STUDIES ON NUTRITIONALLY INDUCED SOFT-TISSUE CALCIFICATION IN THE RAT.

by

G. E. TROUT.

Thesis submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg.

DECLARATION.

This is to certify that the work presented in this thesis is my own, and has not been presented previously for examination.

Signed ..G.E. Trout.....

Date .....7/4/62.....

ACKNOWLEDGEMENTS.

It is with pleasure that I express my sincere appreciation to Professor J. Gillman for initiating and encouraging the present study. Professor Gillman's interest and guidance are in evidence in every aspect of the work, but especially in the interpretation of the data and in the presentation of the thesis. I am deeply grateful for all he taught me.

I should also like to thank Dr. P. R. Levy for the generous way in which he gave his time and advice in criticizing the manuscript. His guidance is largely responsible for the present form of the thesis and I acknowledge it with gratitude.

Numerous people connected with the Joint University/C.S.I.R. Nutrition Research Unit were also of inestimable help at different stages of the work. In particular I should like to thank Dr. C. Gilbert, Miss. M. Tardrew and Mrs. R. Agulhas. The statistical examination of the data was carried out by Mr. W. Lutz of the Dept. of Mathematics and I take pleasure in recording my thanks to him.

It is also fitting that I take this opportunity to thank the C.S.I.R. for permitting me to pursue the study while employed as a Research Officer of the N.C.R.L. seconded to Professor Gillman's unit.

Finally, I should like to thank Professor H. B. Stein for his supervision subsequent to Professor Gillman's departure from the University of the Witwatersrand.

CONTENTS.

TABLES.

FIGURES.

INTRODUCTION.

The presence of vitamin D in the diet is essential for the normal calcification and growth of the bones. In doses greatly in excess of normal requirements the vitamin has the anomalous effect of promoting decalcification of the bones and pathological calcification of the soft tissues.

Several forms of vitamin D are known at the present time (Bills, 1954). The first to be isolated was calciferol, or vitamin D_2, from a solution of ergosterol irradiated by ultra-violet light (Windaus, 1931). Later workers prepared further forms, the best known being vitamin D_3, D_4, and D_5 (Schenck, 1937; Windaus and Trautmann, 1937). Study with the purified vitamins has shown that vitamin D_3 is, at least for chicks, more sensitive than D_2 (Remp and Marshall, 1938). In the present work pure vitamin D_2 - being available commercially - was used exclusively.

With the exception of fish-liver oil used prophylactically the normal requirements of vitamin D - estimated at 1,000 to 3,000 i.u./day (child) - are supplied in the diet mostly by eggs, milk and milk products (Daniel and Munsell, 1937). The dietary intake may also be augmented by the photosynthesis of vitamin D in the skin (Huldschinsky, 1919). This irradiation requires ultra-violet light (Hess et al., 1922), and is mostly of seasonal importance, especially in temperate areas. As a consequence of the relative difficulty in obtaining an adequate supply of vitamin D, especially amongst underpriveleged people, it is much more

common in clinical practice to find cases of hypovitaminosis D (rickets and osteoperosis) than hypervitaminosis D. However, experimental evidence from animal studies as well as from a limited number of clinical case histories (v.i.) in which high doses of vitamin D were administered therapeutically, has proved conclusively that excessive consumption of vitamin D can be harmful. In doses many times in excess of the physiological requirement, vitamin D produces extensive calcification of the soft tissues, an elevated serum calcium concentration and depletion of the calcium content of the bones.

The present work has been concerned with the nature of the soft tissue calcification produced by massive doses of vitamin D (100,000 i.u./kilo body weight). Many workers have studied the calcification so produced and have shown that many organs are susceptible to calcification (v.i.). It has been well established that the serum calcium level is elevated by large doses of vitamin D, but the relationship between this elevated serum calcium concentration and the change in the tissues has not been clarified.

It is probable that a major factor responsible for the paucity of information on the rate of change of tissue calcification has been the difficulty of the estimation of calcium in the minute samples of tissues available from small laboratory animals. The present study was made possible by the development of new methods of analysis for calcium applicable to small samples of tissue and serum from the rat

(v.i.). Thus it was possible to examine tissue samples from groups of rats all treated with vitamin D but killed at defined intervals during the course of the experiment. Several important advantages are to be gained by employing rats as opposed to dogs or rabbits. In the first place, a large number of genetically similar animals may be obtained with greater ease, and thereby, the biological variation in response to the vitamin D is reduced to a minimum. Secondly, the upkeep and cageing of rats rather than dogs or even rabbits presents a greatly reduced economic factor which is frequently of considerable importance. The experiment would therefore be difficult to conduct if older, less sensitive calcium methods were employed.

The study to be described, therefore, takes advantage of ultra-micro chemical techniques for the analysis of calcium and phosphorus to determine the rate of change of tissue mineralisation ingroups of rats during and after a course of treatment of 25,000 i.u. of vitamin D_2 per day for five days. It was also possible to follow the associated changes in serum calcium and inorganic phosphate so as to permit the relationship between serum and tissue to be determined. From such data it was possible to gain further information on the mechanism of soft tissue calcification.

SECTION I.

A review of the methods available for the determination of calcium in biological material.

Introduction.

Adaptations of chemical methods for the analysis of biological material frequently suffer from at least two major disadvantages. In the first place, the scale of operation often needs to be reduced to the micro or ultramicro range particularly in circumstances where the elements investigated occur only in trace quantities or where sampling cannot be undertaken without introducing some change in the organism. Secondly, complex chemical mixtures which characterise biological fluids and tissues frequently contain interfering compounds or elements which need to be identified and thereafter removed before a particular analytical method can be successfully applied. These two disadvantages are particularly evident in the quantitative analysis of the calcium content of the rat aorta, the tissue most closely examined during the present investigation.

As can be judged from the data to be presented later, an ultramicro chemical method for calcium is essential if it is appreciated that in the early stages of calcification the dry weight of the rat aorta does not exceed 20-30 milligrams and may contain only about 20 micrograms of alkaline earth metals. It is not unlikely that the paucity of information concerning the metabolism

of calcium in the aorta of the rat can be attributed, in part at least, to the lack of a micro method sufficiently reliable to estimate small amounts of calcium.

The estimation of calcium in the serum of the rat also presents a problem in so far as the maximum amount of blood that can be obtained by cardiac puncture from rats in particular experimental circumstances rarely exceeds 4-5 ml. yielding approximately 2 ml. of serum. Furthermore, the blood volume of rats apparently contracts at defined stages of vitamin D treatment and the amount of serum available does not permit the estimation of calcium by conventional methods. Moreover, in such instances where it was desirable to follow the fluctuations in the serum calcium of the same rat it was necessary to estimate calcium in small samples of blood not exceeding 0.2 - 0.3 ml. Removal of large samples of blood daily over a week would almost certainly have complicated the investigation. In view of these limiting factors it became necessary to select that technique for the estimation of calcium which could be suitably applied to the kind of problem in mind.

The following well established methods for calcium estimation were assessed by the present author :

1) calcium oxalate precipitation methods.

2) measurement of the emission spectrum of calcium.

3) colour reactions and methods depending upon insoluble complexes other than oxalate.

4) titration of the calcium ion with ethylene diamine tetra-acetic acid.

This scheme largely follows the historical development of methods used to estimate calcium.

Methods dependent upon the precipitation of calcium oxalate.

The author spent considerable time in trying to adapt the classical methods of calcium estimation based on oxalate precipitation to the needs of the present study (Halverson and Berguin, 1917; Kramer and Tisdell, 1921a and b; Clark, 1921; Shohl, 1922; Clark and Collip, 1925; Trevan and Bainbridge, 1926; Van Slyke and Sendroy, 1929; Fiske and Logan, 1931; Sendroy, 1944; Loureiro and Janz, 1944; Kirk, 1950; Kibrick et al., 1951). The minute quantities of calcium could not be estimated accurately by any of the standard methods (Smith et al., 1950; MacIntyre, 1955).

Alternative methods for the estimation of calcium.

Polarographic techniques (Cohn and Kolthoff, 1943; Breyer and McPhillips, 1953) appeared to be more sensitive than the classical methods but since the specialised apparatus was not available these could not be examined.

After most of the present work was complete the author examined the picrolonic acid ~~and~~ method for the estimation of calcium (Erdey and Jankovits, 1954). This involves the precipitation of calcium as calcium picrolonate and the oxidation of picrolonate

by means of potassium persulphate to the intensely coloured and very stable dye pyrazole blue. Although this method gave excellent results in the 100 to 500 microgram range it could not be adapted to the ultra-micro range required in the present study.

More success was achieved with the adaptation of fine mesh ion exchange resin chromatography to the flame photometric estimation of calcium but the method was developed too late for the present study.

The estimation of calcium by ethylenediamine - tetra-acetic acid (EDTA) titration.

The method of estimation of calcium finally employed in the present work depended upon the titration of calcium plus magnesium in a sample with EDTA using Eriochrome Black T as indicator.

The first practical development arising from studies of the EDTA reagent was a method for the determination of the calcium hardness of water (Schwarzenbach, Biederman and Bangerter, 1946). Application of the technique to calcium estimations in biological fluids rapidly followed. (e.g. Buckley et al., 1951; Greenblatt and Hartman, 1951; Fales, 1953; Eldjarn et al., 1955). Today the literature abounds with EDTA methods (q.v.) for calcium in biological samples, especially serum. Most however, are very similar and follow one of the three basic approaches discussed below :-

1) Direct titration of calcium at pH 12 with murexide as indicator. New indicators, superior to murexide, have been described (v.i.).

2) Direct titration of calcium plus magnesium at pH 10.0 - 11.2 with Eriochrome Black T as indicator. The calcium concentration can be computed after independent estimation of the magnesium.

3) The interfering ions can be removed from the solution and the calcium ions estimated by the method employed in (2). Such preliminary treatment eliminates the magnesium interference.

As titration with EDTA appeared to possess many advantages over alternative methods for calcium estimation, it was extensively investigated before selecting a method for the present study. The following discussion indicates the salient features of the three approaches outlined above and their application to biological material.

The original two indicators, murexide and Eriochrome Black T have been studied by Buckley et al. (loc. cit.) and precise conditions for titration with each were established. For murexide, maintenance of the pH at 12.4 was shown to be of crucial importance. Interference from zinc, copper, and ferrous ions on the titration was shown to be effectively eliminated by cyanide. No interference could be detected from the added cyanide, but the anions phosphate, citrate, carbonate and borate can cause considerable error. Buckley and co-workers considered that a more accurate result could

be obtained by using an experimentally determined ratio of EDTA to Ca^{++} rather than the one to one value previously quoted. Murexide, despite its wide use has a very indistinct end-point and fades rapidly (v.i.). Attempts to stabilise the indicator have apparently not been successful. In addition to the above disadvantages, phosphate interferance can be serious in biological samples (Buckly et al., 1951; Horner, 1955). Evidence suggests, however, that the interfering effect of phosphate ions on the titration only arise in excess of a critical level. Kenny and Toverud (1954) could find no significant interference in rat serum in which the P:Ca ratio was 8.09. Similar experiments with human serum having a P:Ca ratio of 3.78 also showed no interference. In larger quantities phosphates need to be removed and as classical analytical techniques cannot be employed because the reagents now introduce new kinds of interference, some procedure of the kind described by Horner (1955) is essential to remove excess phosphates. Alternatively, ion-exchange resins can be used as in the case of milk (Jenness, 1953).

The unsatisfactory nature of the murexide end-point is widely recognised (Buckley, et al., 1951) and numerous attempts have been made to overcome the serious practical difficulties which consequently arise (v.i.). Attention was therefore given to some of these modifications. Probably the most successful of such modifications is detection of the end-point photocolorimetrically. The technique devised by Fales (1953) for serum calcium appears to have

been the first in which the idea was successfully utilized. Without automatic equipment the method is however, very tedious. A simplification of the method has been described by Horner (1955), who also increased the scope of the method by introducing an elegant procedure for the removal of interfering phosphates. Unfortunately, the need for special expensive equipment to permit satisfactory routine use of the method, necessitated rejection of an apparently efficient technique.

Less elaborate modifications retaining the simplicity of the normal titration have been proposed with only moderate success. French workers (Gautier and Pignard, 1953) attempted to improve the end-point by adding an excess of EDTA to the calcium solution and back-titrating with a standard nickel solution. Trial experiments in this laboratory did not show sufficient improvement in the end-point to warrent further consideration of this method. Colorimetric methods with murexide - calcium solutions do not seem to be satisfactory (Buckley et al., 1951).

Recently, new calcium - specific indicators have re-opened consideration of the direct titration of calcium with EDTA (Patton and Reeder, 1956; Diehl and Ellingboe, 1956; Ashby and Roberts, 1957). The change point for "Calcein", the most successful of the new indicators, is best observed under U.V. light (Bett and Fraser, 1958). It appears (Tucker, 1956) that the new indicators, though better than murexide, still do not possess good

change points. General dissatisfaction with the direct titration of calcium has led many workers to take advantage of a less direct but much more satisfactory procedure.

Titration with EDTA at pH 10 - 11.2 quantitatively chelates the sum total of the calcium and magnesium, if any, present in the sample. The end-point can be detected accurately with the indicator Eriochrome Black T even in micro and ultramicro titration. Because of the extreme sensitivity of the method a modification of the above technique was selected as most suitable for the present investigation. Full use of the sensitivity of the method has been made by reducing the scale of the titration to ultramicro levels (Sobel and Hanok, 1951). The extreme micro nature of the technique is indicated by the fact that good titrations are easily obtainable with only 20 microlitres of serum, representing approximately 2 micrograms of calcium. The exact calcium concentration is deduced by subtraction of the magnesium level estimated by an alternative technique. A suitable procedure for serum magnesium has been described by Orange and Rhein (1951).

For the study of serum, the EDTA technique possesses considerable advantage in that the titration can be performed directly on the serum without the removal of the protein. The EDTA - calcium complex is so strong that no error due to competitive complex formation between the protein and calcium arises. It has been noted however, (Liefheit, 1956) that in solutions of high protein

content, the rate of reaction between calcium and EDTA is measurably decreased and for accurate results the end-point should therefore be approached slowly.

Haemolysis in a serum sample also presents a difficult end-point due both to the intense colour of the haemoglobin masking the change point, and an inhibitory effect of the liberated iron on the indicator. Liefheit (1956) studied the range 0 - 0.15% haemolysis on the titration - a range exceeding that found in the average serum sample - and showed that the maximum deviation from the control was only 0.06 mEq/l. (0.12 mg.%). The presence of ferrous and other divalent ions present in biological samples in trace amounts result in high values for the titration. Fortunately, traces of cyanide added to the solution prior to titration (v.s.) effectively remove these ions from the solution by complex formation. The magnitude of the possible error in serum (human) is indicated in the following table.

Table 1.

Trace elements in human serum.

* Zn^{++}	⁑ Cu^{++}	⁑ Fe^{++}
300 ± 160 µg.%	118.6 ± 1.2 µg.%	104.7 ± 3.4 µg.%

* Vallee and Gibson, 1948; ⁑ Cartwright et al., 1948.

In tissue ashes, iron can be particularly troublesome and can completely inhibit the indicator end-point. Removal of iron is therefore obligatory.

The main handicap to the use of Eriochrome Black T lies in the interference of magnesium which, not being easily removed, must be estimated separately. In the present study, estimations of serum magnesium were made, but the results of tissue analysis were uncorrected. It was considered that no great advantage from a physiological viewpoint would be gained from the added refinement of estimating magnesium in each sample.

Procedures have been described in which purification of the calcium has preceded EDTA titration, hence permitting the use of Eriochrome Black T without the simultaneous estimation of magnesium. At the time the present investigation was initiated the only available procedure depended upon the precipitation of calcium oxalate before titration (Harrison and Harrison, 1955; Carr and Frank, 1956). Because of the objections to oxalate precipitation no consideration was given to these methods. Nevertheless, separation of the calcium followed by titration with EDTA in the presence of Eriochrome Black T appears on a theoretical basis to be a very useful approach. Substance was given to the concept by use of ion-exchange chromatography (Griswold and Pace, 1956) whereby complete quantitative separation of calcium from magnesium as well as other interfering ions is readily achieved.

This technique became available when this investigation was already well launched.

Preparation of a solution containing all the calcium ions but freed from interfering organic matter.

Since Ca^{++}; Mg^{++} and PO_4^{---} are not volatile, interfering organic material may be oxidised by relatively violent methods. The residue may be leached and the three ions mentioned above recovered quantitatively.

Two methods have been used for the destruction of organic matter, namely, dry ashing (Stolte, 1911), where oxidation proceeds at high temperature in a muffle furnace, or, alternatively, by wet ashing with a mixture of strongly oxidising acids in hot solution (Neuman, 1902; Mariott and Howland, 1917). The dry-ashing method, if carefully performed, is efficient. The technique consists of heating for several hours a weighed amount of the dry material in a platinum crucible to a temperature of about 700 degrees centigrade in an electrically heated furnace. A good supply of air is necessary for speedy oxidation of carboniferous matter. Two disadvantages have been described (Clark, 1921). First, in the initial stages of heating, loss of material may result from the rapid evolution of gases. Gentle heating at this stage minimises this loss. The second, and more serious disadvantage, arises when the residue is difficult to dissolve completely. Digestion of the residue at 100 degrees centigrade with 6N. hydrochloric acid for one hour is recommended to ensure complete solution of the ash.

The wet-ashing procedure with perchloric, iodic, sulphuric and nitric acids used alone or in combination offers little advantage over the alternative dry combustion. Some saving of time is possible but this advantage is outweighed by two disadvantages. In the first place solution of the calcium in the glass is possible under the action of hot concentrated acid when the wet-ashing is conducted in glass vessels. Secondly, if the method of analysis which follows solution of the material is pH dependent (as is the EDTA titration), destruction of the excess acid remaining at the end of the combustion is troublesome especially if the volume is to be kept small as is frequently the case in micro-work. Accordingly, for the purposes of the present investigation dry-ashing was preferred to wet-ashing. Nevertheless, preliminary trials proved that comparable results could be obtained by either technique.

Estimation of serum calcium and extention of the method to tissue calcium and magnesium (Sobel and Hanok, 1951).

Reagents :- 0.45% disodium ethylenediamine tetra-acetic acid (EDTA).

0.17 M. ethanolamine buffer : 10.2 ml. purified ethanolamine* in 1 litre of calcium free water.

* One hundred ml. of ethanolamine were mixed with approximately 150 ml. of dry benzene and after shaking, the benzene was removed by distillation on a steam-bath. Water is removed by azeotrope formation. The dry ethanolamine was then re-distilled 'in vacuo' collecting only the fraction boiling at 70-72°C/14 mm. Hg.

For serum, the EDTA method has the advantage over some earlier procedures in that analysis can be conducted accurately without prior removal of protein. The calcium determinations were performed directly on the serum in the present work.

Estimation of total calcium and magnesium.

The serum or tissue solution (0.5 ml.) was mixed with an equal volume of 1% sodium cyanide solution in a small tube. An aliquot (20 to 50 microlitres) was transferred to a micro titration dish (made from perspex) containing 0.5 ml. of the buffered indicator solution. The solution in the pipette was rinsed out three times by drawing the solution in the dish in and out of the pipette. The dilution factor was so large that no significant error was introduced from the calcium remaining in the pipette.

By means of a Kirk type ultra-micro burette (35 microlitres capacity) mounted on a special one-piece titration table, the calcium ions were directly titrated with the EDTA solution. The titration table had provision for a rising and falling titration-dish platform and a vibrating reed stirrer. Titration was performed slowly until the indicator changed from red to blue, the exact tint being judged by means of a comparison sample. Replicate titrations were always performed. Simultaneous titrations were carried out on solutions of known calcium ion concentration. Results were calculated by simple proportion.

Estimation of serum magnesium (Orange and Rhein, 1951).

In the following study serum magnesium concentrations were determined by the method given below.

Reagents:- 10% trichloracetic acid.

0.1% polyvinyl alcohol.

0.0075% Titan Yellow (not more than three weeks old).

7.5% sodium hydroxide.

Standard magnesium solutions. (1-5 micrograms /ml. of Mg^{++}).

Procedure :- In a small centrifuge tube (3-4 ml.) 0.1 ml. of serum was diluted with 1.1 ml. of 10% trichloracetic acid and the proteins removed by centrifugation. One ml. of the supernatent liquid was taken for the next stage. Frequently, it was possible to take advantage of the similar first stage of the serum phosphate method (see below), and take 1 ml. of the deproteinised serum from that procedure thus saving both time and material. A small correction factor must be introduced in this case.

To the 1 ml. of solution, 1 ml. of polyvinyl alcohol solution and 1 ml. of titan yellow were added. The volume was then made up to 5 ml. by the addition of sodium hydroxide solution. Colours were read at 560 mμ after five minutes.

Calcium, when present in high concentrations causes interference and high results. The tissues examined by this method may, therefore,

suffer from this error. The estimation of tissue magnesium in all samples was abandoned largely because of this error.

The estimation of micro quantities of phosphorus.

Many variations of the molybdenum blue method for the estimation of phosphorus in biological material exist and the following procedures were selected for the present work.

Estimation of serum inorganic phosphate (Horwitt, 1952).

Reagents:- 15% trichloracetic acid.

10% trichloracetic acid.

1 % ammonium molybdate solution.

0.06% stannous chloride solution in conc. hydrochloric acid (freshly prepared).

Procedure :- A protein-free filtrate of the serum was first prepared by the addition of 0.4 ml. of serum to 3.5 ml. of water and 1.0 ml. of 15% trichloracetic acid in a centrifuge tube. After mixing, the solution was allowed to stand for ten minutes and then centrifuged. One-half ml. of the supernatent was then added to 5 ml. of 10% trichloracetic acid in a second tube and mixed. To this solution 0.5 ml. of 1% ammonium molybdate solution was added and after 7 minutes the colour was developed by the addition of 0.5 ml. of the stannous chloride solution. Maximum colour intensity developed after 15 minutes and was measured at 660 mμ.

Micro-determination of tissue phosphorus.

Reagents :- 60% perchloric acid.

5% ammonium molybdate solution.

"Sulphonic acid" solution. (0.2% 1:2:4 amino naphthol sulphonic acid; 12% sodium metabisulphite; 2.4% : sodium sulphite).

Hydrogen peroxide (30 vol.)

Procedure :- Approximately 10 mg. of the dried tissue was weighed into a small flask (50 ml.) fitted with a condenser, and 1.2 ml. of 60% perchloric acid was added. Organic matter was destroyed by heating to 160-180°C (oil bath) with occasional shaking, and the final trace of brown colour removed by the addition of a drop of hydrogen peroxide. After decolourisation, excess peroxide was destroyed by further heating (at least 5 mins.) and 0.2 ml. perchloric acid added to replace that used in the oxidation. The contents of the flask were then transferred quantitatively to a volumetric flask (25 ml.). Ten ml. of the solution was then taken for each analysis and 0.7 ml. perchloric acid added so that the acid concentration in the aliquot was 1.2 ml. One ml. of ammonium molybdate solution was then added followed by 0.5 ml. "sulphonic acid" reagent. The blue colour which develops reaches a maximum intensity after five minutes and was read in a colorimeter at 700 mμ.

Reliability of methods.

The methods of analysis, described earlier, were checked each day with standard solutions so as to ensure maximum accuracy. Before

accepting a method for use in the investigations its accuracy and reproducibility were checked. In the well-established techniques such as those for serum phosphate and serum magnesium, the method was regarded as satisfactory when reproducible values for standard solutions were readily obtained and the Beer-Lambert relationship confirmed. When a method was modified to suit the conditions of our experiment additional tests were conducted to ensure reliability. Tables 2 and 3 indicate the results of such tests.

Material.

264 male albino rats, selected in such a way that their weights prior to treatment were within the range 210 ± 30 g., were employed in the study. In most cases the age of the animal was thereby restricted to between three and four months. Vitamin D in the form of crystalline calciferol (Glaxo) was dissolved in refined arachis oil and administered orally by means of a standardised dropper. The efficiency of the dropper was tested, and shown to be satisfactory, by weighing twenty separate drops of the vitamin enriched oil, (Table 4). The animals were maintained on the colony's stock diet containing 0.3% calcium and 0.3% phosphorus, and in addition received a daily ration of fresh vegetables and tap-water ad libitum.

The rats were divided into fifteen groups and given a course of treatment with vitamin D of 25,000 i.u. daily for five days. Groups were killed twenty-four hours after the first, second,

TABLE 2.

Variation in a number of replicate estimations of calcium plus magnesium in a sample of liver by EDTA. titration.

Number	Calcium (+Mg)
1	176 mg.%
2	166 "
3	166 "
4	174 "
5	164 "
6	170 "
Mean	167 "

TABLE 3.

Variation in a number of replicate estimations of total phosphorus in a series of samples from the same liver.

Number	Phosphorus.
1	1,571 mg.%
2	1,671 "
3	1,788 "
4	1,649 "
5	1,638 "
6	1,582 "
7	1,590 "
Mean	1,642 "

TABLE 4.

DROP NO.	WEIGHT OF EACH DROP mg.
1	23.8
2	23.1
3	24.3
4	24.3
5	25.2
6	24.4
7	20.0
8	26.1
9	21.9
10	21.6
11	22.0
12	22.0
13	21.8
14	21.8
15	21.0
16	25.3
17	23.0
18	23.1
19	22.6
20	21.8
Mean	22.9
Standard Deviation	± 1.6

Accuracy of Vitamin D dropper as tested by weighing 20 successive drops.

third, fourth, and fifth doses and then two days, three, five, seven, ten, seventeen, thirty-one, fifty, sixty-eight and one-hundred and four days after the last dose. All groups were starved over-night before being killed. A further group of animals was also included as controls.

In all, five experiments were conducted. Experiment 733(A) was a preliminary investigation using only one animal per group, and was immediately followed by Experiment 733(B) taking four animals per group. Two further experiments - 753 and 759 - with five animals per group were undertaken in order to establish that the essential changes were reproducible. Only the serum and the most reactive tissues - namely the aorta, lung and trachea - were considered in these later experiments. As the earlier experiments has demonstrated that the active period of change under our conditions occurs in the first three weeks, Experiment 759 was designed to cover only that period. In Experiment 753, the major interest was the change in serum calcium during the first five days and hence this experiment was of short duration, the necessary data being obtained over seven days. Some disagreement between the serum results of Experiment 733 and the later work appeared at this stage, and hence a further study (Experiment 777) with eight rats per group was undertaken lasting thirty-five days in which serum calcium determinations only were carried out.

In the first series of experiments, blood was taken by cardiac puncture under ether anaesthesia. Later, an improved method was devised by which the abdominal aorta was cannulated with a No. 18 spinal manometer needle and the blood collected directly into a centrifuge tube, usually without the aid of a syringe. The latter technique was cleaner and more reliable for taking a large amount of blood from the rat. Following exsanguination, the lungs, heart, kidneys, stomach, aorta and trachea were excised, washed with distilled water and the excess water removed with blotting paper. After determining the wet weights, the tissue was oven dried at approximately 60-70°C. The dry tissues were re-weighed and stored in a desiccator until required. Blood samples were allowed to clot and were then centrifuged. The serum was stored in the frozen state until analysed.

SECTION II.

The level of calcium in selected soft tissues of the rat before and after vitamin D treatment.

The purpose of the following section is to describe the magnitude of the mineralisation induced in selected soft tissues of the rat by a course of treatment with massive doses of vitamin D. For this purpose it is necessary to describe first the normal calcium* and phosphorus concentrations measured in various tissues and secondly the maximum calcification measured. Substantial agreement between four separate experiments, conducted under similar conditions as described in the experimental procedure, indicate the predictability of the trend of the mineralisation. For convenience, each tissue will be described under a separate heading.

---

* Throughout this thesis reference will constantly be made to calcium values which strictly should be described as calcium plus magnesium values. The approximation is justified by the result of several tissue magnesium determinations undertaken during the course of the experiment. The data showed that the contribution of the magnesium to the composite calcium plus magnesium value was small and hence, for the purposes of the ensuing discussion, could be ignored. It may be noted however, that an elevation of tissue magnesium did develop under vitamin D treatment. The magnitude of the magnesium component may be illustrated by values determined for the aorta and tabulated in table 39 (appendix). The magnesium

(a). Aorta.

Sixteen aortae, divided into two groups and analysed by identical methods, composed the control group of tissues. In six cases the amount of calcium in the aorta was so small that it could not be determined by the method used. The remaining ten determinations ranged in value between 132 mg.% and 600 mg.% with a mean of 237 mg.%. Rat 733/22 (Table 23) appears to have an abnormally high aortic calcium concentration at a level of 600 mg.% and this result tends to raise the mean considerably.

Phosphorus determinations in the same aortae result in 15 values in the range 135 mg.% to 595 mg.% phosphorus with an average of 340 mg.%. The ten results of experiment 733 tended to be higher than the values for the five rats of experiment 759. There appears, however, to be a normal distribution of values on either side of the mean.

The technical limitations in determining trace levels of calcium were again largely responsible for only five results being obtained of 13 rats receiving one dose of vitamin D (Table 24). Calcification did not appear in the aorta at this stage and the results fall between 125 mg.% and 536 mg.% with the arithmetic mean at 268 mg.% calcium.

---

content of this tissue rose from approximately 15 mg.% to 147 mg.% over the course of active calcification representing a ten-fold increase. The latter value of 147 mg.%, however, was associated with a calcium value of 5,748 mg.% and thus was less than 3% of the total at this stage of calcification.

Nine phosphorus determinations were performed 24 hours after the first dose of vitamin D and showed no change from the control values. The mean value was 300 mg.% in the range 205 mg.% to 400 mg.% P. Again, 24 hours after the second dose of vitamin D only 6 out of 13 aortae (Table 25) were satisfactorily analysed and these showed no significant change in either calcium or phosphorus content The mean of the results was 306 mg.% calcium and 342 mg.% phosphorus. However, by the time three doses of vitamin D had been administered, a change became apparent and of 9 determinations, 4 rats (Nos. 733/55, 733/56, 733/57 and 733/58 see Table 26) had concentrations of calcium in the aorta greater than 1,000 mg.%. The calcium content in the aortae of the remaining 5 rats remained within the normal limits.

With further doses of vitamin D and sufficient time to permit the reaction to proceed, extensive calcification of the aorta develops with a high percentage of samples showing values of several grams percent. Thus, 10 days after the fifth and final dose of vitamin D all 9 aortae available for analysis showed calcium values greater than 2,550 mg.% with a mean of 8,437 mg.%. Two rats in this group (Nos. 733/51 and 733/53 see Table 33) possessed levels of 29,170 mg.% and 13,500 mg.% calcium respectively. The remaining results were in the range of 2,550 mg.% to 8,460 mg.% calcium representing a ten to forty-fold increase over the control level.

In time, the level of the mean calcium value increased (Table 17) but this was regarded as the result of an increase in the number of

aortae displaying extensive calcification rather than a real increase in maximum values. Nonetheless, a wide range of calcium values was still found in latter groups. Thus of four aortae taken for analysis 50 days after the last administration of vitamin D the aortae of rats 733/42 and 733/44 contained 5,100 mg.% and 4,775 mg.% of calcium respectively and the calcium content of the aortae of rats 733/43 and 733/45 was 23,900 mg.% and 24,160 mg.%. In the later two rats the calcium content had risen four to five times higher than in the other two rats.

It is concluded that in the aorta the normal calcium (plus magnesium) content is in the region of 237 mg.% and under the action of vitamin D can be increased to 31,000 mg.%. In the same period the phosphorus content of the aorta increases from 340 mg.% to a maximum of 6,000 mg.%. The range of values, especially for calcium, in the aortae of similarly treated rats has been shown to be extremely wide.

(b). Trachea.

The trachea, selected for its high cartilage content, is unique in possessing calcium concentrations greatly in excess of any tissue other than bone. Thus 11 normal tracheae analysed for calcium showed an arithmetic mean value of 3,900 mg.% in the range 1,695 mg.% to 5,510 mg.% (Table 23). Phosphorus determinations on the same tissues showed a mean value 2,505 mg.% P and a range of between 1,200 mg.% and 3,440 mg.%. With progressive mineralisation, both the calcium and

phosphorus of the trachea increased, reaching a maximum mean value of 8,500 mg.% and 4,140 mg.% respectively (Table 19). The greatest amount of calcium - 10,200 mg.% - was determined in the trachea of rat 733/53 (Table 33) while the greatest amount of phosphorus - 5,480 mg.% - was found in rat 733/45.

It was therefore concluded that the high normal mean calcium and phosphorus values of 3,900 mg.% and 2,500 mg.% respectively in the rat trachea can be increased by the action of vitamin D up to a level of 10,000 mg.% calcium and 5,000 mg.% phosphorus. After which, the cartilage is apparently saturated and mineralisation ceases.

(c). Lung.

Eleven samples of normal rat lung were examined for the purpose of assessing the normal range of calcium in the lung. Values so obtained lay between 155 mg.% and 315 mg.% calcium with the arithmetic mean at 218 mg.% calcium. Phosphorus determinations on the same tissues ranged between 1,125 mg.% and 1,700 mg.% P. with a mean of 1,375 mg.% (Tables 18 and 23).

Study of the results presented fully in Tables 23 to 38 and in summary in Table 18 show that the greatest calcification of the lung was measured in the tissue sampled ten days after the fifth dose of vitamin D, the mean concentration of calcium at this time being 1,994 mg.%. Of the nine animals composing the group (Table 33) the maximum calcium concentration in the lung - 4,835 mg.% - was attained in rat 733/53 and the lowest - 345 mg.% - in rat 733/52.

This latter value was almost within the normal range indicating that the rat could not have been affected by vitamin D. The greatest individual calcium concentration in the lung throughout the experiment was for rat 753/44 (Table 31) and did not fall into the group showing maximum mean calcification but developed earlier at the fifth day following vitamin D treatment. It had the value of 5,423 mg.% calcium.

Phosphorus levels in the lung showed not only a smaller group to group change than did the calcium levels, but also a smaller inter-group scatter. The experimental treatment increased the mean phosphorus content of the lung only by about 40 to 50 percent; the maximum value being 1,961 mg.% P. Even if the maximum value in each group is compared the increase is only two-fold, the maximum individual value being 3,050 mg.% for rat 753/44 (Table 31).

(d). Heart.

The determination of calcium and phosphorus in the tissues of normal and vitamin D treated rats has shown that the heart mineralises to a small but measurable degree. Eleven control animals were studied to establish normal limits of the two elements in the heart tissue. The determinations indicated that a range for calcium between 145 mg.% and 290 mg.% (Tables 20 and 23) and a mean of 208 mg.% can be regarded as normal. Similarly, for phosphorus, the range lay between 975 mg.% and 1,295 mg.% P with a mean at 1,171 mg.%.

Throughout the entire experimental period the greatest concentration of calcium in the heart was 800 mg.% representing a four-fold increase over the average control value and was recorded for two different animals on different occasions (rat 733/6 and 733/53 - Tables 29 and 33). In the first animal - rat 733/6 - the 800 mg.% calcium was reached only 48 hours after the last dose of vitamin D, but the second higher value was recorded at the peak of heart calcification ten days after the last administration of vitamin D. The mean result at this time was 713 mg.% calcium and the range of values lay between 510 mg.% and 800 mg.%.

The phosphorus content of the heart was noteworthy for its stability in the face of vitamin D treatment and extensive soft tissue calcification. No significant change was recorded in the heart phosphorus throughout the experiment. Thus the mean value of 1,129 mg.% P at maximum calcification compares with the normal level of 1,171 mg.% as do also the ranges of 880 mg.% to 1,400 mg.% and 975 mg.% to 1,295 mg.% P.

The conclusion from the data cited was that the normal heart calcium content of 208 mg.% on an average can be increased to about 800 mg.% by the action of vitamin D without significant change in the total tissue phosphorus content.

(e) Kidney.

The kidney's vital participation in electrolyte metabolism and the frequency with which earlier investigators found kidney calcific-

ation after vitamin D treatment (Light, Miller and Fray, 1929) suggested that the kidney would be of great interest in the present experiment.

The calcium content of the normal kidney was estimated from the analyses of samples from ten normal rats similar to those employed for the remainder of the experiment. A range of values from 150 mg.% to 304 mg.% with a mean of 212 mg.% calcium was obtained. With increasing doses of vitamin D two changes were observed: the first was a measure of visible damage to the kidney and the second, an increase in the calcium content as determined chemically. The visible kidney change appeared early as paleness and mottling of the surface - signs synonymous with damage. It has been claimed (Taylor et. al., 1931) that an increase in the non-protein nitrogen follows vitamin D treatment.

Analysis of the calcium content of the kidney at defined stages after vitamin D therapy confirms that the vitamin readily promotes calcification in this organ. The maximum mean calcium value under our conditions was 1,041 mg.%, ten days after the final dose of vitamin D. (Table 21). This represents a five-fold increase over normal. The range of values from which the above mean was calculated was from 470 mg.% to 1,915 mg.% calcium. The maximum value of 1,915 mg.% calcium (rat 733/10) in the ten days after vitamin D group (Table 33) was the highest individual value throughout the experiment. At the lower limit of the range at maximum calcific-

ation it can be seen that rats 733/52 and 733/54 had kidney calcium values of 470 mg.% and 500 mg.% respectively which, in comparison with the upper limit for the normal kidney i.e. 304 mg.% shows the weak response in certain individuals. Such variations are typical of the variability observed throughout this study.

The normal mean level of kidney phosphorus was established as 1,357 mg.% in the range 1,125 mg.% to 1,530 mg.% dry weight.(Table 21). It is possible that the high phosphorus levels recorded for the kidney are due in part to small amounts of urine - rich in phosphate ions - being trapped in the tubules at the time of death. An examination of Table 21 indicates that throughout the experiment there was no significant change in the mean phosphorus level despite the occasional individual value showing a slight elevation. This is exemplified by rat 733/10 and rat 733/11 having a phosphorus content of 2,080 mg.% and 1,870 mg.% respectively (Tables 33 and 34).

The evidence therefore permits the conclusion that the normal mean calcium and phosphorus content of the kidney is 212 mg.% and 1,357 mg.%. On treatment with massive doses of vitamin D the calcium content of the kidney increases to mean values of the order of 1,000 mg.% Phosphorus values show no significant change except in the occasional individual. Even in such cases the percentage increase was very small compared with the corresponding calcium changes.

(f). Stomach.

The influence of pH change on the precipitation of calcium phosphate was regarded by early workers as of major importance in the calcification mechanism (Askanazy 1901; Hofmeister 1910; Wells 1925). Consequently, the stomach (with the great changes in pH arising on either side of the gastric mucosa) has been examined for metastatic calcification following treatment with massive doses of vitamin D. In the dog Taylor et al., (1931) have shown that considerable calcification in the stomach can develop in hypervitaminosis D. Further study using the laboratory rat therefore seemed to be worthwhile, and hence justify the inclusion of the stomach amongst the tissues selected for the present study.

In common with many other tissues the rat stomach showed calcification under the influence of vitamin D and the data relating to this tissue have been summarised in Table 22.

The normal range for the calcium content of the stomach, as determined from six samples, lies between 140 mg.% and 192 mg.% with a mean of 164 mg.%. Phosphorus values in the same sample average 831 mg.% between limits of 635 mg.% and 1,019 mg.% (Table 23).

As was the case with the heart and kidney, calcification of the stomach reached a maximum value ten days after the last dose of vitamin D and again, in common with other tissues, each group was characterised by a wide range of individual values suggesting that an additional factor or factors other than vitamin D dosage influence

the development of calcification. Thus of the four rats analysed at the peak of calcification, animals 733/52 and 733/54 showed calcium concentrations in the stomach of only 320 mg.% and 440 mg.% respectively whereas rats 733/51 and 733/53 calcified to levels of 1,045 mg.% and 1,440 mg.% respectively (Table 33).

Some change in tissue phosphorus developed with vitamin D treatment but the significance is doubtful. At most, the increase is approximately 50% in mean values. A mean maximum value of 1,194 mg.% phosphorus was recorded ten days after the last dose of vitamin D.

It was concluded therefore that the rat stomach readily calcifies under the influence of vitamin D and rises from a normal mean value of 164 mg.% calcium to a mean maximum in the region of 811 mg.% representing a three to four-fold increase. Calcification proceeds almost without change in the phosphorus content of the stomach.

Some factors responsible for individual differences in calcification.

Study of the calcification in various tissues has directed attention to the wide variation in response in selected rats treated in the same manner with vitamin D. The question arises whether or not a rat displaying high calcification in one tissue, say aorta, necessarily develops a high degree of calcification in a second tissue such as the lung, or in fact, all soft tissues. The answer to such a question is of importance in deciding whether or not the degree of calcification in a tissue is a function of that tissue only or a function of the rat as an individual. That is to say, is soft tissue calcification dependent upon a local tissue factor or an interplay of the many biological variables determining the constitution of the individual?

Some insight into the problem can be gained from a consideration of individual rats employed in the experiment. Examination will first be made of rats killed ten days after the final dose of vitamin D when many tissues show maximal calcification. (Table 33).

The aorta of rat 733/51 has a calcium content of 29,170 mg.% dry weight, well above the group average. In the same group rat 733/52 has a calcium content in the aorta of 3,135 mg.%, almost minimal for the group (Table 5).

Comparison of tissue calcium content in rats 733/51 and 733/52.

Table 5.

No.	Aorta	Lung	Heart	Kidney	Trachea
733/51	29,170 mg.%	2,505 mg.%	795 mg.%	1,010 mg.%	8,570 mg.%
733/52	3,135 mg.%	1,025 mg.%	510 mg.%	470 mg.%	9,010 mg.%

Comparing the calcium values determined for other tissues in the same rats, lung samples showed a value of 2,505 mg.% for rat 733/51 and 1,025 mg.% for rat 733/52: kidney values were 1,010 mg. % for rat 733/51 and 470 mg.% for rat 733/52. Hence it can be seen that in these two cases the highest calcium value always appears in rat 733/51 suggesting that maximum calcification tends to be a function of the individual. On the other hand, two animals compared 17 days after the final dose of vitamin D show the reverse situation. Thus, rat 733/11 has a relatively low aorta calcium of 2,590 mg.% but has a high lung calcium of 3,720 mg.% in contrast to rat 733/87 of the same group in which the calcium content of the aorta was 29,450 mg.% and the lung calcium 460 mg.%. A smaller but essentially similar disproportionation was observed in the kidney and heart of the same rats (see Table 6). Further, rat 733/88 from the same group, when compared with rat 733/87 has a lower aorta calcium of 13, 920 mg.% but a higher lung calcium of 975 mg.%. In the other tissues from this pair of animals the order of maximum calcification was, in the heart, the reverse of that in the aorta, and in the kidney, the same as in the aorta.

Comparison of tissue calcium contents in Rats 733/11, 733/87, and 733/88.

Table 6.

No.	Aorta	Lung	Heart	Kidney	Trachea
733/11	2,590 mg%	3,720 mg%	450 mg%	1,300 mg%	7,160 mg%
733/87	29,450 mg%	460 mg%	335 mg%	775 mg%	6,460 mg%
733/88	13,920 mg%	965 mg%	460 mg%	560 mg%	5,540 mg%

Other examples are available from the many animals studied. One pair worthy of note are rats 733/42 and 733/43 (Table 36) with calcium values for the aortae of 5,100 mg.% and 23,900 mg.%. In this pair the trachea calcium content in rat 733/42 was 10,070 mg.% and in rat 733/43 was 7,615 mg.%, that is in reverse order from that in the aortae. The calcium of the lungs in the same pair was 590 mg.% in rat 733/42 and 955 mg.% in rat 733/43, that is in the same order as in the aortae. At the same time, however, the calcium of the kidney and stomach were identical within the limits of the experiment.

The weight of evidence suggests that although a general high sensitivity in one rat may express itself in many tissues, such a general effect appears largely fortuitous and need not, indeed often does not arise. Nevertheless, some factor or factors govern first, the extent to which an animal will react to vitamin D and secondly, which tissue will respond most readily to the vitamin. There is much

evidence to suggest that the second factor is governed by a local tissue peculiarity to be considered at a later stage, However, attention can now be drawn to several general aspects of the first factor namely the general variation in the individual response to vitamin D.

The secretions of the endocrine glands are of prime importance in determining metabolic individuality. From data already at hand, it is known that soft tissue calcification can be modified by hormonal treatment and hence it is probable that the degree of calcification in an individual rat after vitamin D treatment is largely a function of the prevailing endocrine pattern. The hormones known to be of importance in soft tissue calcification comprise thyroxin (v.i.), parathormone (v.i.), DOCA (v.i.) and cortisone (v.i.) and it is suspected that the sex hormones (v.i.) may also have a modifying effect on calcium metabolism.

Thyroxin : The thyroid has long been known to have an important role in the metabolism of calcium, especially in growth and bone formation. Thus the absence of the thyroid hormones, as in cretinism, causes marked retardation of growth at the epiphysial cartilage (Wilkins, 1941). Similarly, thyroidectomy in experimental animals results in a slowing of the rate of bone growth (Salmon, 1938). In contrast, hyperthyroidism may result in osteoporosia or osteitis fibrosa generalisata (Hunter, 1930, Aub, Bauer, Heath and Ropes (1929) showed that the thyroid possesses a marked action on calcium excretion;

considerable calcium losses arising in cases of hyperthyroidism. Relatively little is known of the effect of the thyroid on soft tissue calcification for although some experiments have been performed to assess the importance of such a relationship, the results are conflicting. Steck, Deutsch, Reed and Struck (1937) suggested that a hypothyroidic state may condition the severity of the tissue change following the administration of excessive doses of vitamin D. In the same year, Handovsky and Goormaghtigh (1937) concluded that the absence of the thyroid in dogs resulted in much more severe injury to the aorta and large vessels after treatment with large doses of vitamin D. At variance is the work of Schaal (1934) and Gillman and Gilbert (1956) who recorded a marked intensification of the vitamin D lesions in rats simultaneously treated with thyroxin. No quantitive data is as yet available on the degree of change caused by thyroxin treatment in animals suffering from hypervitaminosis D. Such experiments may well clarify the position.

Cortical Hormones: ACTH has been shown to retard osteogenisis in the normal rat (Becks et. al. 1944). Inhibition of calcium resorption from the intestine and a reduction of hypercalcaemia, when present, can be brought about by treatment with cortisone (Anderson et al., 1954). From their results, Anderson and Dent considered cortisone to be a vitamin D antagonist. Clinically, cortisone has been used to reduce hypercalcaemia and the destruction of bone tissue (Fanconi 1956). Pincus et al., (1951) however found

little change in serum calcium levels in normal animals treated with cortisone, but agreed that the tendency was to reduce the serum calcium in those cases in which a change was observed. In nephrectomised animals however, cortisone caused a rapid elevation of the serum calcium to toxic levels.

In soft tissue calcification induced by massive doses of vitamin D, cortisone has been shown to possess considerable protective powers (Gillman and Gilbert 1956) in keeping with its properties of depression of calcification and enhancement of calcium excretion.

Other cortical hormones have also been shown to influence soft tissue calcification. Thus Gillman and Gilbert (1956) demonstrated an intensifying effect of DOCA on vitamin-D-induced soft tissue calcification. Lostroh (1958) has shown that the daily administration of hydrocortisone to female mice caused calcification of the myocardium. Male mice were shown to be less susceptible to this hormone and the simultaneous administration of testosterone to female mice treated with hydrocortisone affords a considerable measure of protection.

Sex hormones: A measure of influence on calcium metabolism by the various sex hormones has long been suspected from such phenomena as the changes in serum calcium levels in the bird during the laying period which is under the control of the sex hormone. It has been shown, for example, that during laying, the serum calcium of the hen rises to values of between 16.3 mg.% and 25.8 mg.% as compared with a

normal value in the range 11.8 mg.% to 13.0 mg.% (Laskowsky, 1933). In a similar vein, Kyes and Potter (1934) found that in the pigeon during the egg laying period, an excess of spongy bone developed in the marrow cavity of the long bones. When calcium was required for shell formation this spongy bone was resorbed. The same effect could be induced in male pigeons and in both male and female mice by oestrogen therapy (Pfeiffer and Gardner, 1938; Gardner and Pfeiffer, 1937).

In man, both oestrogen and androgens cause calcium retention, and defective bone formation and osteoporosis follow primary gonadal insufficiency in both sexes (Albright et al., 1940; Cooke, 1955). In calcium balance studies in elderly people however, it has been claimed that only testosterone causes clear-cut retention of calcium (Toro et al., 1958). The administration of Ca-45 to normal male rats together with oestrogen has however, been shown to result in an elevation in the serum calcium (Manunta, Saroff and Turner, 1957).

Despite the importance of the sex hormones, nothing is known of their function in soft-tissue calcification induced by vitamin D. Experiments were therefore carried out in our laboratories to clarify the situation. Gonadectomised female rats maintained on either testosterone, progesterone or oestradiol benzoate were subjected to a standard treatment of 25,000 i.u. of vitamin D daily for five days and the animals were killed ten days after the final dose. Unfortunately, no significant modification of the soft tissue

calcification pattern could be distinguished nor was there any narrowing of the range of calcification commonly found in rats treated similarly with vitamin D. It was concluded that the function of the sex hormones, if important in vitamin D-induced soft tissue calcification, must be either very subtle or else involve one of the hormones not examined.

Parathormone: While the foregoing discussion indicates the role of several hormones in calcium metabolism, by far the most important hormone modifying the distribution of calcium in the body is parathormone. Three organs of the body namely the bones, kidneys and the intestines are now known to be affected by parathormone. The importance of parathormone for absorption of calcium from the gut has only recently been proved by radiochemical techniques (Talmage and Elliot 1958). The effects of parthormone however, can invariably be detected in the serum for the hormone causes an elevation of the serum calcium as well as changes in other blood constituents. Indeed, it has been postulated by many workers that an inverse relationship exists between blood calcium levels and the size of the parathyroids (eg. Higgins and Sheard 1928; Stoerk and Carnes 1945).

The essential feature for the present discussion however, is the extensive soft tissue calcification following the treatment of normal subjects with the hormone or arising spontaneously in hyperparathyroidism. In such subjects the extensive soft tissue calcification closely resembles the calcification induced by vitamin D. So close

is this resemblance that vitamin D has been considered (Taylor, Wells and Sykes 1934) to act via the parathyroid glands. (The latter authors tabulated eleven points of similarity between the two effects). It is true that vitamin D provides a satisfactory therapy in hypoparathyroidism elevating the hypocalcaemia to normal levels and preventing the tetany developing in extreme cases of hypoparathyroid function. However, as parathyroidectomy does not prevent vitamin D intoxication (Dale, Marbel and Marks 1932) the effects of vitamin D evidently are not mediated by these glands. (see also Collip, Pugsley, Selye and Thompson 1934).

Much evidence is now at hand to support the view that parathormone acts on the kidney and on the bones. A direct action of the hormone on the kidney was apparently first demonstrated by Greenwald and Gross (1925 and 1926) who showed that it caused an increase in the rate of phosphate excretion. Albright and Reifenstein believed that the primary action of parathyroid hormone was an electrolyte balance and renal function. While the importance of the kidney effect is still acknowledged, the view is steadily being accepted that parathormone may also affect the bones via citrate metabolism. It has been shown by Firschein and co-workers (1959) that in dogs treated with parathormone the citrate ion concentration is elevated 2 - 4 hours before hypercalcaemia supervenes. A cause and effect mechanism is believed to be operating. Vitamin D has also been shown to elevate the serum citrate level (Carlsson and Hollinger,1954)

but in this case no lag in the serum calcium level was demonstrated. It would appear that more information is necessary before it can be determined whether or not the relationship between parathormone and vitamin D lies in a common effect on citrate ions.

Whatever the mechanism involved in the action of parathormone, it suffices for the present discussion to state that parathormone will cause vitamin D - like calcification in the soft tissues. Consequently, the tempo of parathyroid activity could influence the soft tissue calcification produced by vitamin D in the individual rat.

From the foregoing discussion it is clear that the metabolism of calcium is dependent in considerable measure upon several endocrine factors. It is therefore suggested that part at least of the wide variations in the degree of the vitamin D - induced soft tissue calcification observed in selected rats is a reflection of the endocrine pattern of each individual animal.

SECTION III.

A comparison of calcification in the aorta, trachea, lung, kidney heart and stomach.

The data presented elsewhere (Tables 23 to 38) clearly indicate that under the action of vitamin D the aorta, trachea, lung, kidney, heart and stomach increase their calcium, magnesium and phosphorus content during the course of the experimental period. It has been shown that the degree and pattern of calcification in each tissue develops in a characteristic manner.

Data are presented in the following section that permit a quantitative comparison of the calcification process in rat tissues thereby enabling an assessment of the differential nature of the calcification to be ventured. Table 7 indicates the maximum degree of change developed in calcium and phosphorus content (mean values) for each tissue.

Comment.

(1). The differential degree of soft tissue calcification.

A study of the limited data quoted above indicates that within any group of rats the soft tissues do not calcify to the same extent. The explanation of this observation probably lies in the tissue itself, such that the factors necessary for binding mineral matter vary from tissue to tissue. Thus, as shown in Table 8, the tissues can be arranged in order of increasing sensitivity to vitamin D.

TABLE 7.

The maximum degree of change in calcium and phosphorus content of the soft tissues of the rat.

	AORTA		TRACHEA		LUNG		KIDNEY		HEART		STOMACH	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
$\overline{Ca}$ mg.%	237	20,560	3,900	8,500	218	1,994	212	1,041	208	713	164	811
P mg.%	340	1,700	2,505	4,140	1,375	1,800	1,357	1,451	1,171	1,129	831	1,194

Tissues in order of sensitivity to vitamin D.

TABLE 8.

Tissue	Initial Calcium	Final Calcium	Increase
Aorta	237 mg.%	20,560 mg.%	100 fold
Lung	218	1,994	10
Trachea	3,900	8,500	2
Kidney	210	1,041	5
Heart	205	713	3.5
Stomach	164	811	4.5

Thus the heart and stomach, which display only a small degree of calcification, form the lower end of the scale; the kidney, trachea and lung, the middle portion; and the aorta, the upper end of the scale. The aorta is unique in the series due to the large amount of calcium which can be deposited in this tissue. When maximally calcified, the calcium content of the aorta (on a weight for weight basis) exceeds by far that of any other tissue except bone.

A number of quantitative studies of the soft-tissue calcification following vitamin D administration have been recorded. Several species have been used (rats; Kreitmar and Moll; Gough, Duguid and Davies, 1933; Cartland et al., 1929; dogs; Kreitmar and Moll, 1928; Taylor, et al., 1931; Steck et al., 1937; Goormaghtigh and Handovsky, 1938; Hendricks et al., 1947; Morgan et al., 1947; Reed et al., 1933; rabbits; Smith

and Elvove, 1929;). Other investigators (eg. Zeek, P.M., 1936; Hoyle, J.C., 1930) have been content to express the calcification in the tissues by a non-quantitative arbitrary scale. In most of the early work the vitamin D was administered as irradiated ergosterol making comparison of the dose with the present work - in which pure calciferol was employed - very difficult. Nevertheless, a comparison of the present values with earlier results for the calcium content of certain tissues may prove useful.

Early investigators of vitamin D-calcification appear to have found the kidney the most sensitive tissue (Gough, Duguid and Davies, 1933; Smith and Elvove, 1929). Smith and Elvove, for example, quote one value depicting a hundred-fold increase in kidney calcium after the administration of irradiated ergosterol in the rabbit. Reed et al. (1933) measured a ten-fold increase in the dog kidney calcium from 64 mg.% to 634 mg.% after excess vitamin D. For the rat, after relatively small doses of vitamin D Cartland et al. (1929) quoted calcium values for the kidney of 19 mg.%. The normal values, as quoted above, for the kidney calcium content are markedly lower than the values obtained in the present study. At the same time, our results, while indicating extensive soft-tissue calcification, do not suggest that the kidney is nearly so reactive as the earlier data would suggest. In the present study, the calcium content of the kidney, as assessed from the average value in each group of similarly treated rats, showed a five-fold increase over normal after vitamin D therapy.

Reed et al. (loc. cit.) have measured the calcium content of the left and right ventricles of the dog heart and their results indicate that calcification is specific to the right ventricle. Vitamin D treatment was shown to increase the calcium content of the right ventricle some five times. As was the case with the kidney, our estimations of the normal level of the heart calcium content (at 208 mg.%) were much higher than values reported by earlier workers.

There appears to be general agreement that the lung is a site of active calcification. In the rabbit, Smith and Elvove (1929) quote an example of a fifty-fold increase in calcium; while in the dog, Reed et al. (1933) measured a five-fold increase. These results are to be compared with a ten-fold increase found in the present experiment in the rat (Table 8). Again, the normal value for the lung calcium in the present study is some three times higher than the value quoted either for the rabbit or the dog (v.s.).

No data on calcification in the trachea after vitamin D treatment have been discovered and hence comparison with the present results is not possible. The trachea was selected for our study because of its large cartilage content and the results obtained show some noteworthy features not observed in other tissues examined. First, the mean for the calcium content of the trachea in normal rats was 3,900 mg.%., a value greatly in excess of that measured in any other tissue.

Although the calcification of cartilage still requires elucidation, the large concentration of chondroitin sulphate, long

known to be present in cartilage (Levene, 1925) is believed to be the polyelectrolyte responsible for the binding of calcium ions. The following evidence favours the foregoing viewpoint. Undoubtedly chondroitin sulphate will bind calcium ions, for equilibrium dialysis experiments have shown (Farber et al., 1957) that one period of chondroitin sulphate can bind 1.3 equivalents of calcium. Boyd and Neuman (1951) have shown that the binding capacity of calf costal cartilage could be correlated with the sulphate content. It was therefore suggested that chondroitin sulphuric acid was the factor responsible for the binding of calcium. Autoradiographic studies of the binding of Ca-45 and S-35 in section of normal and pathological cartilage from various sources (Belanger, 1955) have indicated that there is some spatial relationship between calcium and sulphur. Admittedly, the presence of chondroitin sulphate and calcium ions in the cartilage does not necessarily mean the interdependence of the one upon the other. It is probably, however, that some relationship between calcium and chondroitin sulphate does, in fact, exist.

Not only does the trachea contain a large percentage of calcium, but under vitamin D stimulation there is an immediate incorporation of further calcium into the tissue of the order of two to three times the normalto a maximum of 8,500 mg.%. While the percentage increase is modest in comparison with other tissues studied (eg. aorta and lung), in terms of actual weight the increase is, in fact, large.

The trachea was the only tissue studied in the present investigation in which a 2:1 ratio of calcium to phosphorus was observed, suggesting that a bone-salt type of compound was being laid down (see Section VI). However, Boyd and Neuman (1951), who suggested that calcification of cartilage is an ion-exchange reaction, have shown that 'in vitro', phosphate ions are taken from solution only by cartilage containing appreciable amounts of calcium. If such a reaction takes place 'in vivo' then phosphate can be considered as being of secondary importance only in the binding mechanism.

Calcification of the aorta is known to arise spontaneously in both man (Haythorn et al., 1936; Blumenthal et al., 1944; Buck, 1951) and in animals (Gillman and Gilbert, 1957). Studies in man during ageing have shown that a twenty-fold increase and more in the calcium content of the aorta can arise over an eighty year life-span (v.s.). Under stimulation with vitamin D, the aorta calcifies to much greater limits and is speeded up to such a tempo that within fourteen to twenty-one days values of 20,000 mg.% calcium (dry weight) are obtained. These values are so high that they compare with bone which contains some 24 % calcium (dry weight). In the dog, however, Reed et al. (1933) were able to demonstrate only a modest increase from 67 mg.% calcium to 186 mg.% compared with the hundred-fold increase recorded in our rats. Again, values for the calcium content of normal aortae in our rats were much larger at 237 mg.% than the

values quoted in the literature for either man, dog or rabbit.

Explanation in part, lies in the tissue magnesium (simultaneously estimated by EDTA titration) and hence incorporated into the 'calcium' value. Nevertheless, from a number of estimations of the tissue magnesium (by a slight modification of the technique of Orange and Rhein, 1951) and correction of the calcium plus magnesium value recorded, it is still not possible to explain completely the high values observed during the present study on the basis of magnesium interference. The possibility that low results in earlier experiments employing the oxalate method of calcium estimation cannot be entirely discounted in the light of present knowledge of the limitations of this technique (eg. MacIntyre, 1955).

Despite the large values recorded in many tissues after calcification is established, reconsideration shows that the actual weight of calcium moved into the soft tissues is relatively small. Thus, in an aorta with a calcium content of 20,000 mg.% on a dry-weight basis, the actual weight of calcium is only some 6 mg. for a tissue of 30 mg. in weight. In a similar manner some 3 mg. is sufficient to account for the calcification of the trachea and 5 mg. for the lung. Table 9 indicates the amount of calcium required to produce the measured degree of calcification in the six tissues studied. We realise that after vitamin D - treatment calcification proceeds in most of the soft tissues in the body and not only in those studied in the present investigation. For example, the whole arterial tree and

not only the aorta is particularly susceptible to calcification in hypervitaminosis D. Other tissues known to calcify include the skin, the thyroid, the muscles and the gut (Reed, Struck and Steck, 1939). Clearly then, the tissues studied in the present work are responsible for only a part - though a significant part - of the calcification which follows the feeding of excessive amounts of vitamin D.

Calcium ion concentrations of the above order are readily available both from the bones (Debre et al., 1946) and the diet, and it can be easily appreciated how readily re-distribution of such quantities can take place to give rise to the rapidly developing calcification of hypervitaminosis D.

Further rough calculations are illuminating. Taking the heart rate of the rat as 400 per minute and an estimate of the stroke volume of the rat heart of 0.25 ml., the minute-volume of the rat amounts to 100 ml. per minute. While no value for the arterio - venous difference in serum calcium is available, a hypothetical value of 0.1 mg.% on a normal serum calcium value of 12 mg.% (or up to 17 mg.% in hypercalcaemia) would amount to 0.1 mg. of calcium being lost to the tissues every minute, or 144 mg. per twenty-four hours. It therefore appears that sufficient calcium is being supplied to the tissues at a rate permitting rapid calcification without noticable change in the serum calcium level once a mechanism is established for removing calcium from the serum. It is suggested that the limiting factor in soft-tissue calcification is not so much the availability of the calcium, as a mechanism in the tissues for removing the calcium

from the serum.

TABLE 9

Actual weight of calcium deposited in six soft-tissues after vitamin D.

Tissue	Maximum Ca in mg.%	Wt. of tissue (mg.)	Wt. of Ca (mg.)
Aorta	20,000	30	6
Trachea	8,500	30	2.5
Lung	2,000	250	5
Kidney	1,000	300	3
Heart	800	125	1
Stomach	700	160	1.1
	Total :	895	18.6

We have shown that the mechanism of vitamin D - induced calcification depends in some manner on the tissue undergoing calcification. Without such a condition, all soft tissues would presumably calcify to the same extent. Whether or not an identical calcification mechanism operates in all tissues is, as yet, problematic. While the calcification of cartilage appears to involve chondroitin sulphuric acid, little is known of the importance of this compound in the calcification of other tissues. Vitamin D appears to derange the normal metabolism of the tissue so that calcification ensues, though the nature of the change is still unknown. It has been pointed out, however, that a very small weight of calcium is sufficient to give rise to a marked degree of calcification when bound in the tissues.

(2) The differential rate of soft tissue calcification.

Over and above the individual tissue response, the procedure of mapping the course of calcification as it develops in each tissue has disclosed several noteworth features relevant to the rate of progressive calcification.

In the kidney, heart and stomach the rate of calcium binding is, for two reasons, more difficult to assess than in the lung, aorta and trachea. First, the individual variation between rats assumes a greater importance in the less reactive tissues. Secondly, less data are available for the kidney, heart and stomach as compared with the aorta, lung and trachea. Experiment 733 indicated that aorta, lung and trachea were the most reactive tissues studied and hence were more extensively investigated. However, from the data which is available, the kidney, heart and stomach all showed initially a small degree of calcium uptake within the first 24 hours of vitamin D treatment. Thereafter, the levels rose slightly and then declined to near pre-treatment levels. Table 10 indicates the change during the first three days of the experiment.

TABLE 10

Calcium content of tissues during first three days of the experiment.

Tissue	Day 0	Day 1	Day 2	Day 3
Kidney	212 mg.%	362 mg.%	445 mg.%	257 mg.%
Heart	208	348	498	220
Stomach	164	365	346	282

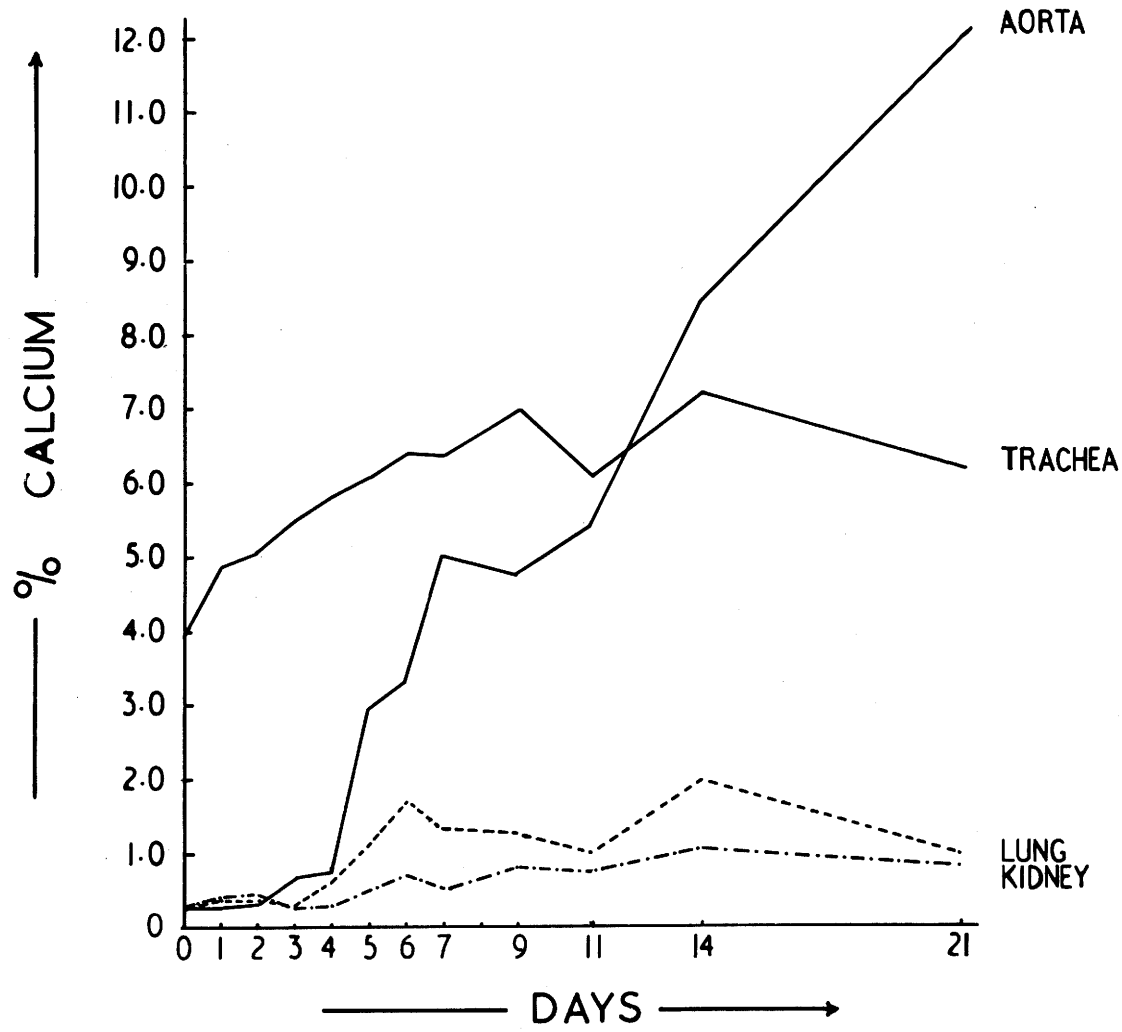
Twenty-four hours after the fourth dose of vitamin D, however, a further increase in calcium in each of the kidney, heart and stomach occurred, reaching a peak ten days after the vitamin D treatment had ceased. In the kidney the maximum calcification (on a mean value basis) was 1,041 mg.%. Corresponding values for the heart and stomach were 713 mg.% and 811 mg.% respectively reached ten days after vitamin D treatment had been withdrawn.

The marked increase in calcium, beginning in the trachea even within 24 hours of the first dose of vitamin D, is readily seen in Table 19. During succeeding days the degree of calcification increased in a steady manner from a normal value of 3,900 mg.% to 6,400 mg.% calcium, or nearly double, 48 hours after the fifth and final dose of vitamin D. Hereafter values fluctuated somewhat, but by the fifth day after the last administration of vitamin D, (or the ninth day of the experiment) calcification was virtually complete at approximately 7,000 mg.% calcium. The higher value of 8,500 mg.% on day 54 of the experiment is probably the result of an individual rat markedly sensitive to vitamin D. The significant feature of tracheal calcification in the present experiment was the large amount of calcium which could speedily be deposited in the tissue under the influence of vitamin D therapy.

The differential nature of the tissue response is emphasised by comparing the rate of calcification in the lung and aorta with the rate in the "low" calcifying tissues and the trachea. Study of sequential calcification showed a distinct inability of the aorta

FIGURE 1

CALCIFICATION OF THE AORTA, LUNG, TRACHEA & KIDNEY



and lung to calcify rapidly. As can be seen from the graph (fig. 1 page 58) a period of three to four days relatively slow calcification precedes the major deposition of calcium in both the lung and the aorta. Thus for the lung the mean calcium values in daily sequence were 218, 336, 347, 293 and 683 mg.%; whereas for the aorta they were 237, 268, 306 640 and 727 mg.% over the same period. Once initiated however, binding was rapid. In the lung the mean calcium values recorded 24 hours after the fourth and fifth dose of vitamin D were 683 and 1,052 mg.% respectively. In the aorta the corresponding values were 727 and 2,397 mg.%. Hence the lung showed a five-fold increase over the normal (and a two-fold increase over the previous day's value) only 24 hours after the fourth dose of vitamin D. Thereafter, a considerable increase in calcium was noted.

In the aorta an essentially similar four day lag in response to vitamin D was followed by a tenfold increase in calcium over the control level and a three-fold rise over the fourth day's value. Once initiated, calcification develops progressively until "saturation" is virtually complete. In the lung maximal calcification was apparently achieved by the tenth day after treatment with vitamin D had ceased. Values of the order of 2,000 mg.% were found. Again, the exceptional nature of the calcification of the aorta was emphasised by its higher maximum value. The mean maximum value for the calcium in the aorta was 20,560 mg.% recorded on the 72nd. day of the experiment although individual higher values were noted earlier (eg. 29,170 mg.% on day 14 - rat 733/51). The data therefore indicates that the period required for maximum calcification to develop in a tissue is a function

of the tissue itself, in much the same way as the range of maximum values obtained, and is in keeping with the concept of tissue specificity.

From the rate of calcification it can be seen that in all the tissues studied, excepting the aorta, "saturation" is usually achieved by the ninth day and complete by the fourteenth day of the experiment. A higher value recorded later is not likely to exceed by much the 9th - 14th day value, a fact illustrated by the trachea in which the value on the 54th day was 8,500 mg.%, an increase over the apparent "saturation" level. The value is, however, only slightly above the "saturation" value of 7,200 mg.% and cannot be likened to the increase of calcium in the aorta achieved after the fourteenth day when the value is 8,437 mg.% rising to 13,571 mg.% by the twenty-first day and to 20,560 mg.% by the seventy-second day of the experiment.

The rate of change of soft tissue phosphorus approximated to that of the calcium though the degree of change was very much smaller. In some tissues there was virtually no increase. Thus, in the heart and kidney only the smallest change - essentially insignificant - in total phosphorus content could be measured. The stomach and lung, however, displayed a small increment in total phosphorus content, developing simultaneously with calcification. Only in the trachea was the anticipated trend of a 2:1 ratio of calcium to phosphorus observed. As was the case for calcium, the aorta showed the largest capacity for phosphorus. No change in the mean phosphorus level of the aorta was noted in the groups of rats killed during the first four days of the experiment and the average value remained at the normal

level of 340 mg.% P. Twenty-four hours after the fifth dose of vitamin D the phosphorus content of the aorta increased rapidly, and by day seven reached a value of 1,590 mg.% at about which level it stabilised for the remainder of the experiment. The binding of phosphorus by the aorta 24 hours after the fifth dose of vitamin D coincided with the first major rise in the calcium content of the aorta. Clearly, the change in phosphorus content during calcification of the aorta and other tissues is much smaller than that of calcium giving rise to the possibility that the role of phosphorus in the calcification mechanism is less important than previously believed. In this connection it is to be remembered that Reed et al. (1939) pointed out that in dogs receiving irradiated ergosterol the phosphorus content of different soft tissues was much lower than was required if the mineral was present as bone-salt.

Whatever the nature of the calcium complex in the tissues, the study of sequential calcification in the rat during and after treatment with large doses of vitamin D has permitted a quantitative assessment of the rate of calcification in each of six tissues. From such an assessment it has been shown that the rate of calcification is dependent upon the nature of the tissue.

(3) The continuing action of vitamin D.

In all of the six tissues examined, calcification has been shown to continue after treatment with vitamin D has been withdra Further, maximum calcification is not achieved in the heart, kid lung and stomach until ten days after the last treatment with v

In the aorta, the time of this maximum calcification is difficult to determine, no doubt due to the wide variation is reactivity of this tissue. Nevertheless, it is clear that calcification continues long after the effective stimulus of vitamin D has ceased to be applied. It is certain, therefore, that the action of vitamin D is continuous and the repercussions of a given dose is not restricted to the period of administration.

In the present section it is our purpose to reconsider the continuing action of vitamin D. In particular, the role of the liver in vitamin D metabolism will be assessed.

Vitamin D, being a fat-soluble vitamin, requires an adequate flow of bile for absorption from the gut. Interference with the free flow of bile from the liver invariably causes osteoporosis and associated disturbances in calcification. Taylor, Weld and Sykes (1935) have shown that vitamin D treatment failed to produce a hypercalcaemia in dogs with a biliary fistula. They subsequently demonstrated that a mixture of sodium taurocholate, sodium glycolate and sodium bicarbonate fed simultaneously with the vitamin D rapidly brings about a hypercalcaemia. It was concluded that bile is necessary for vitamin D absorption. On the other hand, Gilbert and Gillman (1957), working with rats with bile fistulae, were unable to produce calcification with vitamin D even with the simultaneous administration of desoxycholic and cholic acids. Nor did the feeding of bile intragastrically permit vitamin D calcification. It was also shown that if the bile duct was cannulated and arranged so that bile could be recirculated into the duodenum for four hours each day,

vitamin D still failed to induce calcification of the soft tissues.

It appears from the evidence quoted, as well as from additional information (Bowler and Walter, 1924) that bile is a necessary requirement for vitamin D absorption, and to this extent the liver plays an important part in the calcification mechanism. A closer study of the participation of the liver in calcification however, discloses a second, more subtle function in addition to bile formation (Heymann, 1937, 1938). Heymann showed that liver damage inhibited the antirachitic action of vitamin D independently of bile formation. Cirrhosis of the liver produced by both carbon tetrachloride treatment and by ligation of the bile duct reduced by a factor of up to ten times the minimum curative dose of vitamin D administered by intramuscular injection to rachitic rats. Essentially, the same conclusion was reached by Gilbert and Gillman (loc. cit.), though these authors were concerned with producing pathological calcification in contrast to Heymann who studied the calcification of rachitic animals.

In addition to the above two facets of liver participation in vitamin D metabolism and hence in soft tissue calcification, the liver, particularly in the fish, is the major site of vitamin D storage. In animals the reserves of vitamin D appear to be spread over a number of tissues but the liver contains the major proportion. (Coppens and Metz, 1933; Kodicek, 1956).

No further study appears to have been undertaken on the role of the liver in vitamin D induced calcification. It remains, therefore,

an open question whether or not the continuing action of vitamin D is actively related to storage of the vitamin in the liver. While further experiments are therefore required, it is possible to state from data already available (v.s.) that liver participation is necessary for the calcification mechanism to operate effectively.

SECTION IV.

The resorption of deposited calcium and the recovery of the tissues.

The ability of the tissues to lose the calcium accumulated under the influence of vitamin D was regarded as being of importance for two reasons. First, it was thought that a knowledge of the rate of decalcification might assist in the interpretation of the mechanism of the calcification process. Secondly, the degree of recovery possible in the tissues was thought to be a critical factor in determining whether or not the animal will survive, subsequent to treatment, without impaired cardio-vascular and other bodily functions.

Re-presentation of the data from the viewpoint of tissue recovery is therefore essential before comment is possible (Table 11).

Study of the average value for the calcium content of the lung in each group of rats shows that seven days after the maximum value of 1,994 mg.% the calcium content had decreased by half, i.e. to 995 mg.% Ca. Thereafter, values oscillated somewhat and by the 104th day after treatment a value of 650 mg.% was recorded (in this case in one rat only) which was three times the average value for the control group. In the kidney, maximum calcification was denoted by a group average of 1,041 mg.% calcium. When the next group of rats were examined seven days later the average value for the kidney calcium had decreased to 816 mg.%. The final experimental result for the kidney seventy-two days from the initial treatment with vitamin D was 595 mg.% calcium and is to be compared with 212 mg.%

TABLE 11.

The degree of calcium resorption in six soft tissues of the rat fed large doses of vitamin D.

TISSUE	Normal Calcium Value*		Max. Calcium Value*		Calcium Value* 68 days after feeding vit. D	
	Range	Mean	Range	Mean	Range	Mean
Aorta	132-600	237	2,590-31,230**	13,571	6,790-27,130	20,562
Trachea	1,695-5,510	3,900	5,120-10,200	7,204	4,615-9,450	6,540
Lung	155-315	218	345-4,835	1,994	370-3,977	1,370
Kidney	150-3L4	212	470-1,915	1,041	405-980	595
Heart	145-290	208	510-800	713	265-370	334
Stomach	140-192	164	320-1,440	811	180-520	348

* All values in mg.%

** Due to continuous calcification, mean calcium content of aorta was not as high at 21 days as as at 72 days but maximum calcification had occurred in some cases.

in the control. An identical picture was apparent in the heart and stomach where the corresponding values are respectively: maximum calcium, 713 mg.% and 811 mg.%, final value 343 mg.% and 348 mg.% and initial control value of 208 mg.% and 164 mg.%. In the aorta and trachea, both highly calcified tissues, there was no reduction in the calcium content after maximum calcification had been attained. Final and initial calcium values for the aorta are 10,350 mg.% and 237 mg.% and for the trachea, 8,120 mg.% and 3,900 mg.%.

Comment.

In addition to the measurement of the rate of soft tissue calcification, the nature of the experimental procedure permits an assessment of the efficiency of calcium resorption in the period following maximum calcification. A search of the literature revealed that little qualitative or quantitative information is available on the rate of soft tissue de-calcification subsequent to a period of hypervitaminosis D. For the most part, study has been made only of the serum and urinary calcium levels in the post-vitamin D period, together with the results of a few cases in which some correlation is made between serum calcium concentrations and post mortem examination of tissue (Smith and Elvove, 1929). Steck, Deutsch, Reed and Struck (1937) have claimed, without advancing evidence, that the excess calcium is removed via the kidney (see also Dent, C. E., 1954), and not until the urinary calcium has returned to normal can the tissues be expected to have completely recovered.

Further information on the effects and recovery from vitamin D poisoning is available from two sources. The first and most voluminous is a number of clinical case histories of man in whom hypervitaminosis D has arisen in a variety of ways. Usually toxicity develops as a side effect of the clinical administration of large doses of calciferol in the treatment of arthritis, lupus vulgaris and other conditions. Several reports have appeared describing the effects of excess vitamin D used prophyllactically for rickets (Ravine, 1936; Ross and William, 1939; Anning, Dawson, Dolby and Ingram, 1948). Due to the impracticability of examining internal tissues, excepting when death supervened, no information on their degree of calcification is available from these studies in man. However, from the clinical point of view recovery was rapid as judged by the disappearance of the many side effects and symptoms of hypervitaminosis D especially the return to normal of the serum calcium and inorganic phosphate once the vitamin treatment was suspended.

The onset of calcification in the rats used in the present investigation was always associated with a number of side reactions, principally severe loss of weight, kidney damage, diarrhoea and loss of appetite (Gillman and Gilbert, 1956). After withdrawal of the vitamin D, recovery (as defined by recession of these symptoms) was rapid, but the calcium content of the tissues still remained high. Without evidence to the contrary it is probable than in man the

the calcium will remain firmly bound in the tissues in a manner similar to that described for the rat. The deductions made by various authors about resorption of calcium from the tissues, as judged from the serum calcium, are open to criticism and are fully discussed elsewhere (page 96 et seq.).

Histological studies have enabled the stage by stage recovery process in an artery to be mapped (Gillman and Gilbert, 1956). These authors also gave an account of the structural alterations which have taken place. From such work, functional recovery of the arterial wall appears to be a moderately rapid and efficient process in which much of the calcium is clearly not removed as such, but isolated by cellular material and moved towards the adventitia. Complete resorption of calcium is seen as a slow process. Against this histological background it was possible to examine the resorption of mineral matter, by chemical determination, in the aorta, trachea, lung, kidney, heart and stomach of the rat after a short but intensive course of treatment with vitamin D. The data already presented for the rat amplifies that already available to a lesser extent from the dog (Steck et al., 1937) but it is to be emphasised that in the case of the rat, the design of the experiment was such as to provide quantitative data on calcium resorption in tissues heavily mineralised under the influence of vitamin D.

Measurements on animals killed 17 days after the last dose of the vitamin show that in the lung, kidney, heart and stomach some

measure of resorption of the calcium had apparently taken place. The mean value of the results was significantly lower than the equivalent fourteen day peak reading. Measurements performed 31, 50, 68 and 104 days after the last dose of vitamin showed in the case of the lung, kidney, heart and stomach mean values significantly lower than at peak calcification. Nevertheless, there was the occasional individual result which was very high even late in the experiment as, for example, a value of 1,125 mg.% in the kidney fifty days after the last dose of the vitamin (rat 733/45; Table 36). Despite these exceptional results some significant measure of recovery does take place. Comparison of the calcium values determined either sixty-eight or one hundred and four days after conclusion of vitamin D treatment with the control values, however, strongly suggests that recovery was by no means complete. In the kidney and stomach the values recorded sixty-eight days after cessation of vitamin D therapy were approximately twice that of untreated rats of the same age; and in the heart, was one-and-a-half times normal. Table 11 indicates the possible trend in calcium resorption in six tissues.

The contention that the calcium is not completely removed by the sixty-eighth (or one hundred and fourth) day is further supported by a consideration of the range of values at different times. As shown in Table 11 the lower limit of the range of calcium values measured at the end of the experiment compare with the upper limit

of that found in the control rats. The difference appears to be real and not mathematical.

These data do not permit any prediction to be made as to whether or not the calcium is eventually removed from the tissues. In rats treated with large amounts of vitamin D and allowed to live on until they died spontaneously the aorta was still heavily calcified (visible to the naked eye) on post mortem examination. It can be concluded that the removal of calcium from the kidney, heart, stomach and lung is a slow process requiring at least four times as long as the time to produce the calcification. Further, the aorta and trachea showed even less sign of calcium resorption throughout the one-hundred and eight days of the experiment than the tissues mentioned above. In the trachea, all the samples examined after the final dose of vitamin D showed substantial calcification. Notwithstanding some oscillation in the level of tracheal calcium, there was at no time any indication of a major decrease in calcium, and it can only be concluded that practically no resorption of calcium from the rat trachea takes place, at least over a period of one-hundred-and-four days following vitamin D therapy.

In the aorta analysis also failed to disclose any sign of calcium resorption. Only one estimation (10,250 mg.% calcium) was available for the aorta at one-hundred-and-four days after cessation of vitamin D treatment, but numerous rats similarly treated with

vitamin D and dying after a normal life-span all showed on visual examination an extensive calcification of the aorta. It can therefore be claimed with a large measure of confidence that the degree of calcium resorption in calcified aortae - at lease in the rat - is, at most, small. It is probable that the difficulties experienced by the aorta in disposing of excess calcium are the result of the structural changes discussed earlier.

From the foregoing, it appears that the resorption of calcium from a tissue - no less than the deposition - is a process which depends upon the nature of the tissue. Thus the lung, kidney, heart and stomach rapidly display a measure of calcium resorption once vitamin D therapy has been suspended. The calcium of the trachea and aorta, on the other hand, appears to be permanently fixed. The function of the calcified tissue, however, does not appear to be greatly impaired by the calcification. It was noted that even those tissues in which a measure of calcium resorption occurred, failed to return to normal values. It is significant that the repercussions of a five-day course of treatment with vitamin D - on the calcium of various soft-tissues of the rat are to be seen at least a hundred days after treatment has been suspended.

SECTION V.

The influence of vitamin D on the calcium and phosphorus content of the serum.

(1) The degree of hypercalcaemia and phosphataemia observed.

The formation of bone, or the deposition of calcium and phosphorus (as phosphate) in the soft tissues, requires a supply of the necessary constituents to be made available at the relevant sites of calcification. When the normal rate of calcification is drastically modified, as has been frequently demonstrated in hyper- and hypovitaminosis D (e.g. McChesney and Messer, 1942; Steck et. al., 1937) it is customary to follow the progress of calcification by measuring quantitatively the concentration of calcium and phosphorus in the blood. Calcium is almost exclusively confined to the serum for it has been shown (Rymer and Lewis, 1932) that washed red cells contain only about 0.5 mg.% of calcium compared with a range of 8 - 11 mg.% (man) in the serum. It is for this reason that serum calcium was measured in the present investigation. Phosphorus, in contrast to calcium, occurs in large quantities within the red cell as well as in the serum, hence the need for care in avoiding haemolysis when taking samples of blood for serum inorganic phosphate determinations.

In the present section it is our purpose first, to describe the degree of hypercalcaemia and phosphataemia observed in the present experiments, and secondly to compare these results with those described in the literature for both man and animals.

Experimental results.

Four separate experiments were performed, under similar

conditions to test the reproducibility of the vitamin D-induced change in the calcium and phosphorus content of the serum, viz. Experiments 733, 753, 759 and 777. As previously described, all tissues studied in Experiments 733, 753 and 759 - namely the aorta, lung and trachea - showed a large measure of agreement. A significant measure of uniformity was displayed by the sera studied in Experiments 753,759 and 777, but a marked deviation appeared in the case of serum determinations in Experiment 733. We are therefore left with the situation in which the majority of results conform to a pattern, but the exceptional series of results in Experiment 733 must also be considered.

The main change in the serum of rats fed large amounts of vitamin D is one of hypercalcaemia. Individual rats do not react always in the same way but, except for the series 733 in which a hypocalcaemia was observed during the experimental period, the trend towards elevation of the serum calcium was noted in all cases.

The normal level of serum calcium and phosphorus was established from twenty-one rats. All were selected in order to conform to the weight, age and sex of the experimental animals. Examination of the results presented in Table 12 reveal that in Experiment 733 the normal serum calcium concentration was 12.9 mg.%, a result slightly higher than in the other control groups. Because of the general lack of agreement between Experiment 733 on the one hand, and Experiments 753, 759 and 777 on the other, the results of all the experiments could not be pooled. The main change is therefore represented by the

pooled results of Experiments 753, 759 and 777 only. The anomalous Experiment 733 will be considered separately. Thus the control value for serum calcium of the combined group was 12.0 mg.% calcium.

TABLE 12.

Table of normal serum calcium values obtained in four independent experiments.

Expt. No.	No. of rats.	Normal serum calcium in mg.%	
		Range	Mean
733	10	11.5-14.3	12.9
753	3	11.9-12.3	12.3
759	5	10.9-12.4	11.8
777	6	11.7-12.6	12.1

Considering only the combined results, the serum calcium progressively increased with successive doses of vitamin D in such a manner that the average rose from a basal level of 12.0 mg.% calcium to 16.6 mg.% 24 hours after the fifth dose of vitamin D. At this time the calcium began to decline, thus permitting, under our conditions, the determination of the time when the blood calcium reached its maximum.

Examination of the individual animals reveals that the degree of hypercalcaemia varied widely from animal to animal. The maximum degree of hypercalcaemia of 21.9 mg.% appeared in rat 759/29. In the same group of animals a minimum value of 13.5 mg.% - rat 759/27 -

was observed and the remaining fifteen values were distributed between the limits thereby established. Table 13 indicates the serum results at maximum hypercalcaemia.

TABLE 13.

Table of serum calcium values at maximum hypercalcaemia.

Expt. No.	No. of rats.	Serum calcium at maximum hypercalcaemia in mg.%.	
		Range	Mean
733	4	10.4 - 11.4	10.6
753	4	15.7 - 17.9	16.9
759	5	13.5 - 21.9	16.7
777	8	14.8 - 19.0	16.3
Pooled value	17	13.5 - 21.9	16.6

By contrast with the general trend, the serum calcium of Expt. 733 did not rise to a maximum value 24 hours after the last dose of vitamin D. In fact, a lowering of the mean value to 10.6 mg.% calcium (cf. control mean 12.9 mg.% Ca) was noted. High serum calcium values were observed in Experiment 733 but not until five days after the last administration of vitamin D when the mean serum calcium value was 16.0 mg.% ie. in the same range as the mean for the combined experiments at maximum hypercalcaemia. Figure 2 shows the trend of the serum calcium in Experiments 753, 759, and 777.

Throughout the experiment, serum inorganic phosphate estimations

were made on every sample, but again, the serum results of Experiment 733 differed from Experiments 753, 759, and 777. However, in contrast to the serum calcium, the deviation between the different serum phosphate values was, with few exceptions, smaller. Possibly the greatest deviation between experiments, as indicated in Table 14, occurs in the controls. In this group the mean serum phosphate value for Experiment 733 is 11.4 mg.% compared with 6.9 mg.% for Experiment 753, 8.5 mg.% for Experiment 759, and 8.3 mg.% for Experiment 777. It was not possible to pool the results of Experiment 733 with those of the other groups.

TABLE 14.

Table of normal serum inorganic phosphate values obtained in four independent experiments.

Expt. No.	No. of rats.	Normal serum inorg. P in mg. %	
		Range	Mean
733	10	8.0-16.9	11.4
753	3	6.3- 7.9	6.9
759	5	8.1- 8.8	8.5
777	6	7.5-12.0	8.3

Fourteen animals composed the pooled group (Experiments 753, 759 and 777) and the mean serum phosphate value was 8.2 mg.% phosphorus. Concomitant with the administration of vitamin D and the onset of calcification and hypercalcaemia, there appeared a measure of hyper-

phosphataemia. At no time however, were the results as clear-cut as in the case of calcium. An examination of the results does not permit clear identification of any period at which hyperphosphataemia reaches a maximum.

Comment.

A vast literature is available on the level of serum calcium and phosphorus in both normal and pathological conditions in a multitude of species. It is therefore possible to compare the results obtained in the present experiment with values obtained by other workers.

The level of serum calcium and calcification.

The level of the serum calcium has been shown to be, in some measure, species dependent. Thus in man the normal range is 9.0 to 11.4 mg.% calcium with a mean value of 10.3 mg.%, as determined by consideration of 42 observations by different workers (Schmidt and Greenberg, 1935). In the dog, two independent experiments agree closely with mean values of 10.8 mg.% and 10.5 mg.% calcium in the range 9.0-13.0 mg.% and 8.5-12.5 mg.% ascertained from 31 and 14 observation respectively (Schmidt and Greenberg, 1935; Greene and Powers, 1931). In the guinea-pig the mean serum calcium of 9.4 mg.% is slightly lower than in either dog or man. Nine observations recorded by Brown and Ramsdell (1929) indicate a range between 8.4 and 10.4 mg.% calcium. Thirty-three observations of serum calcium in the rabbit (Updegraff et al., 1926) show a mean of 13.1 mg.% between the limits of 11.0 and 16.0 mg.% calcium,

significantly higher than in man. Further studies on the rabbit by Japanese workers (Yanagisawa, Ogasawara and Fujii, 1954) have confirmed earlier results that there is a significant variation between the summer and winter level of serum calcium. In winter the range of values was 14.2 - 14.9 mg.%, higher than the summer range of 11.3 - 12.3 mg.%. In view of the fact that Experiment 733 was undertaken during the summer months and Experiments 753 and 759 in the winter, a seasonal factor was suspected as being related in some way to the anomalous results. However, despite the fact that Experiment 777 was also performed during the summer, the results obtained from Experiment 733 could not be reproduced.

The fluctuations in the serum calcium concentrations in the bird during the egg-laying and non egg-laying periods have excited considerable interest. Five observations by Laskowsky (1933) established a mean serum calcium in the non-laying fowl of 12.2 mg.% in the range 11.8 - 13.0 mg.%. By contrast is the mean value of 20.7 mg.% calcium measured in the laying hen. Seven observations established the range between 16.3 mg.% and 25.8 mg.%. At no time did the mean serum value in our experimental rats under severe and non-physiological doses of vitamin D reach the level measured for the laying hen, and only in a few cases did an individual level exceed 20 mg.%. During the development of hypercalcaemia in vitamin D - treated rats many clinical features such as severe loss of weight, only partially associated with a loss of appetite and diarrhoea, appear which are not evident in the laying bird having even a greater

concentration of calcium in the serum. In a few cases the vitamin D treatment even resulted in death. Apparently, in the laying bird the hypercalcaemia, which in general appears even greater than in hypervitaminosis D, is tolerated with no ill effect, nor does the soft tissue calcification seem to develop. The essential feature for the present discussion is than no such tissue change develops within the short period of hypercalcaemia as already described for rats. Clearly therefore, a hypercalcaemia 'per se' is not the cause of the tissue calcification. The evidence strongly suggests the need for a primary change in the tissue to precede the soft tissue calcification rather than a process of precipitation from a serum supersaturated with respect to calcium phosphate.

An examination of sixty immature rats by Benjamin and Hess (1933) indicated the average serum calcium to be 11.3 mg.% in the range of 10.8 - 12.3 mg.%. Comparison of our results for rats with Benjamin and Hess's standard suggests that our rats have a slightly higher normal serum calcium with a mean for the pooled group of animals of 12.0 mg.% calcium in the range 10.9 - 12.7 mg.%. The difference, however, is of limited significance and may be due to differences in diet, strain or slight differences in analytical methods. The mean value of 12.9 mg.% for Experiment 733 in the range 11.5 - 14.3 mg.% on the other hand, is significantly higher than either Benjamin and Hess's standard or the value established from Experiments 753, 759 and 777. No satisfactory explanation for the discrepancy was forthcoming.

Inorganic serum phosphate and calcification.

Analytical surveys on a number of species have established the normal range of the serum phosphate concentration. As with the serum calcium, a slight species difference is revealed. Thus, while the mean value for man is 4.0 mg.%P. in the range 3.0 - 5.8 mg.% (Schmidt and Greenberg, 1935), for dogs 4.9 mg.% in the range 3.0 - 8.5 mg.% (Schmidt and Greenberg, 1935) and for oxen a mean value of 4.9 mg.% P., the value for rats determined from sixty values has been reported to be 10.3 mg.% in the range 9.4 - 12.2 mg.% (Benjamin and Hess, 1933).

By comparison with the values quoted above, the rats in the present experiment (excepting those of Experiment 733) tended to show lower normal values. In Experiments 753, 759 and 777 the range was between 6.2 mg.% and 12.0 mg.% with a mean value of 8.2 mg.% P. On the other hand, the ten rats comprising the control group in Experiment 733 showed values significantly higher, and in close agreement with Benjamin and Hess's results (v.s.) nine of the values being in the range 8.0 - 12.5 mg.% P and one value exceptionally high at 16.9 mg.% (rat 733/12). The mean serum inorganic phosphate value in Experiment 733 - including rat 733/12 - was 11.4 mg.%.

Serum Ca:P ratio and calcification.

While there is incontrovertible evidence (q.v.) that the normal blood calcium is relatively stable within the ranges quoted (except under physiological conditions of egg formation in birds) it is further known that small changes towards either hypo- or hyper-

calcaemia are of major significance in producing clinically detectable reactions. Thus the serum calcium is relatively resistant to gross change induced by addition of calcium salts to the diet. Deficiencies of calcium salts in the diet for short periods of time will not cause a hypocalcaemia. Nevertheless, diet is important in its long term effect, for if the Ca:P ratio of the diet is not maintained close to the optimum 1:1 value, poor calcium absorption results with eventual rickets or osteoporosis, (Park et al., 1923; Shohl and Wolbach, 1936; Irving, 1946). A high protein content in the diet is said to aid calcium absorption (McCance et al., 1942). A diet high in cereal, and thereby rich in phytic acid, results in poor calcium absorption from the intestine (Bruce and Callow, 1934). Marked changes therefore, in the level of serum calcium are frequently, if not always, associated with some pathological state - a prevailing state of rickets or hypoparathyroidism being signfied by a hypocalcaemia. In extreme cases tetany supervenes.

Vitamin D is known to elevate the serum calcium (e.g. Jones, 1944) probably due to an increased calcium absorption from the gut (Nicolayson, 1937). In rachitic subjects vitamin D rapidly raises the serum calcium and promotes healing of the lesions (Petenyl, 1929). Vitamin D was the first vitamin demonstrated to produce severe toxic effects in normal animals (Kreitmair and Moll, 1928) consisting of extensive calcification of the soft tissues and elevated serum calcium values, in many cases to very high levels. Many other symptoms of hypervitaminosis D have been described by Anning and co-workers (1948).

Experiments have been performed on laboratory animals to assess the magnitude of the hypercalcaemia produced by treatment with vitamin D e.g. Smith and Elvove, 1929; Taylor et al., 1931; Schour and Ham, 1934; Steck et al., 1937; Goormaghtigh and Handovsky, 1938; Reed, Struck and Steck, 1939; Mulligan and Stricker, 1948. In addition, much information has been collected from chemical determinations on human patients (Reed, 1934; Dowling and Thomas, 1946; Anning et al., 1948; Ross and Williams, 1939). One feature common to all the data is that although hypercalcaemia is usually produced by vitamin D feeding, the magnitude of the hypercalcaemia is neither constant nor even of a similar order.

Much of the work on hypervitaminosis D in human patients has arisen from the use of large doses of calciferol in the treatment of certain complaints such as lupus vulgaris (Dowling and Thomas, 1946) and arthritis (Steck, 1937)*. Other information is available from the administration of large doses of vitamin D by error, or failure to appreciate its toxic effects. Ross and Williams (1939) for example, reported four cases of poisoning in children fed large amounts of calciferol, two of whom died from the toxic effects. Anning and associates (1948) have listed the published series of patients fed toxic doses of vitamin D, together with the symptoms noted. Serum calcium levels were summarised and reported as:- untreated cases, 10.9 ± 0.17 mg.%; treated cases, 11.9 ± 2.0 mg.% and toxic cases

* See also Anning et al., 1948.

13.2 ± 0.39 mg.%. Higher values for the serum calcium have been reported by other workers. Dowling and Thomas (loc. cit.) for example, obtained some values as high as 30 mg.%, but these authors draw attention to other cases displaying only a moderate hypercalcaemia. In human subjects complications of control makes detailed study difficult. Consequently, details have been accumulated in animals under controlled conditions.

Smith and Elvove (loc. cit.) examined the degree of calcification in rabbits fed vitamin D (as irradiated ergosterol), and compared the serum calcium value with the degree of soft-tissue calcification at post-mortem. The hypercalcaemia, though frequently present, varied in magnitude, and in a few cases, did not arise at all. Selecting dogs as their experimental animals, Steck and associates (1937) fed large doses of vitamin D and determined the maximum serum calcium values. The highest serum calcium value was 22.1 mg.%. Similar experiments reported by Reed, Struck and Steck (1939) on two dogs receiving doses of 60,000 i.u. vitamin D/kilo/day and 30,000 i.u. vitamin D/kilo/day respectively, showed serum calcium values of 14.0 and 14.89 mg.% when death occurred on the 24th and 33rd day respectively. No results, however, were reported between the first and fifth day of the experiment when hypercalcaemia may have been even greater than reported by these investigators. A similar study by Taylor et al. (1931) in which samples of blood were analyses daily placed the hypercalcaemia at a maximum of 20 mg.% on the seventh day of the experiment.

In experimental work on rats, difficulty is experienced in obtaining serial samples of blood adequate for calcium determinations. The paucity of information on the rate of change of serum calcium in this species is presumably due to such difficulties. Part of the value of the present study lay in the design by which the relation between hypercalcaemia and time could be accurately established. As will be discussed fully in the next section, maximum hypercalcaemia developed 24 hours after the fifth and final dose of vitamin D, and therefore in a discussion of the degree of hypercalcaemia, only the values measured at that time are relevant.

It can be seen from the data quoted that the mean serum calcium of 16.6 mg.% at maximum hypercalcaemia is in general agreement with the results obtained by earlier workers in other species treated with vitamin D. Even the high individual result of 21.9 (rat 759/29) is not exceptional for a severe hypercalcaemia.

The hypercalcaemia in hypervitaminosis D is frequently accompanied by an elevation in the serum inorganic phosphorus (Smith and Elvove, loc. cit.) though not necessarily so (Reed, Struck and Steck, loc. cit.). In our experiment the tendency to hyperphosphataemia was clearly present, though the change was small. Albright and Reifenstein (1948) have outlined the case for and against the numerical product of the calcium and phosphorus concentrations as an indicator of whether or not calcium phosphate can be precipitated from the serum. Quoting from their book (v.s.), the normal human serum has a Ca:P product of 10 x 4 = 40. If a rise in either part of the product develops - as

say in hyperparathyroidism - the product is raised and precipitation takes place. Alternatively, a diminished product - as in rickets - results in a lowered ability to form normal bones. While the calcium phosphorus product concept may be valid in some situations - and certainly in the present study there was a rise from 12.0 x 8 = 96 for the normal rat to 16.6 x 10 = 166 for the vitamin D-treated rat - it cannot be a limiting factor as rickets can be healed by the administration of citrates without concomitant change in the low calcium phosphorus product (Harrison, 1953).

Early workers were impressed by the frequency of hypercalcaemia observed during calcification, and from a knowledge of the solubility of calcium phosphate, suggested that calcification developed as the result of precipitation of calcium phosphate from a serum previously supersaturated with respect to calcium and phosphate ions (Howland, 1923). Certainly the serum calcium can be precipitated by physical techniques 'in vitro'. As example, the experiment of Holt, La Mer, and Chown (1925) may be cited in which the shaking of serum with solid calcium phosphate reduced the calcium level from normal to 1.8 - 2.5 mg.% and the phosphate to 1.5 - 2.1 mg.%. It is doubtful, however, if calcification 'in vivo', especially of the soft tissues after vitamin D therapy, proceeds by some similar mechanism. The following review indicates some of the objections to such a postulate.

In 1913, Rona and Takahashi demonstrated that a large proportion of the serum calcium will not diffuse through a semi-permeable membrane. Estimations on both human (Schmidt and Greenberg, 1935) and animals

Updegraff et al., 1926; Benjamin and Hess, 1933; Brown and Ramsdell, 1929) have shown that approximately 45% of the serum calcium is non-diffusible. It is now believed that the non-diffusible calcium is bound to one or more of the plasma proteins, although other components - such as the serum phospholipids have been suggested (Watchorn and McCance, 1932). The weight of evidence, however, supports the view that the serum proteins are the transporting compounds for calcium. A number of pathological conditions involving low serum protein levels also have low calcium concentrations. The ability of the blood proteins to bind calcium 'in vitro' has been demonstrated by several workers (McLean and Hastings, 1935; Martin and Perkins, 1953). The former investigators constructed a nomograph from which the serum ionic calcium can be determined from a knowledge of the total plasma protein and serum calcium levels. Generally, the higher the blood albumin, the higher the serum calcium. Study of the linkage between the protein and the calcium suggest that the binding is weak, as simple dialysis results in complete removal of the calcium into the dialysing solution. Nonetheless, the existence of a calcium-protein complex in the blood provides a rational explanation of serum calcium levels in excess of those possible in simple solution.

Anning and co-workers (loc. cit.) have studied the total diffusible and ionic serum calcium values in normal and vitamin D-treated subjects and concluded that the diffusible calcium concentration in the serum is the most important index of vitamin D toxicity. No significant variation in the ionic calcium level was found after vitamin D treatment.

A feature of Anning's study was the preliminary report of a calcium - steroid complex in the serum, which presumably is diffusible but non-ionic. It was suggested that a change in the level of such a complex is the major serum change induced by vitamin D.

The estimation of serum inorganic phosphorus by Anning and colleagues showed a comparatively wide variation in values. Scatter diagrams of the diffusible serum calcium and the serum inorganic phosphorus showed no relationship between the two. This later conclusion agrees with the general pattern in the present study. In the present experimental work both high serum calcium and inorganic phosphorus values were recorded but no clear relation between the two was apparent.

The present experiment has provided further quantitative data on the magnitude of the change in the serum calcium and phosphorus levels in normal rats fed a defined course of treatment with vitamin D. Despite the obvious limitations inherent in comparing different species treated with varying amounts of vitamin D, there appears to be a marked similarity in the degree of hypercalcaemia found. Presumably therefore, vitamin D acts on the serum in a similar manner relatively independent of dose and irrespective of species.

Summary

The normal level of calcium (12.0 mg.%) and phosphorus (8.2 mg.%) in the serum of the experimental rats employed in this study are in agreement with values recorded in the literature - with the exception of Experiment 733 where values for the serum calcium tended to be High.

After treatment with a course of vitamin D the serum calcium was shown to rise to a mean value of 16.6 mg.% in agreement with the general trend experienced by other workers (vide supra). The serum phosphorus showed a small increase to approximately 10 mg.% from the normal mean value of 8.2 mg.%.

The importance of an elevation in serum calcium and phosphorus in calcification has been reviewed.

(2) The rate of change of serum calcium induced by treatment with massive doses of vitamin D.

The object of the present section is to indicate the rate of change in serum calcium concentrations during, and subsequent to, a course of treatment with vitamin D. Special consideration will be given to the time of maximum hypercalcaemia, as well as the time required for the level of the serum calcium to return to normal.

Enhanced reliability of the results of the main experiment using groups of animals is forthcoming from a number of serial calcium determinations subsequently performed on the same rats. The success of the latter experiment is due partly to the relative ease with which small (0.5 ml.) samples of blood can be obtained from the tail, and partly to the ultra-micro nature of the analytical technique.

Experimental Results.

In the preceding section, it was shown that the serum results obtained in Experiment 733 differed in most respects from Experiments 753, 759 and 777. As, however, Experiment 733 contributed only a maximum of five animals per group to the total - excepting controls -

the remaining values (numbering between 6 and 9) were sufficient to establish the trend in the changing serum calcium and phosphorus levels. Thus, the data of Experiments 753, 759 and 777 will be pooled for descriptive purposes.

Twenty-four hours after the first dose of vitamin D the mean serum calcium level dropped from the control level of 12.0 mg.% to a value of 11.7 mg.%; a value sufficiently close to the control level to be statistically insignificant. Nevertheless, in terms of other work (Taylor et al. 1931; Carlsson and Hollunger 1954) there may have been a marked drop in the serum calcium approximately six hours after the first administration of vitamin D. With each succeeding dose of vitamin D thereafter, the serum calcium rose as indicated in the graph (Fig. 2). The maximum mean value of 16.6 mg.% was reached 24 hours after the fifth and final dose of the vitamin. A few measurements of the serum calcium were made up to 104 days after treatment, but these values, though fluctuating slightly did not show more than a slight increase over the normal. It is possible that a further period of hypercalcaemia developed during the long intervals between the later determinations. Thus the 35th - day value was 13.4 mg.% calcium - slightly above normal - and may indicate that a further cycle of hypercalcaemia followed the main change measured during the firstten days of the experiment. Further experiment would clarify the point.

Experiment 733, in contrast, developed a measure of hypocalcaemia and is represented in the accompanying graph (Fig. 3). Twenty-four

hours after the fifth dose of the vitamin the mean value for the serum calcium was 10.6 mg.%, at a time when the other experiments were displaying a peak hypercalcaemia. Only four days after the last dose of vitamin D did the rats of Experiment 733 show high serum calcium values. At this time a mean of 16.0 mg.% was recorded. Agreement between the pooled experiments and Experiment 733 was only reached 17 days after the vitamin D treatment was suspended, when serum calcium was within the normal range.

The rate of change of the serum calcium in the individual rat has been shown to be essentially the same as in the group experiments described above. A total of four rats have been studied in order to check the nature of the serum calcium change and the results are presented in Table 15.

In these animals blood was drawn from the tail after removal of about 1-2 mm. of tissue from the tip. No attempt was made to estimate the serum magnesium as in the main experiment, due to the extremely small quantity of serum available. Experience has shown that there is little change in the serum magnesium under our conditions and the change in calcium plus magnesium adequately reflects the change in the serum calcium.

Comment

While many investigators have reported altered serum calcium and phosphorus levels (q.v.) after treatment with various amounts of vitamin D, only relatively few studies of the serial changes have been made (Taylor et al., 1931; Schour and Ham, 1934; Reed, Struck and Steck, 1939). The present study recorded the calcium and

phosphorus levels in rats during and after a defined course of treatment with vitamin D. Determinations were made at 24 hour intervals during the period of rapid change, and at longer intervals thereafter over a total period of 108 days. It is therefore possible to describe in some detail the rate of change in serum calcium. the information deduced from measurements on groups of rats has been confirmed by a number of studies of serum calcium changes in the individual rat.

The investigations of Smith and Elvove (1929) are in many ways typical of the approach adopted prior to the present study. Rabbits were employed by these authors and blood samples were taken prior to vitamin D administration. Treatment with the vitamin continued daily either for thirty days or until death of the animal. Meanwhile, blood samples were taken at ten-day intervals. A few animals lived long enough to permit further samples of blood to be drawn six to ten days after treatment. Thus some four to five values for the serum calcium were obtained during the experiment. It was concluded that although some measure of hypercalcaemia was invariably present, the degree varied in a manner which could not be correlated with other known variables. The serum inorganic phosphate in rabbits was also shown to vary widely, but tended to hyperphosphataemia. General agreement with the results of the present work was therefore obtained. Despite much useful information provided by Smith and Elvove, little data was forthcoming from their experiment on the rate of change in the calcium and phosphorus either in the serum or in the tissues. An attempt to remedy the position, at least in so far as the serum

calcium and phosphorus were concerned, was undertaken by Taylor and co-worker (1931). These authors studied the action of irradiated ergesterol on dogs, and its relationship to parathyroid function. In their experiment a large dose of irradiated ergosterol was injected intravenously over a period of four hours and the blood sampled frequently for several weeks. Serum calcium and phosphate determinations were performed on each sample. Hence a graph illustrating the rate of change of the serum calcium and phosphorus was prepared. In spite of the differences between Taylor's experiment and our own in the amount, mode and time of administration of the vitamin D, there is a large measure of similarity in the rate of change in the serum calcium and phosphorus. Taylor (loc. cit.) showed that after the first twenty-four hours the serum calcium of the dog steadily increased and reached a maximum hypercalcaemia of 20 mg.% by the seventh day. Thereafter, values slowly declined, and by the sixteenth day even showed a transient hypocalcaemia. Values thereafter oscillated around the normal value.

In our rat experiments a hypercalcaemia was observed over the first ten days of the experiment; a peak value developing twenty-four hours after the fifth dose of vitamin D. Thus the only essential difference between the results of the present study and those of Taylor and co-workers, in so far as the serum calcium is concerned, appears to be in the time of maxium hypercalcaemia, amounting to only two days despite the wide differences in details of the experiment.(1

(a) Attention may be directed at this stage to the exceptional series of calcium values recorded for the serum in Experiment 733. In this

So outstanding is the similarity in results between dogs and rats that the question is raised as to whether or not the later doses of vitamin D fed to rats in our experiments contributed to the hypercalcaemia It is relevant to recall that Kodicek (1956) has shown that in rats fed large doses of C-14 labelled calciferol, most of the dose was excreted in the faeces and was probably not absorbed.

A feature of Taylor's work (loc. cit,) requires further comment. In the sample of blood obtained during the first twenty-four hours of treatment, Taylor was able to detect a short-lasting but marked hypocalcaemia. Carlsson and Hollunger (1954), while studying the fluctuations in the serum citrate concentration accompanying the hypercalcaemia produced by vitamin D, were able to confirm that a sharp hypocalcaemia develops during the first six hours of vitamin D treatment. Although no estimations were made in our experimental rats during the first day, the serum calcium measured 24 hours after

---

series, the hypercalcaemia observed in Experiments 753, 759, and 777 twenty-four hours after the fifth dose of vitamin D did not develop until five days after the last dose of the vitamin. Not only did the hypercalcaemia develop four days later in Expt. 733 but prior to the eventual hypercalcaemia, a relative hypocalcaemia of 10.6 mg.% (compared with the initial control value of 12.9 mg.%) was recorded twenty-four hours after the fifth administration of vitamin I Thus, in three experiments (viz. 753, 759, and 777) a total of 17 rats showed a mean serum calcium concentration of 16.6 mg.% whereas in 4 rats of Experiment 733 the mean calcium value was 10.6 mg.% 24 hours after the final dose of vitamin D. By contrast, the effect of vitamin D on the calcium content of the tissues did not appear to differ between experiments with the possible exception of the occasional very high value in (say) the aorta which appeared to be confined to Experiment 733. Whether or not the rate of change of the serum calcium in this group is, in part, responsible for a greater degree of tissue calcification is difficult to confirm as it has not been possible to repeat the serum results of Experiment 733.

the first dose of vitamin D was 11.7 mg.%, that is, slightly lower than th normal level of 12.0 mg.%. The possibility exists that a measure of hypocalcaemia was present some time between the administration of vitamin D and the time blood was drawn 24 hours later.

The level of the serum inorganic phosphorus rose during the period of vitamin D administration. The rise was not, however, nearly so well defined as in the case of the serum calcium and no peak value was distinguished. Consequently, it is impossible to indicate the rate of increase in serum phosphorus in these rats. However, whatever the nature of the change, it is small in comparison with that of the serum calcium.

The present work has therefore established for the rat the rate of change in serum calcium and phosphorus after a course of treatment with large doses of vitamin D. It has consequently supplemented earlier studies using larger laboratory animals such as the dog. Comparing the present data with earlier information a marked similarity is apparent despite differences in dose and species. We have tentatively suggested that once sufficient vitamin D to initiate calcification has been fed, further quantities have only a minor effect on the rate (or magnitude) of development of the hypercalcaemia.

Summary.

The rate of change in the level of the serum calcium and phosphorus during, and subsequent to, a course of treatment with large doses of vitamin D has been described. It has been shown that a hypercalcaemia usually develops during the first five days of treatment,

reaches a maximum 24 hours after the fifth dose of the vitamin and then declines to normal. Normal values were recorded by the fifth day after the last administration of the vitamin. These results, deduced from a study of groups of rats were confirmed by a number of serial determinations in the same rat by analysing small quantities of blood sampled from the tail. An interesting series of anomalous results to vitamin D dosage is described in Experiment 733. The serum phosphorus, on the other hand, showed no regular trend although slightly elevated values were obtained. The results in rats are discussed in the light of work described in the literature.

(3). The relationship between the hypercalcaemia and the calcification in the soft tissues.

While a measure of vitamin D is required daily by all animals, excess of the vitamin produces the widespread soft-tissue calcification described earlier. The margin between the normal dose - estimated for children to be 3,000 i.u. of vitamin D_2 per day or 1,000 i.u. of vitamin D_3 per day - and the amount required to produce pathological effects, appears to be very large (Harris and Moore, 1928). Care must be exercised in deciding what is the minimum toxic dose in view of the known soft-tissue calcification which develops after cessation of treatment with vitamin D.

In assessing the degree of calcification developing in the soft tissues, emphasis has always been placed on the level of the serum calcium. Despite the work of many investigators, information is lacking on the time relationship between the changes in the serum

and the rate of calcification in the various soft-tissues after the administration of vitamin D. Without ignoring the complications and reservations inherent in any experimental procedure dependent upon a statistical treatment, the present experiment illuminates many features of the interdependence of serum and tissues calcium and phosphorus concentrations.

Prime importance is placed on the observation of the relatively wide displacement in time between peak hypercalcaemia and the peak of soft tissue calcification. It has already been shown (page 90) that hypercalcaemia is rapidly produced, and reaches a maximum twenty-four hours after the fifth and final dose of vitamin D. The serum calcium did not return to normal levels before the fifth day after the last administration of the vitamin. Thereafter, despite a small measure of instability, the serum calcium remained within, or close to, normal limits.

Calcification of the soft tissues proved to be a slower process. While some tissues, such as the trachea, heart and kidney, showed an immediate response to the vitamin, others, such as the aorta and lung, barely reacted (as compared with the later accumulation of calcium) during the initial three to four days (see also Laas, 1930). After this time, calcification was rapid and extensive. Whatever the nature of the initial calcification may be, all the tissues examined continued to calcify after the period of maximum hypercalcaemia, and even after the serum calcium values had returned to within the normal range. It was therefore established that the action of the vitamin D on the

soft tissues continued for some time after administration of the vitamin.

It has been shown that the lung, trachea, heart, kidney and stomach all calcify maximally ten days after the final dose of vitamin D. In the aorta the mean maximum calcification did not appear to be reached until seventeen days after the final administration of calciferol. In all the tissues examined the time of maximal calcification did not correspond with the time of maximum hypercalcaemia. On the average, there was a time difference of nine days between the peak value in the tissues and the serum. In the aorta, the time displacement was even wider, being sixteen days. Maximum calcification of the soft tissues even developed against a decline in serum calcium concentrations to normal values, and during the last five days of active tissue calcification the serum level was already within normal limits. It would therefore appear that while the level of calcium circulating in the serum may accentuate the soft-tissue calcification produced by vitamin D, it is doubtful if a hypercalcaemia 'per se' can account for the later calcification of the tissues. A number of investigators, even in the early days of vitamin D study, suspected that a hypercalcaemia was not a 'sine qua non' for soft-tissue calcification despite its frequent appearance. Smith and Elvove (loc. cit.) cited six of twenty rabbits treated with irradiated ergosterol, in which massive calcification was observed with only a 7 to 15% increase in the level of the serum calcium. The possibility still remains that the serum calcium was much greater than that recorded, by virtue

of the long period between sampling in their experiments. The same authors quote a further five cases where a hypercalcaemia of seventeen to forty-eight percent was measured without more than a trace of tissue calcification. Attempts to raise the serum calcium by intravenous injection of calcium salts have been made (Heubner and Rona, 1919) in order to produce soft-tissue calcification. The results of such experiments show that calcification cannot be produced by elevating the serum calcium.

In the light of the foregoing information, it appeared worthwhile to re-consider the case of human patients suffering from vitamin D toxicity. As already briefly mentioned (page 69) it appears common in clinical practice to assume that recovery from vitamin D intoxication is complete when in addition to cessation of the many discomforts attendant on vitamin D poisoning, the serum calcium concentration has returned to normal limits.

In human subjects, it is not possible to say with confidence whether such a conclusion is valid. X-ray examination of such organs as the kidney can indicate gross deposition of calcium, but whether such techniques permit more than an approximate assessment of the degree of the calcification is doubtful. On the other hand, experiments such as those described in rats above, show that in animals, tissue calcification continues long after the serum calcium has returned to normal. Although calcium can be resorbed from such calcified tissues, the serum calcium was never grossly elevated after the normal level was attained five days after the last dose of vitamin D. Whether or

not the small degree of hypercalcaemia occasionally noted is indicative of continued tissue calcification or of resorption of calcium is not clear, but it would appear that in the majority of rats the serum calcium after the initial ten days remained within normal limits. By contraat, the calcium content of the tissues failed to return to normal and despite a small measure of calcium resorption, remained well above the pre-treatment level. In fact, the aorta and the trachea were as heavily calcified at the end of the experiment as at any time after the third day following the last dose of vitamin D. If the situation clearly demonstrated in rats applies to the human subject, the recovery from vitamin D poisoning cannot be assumed to have occurred when the serum calcium has returned to normal and after the many clinical symptoms have subsided. Whether the life-span of such rats is jeopardised remains to be determined.

Anning and co-workers (loc. cit.) in their study of vitamin D intoxication in man, regard the level of the diffusible calcium in the serum to be the critical factor in the production of toxic effects. However, the binding between the proteins of the serum and calcium does not seem to be strong and the relationship :-

$$Ca + Protein \rightleftharpoons Calcium\ Proteinate$$

responsible for the non-diffusible and diffusible levels of calcium in the serum can be expected to move towards the left as calcium is deposited in the tissues.

From the foregoing consideration, there does not appear to be any reason why calcium originally associated with the serum proteins

cannot eventually become diffusible and thereby become available for soft-tissue calcification simply on the basis of the Law of Mass Action. In all events, the amount of calcium required for severe calcification of the soft tissues appears to be small (see page 55), and could be removed from the blood over a period of ten to fourteen days in quantities too small to be detected by existing techniques.

Much has been made, by early workers, of the importance of a measure of hyperphosphataemia as a necessary concomitant to the hypercalcaemia in the production of calcified soft tissues. For example, Smith and Elvove (loc. cit.) point out that though six of twenty rabbits treated with vitamin D displayed heavy calcification of the soft tissues with only a 7 - 15% increase in serum calcium, all were associated with an elevation of the serum phosphate. Re-examination of their data, however, shows that even in the case of the extensively calcified animals the rise of the serum inorganic phosphate was small. Thus, one rabbit (F 12) with a maximum measured rise in serum calcium of 1.8 mg.% (from 13.7 mg.% to 15.5 mg.%) and an increase in the serum phosphorus from 5.9 mg.% to 7.1 mg.% was shown at post mortem, to have a very high lung calcium content of 2,006 mg.% and a kidney calcium content of 9,058 mg.% (dry weight). Our own results have tended to the view that the change in serum phosphate levels is of minor importance. There is evidence from the Ca/P ratio in the tissues that the nature of the calcium deposit is either mostly in a non-phosphate form (e.g. aorta) or is bound to phosphate present originally in the tissue as an organic compound (e.g. kidney) as suggested by Robinson (1923).

If either of the above mechanisms is, in fact, operating, there is little need to postulate removal of phosphate from the serum in order for binding to occur in the tissues.

While the level of the relevant ions in the serum may have some importance in the mechanism of soft-tissue calcification, the weight of current opinion is now shifting to the view-point that a change in the tissue itself precedes calcification (Reaven 1953; Sobel et al. 1954; Levine et al. 1949). The possibility of a tissue factor being responsible for calcification has been repeatedly asserted (e.g. Wells 1925). Although aware of the attractiveness of such a postulate, Wells favoured alternative mechanisms such as precipitation of calcium phosphate by inter-tissue pH changes. Nevertheless, he discussed the two possibilities entertained at that time, namely the precipitation of calcium soaps, and the binding of calcium by free phosphate ions generated by the action of phosphatases on organic phosphorus compounds present in the tissues. The case for a tissue factor is presented fully in Section VII and will not be further examined at this stage. It appears to us, however, that the action of vitamin D in promoting soft-tissue calcification is probably two-fold. First, it is responsible for absorption of calcium from the gut (Nicolayson 1937) and, in toxic doses, from the bones into the blood. Secondly, it initiates a change in the soft tissues permitting the binding of calcium. Thus the level of calcium in the serum represents the balance between the rate of removal by the soft tissues and excretion by the kidney on the one hand, and the rate of solution on the other. In this way a hyper- or hypo-

calcaemia is possible depending on whether or not the rate of supply of calcium from the depots exceeds that of deposition in the tissues. If the mechanism just postulated in fact operates, a basis is provided for the failure to observe a consistent pattern between the tissue calcification and the concentration of calcium and phosphorus in the serum. Such a hypothesis is also consistent with the marked localisation of calcium in certain tissues such as the aorta and lung which are not easily explained if calcification is seen as a precipitation of calcium phosphate from a supersaturated serum. If the latter were the case, a similar degree of calcification would be expected in all tissues and this, as we have shown, is not the case.

In conclusion, while we have confirmed that vitamin D in large concentrations does produce hypercalcaemia, the essential feature of the present experiments has been the clarification of the relationship of this hypercalcaemia to the soft-tissue calcification. It now appears clear that the vitamin D induced calcification is a process which is dependent (to a much greater extent than previously recognised) upon a change in the reactivity of the tissue rather than the direct result of an elevated serum calcium. Earlier work, while indicating both the hypercalcaemia and the deposition of minerals in the soft tissues, was inadequate to illuminate the rate process.

Summary.

This study has permitted a comparison between the rate of soft-tissue calcification and the rate of change of the serum calcium concentration. It has been shown that no close relationship in time

exists between the two phenomena, and in fact the time of peak soft-tissue calcification occurs nine days after maximum hypercalcaemia. In the case of the aorta the difference in time is even greater, namely some 16 days.

It is concluded that both the present data and information from the literature support the belief that hypercalcaemia alone is not the critical factor in soft-tissue calcification. A possible mechanism to explain the serum changes has been discussed in terms of a vitamin D stimulated tissue change which permits removal of calcium by the tissues from normal serum calcium levels. The role of the serum inorganic phosphorate was briefly considered and was not found to be significant. Nevertheless, calcification might be influenced by the initial organic phosphorus present in various tissues, particularly the kidney and heart.

TABLE 15.

Serum calcium and magnesium changes in four individual rats receiving 5 x 25,000 i.u. vitamin D.

Rat	Serum Calcium plus Magnesium in mg.%										
	Day 0	1	2	3	4	5	6	7	8	9	10
4	14.5	13.5	12.7	16.0	16.7	20.6	-	16.4	-	14.2	-
8	12.5	13.1	13.5	-	16.3	17.0	15.8	17.6	14.3	-	-
9	13.2	13.3	-	-	15.1	19.3	-	16.7	-	-	13.1
10	13.2	12.9	-	12.9	17.3	25.7	-	18.3	-	-	13.7

FIGURE 2

MEAN SERUM CALCIUM VALUES

EXPTS. 753, 759 & 777

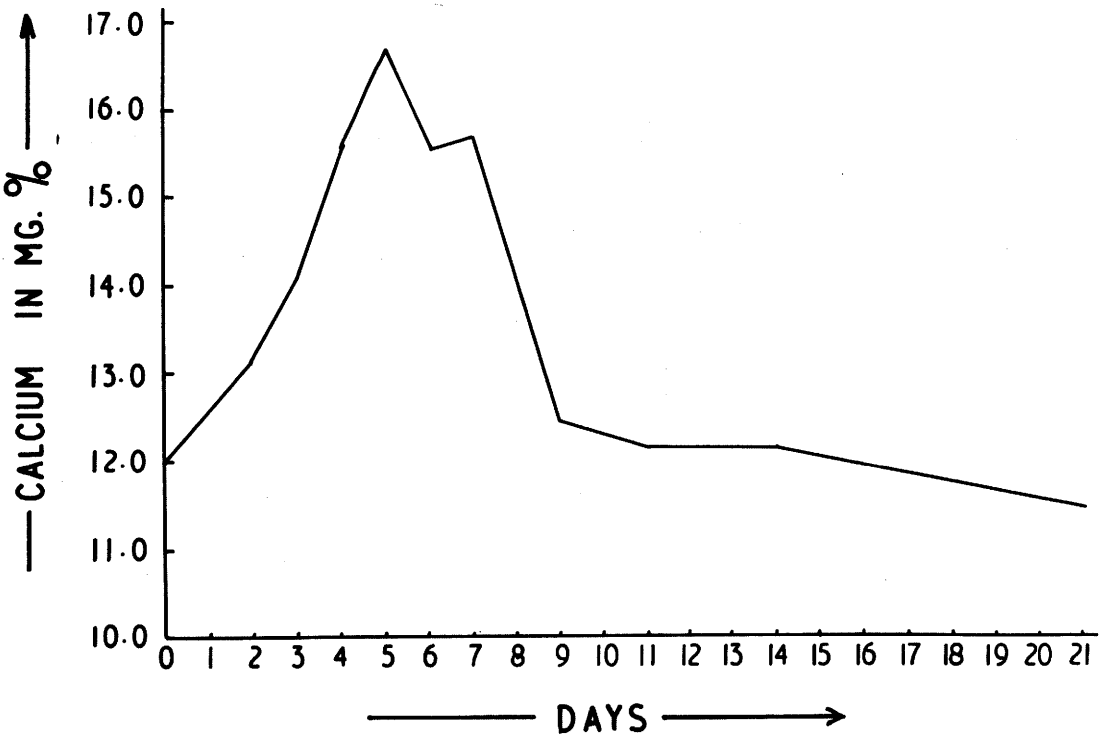
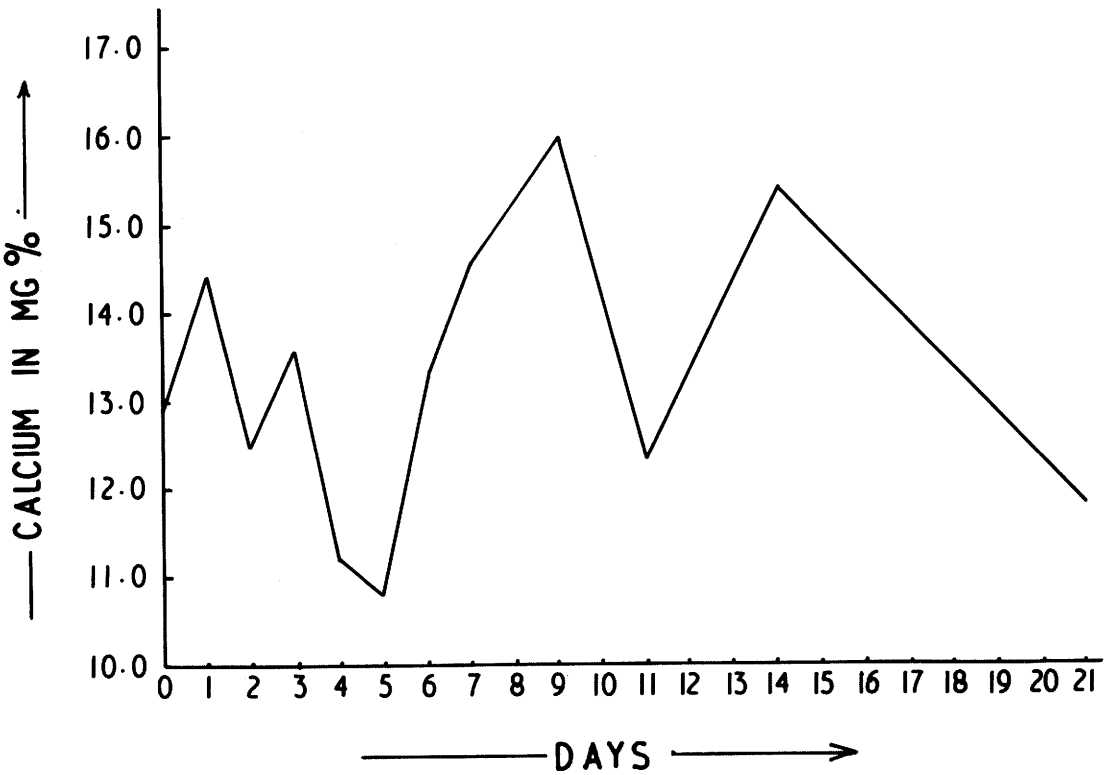


FIGURE 3

MEAN SERUM CALCIUM VALUES

EXPT. 733



SECTION VI

The role of Phosphorus in the calcification mechanism.

Bone is known to consist primarily of an organic matrix saturated with a calcium complex, bound or deposited in this matrix as a part of a solid structure. The mechanism by which 99.0% (Mitchell et al. 1945) of the body's calcium is localised in the bones is still incompletely understood. Nevertheless, it is known that 'bone salt' is composed largely of phosphates. Analysis of the inorganic matter of bone leads to the following composite formula.

$$(Ca_{8.5}\ Mg_{0.25}\ Na_{0.19})\quad ((PO_4)_{5.07}\ (CO_3)_{1.24})\quad (2\ H_2O)^*$$

A small part of the total body calcium is, however, present in the soft tissues. The magnitude of the fraction in the aorta, lung, trachea, heart, kidney, and stomach of the rat is indicated in Tables 17 to 22. It is of importance to discover if pathological calcification develops in a manner similar to ossification, which is a normal and genetically determined process. Pathological calcification is the result of an abnormal metabolism and the deposition of calcium in sites which do not normally undergo calcification.

Two characteristic features of calcification need to be explained. In the first instance, no valid or complete explanation can be given for the specificity of calcium deposition in selected sites. How the normal intake of calcium is directed and fixed in the tissues in an organised manner is, as yet, uncertain. Extension of the question of tissue specificity to the calcification of the soft tissues arising

* Hendricks and Hill, 1942.

pathologically, affords no explanation of the marked differences in "potential" for calcification already described (Section III). In particular, it has been shown that, in the rat, the heart, kidney, and stomach show a significant but small degree of calcification. When compared with the aorta, however, this calcification is small.

The second feature which requires examination is the nature of the calcium complex which is deposited at the site of calcification. Attempts have been made to elucidate the structure of the inorganic complex of bone by X-ray diffraction and other techniques.

Armstrong (1950) has collected some of the possible structures suggested in the literature. Such formulations include mixed calcium carbonate and calcium phosphate $CaCO_3$ n $Ca_3 (PO_4)_2$ where n = 1.86 - 3.33 or m Ca HPO_4 n $Ca_3 (PO_4)_2$ Ca CO_3, and substituted hydrated tricalcium phosphate i.e. $(Ca, Mg, Na)_9 (PO_4, CO_3)_6 (H_2O)$. The full range of possibilities can be obtained from Armstrong's paper.

These structures do not explain all of the properties of 'bone salt', nor do they take into consideration such components as citrate and fluoride which are known to be present. Because of these and other points not discussed, it is unlikely that the above formulations are more than crude approximations. The structures nevertheless, indicate that in bone there is usually a constant ratio of calcium to phosphorus of approximately 2:1.

Study of the complex occurring in calcified soft tissues is less complete than in bone, but due to the presence of phosphate it has been generally accepted that the calcium complex is similar to 'bone

salt', (Kramer and Shear, 1927).

Essentially, the study of the nature of the calcium deposit resolves itself into a study of the anion with which the calcium is associated. It is therefore considered that a study of the phosphate ions both in bone and soft tissues will permit some assessment of the mechanism for the deposition of calcium.

Several mechanisms of both ossification and soft tissue calcification involving phosphates have been proposed at various times and their re-examination is necessary before the results of the present experiment can be discussed. An early theory placed the emphasis on the precipitation of calcium phosphate from the serum (Barr, 1932). It was believed that the serum was supersaturated with respect to calcium and phosphate ions. A local increase in pH by a reduction in the carbon dioxide tension was thought to initiate precipitation (Wells, 1925).

Since about one half of the serum calcium is protein bound (Rone and Takahashi, 1913), and the serum, therefore, not supersaturated with respect to calcium ions, little importance appears to have been given to changes in pH as a causative factor in calcification. Evidence is now forthcoming suggesting that in dental calculus formation, precipitation of calcium phosphate from the saliva is initiated by a change in pH resulting from a loss of carbon dioxide (Hodge and Leung, 1950).

Possibly the mechanism of soft tissue calcification is also dependent upon an antecedent pH change, particularly in such tissues as the lung where a large volume of carbon dioxide is exchanged. The

experimentally determined Ca/P ratio in many soft tissues, however, differs widely from the 2/1 value required by theory if calcium phosphate is deposited.

A further theory of calcification was calcium fixation in the tissues by fatty acids liberated by fat necrosis. Wells and Mitchell (1910) however, demonstrated that calcium soaps implanted in the tissues fail to become fixed and are rapidly absorbed and removed. These results conflict with the observation that calcified tissues do not readily absorb their calcium. More recently, it has been shown (Lansing, 1951) that for intimal thickening with or without fat deposition, at least 1,000 mg.% calcium must be present in the media. Thus the order of deposition of calcium and fat is the reverse of that required by the calcium soap theory. From these and other observations the calcium soap theory has been rejected as untenable.

More convincing are theories of calcification by phosphate binding of calcium within the tissue itself. The original theory as proposed by Robison (1923) suggested that the phosphatases present in many tissues were responsible for the production of phosphate ions from organic phosphate esters present in the tissues. Criticism of the theory arose after it had been demonstrated that calcifiable tissues are frequently deficient in substrate even though the necessary phosphatases are present. A feasible theory explaining calcification on the basis of the local synthesis of substrate material, at least in cartilage, has been developed from investigations in the field of carbohydrate metabolism (Harris, 1932; Gutman and Gutman, 1941).

The transformation of a resting cartilage cell to a hypertrophic cell has long been known to result in the appearance of considerable amounts of glycogen (Harris, 1932) in the cytoplasm. Further observation has shown that immediately prior to calcification, the glycogen disappears. In the period of rapid skeletal growth of the foetus, so great are these glycogen deposits that they become a major repository of the body glycogen. The rather poor blood supply of cartilage makes it unlikely that the deposits are for storage purposes. Nothing is known about the energy requirements of calcification, but it is not generally regarded as a process requiring large amounts of energy. Prima facie, therefore, it is odd that so much glycogen should appear at these sites, and an explanation was not immediately forthcoming. In terms of present knowledge, a valuable clue, not recognised at the time, was provided by Robison and Rosenheim (1934). These workers had demonstrated that calcification of cartilage 'in vitro' can be inhibited by 10^{-3} M cyanide, 10^{-3} M iodoacetate, and 10^{-4} M fluoride. These concentrations are too low to affect phosphatase activity, but are sufficiently high to cause inhibition of several enzymes involved in the classical Embden-Meyerhof breakdown of glycogen to lactic acid. By adding the known intermediate following the inhibited reaction the rate of calcification greatly increases suggesting that the Embden-Meyerhof mechanism is essential for calcification. Further confirmation has been provided by Gutman (1946) who showed also, that phlorrhizin inhibits the 'in vitro' calcification of cartilage and that this inhibition can be overcome by the addition of glucose-1-phosphate.

From these and similar experiments, it appears that the enzyme systems responsible for phosphorylative glycolysis are present in cartilage and are necessary for calcification. It now becomes possible to modify Robison's original theory and explain soft-tissue calcification in terms of the organic phosphate intermediates of glycogenolysis acting as substrates for the alkaline phosphatases. Earlier criticisms of the phosphatases theory are thereby surmounted.

Conflicting evidence, however, has recently been forthcoming in a paper by Sobel et al. (1957) who cast considerable doubt on the importance of the glycogenolytic enzymes in the calcification mechanism. Much of Sobel's evidence rests on the observation that rachitic cartilage loses its ability to calcify 'in vitro' after being refrigerated, despite the observation that the enzymes discussed earlier can be shown to be present. Calcification can be readily initiated once again if, prior to storage, the slice is incubated in calcium chloride solution. There appears to be no explanation of this phenomenon as yet. Further experiments by Sobel showed that demineralised bones, although deficient in glycogenolytic enzymes, were readily re-calcifiable 'in vitro'. Also bone sections heated to 65°C were re-calcifiable 'in vitro' despit destruction of the enzyme system. On the strength of the foregoing experiments, Sobel and co-workers concluded that glycogenolytic enzyme are not part of the minimal system required for calcification.

It has been stressed by Gutman (loc. cit.) that study of the glycolytic enzymes in calcification has been pursued only in cartila and may be a specific mechanism for this tissue. Whether or not s enzymes are relevant to other sites of calcification, and, in part

to vitamin D - induced calcification of the soft tissues, remains to be established.

Further objections to the modified calcification mechanism proposed by Robison (1932) arose from a closer study of the distribution of the phosphatases. In general, the phosphatases are widespread in the body. They appear to be concerned in the hydrolysis of organic phosphate esters to phosphoric acid and the parent alcohol. Two main groups of phosphatases - acid and alkaline - are distinguished on the basis of the characteristic pH for optimum activity. Investigation has shown that it is the alkaline phosphatases which are of importance in the calcification process. The acid phosphatases do not appear to be implicated at all in calcification.

The distribution and activity of the alkaline phosphatases in the tissues of a variety of species has been determined (v.i.). The results, while not in conflict with present theories of the calcification of cartilage and production of bone, give rise to an anomalous situation is the calcification of the soft tissues proceeds by a mechanism dependent upon phosphatases. A consideration of the phosphatases content of the aorta (MacFarlane et al. (1934) typifies the problem: in the dog, rabbit and a variety of other species, the alkaline phosphatases content of the aorta is immeasurably low, whereas in the rat, considerable phosphatase activity has been demonstrated. Nevertheless, under vitamin D treatment all the above species show extensive calcification. In no way does the degree of calcification appear proportional to the level of phosphatase activity in the tissue. A further

observation in the particular situation of hypervitaminosis D needs to be considered. Early workers (e.g. Crimm and Strayer, 1935) showed that in rats treated with high doses of irradiated ergosterol or viosterol, there was a marked reduction in the phosphatase content of the kidney, blood and certain other tissues. Results on bone appear to be inconclusive. No results are available for the aorta. However, there was a three-fold increase in the small intestine, possibly a reflection of the action of vitamin D on calcium absorption. In view of the general lowering of tissue phosphatase activity after vitamin D therapy, the importance of these enzymes is probably small in any mechanism of soft-tissue calcification.

The foregoing review has indicated the role of phosphorus in present theories of calcification. In determining which, if any, of the possible mechanisms appear to operate in the present experiment, considerable information can be gained from a study of the ratio between calcium and phosphorus in each tissue. It is to be recalled that the Ca/P ratio in bone varies little from a value of 2/1. Further, Robison (loc. cit.) has proposed a mechanism by which calcium can be bound to the tissues without change in the total phosphorus concentration. The purpose of the present section is to re-present the data on the calcium and phosphorus content of selected soft tissues of the rat after treatment with a course of vitamin D, in such a way that the importance of the calcium:phosphorus ratio can be assessed. Similarities or differences in composition of the calcium complex from bone, on the one hand, and soft tissues on the other, will thus become apparent. The relevance of the result to the mechanisms of calcification, already described,

will be assessed.

Results:

For convenience of description, no more than three values of calcium and phosphorus concentrations in each tissue will be quoted. These were selected as follows. First, it is necessary that both the initial concentration of the two elements and their ratio be considered as an indication of the prevailing environment under normal conditions. Once vitamin D has been administered and soft-tissue calcification established, the calcium and phosphorus content and their ratio in the tissues at maximum calcification probably yield the greatest information on the nature of the mineral change. While the above two values alone provide sufficient data for an assessment of current theories of the calcification mechanism, further worthwhile deduction can be made from the Ca/P ratio subsequent to peak calcification, a convenient value being the final value measured in each tissue.

Table 16 summarises the data for each tissue.

1. All values and ratios are mean values. It is assumed that the ratio $\Sigma Ca/\Sigma P$ is identical with the ratio Ca/P. If the deposition of calcium and phosphorus occurs in fixed proportion the above assumption is valid irrespective of the proportion of high or low reactive tissues per group.

2. The trachea is the only tissue examined in which the ratio of calcium to phosphorus is similar to that found in bone salt.

3, 4. The heart and kidney showed no significant change in the level of phosphorus throughout the experiment. Thus the change in Ca/P ratio in calcified tissues is due almost entirely to a change in the level of calci

TABLE 16.

The calcium and phosphorus content and their ratio in several different tissues at three selected times during the study.

Tissue	Initial Value[1]			Max. Value[1]			Final Value[1]		
	Ca	P	Ca/P	Ca	P	Ca/P	Ca	P	Ca/P
Trachea[2]	3,900	2,505	1.6/1	7,204	3,421	2.1/1	8,120	3,525	2.3/1
Lung	218	1,375	1/6.3	1,994	1,800	1/1	650	1,340	1/2
Heart[3]	208	1,171	1/5.6	713	1,129	1/1.6	334	980	1/3
Kidney[4]	212	1,357	1/6.4	1,041	1,451	1/1.4	595	1,174	1/2
Stomach	164	831	1/5	811	1,194	1/1.5	348	890	1/2.5
Aorta[5]	237	340	1/1.4	13,571	1,655	8.2/1	20,560	1,701	12/1

5. The aorta showed the greatest change in Ca/P ratio. With progressive calcification the calcium content of the aorta rose to a greater extent than the phosphorus. Thus, 17 days after the final dose of vitamin D had been administered the Ca/P ratio was 8/1. As described earlier (page 67) calcification in the aorta does not appear to regress and very high calcium and phosphorus values, recorded on the 72nd day of the experiment, were 20,562 mg.% and 1,700 mg.% respectively. The calcium/phosphorus ratio was 12/1. As can be seen from the foregoing table no other tissue has a calcium/phosphorus ratio greater than 2.3/1 (trachea).

The significance of the foregoing results is discussed in the following comment.

Comment:

It is now possible to view the Ca/P ratios determined experimentally in the soft tissues of the rat, in the light of current theories of calcification. From such a consideration, an assessment will be made on whether or not one or more of these mechanisms operate in the particular case of vitamin D - induced calcification.

The trachea was the only soft tissue examined in which a 2/1 calcium/phosphorus ratio was observed. Hence, there is a measure of similarity between the calcification of the trachea and osteogenesis in cartilage. The relatively small fluctuation in the ratio of calcium to phosphorus at different stages of calcification in the trachea suggests that there is a parallel relationship between the calcium and phosphorus concentration On the basis of this fact, it appears that a calcium phosphate complex

of some kind was deposited in the tissue during the period of calcification. As shown in Table 19 over the first five days of the experiment the calcium content of the trachea rose in a manner similar to that of the serum calcium. Possibly, therefore, some factor in the trachea binds, or allows the deposition of a 'bone salt' type of complex in the matrix. Even at the end of the experiment the Ca/P ratio was 2.3/1 indicating a large measure of uniformity throughout the experiment in contrast to the situation in the other tissues studied.

An entirely different pattern of calcification was observed in the heart, kidney, stomach and lung. None of these tissues contained as much calcium as the trachea on a weight for weight basis, but invariably the initial calcium content of these tissues was increased by vitamin D by a larger factor. In the heart and kidney, calcification developed without significant change in the total phosphorus content. In the stomach, the increase in phosphorus concentration was very small. A consideration of the heart illustrates the calcification pattern prevailing not only in the heart, but also in the kidney, and, to a lesser extent, in the stomach. While the phosphorus content of the heart remained virtually constant at about 1,100 mg.% P, the calcium content rose from its control level of 208 mg.% to 713 mg.% at peak calcification. Thus, the Ca/P ratio fell from 1/5.6 at the beginning of the experiment to 1/1.6 at maximum calcification without change in the level of tissue phosphorus. If a ratio of 2/1 is "obligatory" for the formation of 'bone salt', then clearly the phosphorus content of the heart greatly exceeds the amount necessary to bind the calcium as 'bone

salt'. It is known that heart muscle is rich in organic phosphorus compounds. The estimation used by the present author did not distinguish between inorganic and organic phosphorus. It is therefore possible that in the heart and tissues possessing a similar calcification pattern, a modified Robison's mechanism operates - that is, the production of phosphate ions 'in situ' by the enzymic hydrolysis of organic phosphate esters and the consequent binding of calcium ions from the serum. The only alternative mechanism is one depending on the binding of calcium by some anion other than phosphate. While this is possible and, in the case of the aorta, likely, there is no apparent need for such a mechanism to be invoked in the case of the heart. Likewise, the Ca/P ratio measured in the kidney and the stomach suggest that a similar mechanism is operating in these tissues during the calcification process.

The ratio of Ca/P in the lung at maximum calcification was 1/1, and while the level of calcium in the lung exceeds that of the kidney, heart and stomach, the ratio indicates that there is also excess phosphorus in the tissues if the calcium is, in fact, bound as a 'bone salt'. In the lung, however, there is some measure of deposition of phosphate as well as of calcium.

As the calcium content of the lung increased more than the phosphorus content, any mechanism dependent upon 'bone salt' deposition can explain only part of the calcification produced by vitamin D. The remainder of the lung calcium can be bound in the tissue in only two other ways. First, part may be bound by a modified Robison's mechanism as has been suggested for the heart, kidney and stomach (v.s.). Secondly,

part of the calcium may be associated with an anion other than phosphate. The present information does not permit a distinction to be made between these two possibilities. Resorption of calcium and phosphorus from the lung also develops in a disproportionate manner, as indicated by the change in Ca/P ratio from 1/1 at maximum calcification to 1/2 after a further fifty-eight days. A re-examination of the values in Table 18 suggests that only a fraction of the calcium resorbed was associated with phosphorus. It follows therefore, that part of the calcium must have been removed from the site of calcification in association with an alternative anion. A valid theory of soft-tissue calcification needs to explain this resorption process.

The aorta showed a marked difference both in its absolute calcium and phosphorus content and its Ca/P ratio as compared with the other tissues studies. Initially, the Ca/P ratio was 1/1.4, but once calcification develops (after four days) Ca ions accumulate in the tissue at a much greater rate than do phosphate ions. Thus, seventeen days after the last dose of vitamin D the Ca/P ratio was 8/1, and sixty-eight days after the last dose was 12/1.

Current theories of phosphate participation in the calcification mechanism clearly cannot explain the calcification noted in the present experiment. The reasons for this conclusion are two-fold. First, the ratio of Ca/P never stabilises at the characteristic 2/1 value, hence eliminating deposition of 'bone salt' as the prime mechanism. Nevertheless, there is some elevation of the total phosphorus of the aorta and part of the calcium may, therefore, be bound by phosphorus.

Secondly, organic/inorganic interconversion of phosphorus cannot explain the aortic calcification as the initial level of phosphorus is too low to be of any significance even if initially it was all organic in nature and during calcification was completely transformed to the inorganic form.

It is concluded that an alternative, and as yet, obscure mechanism of soft tissue calcification is responsible for the binding of large amounts of calcium in the aortae of vitamin D - treated rats. While it is impossible at the present time to offer an explanation of aortic calcification, several histological observations on tissues from animals treated with vitamin D offer an alternative approach. These observations concern the nature of the elastic fibres and the mucopolysaccharides. A discussion of the present state of knowledge of these factors follows in Section VII.

It may be re-stated in summary that the ratio of Ca/P in the trachea after vitamin D treatment supports the hypothesis of a deposition of a calcium phosphate complex of constant composition. In the heart, kidney, stomach and lung the ratio of Ca/P supports, in general terms, the theory of calcium binding by phosphate ions generated by enzymic hydrolysis 'in situ' of organic phosphate esters. The ratio of Ca/P in the aorta (12/1) is very much greater than in any other tissue examined and suggests that the calcium is bound mostly in a non-phosphate form. The nature of the complex however, is still unknown.

SECTION VII.

The local factor in soft-tissue calcification.

The experimental work already discussed, has drawn attention to several important characteristics of calcification in the soft tissues produced by treatment with vitamin D. Under the conditions of our experiment it has been shown that each tissue calcifies in a specific and individual manner. Even those tissues which react only mildly to the vitamin D stimulus (i.e. heart, stomach and kidney) display a detectable degree of variability in the degree of calcification attained. Among the more reactive tissues such as the aorta and the lung, greater variation in the degree of calcification produced by a defined dose of vitamin D was observed. Hence, the soft tissues of the rat were shown to calcify differentially under the action of vitamin D.

We succeeded in demonstrating the rate of calcification in six soft tissues of the rat. The results presented elsewhere (page 56) clearly illustrate that each soft tissue of the rat calcifies at a characteristic rate. The aorta and lung, for example, were shown take 3 to 4 days before the extensive calcification manifested it On the other hand, the trachea started to calcify immediately a the first dose of the vitamin D and continued thereafter to c

stomach and lung rapidly decreased in the seven days following the peak value, although normal values were not reached even 68 days after the last dose of vitamin D.

From the above three considerations of the calcification process it appears that while vitamin D stimulates calcification throughout the soft tissues, the nature of the calcification in any particular tissue is determined by some mechanism within each individual tissue.

The dependence of calcification on a primary tissue change is further emphasised by the relationship between hypercalcaemia and the calcification occurring in the soft tissues. As described earlier, maximum hypercalcaemia develops 24 hours after the fifth and final dose of vitamin D whereas the tissue calcification continues and reaches a maximum after this time. The exact time depends upon the tissue concerned. While the level of calcium in the serum must be of some importance in assisting calcification of the tissues, the data discussed above suggests that the serum calcium level cannot be the determining factor in initiating calcification.

We have already discussed the role of phosphate in the calcification mechanism and theories of phosphate liberation 'in situ' as an explanation of calcification in the soft tissues. The data on calcification of the heart and kidney in the present study support these theories as far as can be seen. It was further shown, however, that calcification of the aorta and lung was not readily explained by theories based on the liberation of phosphate.

This section discusses the role of the mucopolysaccharides and the portein elastin in mediating calcification, particularly in the arteri

(1) The Mucopolysaccharides.

The role of mucopolysaccharide in calcification has been hampered by lack of reliable techniques for the detection and estimation of these compounds. A number of chemical techniques for the estimation of the amino-sugars - an integral and specific part of the mucopolysaccharide molecule - have been proposed (Elson and Morgan, 1933; Dische and Borenfreund, 1950) but the large number of modifications of the original technique (e.g. Blix, 1948; Boas, 1953; Johnston et al., 1951; Schloss, 1951) suggest that improvement in the method is still much desired. Even for histochemical analysis the stains for the mucopolysaccharides are not entirely satisfactory. In part, unsatisfactory results are due to interference from compounds other than mucopolysaccharides, and partly to wide variations in the intensity of staining with minor changes in technique (Heller-Steinberg, 1951). Thus metachromasia, a property of the mucopolysaccharides, can be caused by chemical constituents of the tissue other than mucopolysaccharides especially when gauged by toluidine blue. Marked intensification of metachromasia after toluidine blue staining can be produced by hexametaphosphates (Wiame, 1947). Nevertheless, there is now much evidence that a local tissue factor in calcification is dependent on a complex which in part at least is mucopolysaccharide (e.g. Boyd and Neuman, 1951; Rubin and Howard, 1950; Eisenstein and Groff, 1957).

Histological studies of cartilage (Sylven, 1947; Rubin and Howard, 1950) have shown that the metachromatic effect is most marked in the peripheral uncalcified part of the matrix. If, prior to staining, the

tissue is decalcified, the whole of the matrix displays intense metachromasia (Levine et al. 1949). Prior to the latter experiment it had been noted that as calcification proceeds metachromasia diminishes in both area and intensity (Sylven, 1947).

It was originally concluded that the metachromatic staining substance in cartilage was absent once calcification had occurred. This view has now fallen away for it is more likely that the calcium is bound to active groups in the molecule responsible for the metachromasia. The foregoing possibility has been studied by several workers (v.i.) in attempts to understand further the mechanism of calcification, both 'in vitro' and 'in vivo'. Thus, it has been shown (Miller et al. 1952; Sobel and Berger, 1954) that when a piece of cartilage is incubated in a solution of toluidine blue prior to incubation in a synthetic salt solution containing calcium ions, calcification is inhibited. It was suggested, as a result of these experiments, that there was in cartilage a number of binding sites to which the calcium became attached during the calcification process. The toluidine blue appears to inhibit calcification by occupying the available binding sites before the calcium ions are introduced at the second incubation.

Attempts to inhibit 'in vivo' calcification produced by parathormone by the simultaneous administration of toluidine blue not only failed, but gave rise to enhanced soft tissue calcification. Thus, Baker and co-workers (1953) injected rats with parathormone and toluidine blue. After a given time the animals were killed and compared with rats

treated only with the hormone. Although no quantitative analysis was attempted, it was clear from histological examination that the toluidine blue had greatly potentiated calcification. No explanation of the result has been forthcoming. In passing, it is interesting to observe the contradictions between experiments conducted 'in vitro' and 'in vivo'.

A study of the intensity of metachromasia in parathormone-treated rats led Engel and co-workers (1953) to conclude that prior to calcification depolymerisation of the mucopolysaccharide molecule occurred in the cartilage matrix to yield an increase in the binding sites for calcium. Boyd and Neuman (1951) considered that the binding of calcium by cartilage was associated with the sulphate groups of chondroitin sulphuric acid in the tissues. The nature of the binding is, as yet, uncertain but the above evidence suggests that calcification only occurs where an antecedent chemical change has taken place in the mucopolysaccharides.

Not only is there evidence for the view that the mucopolysaccharides are important for the understanding of calcification of cartilage (v.s.) but also of many soft tissues calcifying pathologically, particularly the stomach, aorta and kidney (Baker et al., 1953). The presence of mucopolysaccharides in the human aorta has been confirmed by extraction and isolation (Dyrbye and Kirk, 1957). The aortae from normal people in the age group 60 to 70 years yielded an average of 4.9 mg. mucopolysaccharide/g. of wet tissue. Relatively little difference with age was noted in the quantity which could be extracted.

Evidence that the mucopolysaccharides are also implicated in vitamin D - induced calcification has been proved by Eisenstein and Groff (1957). These workers repeated earlier studies on the calcification of the heart, kidney and blood vessels and extended their study to demonstrate the presence of the mucopolysaccharides. It was also shown that hypercalcaemia in their animals was accompanied by a hyperseromucoidaemia.

Despite the evidence available and briefly reviewed above, little is known of the function of the mucopolysaccharides in calcification. The presence in the molecule of such acidic groups as sulphate and carboxyl is presumably important, for they would provide suitable electrovalent linkages for calcium ions. If an expansion of the mucopolysaccharide system takes place after the administration of vitamin D, calcification can be readily explained. The evidence of Gillman, Grant and Hathorn (1960) on the amino-sugar content of tissues undergoing calcification suggests that no significant expansion, in fact, occurs. The alternative postulate already raised by Engles et al. (1953) of a depolymerisation appears more probable. The reactive groups in the molecule may be in some way exposed by vitamin D, thus promoting binding of calcium. There is as yet insufficient evidence to suggest more than the above tentative role of the mucopolysaccharides in the mechanism of calcification.

(2) Elastic fibres and elastin in soft-tissue calcification.

Attention has already been drawn to the extreme sensitivity of the arterial tree and especially the aorta, to vitamin D - induced

calcification. The aorta will also show spontaneous calcification as part of the ageing process (Haythorn and Taylor, 1936; Blumenthal et al. 1944). Histological examination of calcified aortae has demonstrated a striking change in the structure of the elastic fibres in the wall as compared with normal aortae. In suitably stained sections it can be seen that much of the calcium deposited in the artery wall lies along the elastic fibres. The affinity of the elastic fibres for calcium in the artery has been stressed by Lansing (1951) after comparing serial sections of arteries stained for elastin with sections micro-incinerated to show only inorganic matter. From such experiments it was concluded first, that there is a marked spatial relationship between inorganic matter, presumably largely calcium, and the elastic fibres. Secondly, that with increasing damage to the elastic fibres there follows a progressive increase in the severity of calcification. The above relationship was observed in studies of human tissues, but an essentially similar pattern can be observed in rats treated with large doses of vitamin D (Vanderveer, 1931; Gillman and Gilbert, 1956). The latter workers have shown that in rats treated with large doses of vitamin D the earliest morphological change appears to be an unwinding of the helical fibril of the elastic fibre. With more extensive damage, fraying and finally lysis of the fibre followed. In severely injured arteries the elastic laminae can be seen to be completely broken, only short lengths being detectable. From these and similar observations lysis of the elastic fibres appears to be a constant feature of arterial calcification. It has been

suggested that reactive groups in the elastin molecule are exposed prior to calcification and subsequently calcium is bound at these points.

Considerable interest has arisen in the elastin solubilising enzyme 'elastase' (Banga, 1952; Balo and Banga, 1954). It has been demonstrated that incubation of unfixed arterial sections with crude elastase produces a lysis of the elastic fibres in the section (Sacks, 1954). This elastolysis is very similar to that appearing in the aorta of the rat fed excessive doses of vitamin D. Possibly arterial calcification involves the presence of an elastase in the tissues.

By incubating a mixture of elastin, elastase and blood, elastolysis - which proceeds rapidly in the absence of the blood - can be seriously inhibited. It was suggested (Banga, Schuler and Laszló, 1954) therefore that blood contains an elastase inhibitor (E.I.). The inhibitory effect of E.I. has been shown to be greatly sensitive to traces of acid. In atherosclerosis in rabbits after an experimentally produced acidosis, a decrease in the blood E.I. was detected simultaneously with the induced hypercholesterolaemia. Banga, Schuler and Laszlo (1954) suggested that elastase and E.I. in the blood forms a system. With the production of an acidosis the E.I. is inactivated and the system is biased in favour of the elastase. Consequently, a lysis of the elastic fibres ensures. As it stands, this hypothesis fails to account for the absence of experimental evidence for calcification in veins. It should be remembered that the pH of venous blood tends to be lower than that of arterial blood and hence the E.I./elastase system should be biased in

favour of more extensive elastolysis in the veins than in the arteries. Structurally, the veins are similar to the arteries, except that having less pressure to withstand, the walls are much thinner.

In pursuance of their studies on elastase, Banga and Balo (1953) estimated the elastase content of the pancreas of human subjects dying of various causes. Three groups were distinguished, namely normals, diseases other than arteriosclerosis and fatal arteriosclerosis. It was shown that arteriosclerosis appears to be associated with a severe reduction in the level of pancreatic elastase from 208 $\pm$ 56 e.u./g. in normal subjects to only 9 $\pm$ 65 e.u./g. in fatal arteriosclerosis. If the level of pancreatic elastase is a critical factor in arterial calcification, the above values indicate a seemingly anomalous situation. Possibly in arteriosclerosis the tissues become hypersensitive to the enzyme and the reduced amount still suffices to cause elastolysis.

At present, the nature of the chemical change in the tissues during and prior to calcification remains obscure. It must, however, bear some relation to the change discernible histologically. A summary suggested by Gillman T and associates (1955) indicated the significant stages of arterial calcification as:-

(1) An alteration in ground substance as shown by changes in the amount and distribution of mucopolysaccharide.

(2) An increase in the amount of collagen present with or without a disturbance in the formation of collagen fibrils, culminating in:-

(3) elastolysis in the affected vascular wall

(4) calcification

So far, investigations into the relation between calcification and elastolysis have been restricted to the arteries. It would be desirable to know whether similar pre-calcification changes in the tissue occur in the lung, tendon and kidney.

However, the important fact which emerges from the foregoing discussion is that a specific change in arterial tissue preceding calcification has been discovered. As such a change has apparently not been observed in calcifying cartilage, a basis has now been found for the difference between normal and pathological calcification of the arteries. Whether a similar change also precedes calcification in other soft tissues normally non-calcifying awaits further investigation

From the foregoing it is clear that elastolysis is vitamin D sensitive. It is suggested that vitamin D is a co-factor in the lysis of the elastic fibres. One of two mechanisms may operate: either the enzyme elastase is activated by vitamin D or the elastase inhibitor of the blood (or possibly in the tissue) is de-activated. Lysis of the fibres may be expected to result in the exposure of reactive groups permitting calcium binding.

The mechanism of elastolysis has already been studied in some detail (Banga and Schuler, 1953; Banga and Balo, 1954). It is clear that elastolysis is not simply a proteolysis, but is a special and specific degradation of the elastin molecule. Analysis of the elastolysate for free amine and carboxyl groups has shown that elastolysis produces a greater proportion of amino to carboxyl groups

in the ratio of two to three times (Banga and Schuler, 1953). An increase in carboxyl groups during lysis might be the basis of the calcification mechanism in the arteries.

Evidence has also been provided of an increase in reducing groups during elastolysis partly correlated with an increase in liberated carbohydrate, the exact nature of which is still unknown. On the basis of their experiments, however, Banga and Schuler (loc. cit.) suggested that the transformation of elastin from a fibrous protein to a globulin form is associated with a release of incorporated carbohydrate. Banga and Schuler appear to believe that elastin is a glycoprotein. If this view is valid, the role of the mucopolysaccharides in arterial calcification may be explained in part in terms of elastin metabolism.

SUMMARY.

The rate and extent of the increase in calcium (plus magnesium), and phosphorus content of the serum and the aorta, lung, trachea, kidney, heart, and stomach was followed in 264 male albino rats by chemical analysis of the tissues during and subsequent to a course of treatment with vitamin D_2 of 25,000 i.u. per day for five days, the groups being killed 24 hours after the first, second, third, fourth and fifth doses and then two days, three days, five days, seven day and at longer intervals up to one-hundred-and-four days after the last dose.

All the tissues examined showed accumulation of calcium and magnesium after treatment with vitamin D. In the aorta the normal mean calcium level of 237 mg.% rose to 31,000 mg.% at maximum calcification. In the remaining tissues the corresponding values are:- lung, 218 mg.% to 1,994 mg.%; trachea, 3,900 mg.% to 10,000 mg.%; kidney, 212 mg.% to 1,100 mg.%; heart, 208 mg.% to 800 mg.% and stomach, 164 mg.% to 811 mg.%. From these results it was concluded that the degree of soft-tissue calcification induced by vitamin D depends upon the nature of the tissue.

Wide variations in reactivity to the vitamin occurred within groups of rats treated identically and was particularly noticable at periods of extensive calcification. By comparing the degree of

calcification in different tissues of the same rat, it was concluded that variations in calcium binding are dependant upon some factor in a particular tissue itself rather than the reactivity of the rat as a whole. Various hormones were assessed as factors determining biological variability in soft-tissue calcification induced by vitamin D.

The study of sequential calcification in the rat during and after treatment with large doses of vitamin D has indicated that the rate of soft-tissue calcification is dependant upon the nature of the tissue undergoing calcification. The trachea calcifies rapidly, reaching a maximum value 48 hours after the final dose of vitamin D. The lung, heart, kidney, and stomach require some ten days after the final dose of the vitamin to calcify maximally. The aorta, in contrast, continues to calcify long after maximum values are reached in the other tissues examined in this study. A value of 31,000 mg.% calcium was recorded 72 days after the final dose of vitamin D for the aorta.

It has been shown that the weight of calcium necessary to produce even the most intense calcification in the tissues is relatively small and readily available from the bones or diet. Attention was directed to the continuing action of vitamin D in producing such calcification subsequent to withdrawal of the vitamin. The period of active calcification can exceed by several times the period of vitamin D administration.

None of the tissues examined showed a complete resorption of calcium during the period studied. Sixty-eight days after the last dose of the vitamin the lung, heart, kidney and stomach still showed a calcium content of two to three times that of untreated animals. The aorta, and to a lesser extent the trachea, showed little, if any, calcium resorption, and in an aorta examined 104 days after treatment the calcium content was 10, 350 mg.%.

The normal level of calcium (12.0 mg.%) and phosphorus (8.2 mg.%) in the serum of the rats studied are in agreement with values recorded in the literature. After treatment with vitamin D the serum calcium was shown to rise to a mean value of 16.6 mg.%, a feature also in agreemen with the general trend experienced by other workers. The serum inorganic phosphorus showed a small increase to approximately 10 mg.%. The importance of these serum elevations in calcification has been reviewed.

The rate of change in the level of the serum calcium and inorganic phosphorus during and subsequent to a course of treatment with large doses of vitamin D has been described. It has been shown that a hypercalcaemia usually develops during the first five days of treatment, reaches a maximum 24 hours after the fifth dose of vitamin and then declines to normal. Normal values were recorded by the fifth day after the last administration of the vitamin. These results, determined from a study of groups of rats, were confirmed by a number of serial determinatio in the same rat by analysing small quantities of blood samples from the tail. An interesting series of anomalous results to vitamin D dosage

is described in Experiment 733. The serum phosphorus, on the other hand, showed no regular trend although slightly elevated values were obtained. The results in rats are discussed in the light of work reported in the literature.

It has been shown that no close relationship exists between the rate of soft-tissue calcification and the rate of change of the serum calcium concentration. In fact, the time of peak soft-tissue calcification occurs about nine days after maximum hypercalcaemia. In the aorta the difference in time was even greater being of the order of 16 days.

It was concluded that both the present data and information from the literature support the belief that hypercalcaemia alone is not the critical factor in producing soft-tissue calcification. A possible mechanism to explain the serum changes has been discussed in terms of a vitamin D-stimulated tissue change which permits removal of calcium by the tissues from normal serum levels. The role of the serum inorganic phosphate was briefly considered and was not considered to be significant, although calcification might be influenced by the initial organic phosphorus in various tissues, especially the kidney.

The ratio of Ca/P in the trachea was 2/1 and supports the hypothesis of a deposition of a calcium phosphate comples of constant composition. In the heart (1/1.6), kidney (1/1.4), stomach (1/1.4), and lung (1/1)

the ratio of calcium to phosphorus supports in general terms the theory of calcium binding by phosphate ions liberated by enzymic hydrolysis "in situ" of organic phosphorus compounds. The ratio of Ca/P in the aorta of 12/1 is very much greater than in any of the other tissues examined and suggests that most of the calcium in the aorta is not bound as phosphate but rather in some, as yet, unidentified form.

The importance of a change in the mucopolysaccharide content of calcifiable tissue and the lysis of the elastic fibres in the arterial wall were discussed as local factors in soft-tissue calcification Elastolysis of arterial elastic fibres, in particular, appear important in providing possible sites for the binding of calcium.

BIBLIOGRAPHY.

ALBRIGHT, F., BLOOMBERG, E., and SMITH, P.H.* Postmenopausal osteoporosis. Trans. Assoc. Am. Phys. 55, 298, (1953).

ALBRIGHT, F., and REIFENSTEIN, E.C. The parathyroid glands and metabolic bone disease. Selected studies. Baltimore, Williams and Wilkins. (1948).

ANDERSON, J., DENT, C.E., HARPER, C., and PHILPOT, G.R. Effect of cortisone on calcium metabolism in sarcoidosis with hypercalcaemia. Possible antagonistic actions of cortisone and vitamin D. Lancet. 2, 720, (1954).

ANNING, S.T., DAWSON, J., DOLBY, D.E., and INGRAM, J.T. Toxic effects of calciferol. Quart. J. Med. 17, 203, (1948).

ARMSTRONG, W.D., Composition and crystal structure of the bone salt. Trans. Conference on Metabolic Interrelations. 2, 11, (1950).

ASHBY, R.O., and ROBERTS, M. Micro determination of calcium in blood serum. J. Lab. Clin. Med. 49, 958, (1957).

ASKANZY, M.* Beiträge zur Knockenpathologie. Festschrift für Jaffe, Braunschweig. (1901).

AUB, J.C., BAUER, W., HEATH, C., and ROPES, M. Studies on calcium and phosphorus metabolism. III. The effect of thyroid hormone and thyroid disease. J. Clin. Invest. 7, 97, (1929).

BAKER, R., REAVEN, G., and SAWYER, J. Effect of toluidine blue on metastatic calcification. Proc. Soc. Expt. Biol. and Med. 83, 281, (1953).

BANGA, I. Isolation and crystallisation of elastase from the pancreas of cattle. Acta Physiol. Acad. Sci. Hung. 3, 317, (1952).

BANGA, I., and BALO, J. Elastin and elastase. Nature, 171, 44, (1953).

BANGA, I., and SCHULER, D., The structure of elastin with special reference to the action of elastase. Acta Physiol. Acad. Sci. Hung. 4, 13, (1953).

BANGA, I., and BALO, J. Studies of the elastolysis of elastase and collagen. Acta Physiol. Acad. Sci. Hung. 6, 235, (1954).

BANGA, I., SCHULER, D., and LASZLO, J. Change of elastase inhibitor in the blood of ammonium hydroxide treated rabbits. Acta Physiol. Acad. Sci. Hung. 5, 1, (1954).

BARR, D.P. Pathological calcification. Physiol. Rev. 12, 593, (1932).

BECK, H., SIMPSON, M.E., LI, C.H., and EVANS, H.M. Antagonism of pituitary adrenocorticotropic hormone (ACTH) to the action of growth hormone on the osseous system of hypophysectomised rats. Endocrinology. 34, 311, (1944).

BENJAMIN, H.R., and HESS, A.F. Forms of calcium and inorganic phosphorus in human and animal sera; normal, rachitic, hypercalcaemic and other conditions. J. Biol. Chem. 100, 57, (1933).

BELANGER, L.F. Autoradiographic visualisation of Ca-45 intake by normal and pathological cartilage in vitro. Proc. Soc. Expt. Biol. and Med. 88, 150, (1955).

BETT, I.M., and FRASER, G.P. A rapid micro method for determining serum calcium. Biochem J. 68, 13p, (1958).

BILLS, C.E. Vitamin D Group. II. Chemistry. page 132. The Vitamins, chemistry, physiology, pathology. vol. 2. Acad. Press, New York. (1954).

BLIX, G. The determination of hexosamine according to Elson and Morgan. Acta Chem. Scand. 2, 467, (1948).

BLUMENTHAL, H.T., LANSING, A.I., and WHEELER, P.A. Calcification of media of human aorta and its relation to intimal atherosclerosis, ageing and disease. Am. J. Path. 20, 665, (1944).

BOAS, N.F. Method for the determination of hexosamines in tissues. J. Biol. Chem. 204, 553, (1953).

BOWLER, J.P., and WALTERS, W. The toxicity and rate of excretion of calcium chloride from the blood stream. Ann. Surg. 80, 545, (1924).

BOYD, E.S., and NEUMAN, W.F. Surface chemistry of bone, ion binding properties of cartilage. J. Biol. Chem. 193, 243, (1951).

BREYER, B., and MC'PHILLIPS, J. Indirect polarographic determination of calcium by chloranilic acid. Analyst. 78, 666, (1953).

BROWN, H.R., and RAMSDELL, S.G. Blood calcium distribution in anaphylaxis in the guinea-pig. J. Expt. Med. 49, 705, (1929).

BRUCE, H.M., and CALLOW, R.K. Cereals and rickets. The role of inositolhexaphosphoric acid. Biochem. J. 28, 517, (1934).

BUCK, R.C. Minerals of normal and atherosclerotic aortas. A.M.A. Arch. Path. 51, 319, (1951).

BUCKLEY, E.S., GIBSON, J.G., and BARTOLOTTI, T.R. Simplified titrimetric technique for the assay of calcium and magnesium in plasma. J. Lab. Clin. Med. 38, 751, (1951).

CARLSSON, A., and HOLLINGER, G. Effect of vitamin D in citric acid metabolism. Acta Physiol. Scand. 31, 317, (1954).

CARR, M.H., and FRANK, H.A. Improved method for the determination of calcium and magnesium in biological fluids by EDTA. titration. Am. J. Clin. Path. 26, 1157, (1956).

CARTLAND, G.F., SPEER, J.H., and HEYL, F.W. Effect of excessive doses of irradiated ergosterol in growing rats and dogs. J. Pharm. and Expt. Therp. 36, 619, (1929).

CARTWRIGHT, G.E., HUGULEY, C.M., ASHENBRUCKER, H., FAY, J., and WINTROBE, M.M. Studies on free erythrocyte protoporphyrin, plasma iron and plasma copper in normal and anaemic subjects. Blood. 3, 501, (1948).

CLARK, E.P., and COLLIP, J.B. A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem. 63, 461, (1925).

CLARK, G.W. The micro determination of calcium in whole blood, plasma and serum by direct precipitation. J. Biol. Chem. 49, 487, (1921).

COHN, G., and KOLTHOFF, I.M. Determination of calcium by precipitation with picrolonic acid and polarographic measurement of the residual picrolonic acid. J. Biol. Chem. 147, 715, (1943).

COLLIP, J.B., PUGSLEY, L.I., SELYE, H., and THOMSON, D.L. Observations concerning the mechanism of parathyroid hormone action. Brit. J. Expt. Path. 15, 335, (1934).

COOKE, A.M. Osteoporosis. Lancet. 1, 877, & 929, (1955).

COPPENS, P.A., and METZ, G.A.* Untersuchungen über die Verteilung des bestrahlen Ergosterins (D-vitamins) im Tierorganismus nach Verbreichung per os. Arch. neerl. de physiol. 18, 407, (1933).

CRIMM, P.D., and STRAYER, J.W. Phosphatase content of blood serum and tissues in the rat following the administration of vitamin D and A. J. Biol. Chem. 112, 511, (1935).

DALE, Sir HENRY, MARBEL, A., and MARKS, H.P. The effect on dogs of large doses of calciferol (vitamin D). Proc. Roy. Soc., London, s. B. 111, 522, (1932).

DANIEL, E.P., and MUNSELL, H.E. Vitamin content of foods, a summary of the chemistry of vitamins, units of measurements, quantitative aspects in human nutrition and occurrance in foods. U.S. Dept. Agr. Misc. Publ. 275, (1937).

DEBRE, R., THIEFFRY, S., BRISSAUD, E., and TRELLU, L. Les troubles morbides determine's par la vitamine D_2 administree a doses trop fortes chez l'enfant. Presse Med. 54, 769, (1946).

DENT, C.E. Some aspects of calcium and phosphorus excretion. Ciba Foundation Symposium: The Kidney. p.242, (1954).

DIEHL, H., and EHLINGBOE, J.L. Indicator for titration of calcium in the presence of magnesium with disodium dihydrogen ethylene-diamine tetraacetic acid. Anal. Chem. 28, 882, (1956).

DISCHE, Z., and BORENFREUND, E. A spectrographic method for the micro estimation of hexosamines. J. Biol. Chem. 184, 517, (1950).

DOWLING, G.B., and THOMAS, E.W.P. Treatment of Lupus Vulgaris with calciferol. Lancet. 919, (1946).

DYRBYE, M., and KIRK, J.E. Mucopolysaccharides of human arterial tissue. I. Isolation of mucopolysaccharides. J. Gerontology. 12, 20, (1957).

EISENSTEIN, R., and GROFF, W.A. Experimental hypervitaminosis D: hypercalcaemia, hypermucoproteinaemia, and metastatic calcification. Proc. Soc. Expt. Biol. And Med. 94, 441, (1957).

ELDJARN, L., NYGAARD, O., and SVIENSSON, S.L. Determination of serum calcium. Scand. J. Clin. and Lab. Invest. 7, 92, (1955).

ELSON, L.A., and MORGAN, W.T.J. A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. 27, 1824, (1933).

ENGEL, M.B., CATCHPOLE, H.R., and JOSEPH, N.P. Effect of parathyroid extract on ground substance and calcium of bone. Trans Macy Conference on Metabolic Interrelations. 5, 119, (1953).

ERDEY, L., and JANKOVITS, L. Determination of small amounts of calcium with pyrazole blue. Acta Chim Acad. Sci. Hung. 4, 235, (1954).

FALES, F.W. A micro method for the determination of serum calcium. J. Biol. Chem. 204, 577, (1953).

FANCONI, G. Variations in sensitivity to vitamin D: from vitamin D resistant rickets, vitamin D avitaminotic rickets and hypervitaminosis D to idiopathic hypercalcaemia. Ciba Foundation Symposium on Bone Structure and Metabolism. (1956).

FARBER, S.J., SCHUBERT, M., and SCHUSTER, N. Binding of calcium by chondroitin sulphate. J. Clin. Invest. 36, 1715, (1957).

FIRSCHEIN, H.E., NEUMAN, W.F., MARTIN, G.R., and MULRYAN, B.J. Studies on the mechanism of action of the parathyroid hormone. Rec. Progs. in Horm. Res. 15, 427, (1959).

FISKE, H.C., and LOGAN, M.H. The determination of calcium by alkalimetric titration-the precipitation of calcium in the presence of magnesium, phosphate, and sulphate with application to the analysis of urine. J. Biol. Chem. 93, 211, (1931).

GAUTIER, J.A., and PIGNARD, P. Novelles methodes pour le dosage du calcium dans le sérum sanguin et les liquides de l'organisme. Ann. pharm. francaises. 11, 438, (1953).

GARDNER, W.U., and PFEIFFER, C.A. Skeletal changes in mice receiving estrogens. Proc. Soc. Expt. Biol. and Med. 37, 678, (1937).

GILBERT, C., and GILLMAN, J. The calcifying action of vitamin D and bile. S. Afr. J. med. Sci. 22, 160, (1957).

GILLMAN, J., and GILBERT, C. (a) Calcium, phosphorus and vitamin D as factors regulating the integrity of the cardiovascular system. Expt. Med. and Surg. 14, 136, (1956).

(b) Periarteritis and other forms of necrotising angeitis produced by vitamin D in thyroxinised rats with an assessment of the aetiology of those vascular lesions. Brit. J. Expt. Path. 37, 584, (1956).

(c) Atherosis in the baboon (Papio ursinus). Its pathogenesis and etiology. Expt. Med. and Surg. 15, 181, (1957).

GILLMAN, T., GRANT, R.A., and HATHORN,M. Histological and chemical studies of calciferol-induced vascular injuries. Brit. J. Expt. Path. 41, 1, (1960).

GILLMAN, T., PENN, J., BRONKS,.D.,and ROUX, M. Abnormal elastic fibres. Appearance in cutaneous carcinoma, irradiation injuries, and arterial disease and other degenerative connective tissue lesions in man. A.M.A. Arch. Path. 59, 733, (1955).

GOORMAGHTIGH, N., and HANDOVSKY, H. Effect of vitamin D_2 (calciferol) in the dog. Arch. Path. 26, 1144, (1938).

GOUGH, J., DUGUID, J.B., and DAVIES, D.R. The renal lesions in hypervitaminosis D. Observations on the urinary calcium and phosphorus excretion. Brit. J. Expt. Path. 14, 137, (1933).

GREENBLATT, I.J., and HARTMAN, S. Determination of calcium in biological fluids. Anal. Chem. 23, 1708, (1951).

GREENE, H.C., and POWER, M.H. The distribution of electrolytes between serum and the 'in vitro' dialysate. J. Biol. Chem. 91, 183, (1931).

GREENWALD, I., and GROSS, J. (a) The effect of thyroidectomy in dogs upon the excretion of calcium, phosphorus, and magnesium. J. Biol. Chem. 66, 185, (1925).

(b) The effect of long continued administration of parathyroid extract upon the excretion of phosphorus and calcium. J. Biol. Chem. 68, 325, (1926).

GRISWOLD, R.L., and PACE, N. Micro determination of calcium and magnesium in tissue ashes. Anal. Chem. 28, 1035, (1956).

GUTMAN, A.B. Relation of phosphorylase and phosphatase to calcification in cartilage. Trans. Macy. Conference on Metabolic Aspects of Convalescence. p20, (1946).

GUTMAN, A.B., and GUTMAN, E.B. Phosphorylase in calcifying cartilage. Proc. Soc. Expt. Biol. and Med. 48, 687, (1941).

HALVERSON, J.C. and BERGUIN, O. The determination of small amounts of calcium, particularly in blood. J. Biol. Chem. 32, 159, (1917).

HANDOVSKY, H., and GOORMAGHTIGH, N. D_2 Vitamin, Schilddruese, und Arteriosklerose. Arch. internat. de pharmacodyn. et de therap. 56, 376, (1937).

HARRIS, H.A. Glycogen in cartilage. Nature. 130, 996, (1932).

HARRIS, L.J. and MOORE, T., "Hypervitaminosis" and "vitamin balance". Biochem. J. 22, 1461, (1928).

HARRISON, H.E. The relationship of vitamin D and parathyroid hormone to citrate metabolism; the treatment of human rickets with citrate. Trans. Macy Conference on Metabolic Interrelations. 5, 307, (1953).

HARRISON, H.E., and HARRISON, H.C. Micro method for the determination of serum calcium. J. Lab. Clin. Med. 46, 662, (1955).

HAYTHORN, S.R., and TAYLOR, F.A. Calcium content of atherosclerotic aortas. Am. J. Path. 12, 303, (1936).

HELLER-STEINBERG, M. Ground substance, bone salts, and cellular activity in bone formation and destruction. Am. J. Anat. 89, 347, (1951).

HENDRICKS, J.B., MORGAN, A.F., and FREYTAG, R.M. Chronic moderate hypervitaminosis D in young dogs. Am. J. Physiol. 149, 319, (1947).

HENDRICKS, S.B., and HILL, W.L. The inorganic constitution of bone. Science. 96, 255, (1942).

HESS, A.F., PAPPENHEIMER, A.M., and WEINSTOCK, M. A study of light-waves in relation to their protective action in rickets. Proc. Soc. Expt. Biol. and Med. 20, 14, (1922).

HEUBNER, W., and RONA, P. Ueber den Kalkgehalt des Blutes bei kalkbehandelten Katzen. Biochem. Ztschr. 93, 187, (1919).

HEYMANN, W. (a). Importance of the liver for the antirachitic efficacy of vitamin D. Proc. Soc. Expt. Biol. and Med. 36, 812, (1937).

(b). Metabolism and mode of action of vitamin D. iii. Importance of the liver for its antirachitic efficacy. Am. J. Dis. Child. 55, 913, (1938).

HIGGINS, G.M., and SHEARD, C. Effect of selective solar irradiation on parathyroid glands of chicks. Am. J. Physiol. 85, 299, (1928).

HODGE, H.C., and LEUNG, S.W.* Calculus formation. J. Periodontol. 21, 211, (1950).

HOFMEISTER, F.* Ueber Ablagerung und Resorption von Kalksatzen in der Geweber. Ergebn. Physiol. 10. 429, (1910).

HOLT, L.E., La MER, V.K., and CHOWN, H.B. Studies in calcification; delayed equilibrium between calcium phosphate and its biological significance. J. Biol. Chem. 64, 567, (1925).

HORNER, W.H. The determination of calcium in biological material. J. Lab. Clin. Med. 45, 951, (1955).

HORWITT, B.N. Determination of inorganic serum phosphate by means of stannous chloride. J. Biol. Chem. 199, 537, (1952).

HOYLE, J.C. The toxic effect of irradiated ergosterol. J. Pharm. and Expt. Therap. 38, 271, (1930).

HOWLAND, J. Etiology and pathogenesis of rickets. Med. 2, 349, (1923).

HULDSCHINSKY, K.* Heilung von Rachitis durch künstliche Hohensonne. Deutsche med. Wchnschr. 45, 712, (1919).

HUNTER, D. The significance to clinical medicine of studies in calcium and phosphorus metabolism. Lancet. 1, 947, (1930).

IRVING, J.T. The effect of dietary restrictions upon the incisor teeth of rachitic rats. J. Physiol. 105, 16, (1946).

JENNESS, R. Titration of calcium and magnesium in milk fractions with EDTA. Anal. Chem. 25, 966, (1953).

JOHNSTON, J.P., OGSTON, A.G., and STANIER, J.E. Modification of the Elson and Morgan method for the estimation of glucosamine. Analyst. 76, 88, (1951).

JONES, J.H. Production of hypercalcaemia with small amounts of vitamin D. J. Nutrition. 28, 7, (1944).

KENNY, A.D., and TOVERUD, S.U. Non-interference of phosphate in EDTA. method for serum calcium. Anal. Chem. 26, 1059, (1954).

KILBRICK, A.C., PALMER, D., and SKUPP, S. Colorimetric determination of calcium in serum. Proc. Soc. Expt. Biol. and Med. 76, 115, (1951).

KIRK, P.L. Quantitative Ultrmicro Analysis. John Wiley and Son. N. York., and Chapman and Hall Ltd., London. (1950).

KODICEK, E. Metabolic studies on vitamin D. Ciba Foundation Symposium on Bone Structure and Metabolism. p 161, (1956).

KRAMER, B., and SHEAR, M.J. Composition of bone II. Analytical results. Proc Soc. Expt. Biol. and Med. 25, 141, (1927).

KRAMER, B., and TISDALL, F.F. (a). A simple technique for the determination of calcium and magnesium in small amounts of serum. J. Biol. Chem. 47, 475, (1921).

(b). Methods for the direct quantitative determination of sodium, potassium, calcium and magnesium in urine and stools. J. Biol. Chem. 48, 1, (1921).

KREITMAR, H., and MOLL, T.* Hypervitaminose durch grosse Dosen Vitamin D. Münsch. Med. Wchnschr. 75, 637, (1928). addendum 1113, (1928).

KYES, P., and POTTER, T.S. Physiological marrow ossification in female pigeons. Anat. Rec. 60, 377, (1934).

LAAS, E.* Die Anfänge der Vigantolschadigung beim Kaninchen. Virchow's Arch. f. path. Anat. 278, 346, (1930).

LANSING, A.I. Changes in the media in human atherosclerosis and hypertension. Macy Foundation Symposium on Factors Regulating Blood Pressure. p 140, (1951).

LASKOWSKI, M.* Ueber den Calciumzustand in Blutplasma der Henne. Biochem. Ztschr. 260, 230, (1933).

LEVENE, P.A* Hexosamines and Mucoproteins. (1925) Longman. London.

LEVINE, M.D., RUBIN, P.S., FOLLIS, R.H. jn., AND HOWARD, J.E. Histochemical studies on calcinosis universalis with respect to the possible relationship between normal and pathological calcification. Trans. Macy Conference on Metąbolic Interrelations. 1, 41, (1949).

LIEFHEIT, H.C. Evaluation of a rapid method for the determination of serum calcium. J. Lab. Clin. Med. 47, 623, (1956).

LIGHT, R.F., MILLER, G., and FREY, C.N. Studies on the effect of overdosage of vitamin D. J. Biol. Chem. 84, 487, (1929).

LOSTROH, A.J., Relationship of steroid and pituitary hormones to myocardial calcification in the mouse. Proc. Soc. Expt. Biol. and Med. 98, 84, (1958).

de LOUREIRO, J.A., and JANZ, G.J. Iodometric and colorimetric methods for the estimation of calcium based on the use of an improved permanganate method. Biochem. J. 38, 16, (1944).

McCANCE, R.A., and WIDDOWSON, E.M. Mineral metabolism of healthy adults on white and brown bread diets. J. Physiol. 101, 44, (1942).

McCHESNEY, E.W., and MESSER, F.H. Metabolism of calcium and phosphorus as influenced by various activated sterols. Am. J. Physiol. 135, 577, (1942).

MACFARLANE, M.G., PATTERSON, L. M. B., and ROBISON, R. Phosphatase activity of animal tissues. Biochem. J. 28, 720, (1934).

MACINTYRE, I. The determination of calcium in biological fluids by flame spectrophotometry. Recueil. 74, 498, (1955).

McLEAN, F.C., and HASTINGS, A.B. Clinical estimation and significance of calcium ion concentrations in the blood. Am. J. med. Sci. 189, 601, (1935).

MANUNTA, G., SAROFF, J., and TURNER, C.W. Relation between oestradiol and parathormone on the retention of Ca-45 in bone and serum of rats. Proc. Soc. Expt. Biol. and Med. 94, 785, (1957).

MARIOTT, W., and HOWLAND, J. A micro method for the determination of calcium and magnesium in blood serum. J. Biol. Chem. 32, 233, (1917).

MARTIN, N. H., and PERKINS, D.J. Calcium binding of human serum albumin in health and disease. Biochem. J. 54, 642, (1953).

MILLER, Z. B., WALDMAN, J., and McLEAN, F.C. The effect of dyes on the calcification of hypertrophic rachitic cartilage in vitro. J. Expt. Med. 95, 497, (1952).

MITCHELL, H.H., HAMILTON, T.S., STEGGERDA, F.R., and BEAN, H.W. The chemical composition of the adult human body and its bearing to the biochemistry of growth. J. Biol. Chem. 158, 625, (1945).

MORGAN, A.F., AXELROD, H.E., and GROODY, M. The effect of a single massive dose of vitamin D on young dogs. Am. J. Physiol. 149, 333, (1947).

MULLIGAN, A.M., and STRICKER, F.L. Metastatic calcification produced in dogs by hypervitaminosis D and haliphagin. Am. J. Path. 24, 451, (1948).

NEUMANN, A.* Eine Verschungsmethode (Säuregemischveraschung) und vereinfachte Bestimmung von Eisen, Phosphorsäure, Salzsäure und anderen Aschenbestandteilen unter Benutzung dieser Säure-gemischveraschung. Z. physiol Chem. 37, 115, (1902-1903).

NICOLAYSON, R. Studies on the mode of action of vitamin D; influence of vitamin D on absorption of calcium and phosphorus in the rat. Biochem. J. 31, 122, (1937).

ORANGE, M., and RHEIN, H.C. Micro estimation of magnesium in body fluids. J. Biol. Chem. 189, 379, (1951).

PARK, E.A., GUY, R.A., and POWERS, G.F. A proof of the regulatory influence of cod liver oil on calcium and phosphorus metabolism. Am. J. Dis. Child. 26, 103, (1923).

PATTON, J., and REEDER, W. Indicator for titration of calcium with (ethylenedinitrilo)-tetra acetate. Anal. Chem. 28, 1026, (1956).

PETÉNYL, G.* Die Speicherung des bestruhlten Ergosterin in menschlichen Organesmes. Orwsi Letil. 2, 871, (1929).

PFEIFFER, C.A., and GARDNER, W.U. Skeletal changes and blood serum calcium levels in pigeons receiving estrogens. Endocrinol. 23, 485, (1938).

PINCUS, J.B., NATELSON, S., and LUGOVOY, J.K. V. Effect of epinephrine, ACTH. and cortisone on citrate, calcium, glucose and phosphate levels in rabbits. Proc. Soc. Expt. Biol. and Med. 78, 24, (1951).

RAVINA, A. L'hypervitaminose D. Presse med. 44, 1471, (1936).

REAVEN, G. Influence of normal and altered connective tissue on metastatic calcification. Bulletin of the Alumni Assoc., School of Medicine, University of Chicago 9, 10, (1953).

REED, C.I. The symptoms of viosterol overdosage in human subjects. J. A. M. A. 102, 1745, (1934).

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. 1939.

REED, C.I., THACKER, E.A., DILLMAN, L.M., and WELCH, J.W. The effect of irradiated ergosterol on the metabolism of normal dogs. J. Nutrition. 6, 355, (1933).

REMP, D.G., and MARSHALL, I.N. The antirachitic activity of various forms of vitamin D in the chick. J. Nutrition. 15, 525, (1938).

ROBISON, R. The possible significance of hexosephosphoric acid esters in ossification. Biochem. J. 17, 286, (1923).

ROBISON, R., and ROSENHEIM, A.H. Calcification of hypertrophic cartilage in vitro. Biochem. J. 28, 684, (1934).

RONA, P. and TAKAHASHI, D. Beitrag zur Frage nach dem Verhalten des Calciums im Serum. Biochem Ztschr. 49, 370, (1913).

ROSS, S.G., and WILLIAMS, W.E. Vitamin D intoxication in infancy. Am. J. Dis. Child. 58, 1142, (1939).

RUBIN, P.S., and HOWARD, J.E. Histochemical studies on the role of acid muco-polysaccharides in calcifiability and calcification. Trans. Macy Conference on Metabolic Interrelations. 2, 155, (1950).

RYMER, M.R., and LEWIS, R.C. Studies on the calcium content of red blood corpuscles. J. Biol. Chem. 95, 441, (1932).

SACKS, N. Further observations on the nature of elastic fibres. S. Afr. J. med. Sci. 19, 166, (1954).

SALMON, T.N. The effect on the growth rate of thyro-parathyroidectomy in newborn rats and of subsequent administration of thyroid, parathyroid and anterior hypophysis. Endocrinol. 23, 446, (1938).

SCHAAL, G.* Die Beeinflussung der Vigantolwirkung durch Schilddrüsenpräparate. Ztschr. f. Kinderh. 56, 55, (1934).

SCHENCK, F. Ueber das kristallisierte Vitamin D_3. Naturwissenschaften. 25, 159, (1937).

SCHLOSS, B. Colorimetric determination of glucosamine. Anal. Chem. 23, 1321, (1951).

SCHMIDT, C.L.A., and GREENBERG, D.M. Occurance, transport and regulation of calcium, magnesium and phosphorus in the animal organism. Physiol. Rev. 15, 295, (1935).

SCHOUR, I., and HAM, A.W. Action of vitamin D and of parathormone on calcium metabolism as interpreted by studying the effect of single doses on the calcification of dentin. Arch. Path. 17, 22, (1934).

SCHWARZENBACH, G., BIEDERMANN, W., and BANGERTER, F. Neue einfache Titriermethoden zur Bestimmung der Wasserhärte. Helv. Chim. Acta. 29, 811, (1946).

SEBRELL, W.H., and HARRIS, R.S. The Vitamins. vol. 2. p. 203. Acad. Press. N. York. (1954).

SENDROY, J. Jnr. Determination of serum calcium by precipitation with oxalate- a comparative study of factors affecting the results of several procedures. J. Biol. Chem. 152, 539, (1944).

SHOHL, A.T. The effect of hydrogen ion concentration upon the determination of calcium. J. Biol. Chem. 50, 527, (1922).

SHOHL, A.T., and WOLBACH, S.B. Rickets in rats; effect of low calcium - high phosphorus diets at various levels and ratios upon production of rickets and tetany. J. Nutrition. 11, 275, (1936).

SMITH, M.I., and ELVOVE, E. The action of irradiated ergosterol in the rabbit. U.S. Public Health Repts. 44, 1245, (1929).

SMITH, R.G., CRAIG, P., BIRD, E.J., BOYLE, A.J., ISERI, L.T., JACOBSON, S.D., and MYERS, G.B. Spectrochemical values for sodium, potassium, iron, magnesium, and calcium in normal human plasma. Am. J. Clin. Path. 20, 623, (1950).

SOBEL, A.E., and BURGER, M. Calcification XIV. Investigations of the role of chondroitin sulphate in the calcifying mechanism. Proc. Soc. Expt. Biol. and Med. 87, 7, (1954).

SOBEL, A.E., BURGER, M., DEANE, B.C., ALBAUM, H.G., and COST, K. Calcification XVIII. Lack of correlation between calcification in vitro and glycolytic enzymes. Proc. Soc. Expt. Biol. and Med. 96, 32, (1957).

SOBEL, A.E., and HANOK, A. A rapid method for the determination of ultra-micro quantities of calcium and magnesium. Proc. Soc. Expt. Biol. and Med. 77, 737, (1951).

SOBEL, A.E., ROCKHEIMER, M., and KRAMER, B. Carbonate content of bone in relation to composition of blood and diet. J. Biol. Chem. 158, 475, (1945).

STECK, I.E., DEUTSCH, H., REED, C.I., and STRUCK, H.C. Further studies on intoxication with vitamin D. Ann. Int. Med. 10, 951, (1937).

STOERK, H.C., and CARNES, W.H. The relation of the dietary Ca:P ratio to serum calcium and to parathyroid volume. J. Nutrition. 29, 43, (1945).

STOLTE, K.* Eine einfache und zuverlässige Methodik der Achen-analyse. Biochem. Ztschr; 35, 104, (1911).

SYLVEN, B. (a) Cartilage and chondroitin sulphate; physiological role of chondroitin sulphate in cartilage. J. Bone and Joint Surg. 29, 745, (1947).

(b) Cartilage and chondroitin sulphate; chondroitin sulphate and physiological ossification of cartilage. Ibid. 29, 973, (1947).

TALMAGE, R.V., and ELLIOT, J.R. Influence of parathyroids on intestinal absorption of radiocalcium and radiostrontium. Fed. Proc. 17, 160, (1958).

TAYLOR, N.B., WELD, C.B., and SYKES, J.F. (a) The relation of the parathyroid glands to the action of irradiated ergosterol. Proc. Roy. Soc., London. s.B. 116, 10, (1934).

(b) The relation of bile to the absorption of vitamin D. Brit. J. Expt. Path. 16, 302, (1935).

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. Canad. Med. Ass. J. 24, 763, (1931).

TORO, G., ACKERMANN, P.G., and KOUNTZ, W.B. Effect of some hormones on calcium balance in elderly subjects. Proc. Soc. Expt. Biol. and Med. 97, 819, (1958).

TREVAN J.W., and BAINBRIDGE, H.W. Estimation of calcium in blood serum. Biochem. J. 20, 423, (1926).

TUCKER, B.M. Calcein as an indicator for the titration of calcium with EDTA. Analyst. 82, 284, (1956).

UPDEGRAFF, H., GREENBERG, D.M., and CLARK, G.W. A study of the distribution of the diffusible and non-diffusible calcium in the blood sera of normal animals. J. Biol. Chem. 71, 87, (1926).

VALLEE, B.L., and GIBSON, J.G. Zinc content of normal human whole blood, plasma, leucocytes and erythrocytes. J. Biol. Chem. 176, 445, (1948).

VANDERVEER, H.L. Hypervitaminosis D and arteriosclerosis. Arch. Path. 12, 941, (1931).

VAN SLYKE, D.D., and SENDROY, J. Jnr. Gasometric determination of oxalic acid and calcium, and its application to serum analysis. J. Biol. Chem. 84, 217, (1929).

WATCHORN, E., and McCANCE, R.A. Inorganic constituents of cerebrospinal fluid. II. The ultrafiltration of calcium and magnesium from human sera. Biochem. J. 26, 54, (1932).

WELLS, H.G. Chemical Pathology. (1925). Chap. XIX. W.B. Saunders and Co. Philadelphia and London.

WELLS, H.G., and MITCHELL, J.H. Studies on calcification and ossification III. J. Med. Research. Boston. 22, 501, (1910).

WIAME, J.M. The metachromatic reaction of hexametaphosphate. J. Am. Che. Soc. 69, 3146, (1947).

WILKINS, L. Epiphyseal dysgenesis associated with hypothyroidism. Am. J. Dis. Child. 61, 13, (1941).

WINDAUS, A. Chemistry of irradiated ergosterol. Proc. Roy. Soc. (London). B108, 568, (1931).

WINDAUS, A., and TRAUTMANN, G. Ueber das Krystallisierte Vitamin D. Ztschr. f. physiol. Chem. 247, 185, (1937).

YANAGISAWA, F., OGASAWARA, K., and FUGII, K. Seasonal fluctuation of serum calcium and magnesium values. Ijaku to Seibutsugaku (Med and Biol.). 30, 130, (1954). Full abstract. Chem. Abstr. 50, 8845^{gh} (1956).

ZEEK, P.M. Chemical analysis of atherosclerotic lesions in human aortae. Am. J. Path. 12, 115, (1936).

Note. Publications marked with an asterisk (*) were unavailable and could not be consulted in the original.

APPENDIX.

AORTA								
DAYS	CALCIUM (in mg.%)				PHOSPHORUS (in mg.%)			
	No. of Samples	Mean	Range	High / Low	No. of Samples	Mean	Range	High / Low
0	10	237	132-600	3/7	15	340	135-595	7/7
1	5	268	125-536	2/3	9	300	205-400	4/4
2	6	306	177-415	3/3	9	342	160-605	5/4
3	9	640	157-1,295	4/5	8	341	180-625	4/4
4	10	727	345-1,070	6/4	14	372	160-610	6/8
5	9	2,397	555-3,900	5/5	14	1,062	200-2,905	8/6
6	14	2,811	1,335-4,375	6/8	15	951	610-1,580	6/9
7	13	5,020	500-10,180	7/6	14	1,590	300-2,700	7/7
9	10	4,758	360-8,925	5/5	10	2,026	956-3,180	4/6
11	8	5,384	2,245-8,655	4/4	8	1,530	900-2,300	4/4
14	9	8,437	2,550-29,170	3/6	9	1,533	1,100-2,240	4/5
21	7	13,571	2,590-31,230	3/4	8	1,655	475-2,620	4/4
35	3	8,189	6,995-8,810	2/1	4	1,486	885-1,965	2/2
54	4	14,484	4,775-24,160	2/2	4	1,544	1,065-2,380	1/3
72	4	20,562	6,790-27,130	3/1	4	1,701	890-2,070	3/1
108	1	10,350	-	-	1	3,035	-	

Mean calcium and phosphorus values in the Aorta during and after vitamin D treatment.

TABLE 17

LUNG								
DAYS	CALCIUM (in mg.%)				PHOSPHORUS (in mg.%)			
	No. of Samples	Mean	Range	High / Low	No. of Samples	Mean	Range	High / Low
0	10	218	155-315	3/7	11	1,375	1,125-1,700	5/6
1	13	336	160-460	7/6	12	1,204	960-1,530	5/7
2	13	347	192-705	4/9	13	1,319	1,086-1,710	5/8
3	11	293	220-410	5/6	11	1,363	1,065-1,710	7/4
4	15	683	350-1,690	3/12	15	1,330	760-2,085	6/9
5	14	1,052	240-2,225	5/9	13	1,523	1,245-1,990	5/8
6	15	1,708	235-4,370	7/8	15	1,961	1,045-3,005	7/8
7	14	1,368	290-2,970	7/7	13	1,714	900-2,800	6/7
9	10	1,357	405-5,423	2/8	10	1,627	1,185-3,050	3/7
11	8	1,268	215-2,835	2/6	8	1,513	1,125-2,140	3/5
14	9	1,994	345-4,835	4/5	9	1,800	1,000-2,860	4/5
21	7	955	175-3,720	2/5	7	1,558	975-3,120	1/6
35	4	749	470-955	2/2	4	1,284	1,075-1,535	2/2
54	4	920	400-1,735	2/2	4	1,375	1,154-1,700	2/2
72	4	1,370	370-3,977	1/3	3	1,245	1,110-1,400	1/2
108	1	650	-	-	1	1,340	-	-

Mean calcium and phosphorus values in the Lung during and after vitamin D treatment.

TABLE 18

TRACHEA								
DAYS	CALCIUM (in mg.%)				PHOSPHORUS (in mg.%)			
	No. of Samples	Mean	Range	High / Low	No. of Samples	Mean	Range	High / Low
0	10	3,900	1,695-5,510	7/3	11	2,505	1,200-3,440	6/5
1	12	4,869	3,230-7,800	5/7	13	2,423	1,655-3,480	6/7
2	13	5,123	4,140-6,935	6/7	13	2,426	1,365-3,225	6/7
3	11	5,469	5,000-6,000	4/7	11	2,911	2,305-3,660	6/5
4	15	5,778	2,935-8,925	8/7	15	2,755	1,615-3,570	9/6
5	14	6,280	4,145-8,070	9/5	14	3,081	1,380-5,320	7/7
6	15	6,400	4,720-7,690	8/7	15	3,089	2,365-4,435	8/7
7	14	6,373	5,055-8,330	6/8	14	3,230	1,750-4,370	7/7
9	9	6,974	4,345-9,745	5/4	10	3,228	2,545-4,080	4/6
11	8	6,074	5,100-7,160	3/5	8	3,481	2,435-4,650	3/5
14	9	7,204	5,120-10,200	4/5	9	3,424	2,490-4,675	5/4
21	8	6,167	4,475-8,630	3/5	8	2,818	2,025-3,440	3/5
35	4	5,931	3,765-7,140	2/2	4	2,952	2,240-3,710	2/2
54	4	8,509	7,615-10,070	1/3	4	4,140	3,590-5,480	1/3
72	4	6,540	4,615-9,450	2/2	4	3,765	3,250-4,440	1/3
108	1	8,120	-	-	1	3,525	-	-

Mean calcium and phosphorus values in the Trachea during and after vitamin D treatment.

TABLE 19

HEART								
DAYS	CALCIUM (in mg.%)				PHOSPHORUS (in mg.%)			
	No. of Samples	Mean	Range	High Low	No. of Samples	Mean	Range	High Low
0	10	208	145-290	3/7	11	1,171	975-1,295	7/4
1	5	347	210-445	3/2	5	1,009	875-1,270	2/3
2	5	498	192-755	2/3	5	1,070	990-1,140	3/2
3	5	220	185-240	3/2	5	1,101	900-1,195	3/2
4	5	323	265-410	2/3	5	1,037	820-1,235	2/3
5	5	459	330-710	2/3	5	1,107	1,040-1,160	3/2
6	5	386	200-800	2/3	5	1,137	1,020-1,385	1/4
7	5	374	260-580	1/4	5	1,070	925-1,235	2/3
9	5	387	305-460	3/2	5	1,046	985-1,160	2/3
11	5	502	365-630	3/2	5	1,024	940-1,260	1/4
14	5	713	510-800	3/2	5	1,129	880-1,400	2/3
21	5	447	335-540	4/1	5	1,188	1,080-1,325	2/3
35	4	411	340-495	1/3	4	998	900-1,080	2/2
54	4	439	250-655	2/2	4	989	830-1,115	2/2
72	4	334	265-370	3/1	4	980	930-1,105	1/3
108	-	-	-	-	-	-	-	-

Mean calcium and phosphorus values in the Heart during and after vitamin D treatment.

TABLE 20

KIDNEY								
DAYS	CALCIUM (in mg.%)				PHOSPHORUS (in mg.%)			
	No. of Samples	Mean	Range	High / Low	No. of Samples	Mean	Range	High / Low
0	10	212	150-304	4/4	11	1,357	1,125-1,530	7/4
1	5	362	190-505	2/3	5	1,126	1,025-1,365	1/4
2	5	445	195-730	2/3	5	1,303	1,130-1,500	3/2
3	5	257	180-350	3/2	5	1,268	1,045-1,540	2/3
4	5	307	270-340	4/1	5	1,102	1,030-1,235	2/3
5	5	482	370-675	2/3	5	1,162	1,010-1,300	3/2
6	4	692	385-1,180	2/2	5	1,141	1,035-1,280	2/3
7	5	495	305-700	2/3	5	1,220	1,035-1,540	2/3
9	5	779	590-1,180	2/3	5	1,363	1,271-1,480	3/2
11	5	746	555-885	2/3	5	1,103	870-1,220	4/1
14	5	1,041	470-1,915	2/3	5	1,451	990-2,080	3/2
21	5	816	605-1,300	2/3	5	1,388	1,165-1,870	2/3
35	4	586	360-820	2/2	4	1,274	1,105-1,415	2/2
54	4	802	380-1,125	3/1	4	1,129	940-1,450	2/2
72	4	595	405-980	1/3	4	1,174	1,090-1,285	2/2
108	-	-	-	-	-	-	-	-

Mean calcium and phosphorus values in the Kidney during and after vitamin D treatment.

TABLE 21

STOMACH								
DAYS	CALCIUM (in mg.%)				PHOSPHORUS (in mg.%)			
	No. of Samples.	Mean	Range	High / Low	No. of Samples.	Mean	Range	High / Low
0	6	164	140-192	2/4	6	831	635-1,019	4/2
1	4	365	310-455	1/3	4	971	905-1,005	3/1
2	4	346	285-440	2/2	4	991	950-1,065	2/2
3	4	282	245-360	1/3	4	1,057	850-1,145	3/1
4	4	298	220-450	1/3	4	1,074	870-1,200	3/1
5	4	642	325-860	2/2	4	1,267	1,030-1,480	2/2
6	3	350	295-430	2/2	4	1,157	910-1,275	1/3
7	4	421	350-490	2/2	4	1,071	1,010-1,165	2/2
9	4	460	355-545	2/2	4	1,091	970-1,200	2/2
11	4	710	410-1,125	2/2	4	1,054	1,015-1,100	2/2
14	4	811	320-1,440	2/2	4	1,194	975-1,325	3/1
21	4	382	300-455	2/2	4	994	795-1,335	1/3
35	4	381	320-445	2/2	4	1,124	900-1,300	3/1
54	4	385	270-475	2/1	4	1,088	880-1,220	3/1
72	4	343	180-520	1/3	4	890	730-1,030	2/2
108	-	-	-	-	-	-	-	-

Mean calcium and phosphorus values in the Stomach during and after vitamin D treatment.

TABLE 22

TABLE 23

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/12	-	400	180	1260	4290	2840	200	1270	210	1365			14.3	16.9
733/13	-	-	315	1365	4250	1955	165	1250	235	1405			13.7	11.9
733/14	132	566	208	1700	5510	2860	204	1270	194	1490			14.3	10.0
733/15	-	430	280	1495	3375	2785	145	1295	304	1330			12.0	10.5
733/16	-	595	-	1433	-	2360	-	1055	-	1530			13.2	12.5
733/17	-	405	210	1130	2080	2055	235	975	230	1125			12.86	11.9
733/18	-	490	195	1125	4530	1850	290	990	210	1150			11.51	10.4
733/19	190	340	190	1130	1695	3440	280	1110	200	1250			11.57	8.0
733/20	300	312	155	1330	4295	1200	200	1175	220	1415			12.32	12.0
733/21	325	170	230	1600	4105	3360	175	1275	170	1380			-	-
733/22	600	420	215	1555	4900	2855	190	1220	150	1490			13.54	10.0
753/1													12.3	6.6
753/3													12.7	7.85
753/4													11.9	6.25
759/1	180	205											11.55	8.8
759/2	175	245											12.05	8.8
759/3	195	155											12.39	8.5
759/4	135	225											11.99	8.5
759/5	135	135											10.88	8.1
777/25											149	774	12.45	7.5
777/26											192	1019	12.37	7.75
777/27											188	836	11.96	7.5
777/28											155	864	11.98	8.05
777/29											140	635	11.98	12.0
777/30											162	858	11.67	9.1

CONTROL ANIMALS

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/1	536	400	160	1260	3715	2840	210	1270	190	1365	-	-	12.84	16.9
733/83	-	300	450	1095	5075	2540	360	910	480	1025	335	990	14.69	13.4
733/84	-	370	305	1155	3230	2850	430	1100	505	1105	360	985	14.98	10.0
733/85	225	360	330	1055	4435	3480	445	890	340	1065	455	1005	13.29	13.4
733/86	300	340	457	960	4580	1945	290	875	295	1070	310	905	12.56	12.0
753/6	-	-	394	1175	-	2410	-	-	-	-	-	-	11.70	9.7
753/7	-	-	219	1170	6880	1950	-	-	-	-	-	-	11.72	8.7
753/8	-	205	175	1205	7800	1655	-	-	-	-	-	-	8.96	6.9
753/9	-	280	360	-	3650	2470	-	-	-	-	-	-	12.02	5.6
753/10	-	-	245	1330	3845	1820	-	-	-	-	-	-	8.96	6.9
759/6	125	210	460	1345	5945	3175	-	-	-	-	-	-	10.92	8.7
759/9	155	225	365	1300	5205	2200	-	-	-	-	-	-	11.55	8.4
759/10	-	-	445	1200	4070	2170	-	-	-	-	-	-	12.14	10.0

1 x 25,000 i.u. Vitamin D_2. Killed 24 hours later.

TABLE 24

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/2	-	466	192	1710	4200	1365	192	1130	195	1500	-	-	13.37	13.7
733/91	415	605	705	1525	5025	2060	755	1140	705	1360	440	950	14.52	14.25
733/92	345	495	450	1275	5440	1670	700	1010	730	1180	350	1035	11.09	13.1
733/93	-	380	520	1180	5400	1400	350	990	355	1130	310	915	11.48	11.4
733/94	-	375	350	1086	6550	2950	495	1080	240	1345	285	1065	12.24	13.1
753/11	-	-	290	1315	4485	3045	-	-	-	-	-	-	-	9.65
753/12	-	-	280	1260	6490	3000	-	-	-	-	-	-	13.0	9.1
753/13	177	230	305	1420	6935	3225	-	-	-	-	-	-	12.08	10.1
753/14	-	-	290	1210	4140	2870	-	-	-	-	-	-	12.96	9.0
753/15	-	-	315	1245	5320	2380	-	-	-	-	-	-	12.96	9.5
759/11	250	180	290	1410	4500	2900	-	-	-	-	-	-	12.25	9.25
759/12	375	160	270	1160	3785	2275	-	-	-	-	-	-	-	-
759/14	275	190	255	1355	4335	2400	-	-	-	-	-	-	-	-

2 x 25,000 i.u. Vitamin D_2. Killed 24 hours after last dose.

TABLE 25

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/3	157	460	220	1710	5325	3160	200	1160	350	1540	-	-	14.87	11.9
733/55	1045	320	250	1560	5320	3360	235	1190	275	1355	360	1145	13.77	11.5
733/56	1295	625	255	1425	5840	3660	240	1195	200	1200	250	1145	13.76	10.9
733/57	1190	345	225	1480	5175	3100	240	900	180	1045	245	1090	13.30	10.0
733/58	1060	350	220	1250	5000	2500	185	1060	280	1200	275	850	13.57	10.9
753/16	195	260	355	1065	5340	2315	-	-	-	-	-	-	13.0	10.6
753/18	170	190	275	1200	5295	2305	-	-	-	-	-	-	15.45	9.2
759/16	435	180	390	1375	5670	3050	-	-	-	-	-	-	-	9.4
759/17	-	-	300	1435	5345	2775	-	-	-	-	-	-	13.82	10.0
759/18	-	-	410	1405	6000	3470	-	-	-	-	-	-	-	-
759/19	210	-	320	1090	5850	2330	-	-	-	-	-	-	13.74	9.25

3 x 25,000 i.u. Vitamin D_2. Killed 24 hours after last dose.

TABLE 26

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	C Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/4	407	225	515	1330	5020	2475	270	1030	312	1030	-	-	13.21	10.0
733/59	802	530	550	855	5700	3100	265	820	305	1060	280	870	11.48	11.6
733/60	985	545	580	1445	8925	2500	410	1005	340	1050	240	1060	-	11.4
733/61	1005	610	580	1720	4280	3060	400	1095	310	1135	450	1165	10.57	11.8
733/62	1070	530	800	1260	2935	2870	270	1235	270	1235	220	1200	11.76	11.4
753/21	-	250	500	1335	5465	3405	-	-	-	-	-	-	16.4	9.6
753/22	-	285	480	1290	6670	3440	-	-	-	-	-	-	15.5	10.2
753/23	-	255	541	1355	5830	2590	--	-	-	-	-	-	16.4	10.2
753/24	-	260	505	760	5700	2905	-	-	-	-	--	-	15.2	9.5
753/25	-	265	530	1200	6250	2275	-	-	-	-	-	-	14.4	9.6
759/21	980	470	450	1240	5950	1615	-	-	-	-	-	-	15.48	9.3
759/22	350	250	1115	1415	6100	1750	-	-	-	-	-	-	16.52	9.3
759/23	345	-	350	1305	6275	2840	-	-	-	-	-	-	17.44	8.4
759/24	935	575	1690	2085	6020	3570	-	-	-	-	-	-	16.13	8.5
759/25	370	160	380	1350	5555	2925	-	-	-	-	-	-	16.90	9.3

4 x 25,000 i.u. Vitamin D_2. Killed 24 hours after the last dose.

TABLE 27

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/5	-	930	240	1380	7225	1380	335	1120	390	1225	-	-	-	11.5
733/67	960	680	530	1540	5150	3700	330	1160	370	1300	325	1370	11.39	10.0
733/68	2570	1110	1550	1450	4580	5320	520	1040	570	1105	780	1190	10.38	10.9
733/69	-	2905	1520	1990	6630	3670	710	1160	675	1170	860	1480	10.44	12.1
733/70	2455	360	800	1510	4145	3290	400	1055	405	1010	605	1030	10.28	10.1
753/26	-	558	615	1395	7470	2555	-	-	-	-	-	-	17.9	8.5
753/27	-	1060	1512	1635	7910	3280	-	-	-	-	-	-	16.7	10.0
753/28	-	685	905	1420	8070	2015	-	-	-	-	-	-	17.2	-
753/29	2255	1290	915	1350	6200	1860	-	-	-	-	-	-	15.7	9.5
759/26	555	200	290	1245	4945	2350	-	-	-	-	-	-	16.46	8.25
759/27	3390	1605	1840	1870	6425	3840	-	-	-	-	-	-	13.47	7.5
759/28	3900	1645	1020	1770	5690	2835	-	-	-	-	-	-	15.88	10.25
759/29	2130	700	770	1245	6110	3620	-	-	-	-	-	-	21.87	10.5
759/30	3360	1145	2225	-	7370	3415	-	-	-	-	-	-	15.80	8.3

5 x 25,000 i.u. Vitamin D_2. Killed 24 hours after the last dose.

TABLE 28

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/6	1335	770	4370	2955	5680	3410	800	1385	1180	1280	-	-	14.22	10.6
733/71	3920	825	1785	1535	5840	3960	420	1025	765	1280	430	1060	14.50	8.9
733/72	1430	700	335	1045	4720	2500	210	1020	385	1070	-	985	11.62	9.0
733/73	3470	760	235	1115	6880	4435	300	1130	440	1040	325	1275	13.21	8.0
733/74	2210	610	265	1195	5935	2410	200	1125	-	1035	295	910	13.69	9.4
753/31	1845	850	2845	2465	6440	3405	-	-	-	-	-	-	14.61	9.0
753/32	-	715	1735	1930	7225	2365	-	-	-	-	-	-	15.07	9.1
753/33	2750	1120	985	1935	6685	2415	-	-	-	-	-	-	14.48	8.7
753/34	2222	660	730	1340	7690	2754	-	-	-	-	-	-	13.95	8.3
753/35	3465	1195	2375	1965	6920	3480	-	-	-	-	-	-	-	8.0
759/31	1850	830	450	1325	5600	3245	-	-	-	-	-	-	15.60	9.0
759/32	4375	1580	2070	2275	5760	3035	-	-	-	-	-	-	15.36	8.5
759/33	2585	1130	3090	3005	6250	2570	-	-	-	-	-	-	17.05	10.2
759/34	4230	1200	2660	2900	7490	3195	-	-	-	-	-	-	15.89	9.0
759/35	3680	1315	1685	2430	6890	3150	-	-	-	-	-	-	16.46	9.25

5 x 25,000 i.u. Vitamin D_2. Killed 48 hours after last dose.

TABLE 29

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/7	500	945	1510	1955	5055	3980	356	1235	700	1540	-	-	18.47	9.4
733/63	4000	300	340	900	6325	2640	340	925	305	1035	490	1165	14.23	10.0
733/64	7250	2235	2070	1935	5845	3840	580	1010	600	1255	480	1075	13.78	11.0
733/65	-	1155	420	-	6565	4370	260	1150	460	1200	365	1010	-	9.15
733/66	5600	595	290	1055	5200	3200	335	1030	410	1070	350	1035	15.52	10.4
753/36	4370	2160	710	1380	7095	3315	-	-	-	-	-	-	16.9	7.5
753/37	4285	2130	2335	1885	8330	3090	-	-	-	-	-	-	15.19	6.9
753/38	4367	2205	1800	1770	6370	3450	-	-	-	-	-	-	15.62	6.5
753/40	5424	2700	722	1440	7300	2500	-	-	-	-	-	-	15.03	7.5
759/36	10180	1840	960	1800	6395	4235	-	-	-	-	-	-	16.03	8.5
759/37	2695	1135	1575	1690	6425	1750	-	-	-	-	-	-	16.64	9.25
759/38	5900	1550	2970	2800	6120	3020	-	-	-	-	-	-	16.87	8.25
759/39	5100	1755	1165	1455	6310	2315	-	-	-	-	-	-	16.59	7.25
759/40	5595	1560	2280	2220	5890	3510	-	-	-	-	-	-	15.20	7.25

5 x 25,000 i.u. Vitamin D_2. Killed 3 days after the last dose.

TABLE 30

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/8	360	965	1210	1995	4345	2545	305	1070	590	1480	-	-	12.49	6.0
733/75	3760	1795	525	1185	9745	4080	370	1160	825	1385	355	1200	14.77	6.4
733/76	5176	2665	680	1395	7535	3520	410	990	1180	1385	505	970	15.88	6.5
733/77	8925	2025	750	1300	7380	3730	390	985	580	1295	545	1200	14.94	6.4
733/78	2950	1790	715	1370	7680	2925	460	1025	720	1271	435	995	17.55	7.0
753/41	4935	1985	2530	1940	7870	3040	-	-	-	-	-	-	13.64	7.2
753/42	2480	1710	575	1255	5380	2650	-	-	-	-	-	-	12.10	8.5
753/43	6885	2240	762	1430	5930	3115	-	-	-	-	-	-	11.68	7.1
753/44	7795	3180	5423	3050	-	3950	-	-	-	-	-	-	12.62	7.1
753/45	4310	1900	405	1385	6900	2725	-	-	-	-	-	-	10.43	6.7

5 x 25,000 i.u. Vitamin D_2. Killed 5 days after the last dose.

TABLE 31

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/9	2245	1105	690	1955	5575	3358	365	1260	555	1150	-	-	15.26	8.7
733/47	7920	1465	2835	1620	7060	4650	630	960	725	870	1125	1100	10.96	7.5
733/48	7230	1825	1235	1160	7160	3480	535	940	885	1220	765	1035	11.07	6.25
733/49	5290	900	1160	1125	5975	3240	425	990	710	1150	410	1015	15.48	7.25
733/50	8655	1875	1195	1190	5630	2435	555	970	855	1125	540	1065	11.87	7.0
753/47	3630	1600	215	1420	5100	3775	-	-	-	-	-	-	13.68	6.6
753/48	2680	1165	330	1495	6385	3140	-	-	-	-	-	-	-	-
753/49	5425	2300	2450	2140	5705	3775	-	-	-	-	-	-	-	-

5 x 25,000 i.u. Vitamin D_2. Killed 7 days after the last dose.

TABLE 32

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/10	5370	1565	2590	2625	5420	3970	750	1360	1915	2080	-	-	14.64	6.7
733/51	29170	2240	2505	1840	8570	2490	795	990	1010	1490	1045	1325	16.17	6.3
733/52	3135	1385	1025	1000	9010	4675	510	1015	470	1195	320	1185	15.18	6.4
733/53	13500	1650	4835	2250	10200	4285	800	1400	1310	1500	1440	1290	14.97	6.5
733/54	3410	1410	1725	1340	7680	2830	710	880	500	900	440	975	-	-
753/51	3975	1665	3095	2860	5120	3530	-	-	-	-	-	-	11.93	9.65
753/52	2550	1100	345	1490	6770	2580	-	-	-	-	-	-	11.6	8.1
753/53	6360	1430	1310	1575	6550	2705	-	-	-	-	-	-	11.17	7.5
753/54	8460	1355	520	1210	5510	3750	-	-	-	-	-	-	11.18	7.0

5 x 25,000 i.u. Vitamin D_2. Killed 10 days after the last dose.

TABLE 33

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/11	2590	1375	3720	3120	7160	3440	450	1325	1300	1870	-	-	14.14	9.4
733/87	29450	2620	460	975	6460	3380	335	1170	775	1270	450	1335	11.35	9.6
733/88	13920	2470	965	1550	5540	2440	460	1160	560	1165	300	870	11.21	10.0
733/89	31230	1330	680	1430	8630	2590	540	1080	840	1200	455	795	12.83	9.6
733/90	-	475	485	1435	6040	2025	450	1205	605	1435	325	975	11.70	9.2
753/56	2185	1185	175	1205	4473	2500	-	-	-	-	-	-	-	8.5
753/58	5895	2045	-	-	5540	3390	-	-	-	-	-	-	11.59	12.3
753/59	9730	1740	200	1195	5492	2775	-	-	-	-	-	-	-	9.5

5 x 25,000 i.u. Vitamin D_2. Killed 17 days after the last dose.

TABLE 34

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/79	-	1965	650	1075	7140	3140	400	900	415	1260	445	1135	12.05	9.9
733/80	8810	885	470	1220	3765	3710	340	1080	750	1315	410	1160	12.2	9.4
733/81	8760	1390	955	1535	7140	2240	495	1065	820	1415	320	900	12.24	10.4
733/82	6995	1705	920	1305	5680	2720	410	945	360	1105	350	1300	12.4	10.2

5 x 25,000 i.u. Vitamin D_2. Killed 31 days after the last dose.

TABLE 35

RAT	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
No.	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/42	5100	1065	590	1200	10070	3670	655	1115	870	970	410	1090	12.32	8.87
733/43	23900	1470	955	1445	7615	3820	380	940	835	940	475	1160	13.51	9.25
733/44	4775	1260	400	1154	8275	3590	250	830	380	1155	270	880	14.30	8.0
733/45	24160	2380	1735	1700	8075	5480	470	1070	1125	1450	385	1220	14.11	8.0

5 x 25,000 i. u. Vitamin D_2. Killed 50 days after the last dose.

TABLE 36

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/25	6790	890	505	1400	4715	3670	350	930	405	1090	330	730	12.61	8.0
733/26	25620	2005	630	1225	7331	3700	350	1105	405	1190	340	1030	11.89	6.2
733/27	22710	2070	3977	-	4615	4440	265	955	980	1285	180	930	12.61	7.9
733/28	27130	1840	370	1110	9450	3250	370	930	590	1130	520	870	12.30	8.4

5 x 25,000 i.u. Vitamin D_2. Killed 68 days after the last dose.

TABLE 37.

RAT No.	AORTA		LUNG		TRACHEA		HEART		KIDNEY		STOMACH		SERUM	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
733/30	10,350	3,035	650	1,340	8,120	3,525	-	-	-	-	-	-	—	—

5 x 25,000 i.u. Vitamin D_2. Killed 104 days after the last dose.

TABLE 38

TABLE 39

The magnesium and calcium content of selected tissues of rats from Expt. 753.

Rat No.	AORTA		LUNG		TRACHEA		TREATMENT.
	Mg.	Ca.	Mg.	Ca.	Mg.	Ca.	
7	-	-	16.8	218	310.0	6,570	1 x 25,000 i.u. vit. D Killed after 24 hrs.
8	18.2	-	18.4	157	250.0	7,550	
9	11.7	-	33.4	327	276.7	3,370	
10	-	-	55.9	245	290.3	3,555	
11	-	-	34.2	256	247.7	4,237	2 x 25,000 i.u. vit. D Killed 24 hrs after
12	-	-	37.7	242	373.9	6,116	
13	50.6	126	36.8	268	302.7	6,632	
14	-	-	22.6	267	349.3	3,791	
15	-	-	34.1	281	306.4	4,914	
16	44.8	150	45.5	309	309.5	5,030	3 x 25,000 i.u. vit. D Killed after 24 hrs.
18	42.6	127	36.0	239	248.4	5,047	
21	26.5	-	56.7	443	257.0	5,208	4 x 25,000 i.u. vit. D Killed after 24 hrs.
22	33.6	-	54.8	425	281.4	6,389	
23	36.6	-	31.4	510	267.7	5,562	
24	33.5	-	37.8	467	247.6	5,452	
25	40.8	-	44.6	485	344.8	5,908	
26	85.8	-	30.9	584	317.0	7,153	5 x 25,000 i.u. vit. D Killed 24 hrs. after.
27	84.2	-	45.6	1,466	356.5	7,554	
28	80.0	-	31.9	873	356.0	7,714	
29	93.6	2,246	43.3	872	325.0	5,875	
31	84.0	1,760	55.8	2,789	314.0	6,126	5 x 25,000 i.u. vit. D Killed after 48 hrs.
32	68.1	-	37.9	1,698	352.0	6,873	
33	98.6	2,652	40.0	945	282.0	6,403	
34	51.8	2,170	31.8	698	331.0	7,359	
35	113.3	3,352	50.2	2,325	320.0	6,600	
36	130.8	4,239	40.2	670	306.0	6,789	5 x 25,000 i.u. vit. D Killed after 3 days.
37	147.4	4,138	49.8	2,285	317.8	8,021	
38	146.9	4,220	44.3	1,757	271.4	6,099	
40	144.7	5,279	30.9	691	292.8	7,007	

TABLE 39 (cont.)

41	130.0	4,805	33.3	2,498	300.0	7,570	5 x 25,000 i.u. vit. D Killed after 5 days
42	69.7	2,410	33.2	542	255.1	5,125	
43	142.8	6,742	27.3	735	310.0	5,620	
44	140.5	7,655	65.3	5,358	-	-	
45	114.0	4,196	32.8	362	265.6	6,634	
47	88.4	3,542	21.2	194	217.8	4,882	5 x 25,000 i.u. vit. D Killed after 7 days.
48	81.2	2,599	41.2	289	320.6	6,604	
49	141.8	5,283	46.5	2,404	294.4	5,411	
51	100.9	3,874	51.1	3,044	337.0	4,784	5 x 25,000 i.u. vit. D Killed after 9 days.
52	65.0	2,485	30.9	314	302.0	6,476	
53	104.4	6,256	40.4	1,270	316.0	6,232	
54	93.1	8,387	25.3	495	306.6	5,203	
56	57.5	2,122	29.8	145	-	-	5 x 25,000 i.u. vit. D Killed after 17 days.
58	146.9	5,748	-	-	-	-	
59	111.4	9,619	32.9	167	-	-	

(All values in mg. %)

TABLE 40.

Serum Calcium Values

Mean values and variance over initial 21 days of the experiment.

Experiment No.	Control			1 day			2 day			3 day		
	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance
733	10	12.9	1.091	5	13.7	1.359	5	12.6	1.869	5	13.9	0.3744
753	3	12.3	0.1601	5	10.7	2.512	4	12.8	0.1998	2	14.2	3.286
759	5	11.8	0.3338	3	11.5	0.2439	1	12.3	-	3	14.2	0.7411
777	6	12.1	0.2158	7	12.5	0.1175	8	13.5	0.1928	7	14.8	0.6998
Combined 753, 759 & 777	14	12.0	0.2486	15	11.7	1.210	13	13.2	0.1963	12	14.6	1.145

TABLE 40 (cont.)

Experiment No.	4 day			5 day			6 day			7 day		
	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance
733	4	11.8	1.042	4	10.6	0.3368	5	13.5	1.223	4	15.5	4.463
753	5	15.6	0.7220	4	16.9	0.9748	4	14.5	0.1154	4	15.7	0.5089
759	5	16.5	0.4119	5	16.7	1.848	5	16.1	0.5478	5	16.3	0.2878
777	7	14.8	2.806	8	16.3	2.377	8	15.6	0.1266	8	15.2	0.3215
Combined 753, 759 & 777	17	15.5	1.526	17	16.6	1.798	17	15.5	0.2917	17	15.6	0.3642

TABLE 40 (cont.)

Experiment No.	9 day			11 day			14 day			21 day		
	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance	No. of rats.	Mean	Variance
733	5	15.1	3.237	5	12.9	5.034	4	15.2	0.4338	5	12.3	1.832
753	5	12.1	1.523	2	13.7	0.0002	4	11.5	0.1342	1	11.6	-
759	-	-	-	-	-	-	-	-	-	-	-	-
777	6	12.8	0.3650	8	11.9	0.0603	7	13.3	0.0436	8	11.5	0.0556
Combined 753, 759 & 777	11	12.5	0.8800	10	12.3	0.0603	11	12.6	0.0889	9	11.5	0.0556

TABLE 41.

Mean Serum Inorganic Phosphorus.

DAYS	EXPERIMENT NUMBER							
	733		753		759		777	
	No. in group.	Mean	No. in group.	Mean	No. in group.	Mean	No. in group.	Mean
0	10	11.4	3	6.9	5	8.5	6	8.3
1	5	13.1	5	7.6	3	9.0	4	10.0
2	5	13.1	5	9.5	1	9.3	4	10.0
3	5	11.0	2	9.9	3	9.6	4	9.3
4	5	11.2	5	9.9	5	9.0	7	9.9
5	5	10.9	3	9.3	5	9.0	5	9.3
6	5	9.2	5	8.6	5	9.2	4	6.9
7	5	10.0	4	7.1	5	8.1	4	7.8
9	5	6.5	5	7.3	-	-	6	7.4
11	5	7.4	1	6.6	-	-	4	8.4
14	4	6.5	4	8.1	-	-	4	9.2
21	5	9.6	3	10.1	-	-	4	7.2
35	4	10.0	-	-	-	-	4	9.0
54	4	8.6	-	-	-	-	-	-
72	4	7.6	-	-	-	-	-	-