EFFECTS OF DIHYDROTACHYSTEROL
IN NEPHRECTOMIZED RATS

by
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INTRODUCTION
In the past few years there has been renewed interest in the physiology of the parathyroids and their relationship to calcium metabolism. This laboratory has been particularly concerned with the actions of the hormone in relationship to kidney function and to bone metabolism. The earlier emphasis of this work pertained to the control of renal thresholds for calcium and phosphate excretion by the parathyroids and re-emphasized the importance of this function of the hormone from these glands. Because of the close relationship, at least clinically, of the vitamin D family to calcium metabolism, the earlier work also included studies of the effects of dihydrotachysterol (a member of the vitamin D2 series) on renal excretion of calcium and phosphate.

Recently the emphasis of this laboratory has turned to a study of the action of the parathyroid on bone. As part of this general interest in the control of calcium mobilization from bone, the work to be reported in the following pages has been an attempt to show clearly the effect of one of the vitamin D series, dihydrotachysterol, on bone metabolism. To provide a background for a better understanding of the results to be presented, it was felt important to review several aspects of past work in this and other laboratories. While these in themselves are distinct and separate entities, all fall into place in the attempt to better
understand the particular work of this thesis. The three reviews that follow deal, in order, with the parathyroids and their relationship to bone and kidney; a short history, chemistry, and physiology of the vitamin D family, and specifically of dihydrotachysterol; and finally the osteoclast and its place as a cytological unit of the structure of bone. It is hoped that from these three brief reviews a better background for the interpretation of the experimental data to follow will be gained.
The Parathyroids

The parathyroids have been the subject of increased interest and investigation ever since the preparation of a potent extract from them by Collip in 1925. Over the last 30 years there has been a divergence of views as to whether the kidney or the bone is the site of action of the hormone. In the last few years, however, there has been increased popularity in the idea that the action of the parathyroids is on bone.

The physiology and function of the parathyroid has been the subject of extensive reviews. For some of these comprehensive studies the reader is referred to the works listed below: Thomsen and Collip, 1932; Shelling, 1935; Pope and Aub, 1944; Albright and Reifenstein, 1948; Greep, 1948; Tornblom, 1949; Fleischmann, 1951; Nicolaysen et al, 1953; Bartels, 1954; Bartter, 1954; Noach, 1954; Reifenstein and Howard, 1954; Greep and Kenny, 1955.

Much of the early work done was on the effect of the hormone on the kidneys. It has long been known that with the administration of the extract there is a marked increase in phosphate excretion, an increase in serum calcium, and a decrease in serum phosphate. This renal effect has been recently emphasized in reports by Albright and Reifenstein (1948), Handler et al (1951), Davies and Gordon (1953), and Bartter (1954).
In contrast to the kidney studies, recent work indicating an action of the parathyroids on bone has been accumulating (Stewart and Bowen, 1951; Talmage, Lotz, and Comar, 1953; Talmage et al., 1953; Grollman, 1954). A paper by Talmage (1956) gives a good summary of the evidence for a dual action of the hormone on the kidney and on bone and presents an hypothesis, based on his work and that of others, as to the mechanisms involved.

Only fragmentary observations of the cytological chemistry of the parathyroid are available. In the Virginia deer, Grafflin (1942) reported well defined granules stainable with iron hematoxylin which he believes to be secretory granules. Similar reports are conspicuously absent from the literature. Among those studying the cytology of the gland have been DeRebertis (1940), Baker (1942), Dempsey (1948), and Groodt (1955).

Within the last five years citric acid has been brought into the study of the physiology of the parathyroids. Chang and Freeman (1950) found that both parathyroid extract and vitamin D would increase the serum citrate level in nephrectomized animals. Talmage and Elliott (1955) found that the citric acid concentration in the extracellular fluids dropped after parathyroidectomy and could be brought back to normal levels with as
little as 6 units of parathyroid extract. Neuman (personal communication) and Talmage and Elliott (1957, in press), in view of recent work on citric acid, have suggested that the parathyroid works through citric acid to remove calcium from bone.

Of interest in connection with the effect of the parathyroids on bone has been the possible relationship of the osteoclasts to this problem. It has long been postulated that the osteoclast is able to dissolve bone. In connection with this aspect there are several recent papers concerning this cell type (Heller et al, 1950; Kroon, 1954; Gaillard, 1955; McLean and Urist, 1955; Talmage et al, 1957, in press). The history of this cell type will be presented in another section.
Vitamin D and Dihydrotachysterol

In the development of the knowledge of the parathyroid gland it was found that the extract which had been prepared by Collip (1925) and purified by Collip and Clark (1925) could not be used clinically to treat hypoparathyroidism. This was because a refractoriness was built up in the patient against the extract after a period of continued use. Therefore it was necessary to turn elsewhere to find a treatment for parathyroid insufficiencies. At this time drugs were also being sought which would cure rickets.

It had been known for years that the sun's rays were beneficial in the treatment of rickets. Later it was found that certain artificial sources of light were just as effective as the sun. In 1926-27 Windaus and Holtz (see Holtz, 1941) produced substances of high antirachitic potency by irradiation of ergosterol, a steroid compound, with ultraviolet light. Angus et al (1931) separated out one of these products and named it calciferol. Today it is regarded as the true vitamin D (vitamin D₂ in the D series). In 1930 Holtz and Schreiber published a paper concerning the further investigation of ergosterin (ergosterol) and its byproducts of irradiation. They found that one of the products, by hydrogenation, lost its antirachitic property but retained what they termed its "Kalcinose Faktor". This had been noted as the cause
of a toxic effect by Windaus and others. Holtz realized that this new product could perhaps be used for the treatment of hypoparathyroidism. He performed many experiments with it and found no toxic effects from continued use of it. Windaus and his co-workers were also testing the new substance. In 1933 Holtz (see Holtz, 1941) published a paper in which he describes the substance as Antitetanic Preparation No. 10 (A.T.10). The name has remained and is used today interchangeably with dihydrotachysterol.

The process of irradiation is actually one involving conversion of the provitamin to its active state. There are several provitamins in the D series but only ergosterol will be considered since it is the precursor of tachysterol, from which dihydrotachysterol is derived.

Ergosterol, or provitamin D₂ (see Plate I), was discovered by Tanret (see Deuel, 1951) in the parasitic fungus ergot (Claviceps purpurea) in 1879. Tanret named the compound ergotinine, and later ergosterine. Ergosterol is present in such lower plants as brown algae, slime fungi, bacteria (Staphylococcus spp.), Mucor spp., yeast, Penicillium spp., and lichens. The most practical source of this sterol is dried yeast, in which ergosterol may make up 90-100% of the total sterols and up to 2% of the total dry weight (Bills, 1935 and Tanret, 1908, see
PLATE I

Figure 1. Structural formula of Ergosterol.

Figure 2. Structural formula for Tachysterol.

Figure 3. Structural formula for Dihydrotachysterol.
Deuel, 1951).

It has been proven that ergosterol comprises 19-25% of the sterols of the red snail, *Arion empri-cornium* (Bock and Wetter, 1938, see Deuel, 1951), while 22% of the sterol content of the earthworm (*Lumbricus terrestris*) has been found to consist of this sterol.

In regard to structure it has been shown that all of the provitamins which are known at the present time are cyclopentanophenanthrene derivatives containing the steroid nucleus (see Plate I). All have an hydroxy group in position 3. They have two double bonds in conjugate positions in the ring B at C5-C6 and C7-C8. The hydrocarbon chain on C17 is of great importance in establishing the nature of the vitamin D activity. Without such a side chain, the molecule cannot be changed to an active vitamin D. Moreover, slight changes in the nature of the side chain will greatly alter the physiological response of the resultant vitamin D formed from the provitamin D (Deuel, 1951).

When the provitamins D are changed into the active forms of vitamin D, the invariable change involves the rupture of the B ring of the steroid nucleus between C9 and C10. The methyl group attached to C10 is changed to a methylene group, while the extra hydrogen thus rendered available is used to saturate C9.

The conversion of the provitamins to the vitamins
D is done using ultraviolet light. The most effective wavelength is 2810 Å. The most effective synthesis of vitamin D occurs when the provitamin D is in solution. The yield is increased when the solution of provitamins is agitated. However, the reaction has been shown to take place at elevated temperatures as well as at the temperature of liquid air (Reed et al, 1939).

The activation of the provitamin involves only a rearrangement of the molecule, and no oxidation occurs. In fact, it has been shown that the presence of oxygen may have a deleterious effect upon this reaction (Bills et al, 1928, see Deuel, 1951).

The series of conversions of ergosterol gives intermediates known as lumisterol, tachysterol, calciferol, and three derivatives of calciferol, namely, toxisterol, suprasterol I and suprasterol II. The pharmacological effectiveness of these intermediates increases with the degree of conversion. Holtz in 1931 (see Holtz, 1941) showed this with a comparison of several of these intermediates as concerns their Kalcinose Faktor (calcium-raising ability). He found in the mouse that the minimum toxic dose was 0.2 mg. of tachysterol, 0.065 mg. of calciferol, and 0.025 mg. of toxisterol.

Tachysterol, \( \text{C}_{28}\text{H}_{43}\text{OH} \) (see Plate I), is the second known product which is formed in the progressive activation of provitamin \( \text{D}_2 \). It was named tachysterin.
The name denotes its outstanding property, i.e., the speed with which it reacts. Lumisterol presumably yields first a protachystersol which is very unstable and which has not been prepared in pure form. It rapidly undergoes a transformation to tachysterol as shown by Windaus and Auhagen in 1931 (see Holtz, 1941). Tachysterol in itself has no undesirable effects and might be used therapeutically were it not for the fact that it is unstable and deteriorates rapidly.

Tachysterol is isolated from the radiation mixture as an addition product of citraconic anhydride. On thermal decomposition the adduct yields tachysterol. It can be further purified by the preparation of tachysterol-3,5-dinitro-4-methylbenzoate, which forms well-shaped crystals. This method was first used by Windaus et al in 1932 (see Holtz, 1941).

Dihydrotachysterol can readily be prepared by reduction of the 3,5-dinitro-4-methyl benzoic ester of tachysterol with sodium and ethanol (Windaus et al, 1932, see Holtz, 1941) and subsequent saponification of the reduced product. Dihydrotachysterol can be crystallized readily, although this is not possible with unreduced tachysterol. Dihydrotachysterol is permanently stable in an oily solution.

On the basis of the absorption spectrum, the
catalytic hydrogenation experiments, and the oxidative degradation reaction, von Werder (1939, see Deuel, 1951) assigned the formula found in Plate I to A.T.10. The pure product melts at 125-127 degrees centigrade and has absorption maxima at 2420, 2510, and 2610 Å.

In the rat, 1 gm. of A.T.10 has a potency of 200,000 I.U. of vitamin D activity. The I.U. of vitamin D is considered to be the effect caused by 0.025 micrograms of pure crystalline vitamin D₂ dissolved in 1 mg. of olive oil. This standard was defined by the International Vitamin Conference held by the League of Nations in 1934.

Calciferol, which is in the same series of compounds as is dihydrotachysterol, has been extensively used in the treatment of rickets (Albright et al, 1939; Correll and Wise, 1942; Harrison and Harrison, 1942; Jolliffe and Most, 1943; Harrison, 1953). A.T.10 has been noted to have a slight antirachitic activity in the rat and chicken (Shohl et al, 1939; Shohl and Farber, 1941).

The various compounds in the vitamin D series exhibit differences in antirachitic and hypercalcemic potencies. Each compound may, in addition, exhibit variable potencies according to the species made subject to it. Vitamin D₂ is equivalent in the rat to about 40,000 USP units/mg. but in the chicken to approximately 1,000 USP units/mg. Vitamin D₃ (activated 7-dehydro-
cholesterol) is equivalent to 40,000 USP units/mg. in both rat and chicken, and crystalline dihydrotachysterol is equivalent to 80 USP units/mg. in the rat and about 360 USP units/mg. in the chicken (McChesney, 1943).

This potency noted above is in terms of antirachitic ability. The dosage for prevention of rickets is in the range of a few units of vitamin D whereas the dosage levels for producing hypercalcemia are in the range of 1000 units of vitamin D.

It is believed that calciferol is taken into the body in an unaltered form since it has been found in several tissues and organs of various animals after ingestion (Nicolaysen and Eeg-Larsen, 1953). Actually calciferol (D$_2$) is in the main vitamin D of yeast while vitamin D$_3$ (7-dehydrocholesterol) constitutes the vitamin D present in animal fats (Moliter and Emerson, 1948).

The main use of A.T.10 has been in the control of the serum calcium levels in hypoparathyroidism. The use of this drug clinically has been somewhat limited as compared to calciferol because of the cost. Albright and Reifenstein (1948) state that when the choice of a drug for hypoparathyroidism comes down to a dollars and cents basis, calciferol is often the drug of choice because it is much less expensive than A.T.10. A.T.10 has twice the hypercalcemic potency of calciferol but is perhaps ten times more expensive. Still, the use of A.T.10 is reported
by many in the literature as the treatment for hypoparathyroidism (Albright et al., 1938; MacBryde, 1938; Albright, 1939; Grette, 1955).

The effects of A.T.10 have been reported by Correll and Wise (1942) who state that A.T.10 works primarily by increasing kidney excretion of phosphate. This view is in accordance with that of Albright (1938, 1939, 1948). Talmage and Dodds (1955) and Herzfeld et al. (1955) found no such effect of the drug. Dodds (1955, Thesis) feels that the action of the A.T.10 is on the bone rather than on the kidney. Harrison and Harrison, 1942, reported that A.T.10 caused an increase in serum phosphate and a decreased phosphate excretion in the normal dog. Albright and Sulkowitch (1938) and Cramer and Steenbock (1956) have shown, as have others, that A.T.10 causes increased absorption of calcium from the gut. It has already been noted that A.T.10 increases the serum calcium in normal and hypoparathyroid individuals. There are several members of the vitamin D series which have this ability. Calciferol and activated 7-dehydrocholesterol as well as dihydrotachysterol have this hypercalcemic factor (McChesney and Kocher, 1942).

Carlsson and Hollunger (1954) have hypothesized that the action of vitamin D on bone is the stimulation of the production of citric acid. They noticed a rapid increase in the citric acid content of the serum, bones,
and incisors after vitamin D treatment. The increase in citric acid following vitamin D treatment has been reported by Elliott and Freeman (1956) also.

Latta and Tristan (1950) found that prolonged treatment of rats with A.T.10 caused a major decalcification of the long bones. Later effects noted were connected with renal failure due to the increased calcium load to be excreted.

The work of Dodds (1955, Thesis) points to the bone as the only site of action of A.T.10. In his work he showed that increases or decreases in the renal excretion of both calcium and phosphate appear to follow similar increases or decreases in the serum levels of these two ions. He also showed that the drug was unable to lower the serum phosphate levels even in the presence of slightly increased renal phosphate excretion. It is hoped that the work to be presented may help to clarify the effect of the drug on bone.
The Osteoclast

Robin, in 1649 (see Hancox, 1949), drew attention to the morphological peculiarities of certain multinucleated giant cells which he named myeloplaques. He was able to describe most of the essential histological attributes of these cells. He observed that the cells had nuclear counts ranging from one or two up to forty. He was not able to make any suggestions as to their functions. In 1670, Rollett (see Hancox, 1949), in discussing the microscopic anatomy of endochondral ossification, remarked upon the occurrence of large masses of protoplasm with many nuclei and associated them with Robin's myeloplaques. Koelliker, in 1873 (see Hancox, 1949), first suggested that these cells were the universal agents of bone absorption and he christened them with the title of "ostoklast". However, in the literature the French term "osteoclast" seems to have been preferred.

The typical osteoclast is a multinucleated cell, usually associated with bone in an area of resorption. It varies greatly in dimensions. Arey in 1919 described and illustrated an osteoclast whose long axis measured 85x105 microns. Estimations of volume made by Hancox (1949) would give such an osteoclast a volume of about 200,000 cubic microns. The shape of the osteoclast varies greatly (see Plate II). Round or oval masses are often seen at a
Figure 4. Section of decalcified bone showing numerous osteoclasts. Note distribution and variation in shape. Stain: Erlich's hematoxylin and phloxin. x600

Figure 5. Section of decalcified bone showing numerous osteoclasts. Note protoplasmic projections of osteoclast on left. Stain: Erlich's hematoxylin and phloxin. x400
little distance from bone tissue and the nuclei of these are generally round and vesicular. Secondly, thin sheets occur. These are found flattened against bone tissue and their nuclei are often flattened. When occupying Howship's lacunae, the cells usually have a roughly semicircular outline which fits the border of the lacuna while the external surface is smooth. In other observations osteoclasts often are composed of two or more lobes connected by fine protoplasmic strands. In 1947, Barnicot, in his supravital studies on mouse osteoclasts, observed adjacent small individuals to be pear-shaped with the stalks pointing toward each other as if they had been joined together. Osteoclasts in tissue culture have been observed to form lobes which move apart and then flow together. Finally, some individuals possess long filiform or short branching processes. The diversity of shapes is better understood when the motility of the cell is borne in mind.

The larger osteoclasts contain upwards of 50 to 100 nuclei while the smaller may contain two or three. Hancox (1956) believes an average figure would be 30 nuclei per osteoclast. The nuclei are rounded or oval in shape with one or two nucleoli. There is considerable variation in their shape. They are sometimes regular in outline, sometimes crenated, and sometimes pyknotic. Many authorities maintain that these differences in form
are due to differences in age of the cells. They associate the irregularities in form with reduced physiological activity.

The cytoplasm of the living cell is refractile and granular. Lipoid granules have been described in the fixed cell by Dubreuil (1910) and by Fell (1925). Weidenreich (1930) demonstrated rounded cytoplasmic bodies by means of gram-Weigert staining. Jaffe (1930) showed the presence of analogous bodies with a silver impregnation method. It is believed that these may be the remains of short rod mitochondria. There seem to be two kinds of vacuoles present in the cytoplasm. The first kind are those concerned with the active cell and are found just beneath the striated border. The others are coarse vacuoles which appear when the cell is in its early stages of degeneration.

Osteoclasts are found apposed to bone in several ways. Often they form a cap over the end of a bone spicule. They may also be found flattened against the bone surface. Possibly they may be seen filling the erosion pits (Howship’s lacunae).

Several authors refer to the most outstanding microscopical feature of the osteoclast as being the striated border. It is found as a striated or fibrillated zone between the cell and the adjacent bone. This border is seen only in a certain proportion of osteoclasts and
then only in the area most closely associated with bone. This border has been shown both by studying stained sections and by phase-contrast. The striations run roughly perpendicular to the surface of the bone. In absorption lacunae the striations appear to radiate from the cell to the curved surface of the bone (Hancox, 1956). The brush border was noticed by Koelliker in 1873 (see Hancox, 1949) who thought it was part of the bone. The evidence to date seems to indicate that both views may be correct. There is indeed a striated border to the cells but also there are exposed bone fibrillae with which these are thought to interdigitate (Ham and Gordon, 1952).

Kroon (1954) pointed out that these striated borders, known in the literature as Koelliker's brush borders, are of a different nature from the brush borders which are found in absorptive epithelia. It is equally incorrect to regard them as mere fringe-like projections formed by collagen fibrils which project from the bone substance at places where destruction of the latter is going on.

These striated structures are formed by the penetration of protoplasmic projections from the osteoclast into the bone substance. The bone substance trapped in the border is broken down and liquified in vacuoles, the number of which, in the border and also in the other protoplasm of the osteoclast, increases steadily through-
out the process. The contents of the vacuoles finally lose all staining properties. These borders in their varying morphological aspects must be regarded as representing functional states of the osteoclast in the process of osteoclastis.

The presence of the previously mentioned vacuoles just beneath the striated border strongly suggests some form of cellular secretion.

Histochemical work in this field is sparse. The distribution of alkaline phosphatase has been studied by several investigators but the results are conflicting and inconclusive. Heller-Steinberg (1951) described small spherical granules in frozen-dried bone with Hotchkiss periodic acid–Schiff procedure. These granules were thought to consist of a polysaccharide–protein complex. However, these granules were also found in osteoblasts and osteocytes, so they cannot be considered specific to the osteoclast.

The origin of osteoclasts has been a great controversy for many years. That they arise by mitotic division of existing cells is doubtful for very few reports of mitoses have been made. Various precursors for the osteoclasts have been proposed. Among the most common are the osteoblast, osteocyte, and histiocyte. Benoit and Clavert (1952) have suggested that more than one source is involved. Heller et al (1950) state that
they have found places in bone where there is clear evidence of fusion of cuboidal or spindle-shaped osteoblasts into multinucleate osteoclasts. They go on to say that there are indications that osteoclasts break down into individual cells that take on a spindle-shaped form. They point out that though there are large numbers of osteoclasts present, no dead ones are ever seen when the numbers are diminished. They have also proposed that osteoblasts may form osteoclasts (in agreement with Arey, 1916-17) and that osteocytes may form osteoclasts. The origin of the osteoclast is still open to question and until some technique is developed whereby the living tissue can be watched the matter will not be settled with finality.

The place of the osteoclast in bone dissolution is likewise an open question. The work of Kroon (1954) most strongly suggests a definite action in the dissolution of bone. It has been shown by Heller in 1950 and by Gaillard in 1955 that bone resorption takes place in the absence of osteoclasts. They have reported the appearance of osteoclasts after several hours of bone resorption have elapsed. McLean and Urist (1955) stated that it was not possible for them to demonstrate any osteoclasts ingesting debris even in an area where there was much debris to be found. Heller et al (1950) reported also that no evidence of phagocytosis by the osteoclasts was
It is hoped by this writer that some new light may be shed on the nature of the osteoclast with a new technique which he will attempt to employ in the near future. This technique is the application of the cryostat as a possible means of sectioning quick-frozen undecalcified bone tissue for histochemical tests and examination.
Statement of Problem

The work of this laboratory over the last few years has opened many avenues of approach to the problem of the parathyroid control of the calcium and phosphate metabolism in the body. The use of nephrectomized animals has been very fruitful in studying effects of the parathyroids on bone.

In view of the work already done with vitamin D and in particular with dihydrotachysterol it was thought that the orientation of work already being used in this laboratory could be profitably applied to an extension of the knowledge of the effects of dihydrotachysterol on the bones.

This study was made, therefore, in an attempt to determine the effects of dihydrotachysterol on those animals in which interference from calcium uptake by the gut and excretion of calcium by the kidney was eliminated.
MATERIALS AND METHODS
MATERIALS AND METHODS

In these experiments a total of 155 male Holtzman albino rats were used. Although the weight range for the entire group was from 131-320 gms., the range for any one experiment was 25 gms. and in the key experiments the weight of the animals was limited to between 225-250 gms.

In all experiments the animals were maintained on a salt-free, calcium-free, low protein diet for two days prior to nephrectomy. This diet consisted of the following ingredients:

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<th>Ingredient</th>
<th>Weight</th>
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<tr>
<td>Alphacel</td>
<td>110 gms.</td>
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<tr>
<td>Dextrose</td>
<td>100 gms.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40 gms.</td>
</tr>
<tr>
<td>Crisco</td>
<td>80 gms.</td>
</tr>
<tr>
<td>Wesson Oil</td>
<td>12 gms.</td>
</tr>
<tr>
<td>Wheat Germ Oil</td>
<td>5 gms.</td>
</tr>
<tr>
<td>Cod Liver Oil</td>
<td>3 gms.</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>115 gms.</td>
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Total weight 465 gms.

In order to study changes in the serum calcium levels it was necessary to adopt a technique which would reveal these changes without grossly altering the animal's physiological state. The direct way to study these changes would have been to remove blood samples and analyze them. However, since the analysis technique
requires at least 2 ml. of blood this would have altered the fluid composition of the animal very greatly after a number of samples had been taken. It was decided to adopt a peritoneal lavage technique. This technique has been used by Talmage and Elliott (1956) to study serum calcium changes without reducing the normal blood volume. The changes which take place in the calcium content of the lavage fluid have been shown by Talmage and Elliott to reflect, relatively, changes in the serum. The lavage technique finally adopted for the main series of experiments was a composite of several techniques used in preliminary experiments. The various methods employed in preliminary work will be discussed below in an attempt to show how and why the final technique was adopted.

Experimental Technique

1) The first lavage method tried was that of Kolff and Page (1954), using a stainless steel tube with a drilled circular flange on the internal end which could be sewn into the animal and having a threaded portion on the other end for securing a stainless steel plug (see Plate III). In this experiment the tube was placed in a mid-ventral incision and sewn to the abdominal musculature. The skin was drawn closed around the tube and secured with skin clamps. The following day the right kidney was removed through a lateral incision. On the
Figure 6. Stainless steel indwelling tube and plug used in peritoneal lavage (see Kolff and Page, 1954). Note holes in flange for suturing. Actual size.

Figure 7. Rat with stainless steel indwelling tube and plug in situ. Incision closed with skin clamps around sutures through flange holes (see above).
third day the left kidney was removed in a like manner.
The three operations were made on succeeding days, each
with ether anesthesia. Some of the rats developed
respiratory congestion and died. However, with those
that survived, 30 ml. of lavage solution were introduced
into the peritoneal cavity through the steel tube with
a rubber cannula attached to a syringe, and the steel
plug reinserted in the tube. After a period of one hour
the cannula was reinserted and the animal manipulated
in a number of positions to facilitate removal of the
lavage fluid. (Hereafter the term 'lavage fluid' will
refer to the lavage material removed from the peritoneum.)

2) In a later experiment a modification of the
operative technique was employed. A mid-ventral incision
was made and both kidneys were removed. The incision
then became the site of the indwelling steel tube. As
in the first experiment, the lavage was performed two
or three times per day until the death of the animals
(two or three days post-nephrectomy).

3) It was desirable to study the hourly changes
in calcium levels and to that end the lavage technique
was again modified. The nephrectomized animals with the
steel tube in place were anesthetized with sodium
nembutal and the left testis removed through an incision
in the posterior portion of the scrotum. A glass cannula
of 3/16 inch outer diameter was inserted into this
incision and passed into the peritoneal cavity. The lavage solution was delivered to the steel tube by gravity flow through a system of glass tubing which coursed through a drip-type flow meter. The constant flow of lavage solution bathed the internal organs and the fluid passed out through the glass cannula. Collections were made at half-hour intervals and analyzed. Sodium nembutal was given as necessary to maintain light anesthesia.

4) At this time a new type of indwelling tube was developed by Talmage, Schooley, and Comar (in press, 1957). This glass tube (see Plate IV) was used in all subsequent experiments. The rats were nephrectomized through the mid-ventral approach as before, but the incision was closed. Twenty four hours after nephrectomy the animals were anesthetized with sodium nembutal given intraperitoneally and an incision made in the inguinal canal just posterior to the penis. The testis was removed. The glass tube was inserted into the canal and "purse-string" type sutures were made through the skin and peritoneal lining around the tube. The flanges of the tube held it in place when the suture thread was tied tightly. The enlarged, flattened base on the tube prevented the viscera from plugging the holes in the side of the tube. A 12-gauge hypodermic needle was then inserted in the peritoneum from a lateral approach.
Figure 8. Glass indwelling tube used for peritoneal lavage. Note holes near base to allow passage of lavage fluid into tube from peritoneal cavity (see Talmage, Schooley, and Comar, 1957, in press).

Actual size.
A piece of polyethylene tubing was then introduced into the peritoneum through the needle and the needle withdrawn, leaving the tubing in place. A wound-clip was fastened to the skin around the tubing to hold it in place and prevent leakage. The lavage solution was put into the peritoneal cavity through the plastic tubing and the fluid withdrawn from the cavity later through the glass tubing in the scrotum by gentle manipulation of the animal.

5) The technique finally adopted for the experimental work was essentially the same as that just mentioned with a change only in the mode of introducing the lavage solution. In this case the solution was injected via the glass tube through which the lavage fluid was later removed. This method proved to be successful and was used for the rest of the experiments.

Lavage Solutions

1) As changes were made in the operative technique, so were they made in the composition of the lavage solution. The lavage solution first used had the following composition:
The solution was buffered at pH 7.4 and contained 2% glucose to aid in its removal.

2) It was found that heating of the lavage solution to 37° C. prior to injection caused a pH shift from 7.4 to as high as 8.9. In an attempt to maintain a constant pH, a solution of 0.84% NaCl was used alone and adjusted to pH 7.4 with NaOH and HCl. One and one-half percent glucose was added to aid in the removal of the lavage fluid from the peritoneal cavity. This lavage solution was used in all subsequent experiments.

<table>
<thead>
<tr>
<th>Ion</th>
<th>meq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
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</tr>
<tr>
<td>K</td>
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<tr>
<td>Ca</td>
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<td>HPO₄²⁻</td>
<td>2</td>
</tr>
<tr>
<td>Lactate</td>
<td>7</td>
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</tbody>
</table>

(Modified from Skeggs, Leonards, and Heisler, 1949, by Kolff and Page, 1954.)

Basic Experimental Technique

In the main series of experiments the following procedure was used. Bilateral nephrectomy was performed through a mid-ventral incision and the wound closed. At this time one half of the animals were given 200 μgms. of A.T.10 (Hytakerol, Winthrop-Stearns brand of dihydro-achyysterol in sesame oil) by stomach tube. A subcutaneous injection of 20,000 units of penicillin was given to
each animal. Twenty four hours after nephrectomy the animals were given another 200 µgms. of A.T.10 and were anesthetized with an intraperitoneal injection of sodium nembutal. The glass indwelling tube was inserted and secured as described above and the animals were filled with 30 ml. of lavage solution. The fluid was removed after half an hour and immediately replaced with fresh solution. This procedure was repeated for 7 to 9 hours. After 2½ hours of lavage, the parathyroids were removed individually from half of the animals which had received A.T.10 and from half of those which had not received A.T.10. There was no interruption in the lavage schedule.

In all of the experiments the lavage fluid removed from the animals was centrifuged to remove any blood cells and other solid material. The sample was then divided for the various analyses.

Calcium Analysis

For the calcium analysis 4 ml. of centrifuged lavage fluid was combined with 4 ml. of 4% ammonium oxalate. This was allowed to stand for 2 hours to insure complete precipitation of the calcium oxalate. After centrifugation and decantation of the supernatant, the precipitate was washed with 2% ammonium hydroxide
and again centrifuged and decanted. The precipitate was allowed to dry completely and was then redissolved in 0.1 ml. of 70% perchloric acid. When solution was complete the volume was increased to 4 ml. by the addition of 3.9 ml. of 10% isopropyl alcohol.

The calcium samples, as prepared, were analyzed with the Beckman, model DU, quartz spectrophotometer with flame attachment. A standard solution of calcium chloride was prepared and several dilutions made of it. The dilution which contained 50 μgms. of calcium per milliliter was taken as the standard used to calibrate the photometer. With the wavelength set at 620 Å, the sensitivity set at 0.1, and the slit width set at 0.3, the transmission dial was set at 50. The machine was calibrated to the standard. The other dilutions were then tested on the photometer. The transmission values obtained were plotted on a graph against the concentration of each dilution and the resulting curve used as a conversion curve for subsequent analyses of the lavages.

Phosphate Analysis

For the phosphate (as phosphorus) determinations 9 ml. of 5% trichloroacetic acid (TCA) were added to 1 ml. of centrifuged lavage fluid. After half an hour the solution was filtered through filter paper (Whatman no. 42) to remove the precipitated proteins. One ml. of
this filtrate was added to 2 ml. of a molybdate-
sulfuric acid mixture (2 parts of 5% sodium molybdate,
1 part of 10 N sulfuric acid, and 1 part of distilled
water). To this was added 6 ml. of distilled water and
1 ml. of Elon (1 gm. p-methylaminophenol sulfate dissolved
in 100 ml. of 3% sodium bisulfate). After 45 minutes
the samples were analyzed with the Bausch and Lomb, model
120, spectrophotometer set at wavelength 660 Å (method
of Gomori, 1942). A set of standard solutions was
prepared as for the calcium determinations and a curve
drawn.

Sodium and Potassium Analysis

In one experiment of the main series and in one
preliminary experiment sodium and potassium determinations
were made on the lavage fluids. For these determinations
9 ml. of 5% TCA were added to 1 ml. of the lavage fluid
and the filtration carried out as for the phosphates.
Two ml. of this supernatant were diluted to 25 ml. with
10% isopropyl alcohol and the analyses were done with
the Beckman spectrophotometer mentioned above. The
following settings were used:
For sodium
Wavelength ------ 590 Å
Sensitivity ------ 0.1
Slit width ------ 0.04

For potassium
Wavelength ------ 768 Å
Sensitivity ------ 0.1
Slit width ------ 0.3

Histological Procedures
At the end of the wash period the animals were sacrificed and the right femur was removed from each and sawed in half and the proximal end discarded. A median sagittal section was made through the distal end and the two halves were fixed in Bouin's fluid for 2 weeks with changes every other day. This decalcified the bone for further histological treatment. After fixation and decalcification the bones were dehydrated in ethyl alcohol, embedded in paraaffin (Fisher's Tissue Mat wafers, melting point 63-65°C.), sectioned at 7 microns on a Spencer rotary microtome, mounted on slides, and stained with Erlich's hematoxylin and phloxin.

Using a Spencer binocular microscope with a 43x objective and 6x matched oculars the sections of bone were examined for osteoclasts. A quantitative count was obtained by surveying successive fields and counting osteoclasts per field. The area examined was the distal end of the diaphysis, and included the row of degenerating cartilage cells of the diaphysoepiphysseal synchondrosis
also known as epiphyseal plate or metaphysis, though some question arises as to the actual area included by each of these alternate terms). This area, as seen in cross section, traverses the section of the femur in the approximate shape of an inverted "W". The total number of osteoclasts per section was divided by the number of fields surveyed for that section to give the average number of osteoclasts per field. These mean values were tabulated with those of similar animals and an over-all mean was obtained for a particular experimental group.

Statistical Analysis

All of the data concerning the calcium, sodium, and potassium values were analyzed with the Student's T test for significance of differences between means (see Treloar, Alan E., Biometric Analysis, Burgess Publishing Company, Minneapolis, Minnesota, 1951).
RESULTS
Preliminary Experiments

In the development of the experimental techniques several preliminary experiments were run in which new procedures were tried and new analyses perfected. These early preliminary experiments were of value for overcoming difficulties in the technique. They also laid the groundwork for successive experiments, and for that reason a brief discussion of some are included here.

In the bulk of the preliminary work the animals which had been given A.T.10 showed a tendency to have higher lavage fluid calcium values than those which had not been given A.T.10. In general there was a drop in the lavage fluid calcium level in those rats which were parathyroidectomized. In those animals which were parathyroidectomized and given A.T.10 there was a tendency for the lavage fluid calcium levels to return to that of the control after several hours.

There were some rats which did not respond to parathyroidectomy and this may have been due to incomplete removal of the glands.

These experiments indicate that A.T.10 is able to raise the lavage fluid calcium level in the nephrectomized rats and that it can perhaps cause a return of the lavage fluid levels toward normal values in parathyroidectomized rats.

In one preliminary experiment the sodium and
potassium values were determined. They were consistent throughout the lavage period. There was no appreciable difference between the different experimental and control groups as regards the levels of these two ions.

Effect of A.T.10 on Lavage Calcium Values

The procedures used in this section are described in detail in the section on materials and methods. The nature of the continuous lavage used creates a continuous calcium drain on the animal. By using only calcium-free lavage solution, allowing time for equilibration of the lavage solution, and removing the fluid, a continuous demand is put upon the rat's calcium stores. These stores must supply more calcium each half hour to maintain the serum values. This must be done in the face of a constant depletion of calcium. The use of this technique permitted comparison of the ability of the animal to continuously supply calcium under the various conditions.

Table I shows the means of the calcium values for the experimental animals throughout the lavage period. Each lavage period was of 30 minutes duration. The figures listed represent the values which were recorded for each group of animals.

Since it has already been stated that all of the animals used in these experiments were nephrectomized, the designation of an experimental group as "nephrectomized,
<table>
<thead>
<tr>
<th>Lavage Period</th>
<th>Control</th>
<th>A.T.10</th>
<th>N,PTX</th>
<th>A.T.10,PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35±2 (11)</td>
<td>40±2 (11)</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>2</td>
<td>36±1 (13)</td>
<td>39±1 (13)</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>3</td>
<td>33±1 (13)</td>
<td>36±1 (13)</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>4</td>
<td>32±1 (13)</td>
<td>37±1 (12)</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>5</td>
<td>33±1 (13)</td>
<td>36±1 (12)</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>6</td>
<td>34±2 (7)</td>
<td>34±2 (8)</td>
<td>29±3 (5)</td>
<td>30±2 (5)</td>
</tr>
<tr>
<td>7</td>
<td>34±2 (8)</td>
<td>35±2 (8)</td>
<td>24±1 (5)</td>
<td>29±3 (5)</td>
</tr>
<tr>
<td>8</td>
<td>33±2 (8)</td>
<td>37±3 (7)</td>
<td>22±2 (5)</td>
<td>27±2 (5)</td>
</tr>
<tr>
<td>9</td>
<td>36±2 (8)</td>
<td>39±3 (8)</td>
<td>22±1 (5)</td>
<td>28±4 (5)</td>
</tr>
<tr>
<td>10</td>
<td>35±2 (8)</td>
<td>37±2 (8)</td>
<td>22±1 (5)</td>
<td>26±2 (4)</td>
</tr>
<tr>
<td>11</td>
<td>39±3 (7)</td>
<td>41±3 (8)</td>
<td>22±1 (5)</td>
<td>24(18-30)*2</td>
</tr>
<tr>
<td>12</td>
<td>39±3 (8)</td>
<td>39±3 (7)</td>
<td>22±1 (5)</td>
<td>26±2 (5)</td>
</tr>
<tr>
<td>13</td>
<td>37±1 (8)</td>
<td>37±2 (7)</td>
<td>20±1 (5)</td>
<td>29±2 (5)</td>
</tr>
<tr>
<td>14</td>
<td>38±1 (7)</td>
<td>39±1 (6)</td>
<td>21±2 (4)</td>
<td>31(23-35)*3</td>
</tr>
<tr>
<td>15</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>34(33-35)*2</td>
</tr>
</tbody>
</table>

Notes: 1) All values given with Standard Deviation of Mean \( \sqrt{\frac{\sum x^2}{n-1}} \). 2) Numbers in parentheses refer to number of animals in each group. 3) Weight range of animals 225-250 gms. 4) Periods each 30 minutes.

* Range
"etc." will not be used. Instead, the animals which were nephrectomized only served as controls and will be referred to as such. For convenience in discussing the groups the following abbreviations will be used:

1) Nephrectomized only ——— control
2) Nephrectomized and given A.T.10 ———— A.T.10
3) Nephrectomized and parathyroidectomized ——— N,PTX
4) Nephrectomized, given A.T.10 and parathyroidectomized ——— A.T.10,PTX

Controls. Upon examination of the data it will be observed that the initial lavage fluid calcium level for the control group was 35 µgms. of calcium per milliliter (35 µgms. Ca/ml.). The calcium values for the rest of the lavage periods were about the same as for the first period, but with a slight decrease after approximately 2 hours, followed by a gradual increase to a final level of 38 µgms. Ca/ml. The trend toward an increase was relatively small and has not been statistically verified.

A.T.10. A parallel situation existed with the animals which had received A.T.10. However, the initial lavage fluid calcium values were higher in this group. The lavage fluid calcium level for the first lavage period was 40 µgms. Ca/ml. and the level for the last lavage period was 39 µgms. Ca/ml. The lavage fluid level in the A.T.10 group tended to approach that of the
control group toward the end of the day.

To determine whether there was a significant difference between the means of the lavage fluid calcium values for the control and for the A.T.10 group, the Student's T test was used. To obtain a mean for each group, the separate lavage fluid calcium values for each animal for the 14 lavage periods were pooled and the mean taken. This, in effect, offered a comparison of the two groups throughout the whole lavage period, or over the entire curve (see graph, Plate V). The difference between the two groups was significant at the 0.1% level; that is, there was only 1 chance in 1000 that the difference between the two curves was due to random distribution (see Table II). It can be seen from the graph that the difference between the two curves, i.e., the A.T.10 group and the control, became less with time. To test this change, the first half of each curve was tested as above. The difference between the two groups for the first half of the lavage period was found to be significant also at the 0.1% level.

The second half of each curve was then tested in a like manner to see of the significance of the difference between the two curves decreased with time. Indeed, the difference was significant only at the 9% level, or 9 chances in 100 of random distribution. For biological
Figure 9. Graph illustrating progressive changes in lavage fluid calcium levels.
<table>
<thead>
<tr>
<th>Test</th>
<th>Group</th>
<th>Mean</th>
<th>No. Animals</th>
<th>T Score</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. A.T.10</td>
<td>A.T.10  control</td>
<td>38±0.5</td>
<td>269</td>
<td>4.3</td>
<td>0.001</td>
</tr>
<tr>
<td>whole curve</td>
<td>A.T.10  control</td>
<td>35±0.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control vs. A.T.10</td>
<td>A.T.10  control</td>
<td>37±0.5</td>
<td>154</td>
<td>4.3</td>
<td>0.001</td>
</tr>
<tr>
<td>1st half</td>
<td>A.T.10  control</td>
<td>34±0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control vs. A.T.10</td>
<td>A.T.10  control</td>
<td>38±0.8</td>
<td>105</td>
<td>1.7</td>
<td>0.09  NS</td>
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<tr>
<td>2nd half</td>
<td>A.T.10  control</td>
<td>36±0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control vs. N₂PTX (per. 7-14)</td>
<td>control</td>
<td>36±0.7</td>
<td>101</td>
<td>14</td>
<td>0.0001</td>
</tr>
<tr>
<td>A.T.10 vs. A.T.10,PTX (per. 7-14)</td>
<td>A.T.10</td>
<td>38±1.0</td>
<td>93</td>
<td>7.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>N₂PTX (per. 7-14)</td>
<td>A.T.10,PTX</td>
<td>28±1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.T.10,PTX vs. N₂PTX (per. 7-12)</td>
<td>A.T.10,PTX</td>
<td>27±1.2</td>
<td>56</td>
<td>3.9</td>
<td>0.0001</td>
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<tr>
<td>(per. 7-14)</td>
<td>N₂PTX</td>
<td>22±0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.T.10,PTX vs. N₂PTX (per. 13-14)</td>
<td>A.T.10,PTX</td>
<td>30±1.8</td>
<td>17</td>
<td>4.1</td>
<td>0.001</td>
</tr>
<tr>
<td>(per. 13-14)</td>
<td>N₂PTX</td>
<td>21±1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means in µgms./ml. with SDM. * Probability of null hypothesis. NS - not significant.
work in general the 5% level is considered the limit of significance. Therefore this difference was not statistically significant (see Table II).

N,PTX. Immediately following parathyroidectomy the lavage fluid calcium levels of the nephrectomized animals began to drop. This decline occurred rapidly as evidenced by the calcium levels of the first three lavage periods after parathyroidectomy which were 29, 24, and 22 μgms./ml. respectively. The calcium level of the lavage fluid stabilized at 22 μgms./ml. and remained essentially unchanged throughout the remainder of the day. In order to compare the effects of parathyroidectomy on the animals which had received A.T.10 and those which had not, a comparison was first made of the difference between the control group and the N,PTX group. These two groups were statistically compared for lavage periods 7-14. The difference between the two groups was significant at the 0.01% level, or 1 part in 10,000 (see Table II).

A.T.10,PTX. Parathyroidectomy in the A.T.10 group caused a fall in the lavage fluid calcium level within a half hour. The downward trend of the calcium values continued for about an hour after parathyroidectomy. The lavage fluid calcium levels then tended to stabilize at about 27 μgms./ml. In the last hour of lavage the calcium content of the lavage fluid began to increase
and by the last lavage period had reached 34 μgms. Ca/ml.

A statistical comparison of the A.T.10 group and the A.T.10,PTX group (periods 7-14) showed that the difference between them was significant at the 0.01% level (see Table II).

As a result of the preceding relationships it was possible to compare the A.T.10,PTX group with the N,PTX group. The Student's T test showed that the significance of the differences between the two groups was marked (see Table II). The lavage fluid calcium level of the A.T.10,PTX group had not reached, at any time, as low a value as had that of the N,PTX group.

In the last two lavage periods there was a decided increase in the lavage fluid calcium values of the A.T.10,PTX group. This trend was not paralleled by the N,PTX group (see graph, Plate V).

Effect of A.T.10 on Lavage Potassium Values

In one experiment the potassium content of the lavage fluid of the four rats was determined. The average value for all of the animals was about 0.17 mgs. potassium per milliliter of lavage fluid (see Table III). It was found by comparing the potassium values of each of the animals to the mean potassium values of the other three that there were not significant differences between any of the animals. Therefore, there was no
<table>
<thead>
<tr>
<th>Lavage Period</th>
<th>Control</th>
<th>A.T.10</th>
<th>N,PTX</th>
<th>A.T.10,PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.155</td>
<td>0.150</td>
<td>0.179</td>
<td>0.176</td>
</tr>
<tr>
<td>3</td>
<td>0.146</td>
<td>0.129</td>
<td>0.157</td>
<td>0.162</td>
</tr>
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<td>5</td>
<td>0.154</td>
<td>0.161</td>
<td>0.179</td>
<td>0.150</td>
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<tr>
<td>7</td>
<td>0.168</td>
<td>0.176</td>
<td>0.188</td>
<td>0.190</td>
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<td>9</td>
<td>0.176</td>
<td>0.170</td>
<td>0.205</td>
<td>0.183</td>
</tr>
<tr>
<td>11</td>
<td>0.160</td>
<td>0.171</td>
<td>0.183</td>
<td>0.125</td>
</tr>
<tr>
<td>13</td>
<td>0.145</td>
<td>------</td>
<td>0.184</td>
<td>0.179</td>
</tr>
</tbody>
</table>

Means: 0.158±.001 0.160±.004 0.182±.012 0.166±.009

Note: The means are given with Standard Deviation of the Mean ($\sqrt{\frac{\sum(x^2)}{n}}$).
significant difference between any of the experimental
groups. There was also no indication of a significant
change in the potassium content of the lavage of any
animal throughout the day.

Effect of A.T.10 on Lavage Sodium Values

In the same experiment the sodium values of the
lavage fluid for the four rats, representing the four
experimental groups, were also determined. The average
value for the four was 3.2 mgs. of sodium per milliliter
of lavage fluid (see Table IV). When each animal was
compared to the other three as above, a significant
difference was found between the mean of the control and
that of the other three. There was also a statistical
difference between the N,PTX animal and the other three.
However, these differences do not reflect any effect of
A.T.10 on the animals, for they both represent the
animals which had not received A.T.10 and their means
represent the extremes of the values for the four
animals.

Phosphate Determinations

The phosphate (as phosphorus) determinations
which were made gave such inconsistent and widely
varying results that they have been of no value in
examining these relationships between experimental
groups and so have not been included.
### TABLE IV

**Sodium (in mEq/mL) Removed by Peritoneal Lavage**

<table>
<thead>
<tr>
<th>Lavage Period</th>
<th>Control</th>
<th>A.T.10</th>
<th>N,PTX</th>
<th>A.T.10,PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.23</td>
<td>3.18</td>
<td>3.50</td>
<td>3.21</td>
</tr>
<tr>
<td>3</td>
<td>3.15</td>
<td>2.63</td>
<td>3.33</td>
<td>3.26</td>
</tr>
<tr>
<td>5</td>
<td>2.88</td>
<td>3.25</td>
<td>3.38</td>
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<td>3.25</td>
<td>3.15</td>
<td>3.50</td>
<td>3.12</td>
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<td>3.49</td>
<td>3.60</td>
<td>3.44</td>
</tr>
<tr>
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<td>3.12</td>
<td>3.49</td>
<td>3.41</td>
<td>3.50</td>
</tr>
<tr>
<td>13</td>
<td>2.75</td>
<td>----</td>
<td>3.48</td>
<td>3.38</td>
</tr>
</tbody>
</table>

Mean 3.11±.02 3.20±.11 3.43±.06 3.30±.08

Note: The means are given with Standard Deviation of the Mean (SDM = \(\sqrt{\frac{\sum d^2}{n(n-1)}}\)).
Effect of A.T.10 on Small Rats

A series of experiments was performed on rats weighing 185-205 gms. This was done because, at the time, heavier rats were not available. When the first experiment had been analyzed the results differed greatly from the ones of previous experiments (using heavier rats). To find the discrepancies the experiment was repeated several times with small rats and the results tabulated (see Table V). These experiments were carried out exactly as were the basic ones. The results, however, did not show the uniformity of the results with the larger rats, nor did they show the same relationships between the experimental groups as found in the larger rats.

A comparison of the control group of each weight range shows a difference in the behavior of the two. In the heavy animals there is the tendency for the lavage fluid calcium values to increase throughout the day. In the lighter animals this trend is reversed and there is a trend toward a decrease in calcium values through the day. While the calcium values for the large A.T.10 group remained nearly stable throughout the day, the calcium levels of the small A.T.10 group again showed the tendency to decrease.

The calcium level of the lavage fluid dropped to 22 μgms./ml. in the N,PTX group of larger animals
### TABLE V

**Effect of Age of Rat on the Calcium Removed by Peritoneal Lavage**

<table>
<thead>
<tr>
<th>Lavage Period</th>
<th>Control</th>
<th>A-T.10</th>
<th>N.PTX</th>
<th>A-T.10,PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27±2 (7)</td>
<td>30±2 (8)</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>33±1 (8)</td>
<td>37±2 (8)</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>3</td>
<td>33±2 (8)</td>
<td>35±2 (8)</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>4</td>
<td>33±2 (8)</td>
<td>31±2 (8)</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>5</td>
<td>30±3 (4)</td>
<td>31±1 (4)</td>
<td>34±2 (4)</td>
<td>34±3 (4)</td>
</tr>
<tr>
<td>6</td>
<td>29±4 (4)</td>
<td>27±2 (4)</td>
<td>28±3 (4)</td>
<td>30±2 (4)</td>
</tr>
<tr>
<td>7</td>
<td>31±5 (4)</td>
<td>29±3 (4)</td>
<td>22±1 (4)</td>
<td>26±3 (4)</td>
</tr>
<tr>
<td>8</td>
<td>27(25-30)* (3)</td>
<td>30(26-36)* (3)</td>
<td>22(19-26)* (3)</td>
<td>28±2 (4)</td>
</tr>
<tr>
<td>9</td>
<td>28±4 (4)</td>
<td>32(29-34)* (2)</td>
<td>26(21-31)* (3)</td>
<td>23(15-28)* (3)</td>
</tr>
<tr>
<td>10</td>
<td>27(20-34)* (2)</td>
<td>28(17-39)* (2)</td>
<td>23(19-31)* (3)</td>
<td>24(20-29)* (3)</td>
</tr>
</tbody>
</table>

**Notes:**
1) All values given with Standard Deviation of Mean except those marked (*) which represents the range. 2) Numbers in parentheses refer to number of animals in each group. 3) Weight range 185-205 gms. 4) 30 minute lavages. 5) in μg/mg. Ca/ml.
and this was also the case in the comparable group of small animals. However, the small animals did not maintain this low level and showed an increase in calcium levels. The lavage fluid calcium levels for the A.T.10,PTX group of small animals showed an instability not noted in the larger comparable group.

If the calcium values and their standard deviations or ranges are plotted, it will be seen that there is no significant difference between the curves of any of the four groups except in a few individual instances.

The variations may be due, in part, to the fast growth rate of the smaller rats. There is a more rapid turnover of bone calcium in the younger rats due to reorganization of bones under the stimulus of increasing muscle mass and body weight. Thus a greater amount of calcium might be in a state readily available for the serum needs.

In other experiments with small rats in this laboratory lavage periods of one hour have demonstrated significant differences between the control and the N,PTX groups.

The problem of differences in calcium in relation to size variation is being followed up in this laboratory, as is the problem concerning the ability of the bones to supply calcium to the serum in the parathyroidectomized rats.
Effect of A.T.10 on Concentrations of Osteoclasts

When the lavage was completed the femurs of the rats were prepared as noted earlier and examined for osteoclasts. An attempt was made to determine if there were differences between the experimental groups on the basis of the numbers of osteoclasts per area of bone. The general distribution of the osteoclasts throughout the area examined was the same for each of the experimental groups even though the numbers varied (see Plate II).

The results of the osteoclast counts are given in Table VI. The various experimental groups are listed in addition to several animals which represent different physiological conditions and are thus added in the nature of controls for these counts.

The two normal animals which were sacrificed had the least number of osteoclasts per area of bone of any animal examined. The two animals each had only 1.8 osteoclasts per field.

It was found that the animal which had been parathyroidectomized for one week had approximately the same number of osteoclasts per field as did the normal.

The work of Elliott and Freeman (1956) has shown that nephrectomy causes a transient increase in the serum calcium levels. This may explain the increase in the number of osteoclasts found in the animal which had been nephrectomized for 24 hours but had not been lavaged.
TABLE VI

Effect of Continuous Peritoneal Lavage on Numbers of Osteoclasts in the Long Bones of Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Osteoclasts/Field*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal animal</td>
<td>1.8 (1.8-1.8) (2)</td>
</tr>
<tr>
<td>PTX for 1 week</td>
<td>2.0 (--------) (1)</td>
</tr>
<tr>
<td>Nephrectomized for 24 hours, no lavage</td>
<td>4.7 (--------) (1)</td>
</tr>
<tr>
<td>Nephrectomized control with lavage</td>
<td>11.5 (10.4-12.5) (3)</td>
</tr>
<tr>
<td>A.T.10, lavaged</td>
<td>10.5 (8.7-11.6) (3)</td>
</tr>
<tr>
<td>N,PTX, lavaged</td>
<td>3.9 (3.3-4.5) (3)</td>
</tr>
<tr>
<td>A.T.10, PTX, lavaged</td>
<td>12.4 (10.7-13.3) (4)</td>
</tr>
</tbody>
</table>

*Spencer binocular microscope with 43x objective and matched 6x oculars used.

Notes: 1) Ranges are given after number. 2) The last column on the right represents number of animals in each group.
However, recent work from this laboratory suggests that this increase may be due to the calcium-free diet on which the animals are maintained prior to nephrectomy. More work must be done to clarify this situation.

In contrast to the last mentioned animal, it was found that in the nephrectomized rats subjected to lavage the average number of osteoclasts per field was 11.5. The A.T.10 group had approximately the same number of osteoclasts per field. The N,PTX group had about the same number of osteoclasts as did the nephrectomized animal which was not lavaged. In the A.T.10,PTX group the number of osteoclasts per field was in the same range as for those with their parathyroids.

The figures presented here represent only a small area of the bone. In order to get a truly quantitative count of the osteoclasts, serial sections would have to be made and examined. However, the areas counted did show a fairly consistent count for any one group and it is upon this consistency that the results depend.
DISCUSSION
DISCUSSION

The objective of this work was to determine whether or not A.T.10 had an effect on bone. In the work presented, any effect of A.T.10 on calcium absorption from the gut was eliminated by the calcium-free diet. The possibility of the A.T.10 exerting any effect on the animal through the kidneys was dismissed because of its administration after nephrectomy. By ruling out the effect of the other two sites of action of A.T.10 any experimental changes would of necessity be of extra-renal origin and would indicate a direct effect on bone. Therefore, the fact that the lavage fluid calcium levels of the A.T.10 group were statistically higher than those of the control makes valid the conclusion that the A.T.10 is able to mobilize calcium from the bones.

The work which has been presented in this thesis does not, in itself, rule out a renal action in addition to this extra-renal action. However, it would seem from evidence presented in this and other papers that the primary site of action of A.T.10 in the normal animal is the bone rather than the kidney. In support of this hypothesis is the work of Talmage and Dodds (1955). They showed that, although there were increases in phosphate and calcium excretion following A.T.10 administration,
there were, preceding these changes, increases in serum levels for these ions. This would certainly indicate a primary extra-renal effect.

Having accepted the idea of an extra-renal action of A.T.10 separate also from an effect on intestinal absorption, the next aim of these studies was an attempt to gain some insight into the actual mechanism through which the drug produced its action on bone. Before discussing its effect on bone, the possibility that A.T.10 produces its effect by stimulation of the parathyroids must be considered. If the lavage values of only the A.T.10 treated animals and their controls were compared, one might argue that the effect of A.T.10 was to activate the gland to increased production of hormone and thereby increase the serum and extracellular calcium level. Indeed, the decrease in the difference between the controls and the animals treated with A.T.10 as the lavage progressed might be explained by a "using up" of the A.T.10 since this was of exogenous origin and not increased on stress. However, the ability of A.T.10 to produce an effect in the parathyroidectomized animals does not substantiate this view.

Parathyroidectomy is normally followed by a drop in the calcium values of the lavage to a level approximately half that of the controls. This reduced level, however, remains fairly constant thereafter. This pattern was
noted in these experiments. However, if the animal was pretreated with A.T.10 prior to parathyroidectomy, the drop in calcium values was significantly less, and after about two hours tended to rise toward the control level. This indicates that the A.T.10 is affecting some mechanism for the mobilization of calcium from bone, exclusive of the parathyroids.

It appears that dihydrotachysterol (and possibly vitamin D) and the parathyroid hormone work on different, but closely related, mechanisms. This is borne out by the drop in calcium level in the animals pretreated with A.T.10 and parathyroidectomized. If A.T.10 were working on the same mechanism as the hormone, the parathyroids should be practically non-functional during A.T.10 administration and therefore parathyroidectomy should elicit no calcium drop. The relationship between the lavage calcium levels and osteoclast concentrations of the control group and between the calcium levels and osteoclast concentrations of the parathyroidectomized group pretreated with A.T.10 is similar. This points to a similarity between exogenous A.T.10 and endogenous parathyroid hormone.

The specific mode of action of either the parathyroid hormone or A.T.10 (and the vitamin D "family" in general) presents a problem not easily resolved. At the present time there is not sufficient evidence to
distinguish the sites of the two substances. However, a theory has been advanced which relates the action of both the hormone and the vitamin to the citric acid cycle. Neuman (personal communication) proposes that both work as partial blocks on different parts of the citric acid cycle and by their blockage cause the accumulation of citric acid. The citric acid in turn causes the mobilization of calcium from bone. Evidence for this theory is circumstantial and more concrete results will have to be noted in order to verify this proposal.

In regard to the place of action in the bone, it is the opinion of this author that the osteoblasts and osteoclasts are the sites of dissolution of bone. It has been established by Kroon (1954) that the osteoclast is capable of resorbing bone. Heller et al (1950) reported that osteoblasts become osteoclasts and that the change is reversible. In the light of these two sets of findings and considering the ubiquity of the two cell types it is believed by this author that the actual resorption of bone takes place through these two cell types. It would seem that the stimulus to greater dissolution of bone could also be the stimulus for a cytoplasmic response resulting in the fusion of the osteoblasts into osteoclasts.

The question of the action of either the hormone or vitamin D is far from being fully answered. The most promising avenue of research at the present time seems
to be in the study of the connection of citric acid to the process of bone dissolution and calcium mobilization. It is hoped that continued study of the osteoclasts may reveal something more of the process of resorption.
SUMMARY
SUMMARY

Nephrectomized male rats maintained on a calcium-free diet and pretreated with dihydrotachysterol (A.T.10) were subjected to continuous peritoneal lavage and the lavage fluid analyzed for calcium content. Half of the animals were parathyroidectomized (PTX) during the lavage. At the termination of the experiment the distal head of the femurs was fixed, sectioned, and examined for concentration of osteoclasts.

The A.T.10 treated animals had calcium levels significantly higher than the controls. The A.T.10 treated PTX animals exhibited a drop in calcium followed by a subsequent rise. The PTX control group maintained a constant low calcium level.

The osteoclast concentration of the A.T.10 treated animals, their controls, and the A.T.10 treated, PTX animals was at about the same high level. The PTX control group had a concentration of osteoclasts less than half that of the above groups.

The sites of action of the A.T.10 and the endogenous parathyroid hormone seem to be different, but closely related. A theory concerning the citric acid cycle as the possible site of action is described.
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