		10	13.20	15.30	17.10
10		1	"	200,000	"		10	12.80	14.00	15.91
11		1	"	100,000	"		10	12.30	discontinued	
12		1	"	100,000	"		10	13.00	discontinued	
13		1	"	80,000	"		10	10.56	disabled	
14		1	"	80,000	"		10	11.50	died 7-24-44	
15		1	Std. Brands IE D ₂ inVO	20,000	"		10	13.60	14.00	
16		1	"	10,000	"		10	12.78	13.01	

IE = Irradiated ergosterol
 WO = Wesson oil
 VO = Vegetable oil

TABLE VIII

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Wt. Kg.	Controls or o days	mgms Ca/100 ml serum Days 7
Dogs								
1		1	DHT	1 mgm	K	10	10.30	18.41
2		1	"	.5 mgm	"	"	11.70	16.90
3		1	"	.5 mgm	"	"	13.89	19.81
4		1	Pure Calci- ferol D ₂ WO	500,000	"	"	12.94	17.72
5		1	"	400,000	"	"	11.13	died 8-12-44
6		1	"	200,000	"	"	10.10	13.12

DHT = Dihydrotachysterol
WO = Wesson Oil

TABLE IX

Two 10 kg dogs placed on Hydee yeast extract containing 24,000 u Vitamin D/ml. Administered the oil in ground meat mixed with small amount of feed.

	0 Days	5 Days	10 Days (Supplements stopped here)	15 Days
Boots 200,000 u of Vitamin D/ 10 kg/day for 10 days	14.01	17.40	19.87	19.84
Mike 400,000 u of Vitamin D/ 10 kg/day for 10 days	14.82	21.91	25.63	20.96

III Chickens

TABLE X

Group	Sex	No. of Animals	Supple-ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Kg.	Controls or o days	mgms serum	Ca/100 Days	ml 14 or 15
Chickens									7	10	
1	4	DHT	1mgm/10kg	2% Ca	Laying Hens	3	13.91			24.30	
2	4	"	1mgm/10kg	"	"	3	8.92			17.13	
3	4	"	1mgm/10kg	"	"	2	19.67		27.92		
4	4	"	1mgm/10kg	"	"	2	8.30				17.03
5	4	"	1mgm/10kg	"	"	2	19.67				28.60
6	4	"	1mgm/10kg	"	"	2	8.30		17.11		
7	3	"	1mgm/10kg	"	Non-laying	3	13.47				21.31
8	3	"	1mgm/10kg	"	"	3	8.30				15.89
9	4	"	.5mgm/10kg	"	Laying Hens	3	13.91			19.56	
10	4	"	.5mgm/10kg	"	"	3	8.92			12.56	
11	4	"	.5mgm/10kg	"	"	2	19.67				24.13
12	4	"	.5mgm/10kg	"	"	2	8.30				14.90
13	4	"	.5mgm/10kg	"	Laying Hens	2	19.67		24.08		
14	4	"	.5mgm/10kg	"	"	2	8.30		14.92		
15	2	"	.5mgm/10kg	"	Non laying	3	10.71			16.11	
16	2	"	.5mgm/10kg	"	"	3	9.68			13.93	
17	3	"	.5mgm/10kg	"	Non laying	3	13.47				18.73
18	3	"	.5mgm/10kg	"	"	3	8.30				13.09
19	2	"	.25mgm/10kg	"	"	3	10.71			11.87	
20	2	"	.25mgm/10kg	"	"	3	9.68			11.41	

DHT - Dihydrotachysterol

TABLE XI

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Kg.	Controls or o days	mgms 100 ml serum Days 10
Chickens									
1		3	Irrad. erg D ₂ Ew Std.	400,000	2% Ca	Young birds	2	13.10	13.20
2	"	3	Brands D ₂ Irrad.	400,000	"	"	2	13.10	13.64
3	"	3	erg D ₂ Ew Std.	200,000	"	"	2	13.10	13.08
4	"	3	Brands D ₂ Tuna	200,000	"	"	2	13.10	13.19
5	"	3	Oil D ₃ Ew Tuna	200,000	"	"	2	13.10	14.90
6	"	3	Oil D ₃ Ew HLO	100,000	"	"	2	13.10	13.96
7	"	3	D ₃ Ew	4,500	"	"	2	13.10	14.52
8	"	3	DHT	1 mgm	"	"	2	13.10	23.23
9	"	3	DHT	.5 mgm	"	"	2	13.10	18.10
10	"	3	DHT	.25 mgm	"	"	2	13.10	13.50

IE = Irradiated Ergosterol
 HLO = Haliver Oil
 EW = Obtained from Dr. Ewing

IV Cows

TABLE XII

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Kg.	Controls or o days	mgms Ca/100 ml Days	serum 14 or 15
Cows									3 5 7 10	
1		1	DHT	.5 mgn	Hay & Grain	Supple- ments for 10 days	500	10.88	11.2011.6814.2415.91	11.29
2	"	1	DHT	.5 mgn	"	"	"	12.01	12.0812.4114.0116.49	11.81
3	"	1	DHT	.5 mgn	"	"	"	11.03	11.8314.0616.4216.53	
4	"	1		Control	"		"	10.18	10.2810.2310.1010.19	
5	"	1	Hydee Yeast Std.	500,000	"	"	"	12.79	12.7213.0415.9816.03	14.30
6	"	1	Brands D ₂ IE	500,000	"	"	"	10.40	10.4013.3614.4815.98	12.16
7	"	1	"	500,000	"	"	"	13.32	13.3616.0816.8817.76	13.61
8	"	1	Hydee Yeast	250,000	"	"	"	12.81	12.9213.1613.9014.32	13.09
9	"	1		Group Control	"		"	12.02	12.0812.1312.0812.10	12.08

B. Phosphorous

I Rats

TABLE XIII

Group	Sex	No. of Animals	Supplements	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Kg.	Controls or mgms blood 30 days	P/100 ml
Rats									
1		5	DHT	1 mgm	Lo P (basal)	Young Rats	.05	2.39	3.79
2	"	5	DHT	1 mgm	Sp Lo P	"	.05	4.43	5.81
3	"	4	DHT	1 mgm	Lo P	Mature Rats	.165	3.77	6.38
4	"	4	DHT	1 mgm	Sp Lo P	"	.165	5.69	7.08
5	"	5	DHT	.5 mgm	Lo P	Young Rats	.05	2.39	3.28
6	"	5	DHT	.5 mgm	Sp Lo P	"	.05	4.43	5.28
7	"	4	DHT	.5 mgm	Lo P	Mature Rats	.165	3.77	5.08
8	"	4	DHT	.5 mgm	Sp Lo P	"	.165	5.69	6.63

DHT = Dihydrotachysterol

Lo P = Low Phosphorus

Sp Lo P = Special low phosphorus

TABLE XIV

Thirty rats about 50 grams started on stock ration and fed DHT levels as follows:

	No. of Animals on each level and time interval	10 Days	30 Days
Level B .25 mgm/10kg/day/10 days	3	5.92	6.15
Level A .5 mgm/10kg/day/10 days	3	6.34	6.47
Level D .75 mgm/10kg/day/10 days	3	7.27	7.51
Level D 1 mgm/10kg/day/10 days	3	8.28	7.69
Controls 6 rats on stock only	3	3.91	4.55

TABLE XV

Four large mature stock animals weighing 200 gms
each were fed DHT as follows:

Rats	No. of Animals	mgms P/100 ml blood 30 Days
4 Stock 1 mgm/10kg/day/30 days	4	5.71
4 Stock Controls	4	3.49

II Dogs

TABLE XVI

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units /10 kg body wt.	Ration	Wt. Kg.	Controls mgms or days	P/100 ml blood Days	4	7	14	30
Dogs												
1		1	DHT	1 mgm	K	10	2.72	3.21	5.25			
2		1	"	"	"	"	3.08	3.31	3.70			
3		1	"	.5 mgm	"	"	4.56	3.52	4.53			
4		1	"	.5 mgm	"	"	4.50	2.80	4.70			
5		1	"	.5 mgm	"	"	4.00	5.07	7.55			
6		1	"	.5 mgm	"	"	3.70	4.50	5.52			
7		1	"	.5 mgm	"	"	2.77	3.03	4.08			
8		1	"	.5 mgm	"	"	3.63	4.22	6.66			
9		1	"	.5 mgm	"	"	3.52	4.14	4.38			
10		1	"	.5 mgm	"	"	6.38			6.47	6.47	
11		1	"	.25 mgm	"	"	7.05			7.03	6.90	
12		1	"	.25 mgm	"	"	3.14	2.69	2.93			
13		1	"	.25 mgm	"	"	3.03	3.59	3.10			
14		1	"	.25 mgm	"	"	2.55	5.17	6.52			
15		1	"	.25 mgm	"	"	2.24	4.14	4.28			
16		1	"	.25 mgm	"	"	3.89	4.05	4.14			

DHT = Dihydrotachysterol

TABLE XVII

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units /10 kg body wt.	Ration	Wt. Kg.	Controls or o days	mgms 4	P/100 ml blood Days 7	20
Dogs										
1	1		Std. Brands IE D ₂ in VO	500,000	K	10	4.00		6.77	
2	1		"	500,000	"	10	5.49		6.70	
3	1		Pure Calci- ferol D ₂ in WO	400,000	"	10	3.14	3.28	3.62	
4	1		"	400,000	"	10	3.01	4.52	4.92	
5	1		"	200,000	"	10	3.04	5.07	5.25	
6	1		"	200,000	"	10	5.52	5.78	6.00	
7	1		"	200,000	"	10	3.49	4.49	5.37	
8	1		"	200,000	"	10	2.23	3.77	4.63	
9	1		"	200,000	"	10	3.14	4.02	5.25	
10	1		"	200,000	"	10	1.84	3.56	4.07	
11	1		"	100,000	"	10	1.12	discontinued		
12	1		"	100,000	"	10	1.10	discontinued		
13	1		"	100,000	"	10	7.14	discontinued		
14	1		"	100,000	"	10	5.43	died		
15	1		Std. Brands IE D ₂ in VO	20,000	"	10	4.35			4.25
16	1		"	20,000	"	10	4.24			4.26
17	1		"	10,000	"	10	4.91			4.89
18	1		"	10,000	"	10	5.00			4.90

IE = Irradiated Ergosterol
VO = Vegetable Oil
WO = Wesson Oil

TABLE XVIII

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units /10 kg body wt.	Ration	Wt. Controls Kg. or days	mgms 7	P/100 ml blood Days 10
Dogs								
1		1	Std. Brands IE D ₂ in VO	500,000	K	10	3.25	5.66
2		1	Pure Calci- ferol D ₂ in WO	400,000	"	"	4.07	died 8/4/44
3		1	"	200,000	"	"	3.91	5.81
4		1	DHT	1 mgm	"	"	2.95	3.84
5		1	"	.5 mgm	"	"	3.98	4.45
6		1	"	.5 mgm	"	"	1.72	2.12

VO = Vegetable Oil
 IE = Irradiated Ergosterol
 DHT = Dihydrotachysterol
 WO = Wesson Oil

TABLE XIX

Two dogs fed supplements of Extracts of Hydee Yeast in W.O. containing 24,000 u/ml of D as follows, and bled at the end of 0,5,10, and 15 days.

	mgms P/100 ml blood		Supplements stopped	
	0 Days	5 Days	10 days	15 Days
Boots 200,000 u/day for 10 days	3.77	4.16	4.74	5.14
Mike 400,000 u/day for 10 days	4.09	4.56	4.87	6.03
W.O. = Wesson Oil				

III Chickens

TABLE XX

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units /10 kg body wt.	Ration	Remarks	Wt. Controls Kg. or o days	mgms P/ 100 ml blood 10 Days
			Chickens					
1		4	Std. Brands IE D ₂ in VO	400,000	2% Ca	Young non laying	2 6.92	8.87
2		4	"	"	"		2 6.04	8.46
3		3	IE PD Ewing D ₂	400,000	"	"	2 6.92	8.37
4		3	"	"	"		2 6.04	7.43
5		3	"	200,000	"	"	2 6.92	7.96
6		3	"	200,000	"		2 6.04	6.87
7		4	Std Brands IE D ₂ in VO	200,000	"	"	2 6.92	7.19
8		4	"	200,000	"		2 6.04	7.10

IE = Irradiated Ergosterol
VO = Vegetable Oil
PD = Parke-Davis-Ewing

TABLE XXI

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Kg.	Controls or days	mgms blood Days	P/100 ml	14 or 15
		Chickens							7	10	
1		3	DHT	1 mgm	2% Ca	non- laying	4	3.21			4.53
2		3	"	"	"		4	2.11			3.62
3		4	"	"	"	young non-laying	2	6.92		7.84	
4		4	"	"	"		2	6.04		7.60	
5		3	"	.5 mgm	"	non- laying	4	3.21			3.62
6		3	"	.5 mgm	"	old	4	2.11			3.49
7		1	"	"	"	non-laying	5.5	3.21		4.92	
8		1	"	"	"		5.5	2.11		2.79	
9		1	"	"	"	laying	4	3.26			7.14
10		1	"	"	"	hen old	5	3.26	9.07		
11		1	"	"	"		4	2.51	7.42		
12		1	"	"	"	laying hen	4	3.26			8.55
13		1	"	"	"	young non-laying	2	6.28		4.99	
14		1	"	"	"		2	6.35		7.41	
15		4	"	"	"		2	6.04		7.49	
16		4	"	"	"	young non-laying	2	6.92		7.72	
17		1	"	.25 mgm	"	old non- laying	5.5	3.21		5.81	
18		1	"	.25 mgm	"		5.5	2.11		2.21	
19		1	"	.25 mgm	"		4	3.26		6.77	
20		1	"	.25 mgm	"		5	2.51	9.39		
21		1	"	.25 mgm	"		4	3.26	8.12		
22		1	"	.25 mgm	"	laying hen	4	3.26		9.53	
23		1	"	.25 mgm	"	young non-laying	2	6.28		6.03	
24		1	"	.25 mgm	"		2	6.35		6.29	
25		4	"	.25 mgm	"	young non-laying	2	6.04		7.33	
26		4	"	.25 mgm	"		2	6.92		7.57	

TABLE XXII

Group	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Controls Kg. or o days	mgms P/100 ml blood 10 days
		Chicken				Young Birds		
1	4		Tuna Liver Oil D ₃	200,000	2% Ca		2 6.92	7.67
2	4		"	200,000	"	"	2 6.04	6.77
3	4		"	100,000	"	"	2 6.92	7.48
4	4		"	100,000	"	"	2 6.04	6.35
5	3		Haliver Oil D ₃	9,000	"	"	2 6.04	6.38
6	3		"	4,500	"	"	2 6.92	7.20

IV Cows

TABLE XXIII

Gr- oup	Sex	No. of Animals	Supple- ments	Daily Level mgms or units/10kg body wt.	Ration	Remarks	Wt. Kg.	Controls or Days	mgms P/100 ml blood	Days	3	5	7	10	15
			Cows					0 days							
1		1	DHT	.5 mgms	Hay & grain		500	4.81	5.07	5.86	5.94	7.14			
2	"	1		Control	"	Control	500	4.47	4.57	4.77	4.03	4.47			
3	"	1	DHT	.5 mgms	"	Supple- ments 10 days	500	5.09	5.81	6.18	6.58	7.14	5.63		
4	"	1	DHT	.5 mgms	"	"	500	3.18	3.70	4.22	5.04	5.91	4.07		
5	"	1		Control	"	Control	500	5.17	5.01	5.28	5.01	4.95	5.17		

DHT = Dihydrotachysterol

TABLE XIV

Two cows fed Hydee yeast (Std. Brands) 24,000
units/gm. Both were fed 500,000 and 250,000
units/day for 10 days respectively.

	0 Days	3 Days	5 Days	7 Days	Supplement stopped 10 Days	15 Days
Cow A-47 500,000 u/day/10kg or 1.04 kg Hydee yeast mixed with grain (ground corn)	3.67	4.06	4.55	6.20	6.79	3.98
Cow A-50 250,000 u/day/10kg Hydee yeast as above	3.31	3.83	4.27	4.69	5.34	4.12
#426 Control Hay & grain	5.17	5.01	5.28	5.01	4.95	5.17

TABLE XXV

Two cows fed supplements of Std. Brands Irradiated
 Ergosterol (Vitamin D₂) Viosterol as follows:
 Fed by #10 Vet Capsule
 Potency = 400,000 u/g

	mgms P/100 ml blood				Supplements Stopped	
	0 Days	3 Days	5 Days	7 Days	10 Days	15 Days
A-53 500,000 u/10 kg/day/ 25,000,000/ for 10 days (250,000,000	5.71	5.82	6.34	7.02	7.46	6.18
A-55 Same as above	5.17	5.25	5.82	6.29	7.02	5.53
#426 Control Hay & grain	5.17	5.01	5.28	5.01	4.95	5.17

C. Phosphatase

I Rats

TABLE XXVI

Thirty-two large animals were placed on stock ration and fed supplements of Vitamin D₂ (Std. Brands Viosterol) as follows:

Source	Rats	Group	Units/10kg body wt.	No. of Animals	Units/100 ml serum Acid	Alk
		1			8.2	62.1
Std. Brands		1	1000	4	7.6	67.8
Irrad.		2	2000	4	11.2	91.1
Ergosterol		3	4000	4	9.2	80.9
Viosterol-Vit. D ₂		4	6000	4	9.2	70.4
		5	8000	4	7.2	66.1
		6	10,000	4	3.2	46.5
		7	20,000	4	3.8	44.0

TABLE XXVII

Sixty-four young animals (60-70 grams) were placed on Vitamin D₂ on the following levels:

Source	Units/10kg body wt.	No. of Animals	Units Acid	Alk
Std. Brands	1000 units D	16	3.3	70.4
Vit. D ₂ (Viosterol)	100	16	7.9	75.7
	10	16	10.6	77.8
	Controls	16	10.1	78.7

TABLE XXVIII

Twenty animals (young 60-70 gms) were placed on a low calcium ration supplemented by various percentages of Hydee Yeast as follows:

Ration & Supplement Low Ca + % Hydee Yeast	No. of Animals	Rats	Acid	Units/100 ml serum Alk
2% (48,000 u/100 gms) feed	5		12.9	59.2
5% (120,000 u/100)	5		9.5	48.5
10% (240,000 u/100 gms)	5		6.0	44.6
Controls	5		13.5	57.5

TABLE XXIX

One hundred and twelve young animals (60-70 grams) were placed on a low Ca and stock ration and fed supplements of calciferol (Vitamin D₂) and Haliver Oil (Vitamin D₃) as follows:

Supplement	Ration	Group	Units	No. of Animals	Trial I Units		Trial II Units/100 ml serum	
					Acid	Alk	Acid	Alk
Calciferol	Stock	A	10	4	4.0	72.1	4.7	71.5
Vitamin D ₂	Ration							
"	"	B	200	4	2.5	97.4	2.2	55.35
"	Low Ca							
"	Ration	C	10	4	--	115.4	9.3	90.0
"	"	D	200	4	1.0	85.4	6.3	82.5
Hydee								
Yeast	Stock	E	10	4	7.0	72.1	7.8	78.0
"	"	F	200	4	7.0	58.6	9.3	90.0
"	Low Ca							
"	Ration	G	10	4	4.0	111.6	6.3	60.0
"	"	H	200	4	2.5	84.6	22.8	33.0
Haliver Oil	Stock	J	10	4	2.5	115.7	13.8	13.5
"	"	K	200	4	2.5	84.6	4.7	82.5
"	Low Ca	L	10	4	--	111.4	--	111.0
"	"	M	200	4	--	105.7	15.3	84.8
Control	Stock	N	Controls	4	7.0	102.7	6.3	60.0
"	Low Ca	P	"	4	7.0	101.0	7.8	84.0

II Cows

TABLE XXX

Two cows placed on experiment, one being fed DHT
and the other used as control (1300# each - 600 Kg).

Cow #	Daily Level mgms/10kg body wt.	0 Days		3 Days		5 Days		7 Days		10 Days	
		Units		Units/100 ml serum		Units		Units		Units	
		Acid	Alk.	Acid	Alk.	Acid	Alk.	Acid	Alk.	Acid	Alk.
A-15	.5mg/10kg	3.42	16.61	—	4.26	1.26	6.45	1.02	3.80	4.71	7.23
A-33	Control grain & hay	.6	16.88	—	20.70	9.18	19.02	.92	9.50	4.02	21.10

DISCUSSION

A. Calcium

1. Rats--

The blood calcium values in the studies on rats are given in Tables I - V.

Table I contains the results of studies on rats fed a stock ration (complete ration) supplemented with various levels of irradiated ergosterol in vegetable and sesame oils. Young animals about 50 grams in weight were used. The doses were administered on basis of body weight (units per 10 kg.).

In comparing the experimental animals with the controls a very slight blood calcium rise is shown on all levels. This rise gradually decreases from an average 1.25 mgm increase in the case of the 8,000,000 unit level to a .75 mgm rise on the 400,000 unit level. The increases were not as high as one would normally expect where such massive doses were administered. Continued administration of the supplement apparently did not cause any further increases in the calcium level.

Table II shows the results on several groups of young and mature rats placed on stock and low phosphorous diets and administered supplements of various levels of dihydrotachysterol. The low phosphorous diet consisted of a regular basal rachitogenic diet containing about .16% phosphorous. The special low phosphorous ration was the same as the basal except the CaCO_3 was excluded. The rise in blood calcium on each level is

directly proportional to the dose administered. Young animals on the low phosphorous (basal) diet exhibited 6.0 and 4. mgm rises respectively for the 1 mgm and .5 mgm levels, whereas the mature animals on the same ration gave 2.4 and 1.11 mgm rises for the 1 mgm and .5 mgm levels. Young animals placed on the special low phosphorous ration (less CaCO_3) gave rises of 4.4 and 3.04 on the 1 mgm and .5 mgm levels. The mature rats on the same ration exhibited calcium rises of 2.1 mgms and 1.16 mgms on the 1 mgm and .5 mgm levels respectively.

The young animals showed the greater uses in calcium than mature animals on all rations. The low phosphorous, special low phosphorus, and stock rations exhibited the greatest rises on all levels in the order listed both in the experimental and control animals. The length of time of supplement feeding is insignificant after 15 days.

In Table III are the results of both young and mature animals fed a stock and low phosphorus (basal) rations mixed with irradiated brewers yeast. The results on these levels of Vitamin D show no significant calcium changes on either of the rations used. These studies on the rat's resistance to changes in blood calcium seem to be in agreement with such workers as Taylor, Weld, Branion, and Kay (83).

The effect of Cod Liver Oil on mature rats feed on a stock diet may be found in Table IV. On levels of Vitamin D_3 ranging from 50,000 to 150,000 units per 10 kg of body weight there were no significant changes in blood calcium.

The effect of a low calcium and stock diet fed with supplements of pure calciferol, Hydee Yeast, and Haliver Oil may be seen in Table V. Two vitamin D levels were used namely 40,000 and 2,000 units per 10 kg of body weight. Two groups were covered through a 15 day experimental period. No significant differences were found on either the stock or low calcium ration on either level of vitamin D intake regardless of source.

2. Dogs--

Table VI contains the results with dogs fed a complete diet and supplements of dihydrotachysterol. The average blood calcium rise found were 8.6 on the 1 mgm level, 5.1 mgms on the .5 mgm level, and 1.1 on the .25 mgm level. On the basis of antirachitic activity these levels of dihydrotachysterol represent 100, 50, and 25 units U.S.P. of vitamin D respectively. There were no apparent toxic effects observed in these studies. Dihydrotachysterol is obviously very potent in raising blood calcium level. Compared with vitamin D₂ and D₃ these levels (weight basis) would represent 40,000, 20,000, and 10,000 units respectively.

A group of dogs were fed supplements of irradiated ergosterol and pure calciferol. The levels fed varied from 10,000 to 500,000 U.S.P. units per 10 kg. The results are shown in Table VII.

The dogs receiving 500,000 units per 10 kg showed an average 11 mgm rise in calcium (severe hypercalcemia) with marked toxic effects such as loss of weight, emaciation, and death. On autopsy tissues such as the liver, spleen and kidneys showed extreme

calcification. The dogs on 400,000 units level of pure calciferol showed a 6 mgm rise and loss of weight but recovered. At the 200,000 level an average rise of 3 mgms was observed. At lower levels no significant calcium changes were noticed. The 500,000, 400,000, and 200,000 levels represent on a weight basis 12.5, 10, and 5 mgms of vitamin D₂. Thus on a weight basis 20 times more vitamin D₂ than dihydrotachysterol are required to produce the same blood calcium rise.

Similar results were obtained in further work, the results of which are shown in Table VIII.

Two dogs were fed supplements of an extract of Hydee Yeast. This was prepared by extracting several times with ether, evaporating and taking up in oil. It was fed at levels of 200,000, and 400,000 unit (vitamin D₂ in the oil was assayed and found to contain 24,000 units per ml). Calcium rises of 11 and 6 mgms were found at the 400,000 and 200,000 unit levels respectively. This yeast extract seemed to be a little more potent on the same unit basis than pure calciferol or irradiated ergosterol. This may indicate that certain irradiation products other than vitamin D₂ might have contributed to the rise in blood calcium.

3. Chickens--

In these studies young and old birds were fed a complete ration supplemented with dihydrotachysterol at levels of 1, .5, and .25 mgms. The results of the blood calcium changes are contained in Table X. From this it can be seen that chickens

respond like dogs to the various levels of dihydrotachysterol. Blood Ca rises of 8.6, 5.1, and 1.3 mg were observed at the above three levels of dihydrotachysterol. Laying hens showed a somewhat smaller use which might be expected inasmuch as they had a higher initial blood calcium level. The males showed a slightly greater response. The old birds showed about the same response as did the young birds on the various levels.

Other groups of birds were given supplements of irradiated ergosterol (vitamin D₂), Haliver oil (vitamin D₃), and Tuna liver oil (vitamin D₃) at levels ranging from 4500 units per kg to 400,000 units per 10 kg body weight. The results are given in Table XI.

The birds showed no significant calcium rises on 400,000 or 200,000 units of vitamin D₂. With vitamin D₃ from tuna liver oil a rise of 1.8 mgms on a 200,000 unit level represented a 1 to 36 ratio between the vitamin D₂ and D₃ or the vitamin D₃ was 36 times more effective on the same unit level. Results of vitamin D₃ from Haliver oil indicated the same effectiveness as that of Tuna oil.

4. Cows--

A group of cows were fed supplements of D.H.T., Hydee Yeast, and irradiated ergosterol. The results are given in Table XII.

With dihydrotachysterol an average rise of 5.00 mgms was found on the basis of the .5 mgm level fed. This is in surprisingly close agreement with that of dogs and chickens for this level.

The cows fed Hydee Yeast supplement showed a rise of 5.2 and 1.5 mgms respectively at the 500,000 and 250,000 unit levels. These results were found to be lower than the Hydee Yeast extract fed dogs in which a rises of 11 and 6 respectively were found at the 400,000 and 200,000 unit levels.

In cows given supplements of irradiated ergosterol at a 500,000 units /10 kg level a rise of 5 mgms was obtained which was lower than the response in both dogs and chickens.

B. Phosphorus

1. Rats--

Results of studies with young and mature rats placed on low phosphorus and special low phosphorus diets supplemented with dihydrotachysterol are given in Table XIII. Referring to the 1 mgm and .5 mgm levels on the low phosphorus diet, one can observe definite blood phosphorus rises at both levels with young and mature animals. The somewhat larger increases in blood phosphorus observed with the diet containing CaCO_3 are undoubtedly due to the abundance of calcium in this diet.

Groups of young rats were placed on stock ration and D.H.T. supplements ranging from 1 mgm to .25 mgms. The results found in Table XIV, indicate blood phosphorus elevations ranging from 3.14 to 1.60 for the four levels of D.H.T. administered. The notable difference here is that on the stock diet (.62-.64% P content) the elevations were much greater than with the low phosphorus diets on either young or mature animals. This may indicate

that the food supplies of phosphorus as well as calcium have an important influence on blood changes involving these elements. This is further supported by the results shown in Table XV in which higher phosphorus rises of the blood were observed in mature rats receiving D.H.T. supplements in conjunction with the stock ration.

2. Dogs--

Table XVI contains the blood phosphorus values of dogs fed a complete ration supplemented by the daily administration of D.H.T. on 1, .5, and .25 mgm levels. Increases of 2.53, 1.75, and 1.05 mgms were observed on the above three levels administered.

Although the phosphorus changes are not as consistent as the calcium they are nevertheless in harmony with other results of the investigation.

The results of feeding supplements of irradiated ergosterol and pure calciferol are shown in Table XVII. A phosphorus rise of 2.6 mgms was obtained at the 500,000 level of irradiated ergosterol and one of 1.6 mgms at 200,000 units of pure calciferol.

Table XVIII contains the results of further studies on high levels of irradiated ergosterol and pure calciferol. Here again the results show about the same order of blood phosphorus rise as previous experiments at the same levels.

The results with an extract of Hydee Yeast are given in Table XIX. Blood phosphorus rises of 1.4 mg on the 200,000 unit level and about 2.0 mg on the 400,000 level were observed.

3. Chickens--

Studies with young male and female birds placed on a complete ration supplemented with two sources of irradiated ergosterol at 400,000 and 200,000 unit levels gave significant blood phosphorous rises. Overall (male and female) averages of 2.18 mg and 1.41 were obtained at the 400,000 unit level of irradiated ergosterol sources I and II respectively, and rises of .94 and .63 at the 200,000 unit level. The results are shown in Table XX.

In Table XXI results are shown for young and old birds fed supplements of D.H.T. At all levels an increase in blood phosphorus was noted. The old males and females show higher blood calcium rises throughout than do the younger birds. The males both young and old show smaller rises throughout these studies except at the lower levels where there seems to be no significant differences.

The results of feeding supplements of Vitamin D₃ in the form of Tuna and Haliver oil on various levels are shown in Table XXII. The phosphorus rises here are smaller but more uniform within the various levels. No significant differences are observed between the two vitamin D₃ sources used in these studies.

4. Cows--

The studies with cows administered D.H.T. supplements at a .5 mgm per 10 kg level gave blood phosphorus rises which averaged 2.04. All of them were fairly consistent at this level. The control cows used served as a double control and exhibited no blood

phosphorus changes. The cows were bled initially before supplements were administered and 5 days after the last supplement was fed. Here in contrast to the lasting effect of the compound, we find a drop in blood phosphorus back towards normal or the initial values. The results are shown in Table XXIII.

Supplements of Hydee Yeast administered in grain at levels of 500,000 and 250,000 units of Vitamin D₂ per 10 kg, were observed to exert a blood phosphorus rise of 3.12 and 2.03 respectively. The control cow showed no rise. These values were practically the same as those observed on dogs fed the Hydee Yeast extract at lower levels (400,000 and 200,000). These values are shown in Table XXIV.

Cows administered irradiated ergosterol at a 500,000 units per 10 kg level exhibited an average blood phosphorus increase of 2.09, as shown in Table XXV. These values were of about the same order as those exhibited by dogs at the same irradiated ergosterol level.

C. Phosphatase

1. Rats—

The phosphatase values on mature rats placed on stock ration and administered irradiated ergosterol on levels varying from 1,000 to 20,000 units per 10 kg of body weight are shown in Table XXVI. The acid phosphatase values show significant decreases in the ascending order of vitamin D₂ dosage. The alkaline phosphatase values decreased as the vitamin D₂ level increased.

Table XXVII shows the same decrease in activity in the case of both the acid and alkaline phosphatase, altho the latter was not as pronounced. The animals were fed a normal stock diet and given supplements of irradiated ergosterol and pure calciferol.

Young rats placed on low calcium diet showed an initially high acid and alkaline phosphatase activity. Significant decreases in both acid and alkaline phosphatase activity was observed when Hydee Yeast was administered as shown in Table XXVIII.

Young rats placed on a low calcium and stock diets supplemented with pure calciferol Hydee Yeast and Haliver Oil exhibited lowered acid and alkaline phosphatase values at all levels. There were no significant differences in effect between vitamins D₂ and D₃ as shown in Table XXIX.

2. Cows--

Table XXX shows the serum phosphatase results on two cows, one fed .5 mgm/10 kg level of DHT and the other used as a control. A gradual decrease in the alkaline phosphatase was observed in the cow receiving the supplements. The acid phosphatase activity showed no significant changes. In the control cow no significant variations were observed in either the acid or alkaline phosphatase activity.

CONCLUSIONS

1. Rats fed massive doses of vitamin D from various sources showed characteristic resistance to blood calcium changes.
2. Dogs to which massive doses of various vitamin D products were administered were observed to show a hypercalcemia, and, in certain cases, a general emaciated condition, followed by death. Autopsy showed a general calcification of various tissues.
3. Chickens administered varying doses of vitamin D products showed lower rises on vitamin D₂ sources than with vitamin D₃. Vitamin D₃ was found to be about 36 times more effective than Vitamin D₂.
4. Massive doses of various Vitamin D products administered to cows caused a definite hypercalcemia.
5. On the basis of limited studies, it would appear irradiated yeast or an extract of irradiated yeast was somewhat more effecting than a corresponding amount of pure Vitamin D.
6. Dihydrotachysterol produced significant blood calcium elevations in young and mature rats fed normal and low phosphorus diets.
7. The average blood calcium use exhibited by dogs on dihydro-tachysterol supplements of 1 mgm., .5 mgms., and .25 mgms per 10 kg. of body weight were 8.6 mgms 5.1 mgms, and 1.1 mgms respectively.

8. Chickens fed supplements of dihydrotachysterol were observed to show calcium uses of 8.6 mgms., 5.1 mgms., and 1.3 mgms., for levels of 1 mgm, .5 mgm., and .25 mgm. respectively. These results agree well with those obtained on dogs.
9. Dihydrotachysterol on a .5 mgm level produced a 5.0 mgm blood calcium rise in cows. These effects are comparable to those obtained with dogs and chickens.
10. Dogs administered massive doses of various vitamin D products were observed to attain blood phosphorus rises of between 1.4 mgms and 2.6 mgms.
11. Young and mature chickens were observed to show significant blood phosphorus elevations when fed massive doses of various vitamin D products. Here again the vitamin D₃ was found to be more effective than D₂
12. Cows fed supplements of various vitamin D products in massive doses produced marked blood phosphorus rises ranging from 2 to 3 mgms.
13. Young and mature rats fed normal and low phosphorus diets were shown to have elevated phosphorus levels when various doses of dihydrotachysterol were administered. The rises were greatest in the case of the normal ration.
14. Dogs to which dihydrotachysterol was administered showed phosphorus elevations of 2.53 mgms, 1.75 mgms, and 1.05 mgms at levels of 1.0 mgm., .5 mgm., and .25 mgm., per 10 kgm body weight respectively.

15. Chickens administered supplements of D.H.T. showed blood phosphorus increases at all levels used. The rises were somewhat higher in the males than in the females.
16. Cows fed dihydrotachysterol on a basis .5 mgm per 10 kg of body weight showed an average phosphorus rise of 2.04 mgms.
17. Acid and alkaline serum phosphatase values on rats fed on 1,000 to 20,000 unit levels of vitamin D (I.E.) per 10 kg body weight were shown to be significantly lowered. Normal and low calcium rations were used. The low calcium ration gave higher initial and final values in these studies.
18. Serum phosphatase values on cows fed dihydrotachysterol, showed a gradual decline in the alkaline phosphatase values whereas the acid phosphatase remained unchanged.

BIBLIOGRAPHY

1. Drummond, J. C., The Fat-soluble Vitamins in Nutrition. Lane Medical Lect., (Stanford University Press, 1934).
2. Metz, G. A., and Coppens, A., Long en Vitamin D. Nederl. tydscher. v. geneesk., 78, 769, (1934).
3. Rosenheim, O., and Webster, T. A., The Relation of Cholesterol to Vitamin D. Biochem. J., 20, 537, (1926).
4. Windaus, A., and Bock, F., Uber das Pro-vitamin aus dem Sterin der Schweinschwarte, Ztschr, f. Physiol. Chem., 245, 168, (1937).
5. Windaus, A., Linsert, C., Luttringhaus, A., and Weidlich, G., Uber das Krystallisierte Vitamin D₂. Ann. Chem., 492, 226, (1932).
6. Lettre, H., Zur Kenntniss der Bestrahlungs-producte des Ergosterins. Ann. Chem., 511, 280, (1934).
7. Friedmann, E., Sterols and Related Compounds pp. 45-47. (Chemical Publishing Company of N. Y. Inc., 1937).
8. Windaus, A., Werder, F. von, and Gschaider, B., Uber die Zahl der Kohlenstoffatome im Molekul des Sitosterins and einiger anderer Sterine., Ber. deutsche Chem. Gesellsch., 65, 1006, (1932).
9. Holtz, F., Gissel, H., and Rossmann, E., Experimentelle und klinische Studien zer Behandlung der postoperativen Tetanie mit A.T. 10 Deutsche Ztscher. f. Chir. 242, 521, (1934).
10. Albright, F., Bloomberg, E., Drake, T., and Sulkowitch, H. W., A Comparison of the Effects of A.T. 10 (dehydratac-hysterol) and Vitamin D on Calcium and Phosphorous Metabolism in hypoparathyroidism. J. Chem. Ivestg., 17, 317, (1938).
11. Albright, F., Sulkowitch, H. W., and Bloomberg, E., A Comparison of the Effects of Vitamin D, dihydrotachysterol (A.T.10) and parathyroid Extract on the Disordered Metabolism of Rickets. J. Chem. Investig., 18, 165, (1939).

12. Shohl, A. T., Fan, H. T., and Farber, S., Effect of A.T. 10 (dihydrotachysterol) on Various Types of Experimental Rickets in Rats. Proc. Soc. Exp. Biol. and Med., 42, 529, (1939).
13. Harnapp, G. O. Der Pathogenese der Spasmophilie Behandlungsoersuche mit A.T.10 Monatscher. F. Kinderh., 63, 262, (1935).
14. Shohl, A.T., and Barber, S., Effect of A.T.10 (dihydrotachysterol) on Rickets in Rats Produced by High Calcium, Low-Phosphorus Diets. J. Nutrition, 21, 147, (1941).
15. Hess, A. F., and Supplee, G. C., The Action of Irradiated Ergosterol on Rats and Chickens, Pro. Soc. Exper. Biol. and Med., 27, 609, (1930).
16. Correll, J. T., and Wise, E. C., Antirachitic Properties of A.T.10 for the Rat and Chicken, J. Nutrition, 23, 217, (1942).
17. Massengale, O. M. and Bills, C. E., A Quantitative Method for the Assay of Vitamin D with Chickens, J. Nutrition, 12, 429, (1936).
18. Caward, K. H., The Relative Antirachitic Potencies of Vitamin D₂ (Calciferol from Ergosterol) and of Vitamin D₃ (from Irradiated 7-dehydrocholesterol). Bull. Health Organ. (League of Nations), 9, 425, (1940-41).
19. Renys, D. G., and Marshall, I. H., The Antirachitic Activity of Various Forms of Vitamin D in the Chick, J. Nutrition, 15, 525, (1938).
20. Russell, W. C., Taylor, M. W., and Wilcox, D. E., The Fate of the Antirachitic Factor in the Chicken. II The Effect of the Factor Administered by Mouth and Intraperitoneally, J. Biol. Chem., 99, 109, (1932).
21. Russell, W. C., Taylor, M. W., and Wilcox, D. E., The Fate of the Antirachitic Factor in the Chicken. III The Effective Levels and Distribution of the Factor from Cod Liver Oil and from Irradiated Ergosterol in Certain Tissues of the Chicken, J. Biol. Chem., 107, 735, (1934).
22. McChesney, E. W., and Homburger, E., A Modification of the Line Test Applicable to Chicken Vitamin D Assay, J. Nutrition, 20, 339, (1940).

23. McChesney, E. W., and Kacher, H., An Evaluation of Various Methods for Bioassay of Dihydrotachysterol, *Endocrinology*, 30, 787, (1942).
24. McChesney, E. W., Studies of Calcium and Phosphorus Metabolism in the Chick. I The Comparative Effects of Vitamins D₂ and D₃ and Dihydrotachysterol Given Orally and Intramuscularly, *J. Nutrition*, 26, 81, (1943).
25. McChesney, E. W., Studies of Calcium and Phosphorus Metabolism in the Chick. II Relative Antirachitic Effectiveness of Vitamins D₂ and D₃ and Dehydrotachysterol Administered Parenterally. *J. Nutrition*, 26, 487, (1943).
26. Correll, J. T., and Wise, E. C., The Comparative Toxicity of Calciferol, A.T. 10, and Cod Liver Oil Concentrate for Chicks, *J. Nutrition*, 26, 641, (1943).
27. Jones, J. H., Rapaport, M., and Hodes, H. L., The Effect of Irradiated Ergosterol on Thyroparathyroid-ectomized Dogs, *J. Biol. Chem.*, 86, 267, (1930).
28. Schneider, H., and Steenbock, H., Differences in Response to Vitamin D₂ of Rats on Cereal Low Phosphorus Diets and Synthetic Low Phosphorus Diets, *J. Biol. Chem.*, 123, Proc. CV, (1938).
29. Correll, J. T., and Wise, E. C., Studies on the Relative Efficiency of Vitamin D from Several Sources. II Influence of Vitamin D of different Origins on the Serum Phosphatase of the Chick, *J. Biol. Chem.*, 126, 581, (1938).
30. Rapaport, S., and Guest, G. M., Changes of Organic Acid-soluble phosphorus, Diphosphoglycerate, Adenosinetriphosphate and Inorganic Phosphorus in the Blood Cells of Rats During the Development and the Healing of Rickets, *J. Biol. Chem.*, 126, 749, (1939).
31. Jones, J. H., The Production of Hypercalcemia with small Amounts of Vitamin D, *J. Nutrition*, 28, 7, (1944).
32. Waddell, J., The Provitamin D of Cholesterol. I. The Antirachitic Efficacy of Irradiated Cholesterol, *J. Biol. Chem.*, 105, 711, (1934).
33. Bethke, R. M., Record, R. R., Kennard, D. C., The Comparative Antirachitic Efficiency of Irradiated Ergosterol Irradiated yeast, and Cod Liver Oil for the Chicken, *J. Nutrition* 6, 413, (1933).

34. McChesney, E. W., Comparison of Hypercalcemia Effects of Activated Sterols in the Chick, *Prox. Soc. Exp. Biol. and Med.*, 52, 147, (1943).
35. Coward, K. H., The Biological Standardization of the Vitamins, (William Wood and Co., Baltimore, Md. 1938).
36. Russell, W. C., and Taylor, M. W., The Relation Between the Vitamin A and D Intake by the Hen and the Output in Eggs, *J. Nutrition*, 10, 613, (1935).
37. Morgan, A. G., and Shimotori, N., The Absorption and Retention by Dogs of Single Massive Doses of Various Forms of Vitamin D., *J. Biol. Chem.*, 147, 189, (1943).
38. Sherman, H. C., and Booker, L. E., The Calcium Content of the Body in Relation to that of the Food, *J. Biol. Chem.*, 93, 93, (1931).
39. Lanford, C. S., and Sherman, H. C., Further Studies of the Calcium Content of the Body as Influenced By That of the Food, *J. Biol. Chem.*, 126, 381, (1938).
40. Jones, J. H., Rapaport, M., and Hodes, H. L., The Source of Excess Calcium in Hypercalcemia Induced by Irradiated Ergosterol, *J. Biol. Chem.* 86, 267, (1930).
41. Greenwald, J., The Relation of the Concentration of Calcium to that of Protein and Inorganic Phosphate in the Serum, *J. Biol. Chem.*, 93, 551, (1931).
42. Klein, D., and Russell, W. C., The Fate of the Antirachitic Factor in the Growing Chick. I The Antirachitic Factor Balance in the Growing Chick, *J. Biol. Chem.* 93, 693, (1931).
43. Outhouse, J., Smith, J., and Twomey, J., The Relative Effects of Certain Sacharides and of Vitamin D on mineral metabolism of Rats, *J. Nutrition*, 15, 257, (1938).
44. McClean, F. C., Activated Sterols in the Treatment of Parathyroid Insufficiency, *J. Am. Med. Assn.*, 117, 609, (1941).
45. Albright, A., Sulkowitch, H. W., Bloomberg, E., A Comparison of the Effects of Vitamin D, Dihydrotachysterol (A.T. 10), and Parathyroid Extract on the Disordered Metabolism of Rickets, *J. Clin. Invest.*, 18, 165, (1939).

46. Cohn, W. E., and Greenberg, D. M., Studies in Mineral Metabolism with the Aid of Artificial Radioactive Isotopes. III The Influence of Vitamin D on the Phosphorus Metabolism of Rachitic Rats, J. Biol. Chem., 128, Sci. Proc. XVI, (1939).
47. McDonald, F. G., and Massengale, O. N., The Antirachitic Potency of Eggs From Hens Receiving Massive Doses of Activated Ergosterol, J. Biol. Chem., 99, 79, (1932).
48. Russell, W. C., Taylor, M. W., and Wilcox, D. E., The Fate of the Antirachitic Factor in Chicken. II. The Effectiveness of the Factor Administered by Mouth and Intraperitoneally, J. Biol. Chem. 99, 109, (1932-33).
49. Reed, C. I., Struck, H. C., and Steck, I. E., Vitamin D; Chemistry, Physiology, Pharmacology, Pathology, Experimental and Clinical Investigations, (The University of Chicago Press, Chicago, Illinois 1939).
50. Eddy, W. H., and Dalldorf, G. The Avitaminoses. The Chemical, Clinical and Pathological Aspects of the Vitamin Deficiency Diseases. (Williams and Wilkins, Baltimore, Md., 1937).
51. Kern, R., Montgomery, M., and Still, E. U., The Effect of Large Doses of Irradiated Ergosterol Upon Nitrogen, Calcium, and Phosphorus Metabolism in Rats. J. Biol. Chem. 93, 365, (1931).
52. Taylor, N. B., and Weld, C. B., The Mobilization and Excretion of Calcium Following Overdosage with Irradiated Ergosterol, Brit. J. Exper. Path., 13, 109, (1932).
53. Bauer, W., Marble, A., and Claflin, D., Studies on the Mode of Action of Irradiated Ergosterol. IV In Hyperparathyroidism, J. Chem. Invest., 11, 47, (1932).
54. Duncan, C. W., and Huffman, C. F., The Effect of Daily Massive Doses of Viosterol Upon Calcium and Phosphorus Metabolism and Blood Calcium and Inorganic Phosphorus in Calves, J. Dairy Sci., 17, 83, (1934).
55. Wallis, G. C., Palmer, L. S., and Gullickson, T. W., The Relation of Vitamin D to Calcium and Phosphorus Retention in Cattle as Shown by Balance Trials, J. Dairy Sc., 18, 213, (1935).

56. Patwhordan, V. N., and Chitre, P. G., The Effect of Hyper-vitaminosis D on the Calcium and Phosphorus Metabolism in the Albino Rats, *Proc. Soc. Exp. Biol. and Med.*, (India) 3, 63, (1938).
57. Reed, C. I., On the Nature of the Toxic Action of Vitamin D. *Proc. Soc. Exper. Biol. and Med.* 38, 791, (1938).
58. Nicolaysen, R., Studies on the Mode of Action of Vitamin D. I. Investigations Upon the Phosphorus Compounds in Muscles, Liver, and Kidneys as Influenced by Different Levels of Vitamin D and Phosphorus in the Diet, *Biochem. J.*, 30, 1329, (1936).
59. Nicolaysen, R., II. The Influence of Vitamin D on the Faecal Output of Endogenous Calcium and Phosphorus in the Rat, *Biochem. J.* 31, 107, (1937).
60. Nicolaysen, R., III. The Influence of Vitamin D on the Absorption of Calcium and Phosphorus in the Rat, *Biochem. J.*, 31, 122, (1937).
61. Nicolaysen, R., IV. The Absorption of Calcium Chloride, Xylose, and Sodium Sulfate From Isolated Loops of Small Intestine and of Calcium Chloride from the Abdominal Cavity of the Rat, *Biochem. J.*, 31, 323, (1937).
62. Nicolaysen, R., V The Absorption of Phosphates from the Isolated Loops of the Small Intestine in the Rat, *Biochem. J.* 31, 1086, (1937).
63. Nicolaysen, R., The Carbon Dioxide Output in Awake and Narcotized Rats in Rickets and When Rickets Has Been Cured, *Skandinav. Arch. f. Physiol.* 82, 79, (1939).
64. Schmidt, C.L.A., and Greenberg, D. M., Occurrence, Transport, and Regulation of Calcium, Magnesium, and Phosphorus in the Animal Organisms, *Physiol. Rev.*, 15, 297, (1935).
65. Phillips, R. A., Robertson, D. F., Carson, W. C., and Irwin, G. E., The Effect of Irradiated Ergosterol on the Thrombocytes and the Coagulation of Blood, *Ann. Int. Med.*, 4, 1134, (1931).
66. Sure, B., and Kik, M. C., Hematopoietic Function in Hyper-vitaminosis. V. Vitamin D Deficiency, *Proc. Soc. Exp. Biol. and Med.*, 28, 496, (1931).

67. Taylor, N. B., Weld, C. B., Branion, H. D., and Kay, H. D., A Study of the Action of Irradiated Ergosterol and its Relationship to Parathyroid Function, *Canal, M.A.J.*, 24, 763, (1931).
68. Leenhardt, E., Baucumont, J., and Cayla, J., Recherches Sur le Phosphore et les Glycerophosphatases du Sang des Enfants Rachitiques. Variations Observees au Cours du Traitement par les Rayons Ultra-violets, *Rev. Franc. de Pediat.*, 13, 144, (1937).
69. Dreyer, I., and Reed, C. I., Treatment of Arthritis with Massive Doses of Vitamin D. *Arch. Phys. Therapy*, 16, 537, (1935).
70. Reed, C. I., and Thacker, E. A., The Effect of Intravenous and Intraperitoneal Injections of Irradiated Ergosterol, *Am. J. Physiol.*, 96, 21, (1931).
71. Bassett, S. H., Mineral Exchanges of Man. V. Balances of Electrolytes in a Case of Hyperthyroidism, *J. Nutrition*, 9, 323, (1935).
72. Freudenberg, E., Rachitis und Spasmophile., *Monatschr. f. Kinderh.*, 69, 303, (1937).
73. Widenbauer, F., Korrelation der Vitamin A and B mit dem Calcinose-faktor, *Klin. Wchnscher.*, 14, 901, (1935).
74. Correll, J. T. and Wise, E. C., Anti-rachitic Properties of A.T. 10 for the Rat and Chicken, *J. Nutrition*, 23, 217, (1942).
75. Goormaghtigh, N., and Handovsky, H., Effect of Vitamin D₂ (Calciferol) on the Dog, *Arch. Path.*, 26, 1144, (1938).
76. Fieser, L. F., The Chemistry of Natural Products Related to Phenanthrene. 2nd ed. (Rheinhold Publishing Corp. New York, N. Y. 1937).
77. Bills, C. E., and McDonald, F. G., Experiments on the Synthesis and Isolation of Vitamin D. Read before the American Association for the Advancement of Science, New Orleans, December 30, (1931).
78. Heinbron, I. M., Kamm, E. D., and Morton, R. A., The Absorption Spectrum of Cholesterol and Its Possible Biological Significance with Reference to Vitamin D. I. Preliminary Observations. *J. Soc. Chem. Indust.*, 45, 932, (1926).

79. Bills, C. E., Physiology of the Sterols, Including Vitamin D, *Physiol. Rev.*, 15, 1, (1935).
80. Smith, E. E., and McLean, F. C., Effect of Hyperthyroidism upon Growth and Chemical Composition of Bone, *Endocrinology*, 23, 546, (1938).
81. Benjamin, H. R., and Hess, A. F., The Forms of the Calcium and Inorganic Phosphorus in Human and Animal Sera. I. Normal, Rachitic, Hypercalcemic, and other Conditions, *J. Biol. Chem.*, 100, 27, (1933).
82. Gelfan, S., Effect of Viosterol Upon Oxygen Consumption of Frog's Muscle, *Am. J. Physiol.* 113, 464, (1935).
83. Taylor, N. B., Weld, C. B., and Sykes, J. F., Observations Upon the Absorption of Calcium in Normal Animals, *Brit. J. Exper. Path.*, 14, 355, (1933).

ACKNOWLEDGEMENTS

1. To Dr. Huffman for his kind support in supplying feeding and maintaining the cows for these studies.
2. To Dr. Davidson for his generosity and help in obtaining, feeding and housing the birds used in these studies.
3. To Standard Brands Company for supplying our irradiated ergosterol (1,000,000,000 units) used in these studies.
4. To E. R. Squibb and Company for their kindness in preparing and donating some of the various vitamin D products used in these studies.
5. To senior problem students Robert W. Stipek, James M. Isbester and Charles Leng, for their help in the carrying out of some of the analytical and animal work.
6. To the Veterinary and Poultry and Physiology Departments in furnishing housing quarters for the dogs used in these studies.
7. To the assay laboratory for the assays run on various materials used.
8. To William H. Gill for the many kindnesses and help in obtaining equipment for maintenance of the various animals used in these studies.

ACKNOWLEDGEMENTS

1. To Dr. D. T. Ewing of the Chemistry Department for his kind assistance and generosity in supplying of various vitamin D sources.
2. To Dr. Huffman for his kind support in supplying feeding and maintaining the cows for these studies.
3. To Dr. Davidson for his generosity and help in obtaining, feeding and housing the birds used in these studies.
4. To Standard Brands Company for supplying our irradiated ergosterol (1,000,000,000 units) used in these studies.
5. To E. R. Squibb and Company for their kindness in preparing and donating some of the various vitamin D products used in these studies.
6. To senior problem students Robert W. Stipek, James W. Isbester and Charles Lenz, for their help in the carrying out of some of the analytical and animal work.
7. To the Veterinary and Poultry and Physiology Departments in furnishing housing quarters for the dogs used in these studies.
8. To the assay laboratory for the assays run on various materials used.
9. To William H. Gill for the many kindnesses and help in obtaining equipment for maintenance of the various animals used in these studies.