XLV. THE MODE OF ACTION OF VITAMIN D. STUDIES ON HYPERVITAMINOSIS D. THE INFLUENCE OF THE CALCIUM-PHOSPHATE INTAKE.

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The production of toxic symptoms by overdoses of irradiated ergosterol was first recorded by Pfannenstiel [1927] and Kreitmair and Moll [1928]. A number of workers were unable to confirm this result [Dixon and Hoyle, 1928; Hoyle, 1929; Hoyle and Buckland, 1929; Comel, 1929; Cartland, Speer and Heyl, 1929; Lesné and Clément, 1929] and discredit was thrown by some on the theory of hypervitaminosis, it being suggested that the ill-effects in question must have been caused by toxic by-products, formed it was supposed from the alcohol used as solvent during the process of irradiation [Dixon and Hoyle, 1928; *cf.* Underhill, 1928]. Evidence in support of the opposing view that excess of vitamin D is toxic *per se*, so that the phenomenon genuinely merited the description of hypervitaminosis, was brought forward by Harris and Moore [1928; 1929, 1]. Without denying the possibility that secondary products might also be toxic, it was shown that the severity of the ill-effects ran parallel with the amount of vitamin ingested, that specimens of ergosterol irradiated in various solvents or no solvent were equally toxic when fed at the same level of antirachitic activity, that destruction of vitamin D by over-irradiation entailed a corresponding loss of toxicity, and that transformation products of ergosterol produced by resinification without irradiation were devoid of both antirachitic activity and of toxicity. These conclusions have since been confirmed by the experiments of other workers [*e.g.* Scheunert and Schieblich, 1929][2].

[1] In the whole time service of the Medical Research Council.

[2] Holtz and Schreiber [1930] similarly find that all preparations of irradiated ergosterol which display any antirachitic activity always possess a parallel degree of toxicity, and they are unable to obtain antirachitic but non-toxic products. On the other hand, they claim that they have been able to destroy the antirachitic activity and leave unimpaired the toxic (or "calcinosing") factor. However, the toxicity of the supposed non-antirachitic preparations was typified by its power of raising the blood-P or -Ca (*e.g.* in tetany) and (by definition) of producing calcification. Since these very effects are not infrequently taken as actual indices of antirachitic action, it is difficult to see how the positive result for toxicity, which was demonstrated by tests on dogs, can be reconciled with the reported negative result for antirachitic activity, obtained by tests on rats. Possibly the technique adopted (prophylactic method) may explain the anomaly. It may be

Perhaps the most convincing evidence as to the reality of hypervitaminosis accrued from a consideration of the very nature of the abnormalities produced. We argued [Harris and Moore, 1928; 1929, 1] that since in hypovitaminosis D (*e.g.* in rickets) the typical defective calcification at the growing end of the bone is associated with a diminished blood-P or -Ca, or both, so we might reasonably anticipate finding the reverse condition in hypervitaminosis D, where the characteristic lesion is an excessive calcification of the bone ending [Harris and Innes, 1928–1929], arteries, non-striated muscle[1], *etc.* [Kreitmair and Moll, 1928; Harris and Moore, 1929, 1]. The prediction was found to be justified, the administration of excess of vitamin D giving rise to hyperphosphataemia or hypercalcaemia or both in experimental animals [Harris and Stewart, 1929]. (This result has been confirmed in an extended series of tests [see Harris, 1930] and also by other workers. Hypercalcaemia or hyperphosphataemia has been seen likewise in infants receiving excessive doses of vitamin D [Hess, Lewis and Rivkin, 1928], while Hess, Weinstock and Rivkin [1928] had indeed earlier recorded that the blood-Ca reduced to a low level by a low-Ca, high-P diet could be brought back to a more normal figure by administration of very large amounts of irradiated ergosterol.) In the second place we suggested that just as the low blood-P or -Ca of rickets appeared to be associated with a diminished net absorption from the gut [see Harris, 1928] so again with very large doses of vitamin D an abnormally high net absorption might be expected[2]. Finally it was predicted that just as the severity of the hypovitaminosis is influenced by the Ca-P intake, so also this would probably prove to be a controlling factor in hypervitaminosis. These suppositions have proved to be justified, and a preliminary report showing that the severity of the hypervitaminosis is governed by the calcium intake appeared a year ago [Harris, 1930].

added that Windaus now employs the minimal lethal dose as a measure of the vitamin D activity of irradiated ergosterol products [Jephcott and Bacharach, 1930]. We must point out, however, how large is the number of workers who have been led to adopt the view that toxicity is associated entirely with by-products and not vitamin D [Adam, 1928; Reyer and Walkoff, 1928; Heubner, 1929; Steudel, 1929; Simmonet and Tanret, 1929, 1930; Holtz and Brand, 1929; Haendel and Malet, 1929; Vara-Lopez, 1930; Borghi, 1929]. A later view of Dixon [1929] and Hoyle [1930], that hypervitaminosis could only be produced on synthetic diets and not on a "natural" diet such as bread and milk, is accounted for in the present paper [see also Harris, 1930], where it is shown that the exact level of overdose of irradiated ergosterol needed to produce toxic symptoms varies with the Ca/P ratio of the diet.

[1] Similarly in rickets there is a deficient calcium content of muscle [Aschenheim and Kaumheimer, 1911; Haury, 1930].

[2] *Net absorption* = Ca [or P] intake *minus* faecal output = absorption from gut *minus* excretion into gut. In earlier papers the alternative phrase "retention from gut" was used in order to indicate that we were taking into account this factor of a possible excretion into the gut, *i.e.* that we were dealing with the "net" and not the "gross" absorption. The use of this phrase was perhaps unfortunate, since it may have led [*e.g.* Watchorn, 1930, 1] to confusion with the quite different concept of the retention by the animal as a whole (*i.e.* intake less urinary and faecal output). Of course we nowhere postulated that the retention as a whole was increased in hypervitaminosis, in fact we were the first to draw attention to the enormously increased urinary Ca excretion.

In the present communication our experiments, begun in 1928 and concluded in the spring of 1930, are described in greater detail and discussed more especially in relation to the mode of action of the vitamin. Convincing evidence is furnished to substantiate the theory that vitamin D exerts its action by raising the blood-Ca or -phosphate, so giving rise to an increased calcification. It is shown that the increase in the calcium and phosphate is associated with an increased net absorption from the gut (this is of special consequence in hypervitaminosis with diets rich in Ca), but also that the shaft of the bone may provide an important additional source under certain circumstances, for example on low Ca diets and with larger excess of vitamin D. The theory that vitamin D might function by drawing Ca and P out of the bone was put forward by Hess, Weinstock and Rivkin [1929, 2] and is supported by work in other laboratories carried out simultaneously with our own [Light, Miller and Frey, 1929; Hess, Weinstock and Rivkin, 1930; Baumgartner, King and Page, 1929; Brown and Shohl, 1930; Weinmann, 1929] as well as by that of Watchorn [1930, 2] in this laboratory. Jones, Rapoport and Hodes [1930, 2] on the other hand have concluded that the Ca comes from the food and not the bone. It does not appear to have been realised that these two methods of calcium mobilisation may both be involved, and to varying extents dependent on the Ca-P content of the diet and the degree of excess of the vitamin.

Experimental results.

All the experiments to be described here have been carried out on rats; we have obtained similar results with rabbits. The strain had been inbred for many generations, and carefully matched litter mates were always evenly distributed between the several groups in each experiment. Both piebalds and albinos (the latter originating from Wistar Institute stock) were used.

The irradiated ergosterol used in the later experiments[1] had an antirachitic activity of approximately 10^7 (Pharmaceut. Soc.—Med. Res. Council) units per g. and in the earlier experiments of approximately one-half to one-fifth as much.

In general, the degree of severity of the hypervitaminosis in a given experiment is reflected in a proportional impairment of growth, or loss in weight, so that the growth curves presented below convey a very fair impression of the relative toxicity of the various diets.

1. *Influence of variations in the calcium phosphate content of the diet.*

The same basal diet (Steenbock's rachitogenic diet [Steenbock and Black, 1925] with the calcium carbonate omitted) was fed to all groups, but with varying graded additions of calcium phosphate.

It was found (Fig. 1) that each increase in the calcium phosphate allowance gave rise to an increased severity of hypervitaminosis. By sufficiently re-

[1] We are indebted to Messrs J. Nathan and Co. for generous gifts of irradiated ergosterol.

ducing the calcium phosphate a level of vitamin D excess which was otherwise definitely toxic became virtually innocuous.

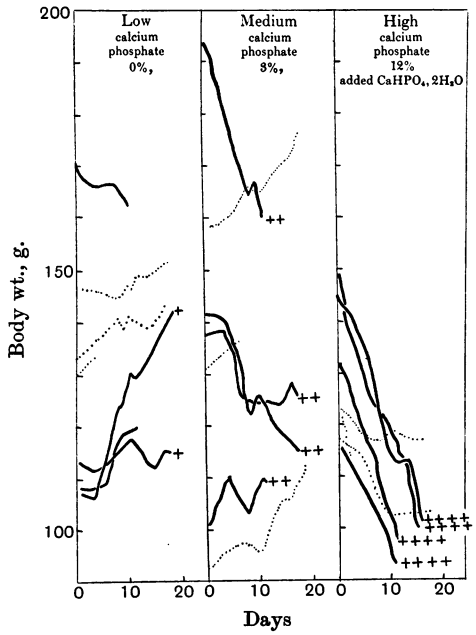

Fig. 1. Dependence of severity of hypervitaminosis D upon amount of calcium phosphate in diet.

\+ Microscopic calcium deposits (in kidney only).
\+ + Definite calcareous deposits.
\+ + + + Very extensive calcareous deposits.
—— 10 mg. irradiated ergosterol/rat/day.
..... Controls without vitamin D excess.

2. *Comparison of natural and synthetic diets with varying content of calcium phosphate.*

A series of tests was carried out to determine quantitatively the comparative effects of diets of Hovis bread and milk on the one hand, and synthetic diets, rich in calcium and phosphate, on the other, when fed in conjunction with various graded excesses of vitamin D. The results show that doses which are just on the border line of toxicity for the synthetic, salt-rich diet may be practically harmless for the bread and milk diets. However, when slightly larger excesses of vitamin D are given the difference becomes less noticeable, and with still larger excesses the difference virtually vanishes (Fig. 2). Next it was shown that the addition of an inorganic salt mixture (consisting principally of calcium salts and phosphates [Harris and Moore, 1928]) to the bread and milk diet sufficed to render it indistinguishable from a synthetic diet, in its power to permit hypervitaminosis at a given level of vitamin D excess (Fig. 3). Finally, we were able to demonstrate that on a diet consisting wholly of dried milk, and therefore very rich in Ca, an even more severe hyper-

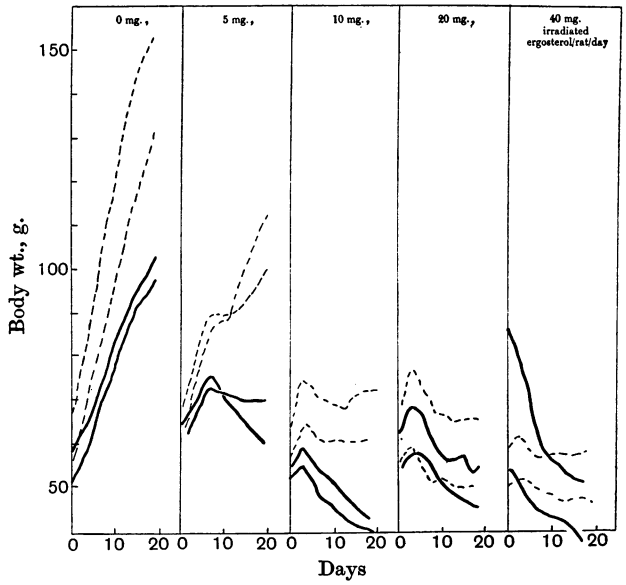

Fig. 2. Relative toxicity of vitamin D excess, on bread and milk and synthetic diets.
– – – – Hovis bread and milk (50:50).
——— Synthetic diet.

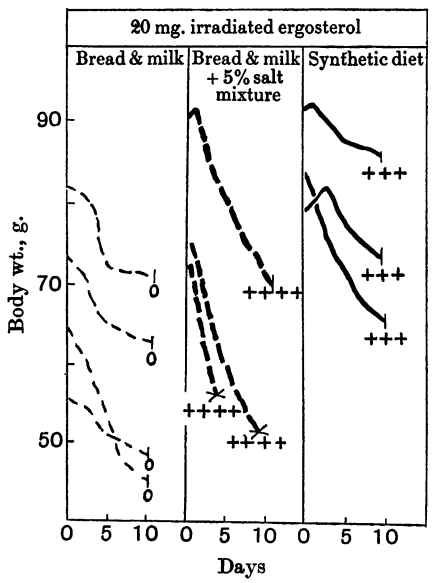

Fig. 3. Bread and milk diet *plus* added salt mixture permitting hypervitaminosis as readily as synthetic diet.

× Animal died. | Animal killed.
0 No calcareous deposits yet appeared.
+ + + Considerable calcareous deposits.
+ + + + Most extensive calcareous deposits.

vitaminosis was produced than on the usual synthetic diet, at the various graded levels of vitamin excess[1] (Fig. 4).

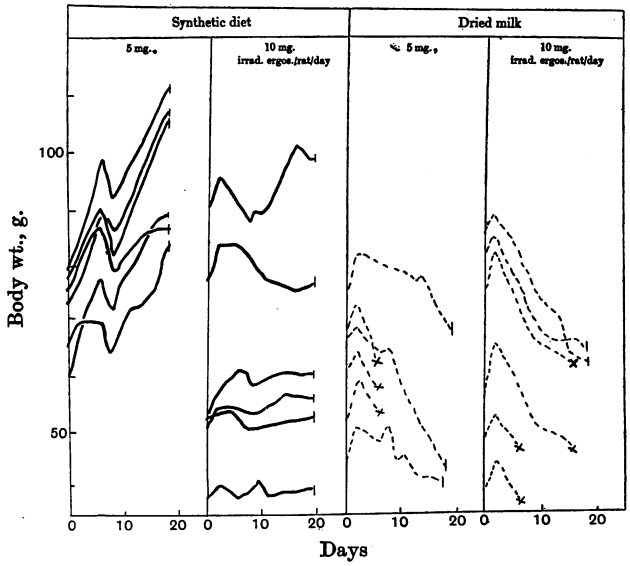

Fig. 4. Sole diet of milk even more favourable for production of hypervitaminosis than synthetic diet (4–5 % salts).
× Animal died. | Animal killed.

3. *Influence of variations in Ca/P ratio.*

These experiments were planned with the object of comparing the effects of large doses of irradiated ergosterol when administered in conjunction with

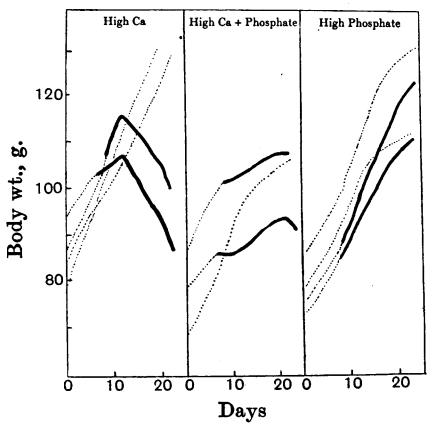

Fig. 5. Increased toxicity of irradiated ergosterol with increased Ca/P ratio of diet.
—— Irradiated ergosterol (0·05 % of diet).
..... Controls without vitamin D excess (1 drop "radiostol" per day).

[1] These results afford an explanation of the results of Dixon [1929] but fail to support his main conclusion:—"the same experiments made on animals living on an ordinary diet show that excess of the 'poisonous' food is harmless."

diets (1) rich in calcium but deficient in phosphate (2) rich and well-balanced with respect to both calcium and phosphate, and (3) deficient in calcium but rich in phosphate. The same basal diet was used throughout (based on Steenbock's formula [Steenbock and Black, 1925]: yellow corn, 60 g., gluten flour, 200 g., sodium chloride, 10 g.); but while the calcium-rich diet contained 38 g. of calcium lactate, the calcium- and phosphate-rich diet contained 30 g. of $CaHPO_4, 2H_2O$ and the high-phosphate, low-calcium diet contained 24·8 g. of Na_2HPO_4.

Our results (Fig. 5) showed that a given overdose of irradiated ergosterol, just sufficient to cause rapid loss of weight with diets rich in calcium and deficient in phosphate, loses much of its toxicity when the calcium is balanced by the addition of phosphate, and that it becomes relatively harmless when the calcium is omitted and the phosphate remains high. This held true even when the feeding was prolonged for many weeks (Fig. 6). In further experi-

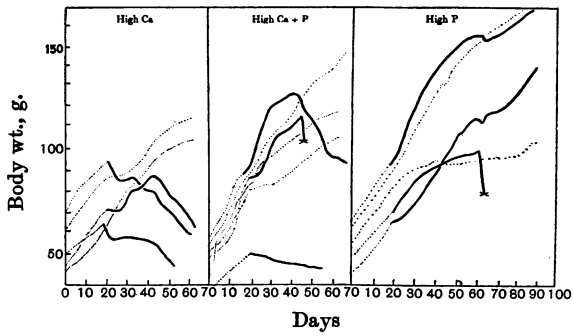

Fig. 6. Long-time feeding experiments, comparing high-calcium, high-phosphate, and high-calcium-*plus*-phosphate diets.
—— Irradiated ergosterol. ..... Controls.

ments (Fig. 7; *cf.* Fig. 8) in which the ergosterol was administered at higher levels or more fully-grown animals were used, a similar difference between effects of the high-calcium and the high-phosphate diets could still be detected.

Measurements of the calcium and phosphate metabolism of these animals are reported separately [Watchorn, 1930, 2]. The *post mortem* abnormalities characteristic for each group are discussed in Sections 6 and 7.

4. *Production of a distinctive type of hypervitaminosis on calcium-free or calcium- and phosphate-free diets.*

Since it had been shown (Figs. 5, 6, 7) that normal growth was permitted on high-P, low-Ca diets with such levels of irradiated ergosterol as were sufficient to cause rapid loss in weight and death when fed with diets containing both calcium and phosphate (or high-Ca, low-P), it was decided to ascertain whether even larger doses of irradiated ergosterol would still prove harmless under these conditions.

The results show that a toxic effect can be produced (at least eight times

as much irradiated ergosterol being needed when fed with a high-P, Ca-free diet as with a normal Ca-P diet, in order to produce loss in weight at an equal rate (Fig. 8)), but that there is a striking difference in the nature and

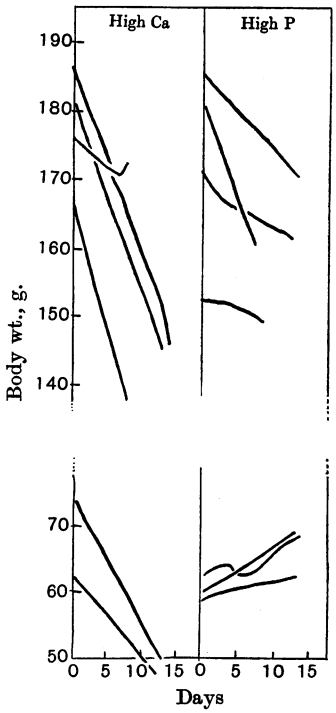

Fig. 7.

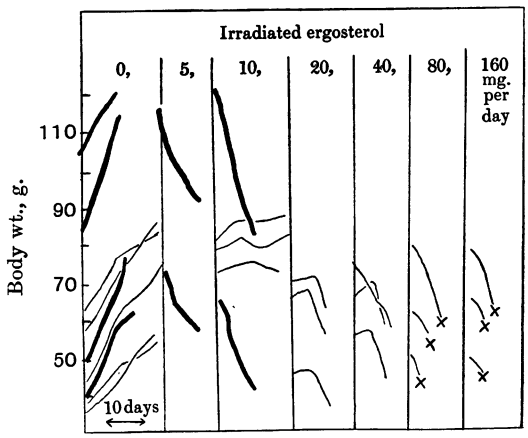

Fig. 8.

Fig. 7. Comparison of high-calcium and high-phosphate diets at higher level of vitamin D excess. Adults shown above. Representative curves from 50 animals.

Fig. 8. Hypervitaminosis induced on high-P, low-Ca diet by sufficiently increased vitamin D intake. —— Ca-deficient diet. —— Control diet, normal Ca and P. × Animal died.

symptoms of the hypervitaminosis in the two cases. Whereas the controls, on the diet containing both calcium and phosphate and 10 mg. irradiated ergosterol, showed the classical picture of hypervitaminosis, with calcification of vessels, heart and kidney, stimulation of bone formation, and, in the early stages, absence of noticeable osteoporosis in the cortex of the bone shaft or in the jaw bone, the experimental animals on the calcium-free diet with vitamin excess showed a rickety appearance in the bones, a pronounced resorption of compact bone together with almost complete absence of the usual calcification of vessels, kidneys, *etc.* In short, the changes in hypervitaminosis on the Ca-free diet resemble those on the control Ca-free diet without any vitamin excess, except that loss of mineral substance from the spongiosa is more extensive[1].

[1] The high-phosphate diet *per se* always tends to give rise to a characteristic kidney lesion, entailing focal necrosis of the tubules of the boundary zone, both in the hypervitaminosis and in the control groups.

Diets deficient in Ca and P. On a diet deficient in both calcium and phosphate, similar results were obtained (Fig. 9). Loss of weight and death resulted if sufficiently augmented doses of irradiated ergosterol were given, but

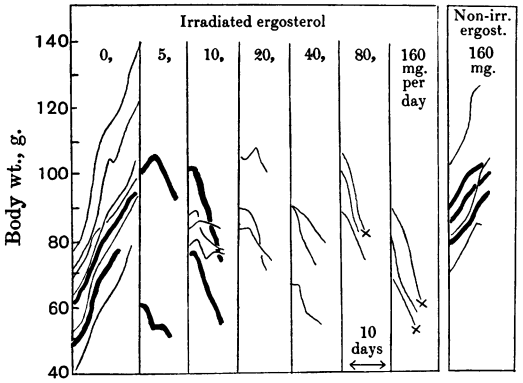

Fig. 9. Hypervitaminosis produced on diets deficient in Ca and phosphate.
—— Ca- and P-deficient diet. ▬▬ Control diet, normal Ca and P. × Animal died.

no appreciable deposits of calcium salts had appeared in the organs, vessels or bone-endings, while the bones were soft and fragile with marked evidence of resorption and a rickets-like beading at the costo-chondral junction (again resembling the controls in the Ca- and P-deficient group without the vitamin D excess but with hastened resorption of spongiosa).

5. *Radiographs of bones.*

In view of the well-known effects upon bone structure of deficiency of vitamin D or of abnormal Ca-P intake, a detailed X-ray study was made of animals receiving excessive vitamin D, in the various groups and sub-groups already described[1]. The typical effects of hypervitaminosis upon the bony skeleton were best seen at the growing end of the long bone, and with diets containing adequacy of both Ca and P in normal ratio. Again the picture in hypervitaminosis may be contrasted with that in rickets (Plate I). In the early stage of hypervitaminosis, or when only moderate overdoses were given, an unmistakable narrowing of the metaphyseal cartilage was always seen. The epiphyseal end of the diaphysis became highly calcified, forming a characteristic dense band which rapidly broadened and extended to the marrow cavity. When the irradiated ergosterol was fed at very high levels of toxicity and the observations were continued for a sufficiently long period until the animal had lost weight considerably, it was found that the dense area continued to recede into the marrow cavity but left behind a distinct gap of lessened density, at the site which would normally correspond with the growing end of the bone.

[1] The exposures were made in the University Radiological Department, under the general supervision of Dr A. E. Barclay.

In contrast with the foregoing we found that when equally large overdoses of vitamin D were fed, but in conjunction with a diet such as is normally able to retard calcification (*e.g.* Steenbock's rachitic ration), excessive calcification of the bone-ending was in fact much less evident, or absent, although the production of calcareous deposits in the soft tissues (see below) was appreciably intensified.

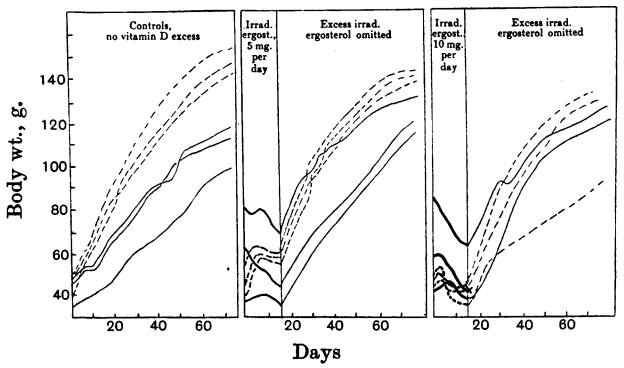

Fig. 10. Hypervitaminosis and cure (normal synthetic diet, and Steenbock rachitogenic diet).
Broken line, normal diet.
Continuous line, Steenbock diet.
Thickened line denotes excess of vitamin D.

6. *Factors favouring the formation of the calcareous deposits.*

An examination of our *post mortem* records for the various experiments described above makes it clear that the formation of the calcium deposits in the soft tissues (kidneys, arteries, non-striated muscle, *etc.*) is greatly increased when there is an abundance of calcium salts in the diet. As will be shown below the ingestion of large doses of vitamin D under such conditions gives rise to a greatly intensified hypercalcaemia (or hyperphosphataemia), associated with an increased net absorption from the gut. Examples of the influence of the calcium content of the diet in inducing calcareous depositions are seen in the following instances: (1) animals receiving Hovis bread and milk with 20 mg. of irradiated ergosterol lost weight rapidly but showed no calcium deposits after 10 days, whereas animals receiving the same diet *plus* 5 % added salt mixture had extreme and extensive calcium deposition, and animals on synthetic diet containing 5 % salt mixture showed fairly severe calcification; (2) after 12 days on 10 mg. of irradiated ergosterol and synthetic diet there were no naked-eye calcium deposits in animals given the low calcium phosphate diet, but there were definite calcium deposits in animals given medium calcium phosphate, and very extensive calcium deposits in animals given high calcium phosphate; (3) there was a virtual absence of calcareous deposits in animals having excess vitamin D with diets devoid of calcium, or devoid of calcium and phosphate (Figs. 8, 9); and (4) the calcium deposits were more readily produced on a diet of dried milk (Fig. 4) than on a diet

of bread and milk (Fig. 3). With the low-calcium diets, as will be shown, a relatively greater amount of calcium is drawn into the blood from the bone.

Two other factors concerned are the duration of the hypervitaminosis and the degree of excess of vitamin D in the diet. With insufficient excess, no calcium deposits are seen; with very large excess, the animal may die before the deposits have had time to develop. An animal may lose weight with hypervitaminosis for 15 days or longer before showing calcareous deposits (*e.g.* on the various diets referred to in Fig. 5; bread and milk, Fig. 3; etc.). Hence, the most favourable condition for the development of the calcareous deposits is a long-drawn-out hypervitaminosis, with no greater excess of vitamin D than is needed to cause a slow loss in weight, and an abundant supply of calcium salts in the diet[1].

7. *Histology of bone and teeth; osteogenesis and resorption in hypervitaminosis.*

The abnormalities seen in the bony skeleton[2] in hypervitaminosis D vary somewhat according to the stage of development, and also with the mineral salt content of the basal diet.

The effects seen with normal basal diets (complete synthetic diet; bread and milk; milk; *etc.*) may be described first. In the *teeth*, the most noteworthy feature is a remarkable overgrowth of cement, which extends to three or four times its normal thickness, shows much cellular proliferation and invades the marrow spaces of the jaw bone. The inner part of the dentine, to the extent of perhaps half its transverse thickness, is transformed into a distinctive type of secondary dentine, possessing a roughly granular and irregular laminated structure and staining intensely with haematoxylin (Plate II A).

In the *long bones* (or *ribs*) (Plate II B) the first change, observed with moderate over-doses of the vitamin and before toxic symptoms have appeared, is a marked stimulation of new bone formation. Osteogenesis is very active, and abnormally long and slender trabeculae are produced, extending far into the marrow cavity and already fusing to some extent. This is in conformity with the early radiograms (Section 5), which shows a dense shadow at the sub-epiphyseal region. At this stage the bones are solid and compact and there is little evidence of loss in density in the cortex of the shaft of the long bones or in the jaw bone. This is the picture seen in rats receiving bread and milk *plus* 5–10 mg. of irradiated ergosterol after 27 days (Fig. 2), or

[1] The contention that the calcium deposition in hypervitaminosis is subsequent to necrosis of the tissues is discussed elsewhere [Innes, 1930, *Ann. Rep. Dept. Animal Path., Camb.* (in press)].

[2] The specimens examined embrace (*a*) transverse sections of the lower jaw across the level of the root of the incisor teeth, (*b*) longitudinal sections of the costochondral junctions, and (*c*) longitudinal sections through the distal end of the femur and the proximal end of the tibia. These were fixed in formaldehyde saline and decalcified in 7 % HNO_3, embedded in paraffin in the usual way, and stained with haematoxylin and eosin.

synthetic diet *plus* 5 mg. irradiated ergosterol after 10 days (Figs. 8 and 9)[1]. As the process advances, the fusion of trabeculae continues so that a great mass of spongy bone is formed, which extends some considerable distance up the marrow cavity and occupies its entire width (Plate II B, no. 2). In the advanced degree of hypervitaminosis (Plate II B, no. 3), however, the most prominent feature is the evidence of excessive resorption. Macroscopically the bones become soft and fragile. The cortex of the shaft of the long bones and the jaw bone also, instead of remaining compact, is extensively osteoporosed and osteoclastic activity is prominent. Similarly a large marrow space appears at the epiphyseal end of the abnormal mass of spongiosa. This finding is in agreement with the radiograms taken during the late stages of hypervitaminosis, in which the dense shadow appears to have receded from the epiphysis. The picture here is complicated by the cessation of bony growth. The intermediate growth cartilage is very narrow and lined on the diaphyseal side by a thin transverse plate of bone. (This account applies, for example, to rats receiving bread and milk and the higher levels of irradiated ergosterol.)

The resorption from bone is much lessened when the diet is rich in calcium. Thus on a high-calcium, low-phosphate diet (Fig. 6), the animal may die with extensive calcareous deposits, but the bones and teeth will show no osteoporosis or other abnormalities, except those due to cessation of growth. On low-calcium diets, on the other hand, the resorptive changes become intensified. For example, the cortex is less compact on a bread and milk diet with hypervitaminosis than it is on a Ca-rich synthetic diet with the same excess of vitamin D (Fig. 2). Finally on Ca-free diets, or diets rich in phosphate and deficient in calcium, resorption is very extensive. A rickets-like appearance is seen resembling that in the controls on the same diets without the excessive vitamin, but in addition there is also a disappearance of the sub-epiphyseal spongiosa. Thus we have evidence of the bones acting as the main source of the extra calcium drawn into the blood stream when there is an insufficient supply from the gut.

8. p_H *of faeces.*

It has been shown that on certain rachitogenic basal diets the faeces of the rat tend to become abnormally alkaline when there is a deficiency of vitamin D, and to revert to a more normal reaction when the vitamin is restored to the diet [Zucker and Matzner, 1923; Jephcott and Bacharach, 1926, 1928; Bacharach and Jephcott, 1929; Grayzel and Miller, 1928]. An acid reaction in the gut favours solubility of Ca and hence ease of absorption. By analogy it seemed possible that feeding of excessive vitamin D might give rise to faeces with an abnormally low p_H figure. It has already been reported [Harris and Moore, 1929, 1] that, on an ordinary synthetic diet, no very decisive increase in acidity is in fact obtained. In continuation, we have examined the effects of excessive vitamin D given in conjunction with a

[1] These changes were not seen in adult animals where bony growth has ceased, but the formation of calcareous deposits was very marked.

Zucker diet [Jephcott and Bacharach, 1926], such as readily permits the change of faecal p_H in vitamin D deficiency[1]. Here again a similar result was found (Fig. 11).

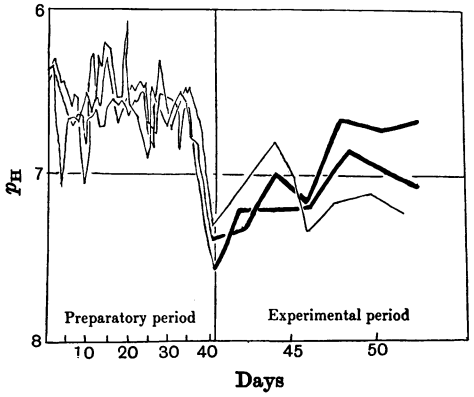

Fig. 11. p_H of faeces in hypervitaminosis.
Excessive vitamin D.
Control, or preparatory, diet (low in, or devoid of, vitamin D).

9. "*Vitamin balance.*"

In earlier papers [Harris and Moore, 1928; 1929, 1] we showed that the degree of severity of the symptoms of hypervitaminosis was sometimes diminished by an increase in the vitamin B (or B + C) allowance. This result has been fully confirmed [*e.g.* Duguid, Duggan and Gough, 1930].

A somewhat similar phenomenon was the production of symptoms resembling avitaminosis B by feeding excessive cod-liver oil [Harris and Moore, 1928] or cod-liver oil concentrate [Harris and Moore, 1929, 2]. These symptoms could be cured or prevented by a slight increase in the vitamin B allowance. The same result has since been obtained independently by American workers [Norris and Church, 1930]. However, we thought it necessary to emphasise that this balancing effect could only be readily seen when the vitamin B allowance was already low, and the cod-liver oil excess very considerable.

Further work on hypervitaminosis D confirms the view that similar conditions apply here. If the diet is already rich in vitamin B, further small additions fail to show any appreciable alleviating effect in protecting the animal against massive overdoses of vitamin D (Fig. 12). It is clear therefore that in interpreting such results, a limited significance only can be attached to the conception of a balance between vitamins D and B.

Discussion.

The observations recorded in the above experimental section, taken in conjunction with results described in earlier papers, appear to furnish us with

[1] The electrometric determinations of p_H were kindly carried out by Miss E. Allchorne of the Glaxo laboratory.

a logical and consistent picture of an orderly sequence of changes induced by the action of vitamin D in the animal organism. As we pass from a deficient intake of vitamin D to an adequate level, then to an abundance, and so to excess, and finally to very great excess, a series of phenomena is seen in increasing intensity. Our main deductions may first be summarised briefly as follows, and we can then proceed to a more detailed critical analysis. An increased vitamin D intake promotes increased absorption of Ca and/or phosphate from the gut (or diminished excretion into the gut), so tending to raise the level in the blood. With inadequacy of vitamin D, the blood fails to

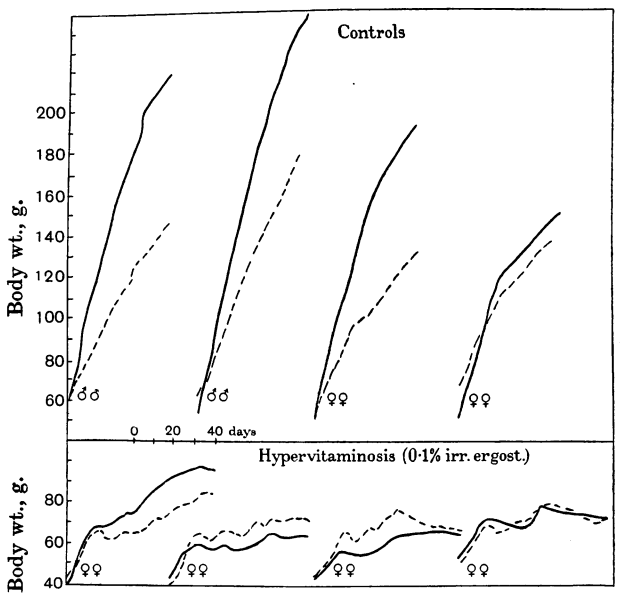

Fig. 12. Hypervitaminosis on (1) synthetic diet, and (2) with the sugar (60 % of diet) replaced by equivalent of concentrated orange juice (note remarkable growth rates of controls on the orange juice regimen).

--- Orange juice diets
—— Synthetic diets
} matched litter mates.

secure sufficient Ca and/or phosphate; with moderate amounts the blood succeeds in maintaining its approximate constancy of composition; while with large overdoses, hypercalcaemia and/or hyperphosphataemia cannot be averted. In the latter condition the kidney responds by an abnormal urinary excretion of Ca and/or P. If on the other hand the excessive vitamin D be now suddenly removed, a suitably increased excretion of Ca and phosphate is permitted to the gut [Watchorn, 1930, 1]. The "retention" of Ca or phosphate by the animal as a whole is seen to be the resultant of two factors, working in opposite directions, *viz.* (i) the increased absorption from (or diminished excretion into) the gut, and (ii) the increased excretion by the kidney. As more and more vitamin D is given the latter factor overtakes the former, so that the resulting retention, while it is increased by moderate doses

Page 380. Key to Fig. 12

for ------ Orange juice diets
——— Synthetic diets

read ——— Orange juice diets
------ Synthetic diets

of the vitamin, is diminished by very large doses. The deposition of calcium salts in certain sites is associated with a withdrawal from others; in hypervitaminosis this transference process in the bone is apt to be excessive; in rickets there is evidence of a defective transfer [Morelle, 1930]. As has been shown in the experimental section, when the diet is deficient in calcium the withdrawal from the bone stores becomes the noteworthy feature of the hypervitaminosis. When on the other hand the diet is rich in calcium the bone is less called upon, but there is increased liability to calcareous deposition as a result of an increased net absorption from the gut, and partly also no doubt because the amount stored by the bone is less than normal. Each addition of calcium to the diet intensifies the hypercalcaemia and calcareous deposition with a given overdose of vitamin D.

It is clear, therefore, that the precise level of Ca and P in the blood as influenced by the vitamin D intake depends on a greater variety of complex factors than has been generally realised in the past, including gut absorption (or excretion), kidney excretion, and deposition and dissolution of calcium deposits.

The experimental results are not inconsistent with the simple view that vitamin D acts by increasing the apparent solubility of the Ca and phosphate in the blood, an increased content in the blood facilitating deposition in the sites to be calcified. The latter assumption seems justified in view of the observation [Shipley, 1924; Shipley, Kramer and Howland, 1926] that non-calcified cartilage from a rachitic rat will rapidly calcify once it is put in blood-serum or inorganic saline containing adequate Ca and P (or Ca × P product). Our evidence also indicates that vitamin D can serve to promote osteogenesis. This could be explained readily enough, however, by supposing that the rate of formation of fresh osteoid tissue depends on the rate at which the old becomes calcified, in which case clearly the precipitation of extra calcium would mechanically serve to stimulate fresh osteogenesis.

It is obvious that much information has still to be acquired before a full explanation can be given for the preferential deposition of calcium salts in certain types of tissue to the total exclusion of others; this is seen as well in hypervitaminosis as under normal physiological conditions. The presence of phosphatase [Martland and Robison, 1924] is doubtless of special importance, so too perhaps the alkalinity.

The suggestion that vitamin D mobilises Ca (or P) by stimulating the parathyroid demands consideration. Obviously the theory could be tested by determining the influence of vitamin D after parathyroidectomy. The results of such tests hitherto recorded are very conflicting [Hess, Weinstock and Rivkin, 1929, 1, 1930; Jones, Rapoport and Hodes, 1930, 1; Taylor, Branion and Kay, 1930]. Nevertheless it seems probable that vitamin D can function independently of the parathyroid, for the parathyroid hormone raises the calcium in the blood solely by withdrawing it from the bones and without increasing the net absorption from the gut.

Certain points in the main conclusions outlined above appear to run counter to current views or to recent observations by other workers, and the evidence in their favour will now be analysed in greater detail.

Gut absorption.

The measurements taken by Miss Watchorn of calcium and phosphate intakes and faecal outputs upon the animals dealt with in Fig. 7 (Steenbock basal diet, (1) with added calcium and no phosphate, and (2) with added phosphate and no calcium) appear at first sight to contradict our assumption of an increased stimulation of gut absorption through the agency of vitamin D. However, in interpreting her results [Watchorn, 1930, 2], several complicating factors have to be borne in mind, most notably that of starvation, or general failure. Once a severe degree of hypervitaminosis has supervened (so that the animal is consuming only a very small fraction of its normal food intake and body processes are failing and perhaps calcium deposits already appearing in kidneys, muscles, vessels, intestinal tract, *etc.*) the gut function appears to break down and the effect of vitamin D in increasing the absorption can now no longer be demonstrated. This was illustrated clearly enough in Watchorn's earlier paper [1930, 1], in which she showed, working with normal basal diets, that vitamin D excess at first caused an increased net absorption (in every instance but one), particularly when the absorption had previously been low, but that this absorption ultimately fell off, as the animal ceased to eat. Partial starvation, in fact, was shown in a control experiment to be sufficient in itself to diminish Ca and P absorption. If now we review in the light of these considerations the results with the Steenbock high-Ca diet [Watchorn, 1930, 2], we find that the analyses refer to the period when these very complications had already set in and the animals were losing weight and taking very little food (in the second week of hypervitaminosis). An unequivocal demonstration of increased net absorption could not therefore be expected. Even so, it can be shown that some effect on the Ca is still apparent, when figures are recalculated upon a percentage basis, in the case of two animals out of the three. The third animal may be fairly neglected for its food intake had dropped 75 %, compared with only 46 % and 54 % for the other two, and absorption from the gut had indeed virtually ceased.

The phosphorus absorption in this test showed immense fluctuations between the three animals in the group, varying from 56 to 77 % in the preliminary period, from 64 to 15 % in the hypervitaminosis period, and from 57 to 15 % in the recovery period[1]. However, a similar investigation has been carried out by Brown and Shohl [1930], but embracing a series of different levels of vitamin D feeding; and since their results have the advantage of showing greater uniformity than those just discussed, it has been thought

[1] This relates to the organically bound P of the phosphate-free Steenbock diet. Since in vitamin D metabolism we are primarily concerned with inorganic phosphate, the meaning of the figures might conceivably be disputed, but the results of Brown and Shohl (*v. infra*) definitely show its increased absorption under the influence of vitamin D.

worth while to recalculate their figures, also upon a percentage basis, in order to demonstrate the remarkable consistency and regularity with which each increase in vitamin D does in fact give rise to an increased net absorption from the gut, until finally the toxic complications supervene. Thus on Steenbock's rachitogenic diet (high-calcium, low-phosphate) the addition of 10,000 times the minimal effective dose of vitamin D increased the net absorption of phosphorus by no less than 300 %, and of Ca by about 50 %. With still larger doses, and the appearance of toxic symptoms and loss of appetite, the absorption begins to fail (Table I). Brown and Shohl themselves, although drawing attention to the increased urinary elimination of Ca and P with large doses of vitamin D (the excretion as they say shifting from the faeces to the urine, and the retention decreasing), did not attach special significance to the question of gut absorption, in fact they stated that the amount of Ca and P excreted remained relatively constant (in the absence of toxic symptoms).

Table I. *Increase in net absorption of Ca and P resulting from increasing doses of irradiated ergosterol. Recalculated from Brown and Shohl [1930]. (Steenbock high-Ca, low-P diet).*

Daily dose of irradiated ergosterol (mg.)	0	0·01	0·1	0·5	1·0	2·0
P "absorbed," % of intake	8, 12	22, 27	26, 26	33, 37	41, (21)*	(27)*
Ca "absorbed," % of intake	31, 36	42, 43	43, 49	48, 48	49, (34)*	(34)*

* Marked loss of appetite.

A similar examination of Brown and Shohl's figures for a diet (Sherman) with a normal Ca/P ratio, as opposed to the rachitogenic, high-Ca, diet, shows a corresponding if less intense effect. An increase of vitamin D by about 2000 times above the minimal protective dose gives rise to a well-marked increase in the weight of Ca and P absorbed (Fig. 13), while at higher levels the complicating influence of the lowered food intake is again evident, even when results are calculated on a percentage basis (Fig. 14).

With our diet rich in phosphate but free from Ca (Fig. 7) the net absorption of P was already at so high a level (83 to 93 %) in the control that the demonstration of any increase under the action of vitamin D was not to be anticipated. It may be recalled indeed that at the level of vitamin D excess employed no toxic ill-effects were seen with this diet. As has already been suggested the effect on phosphate may be secondary to that on Ca.

But perhaps the most striking argument that can be deduced in support of our view, that large doses of vitamin D tend to raise the absorption of Ca and phosphate (or to diminish their back excretion into the gut), is obtained from a study of the effects of a cure in hypervitaminosis. An animal having the typical abnormally high blood-phosphate or -Ca induced by excessive doses of vitamin D is suddenly put back on to a diet free of any such excess. The curative process begins immediately. The absence of vitamin D, it can

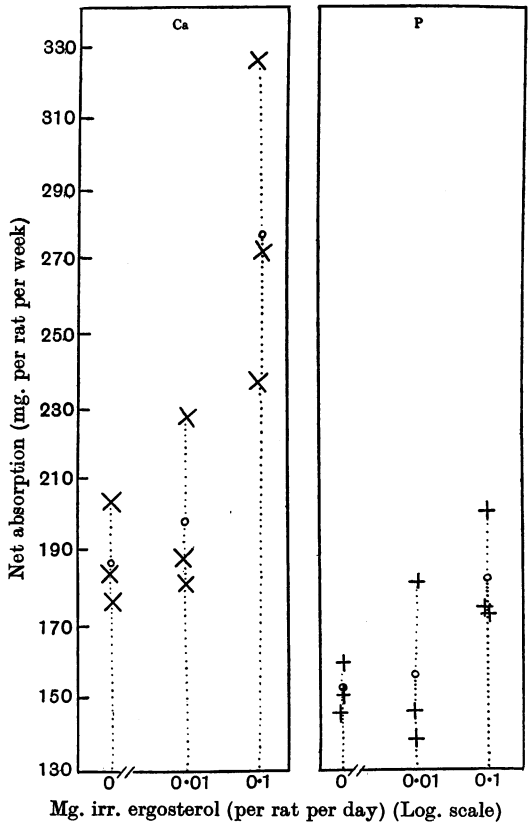

Fig. 13. Increase in Ca and P net absorption with increasing doses of irradiated ergosterol. Recalculated from Brown and Shohl. Normal diet (Sherman).
0 = mean readings.

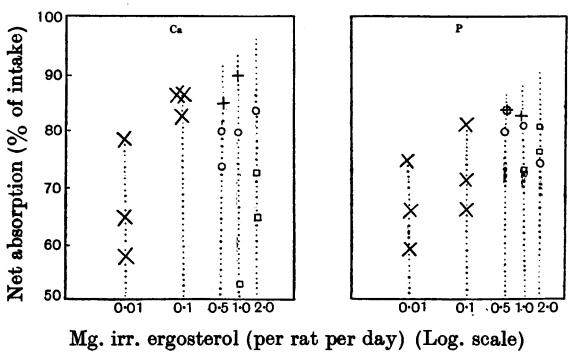

Fig. 14. Complicating influence of failure and loss of appetite with high levels of toxicity.
X Intake approx. normal, 240–390 mg. Ca per week.
\+ ,, below normal, 170 ,,
o ,, low, 110–150 ,,
▫ ,, very low, under 100 ,,

be shown, now permits the animal to excrete in its faeces a vastly increased amount of Ca or P, more suitable to the excessive blood level and tending to reduce it towards the normal. The faecal calcium now reaches figures as high as 65–90 %, compared with only 10–20 % in the presence of the excessive vitamin D, or 20–60 % for the appropriate controls (expressed as percentage of intake) (Fig. 15). Thus the point of view we are here advocating will allow

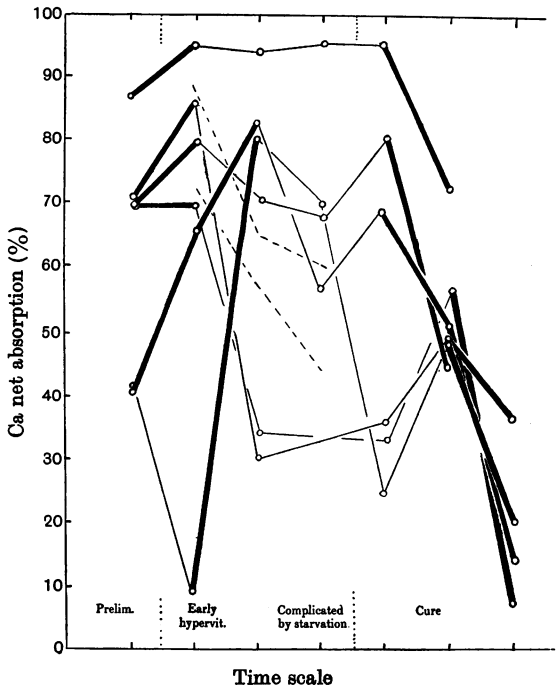

Fig. 15. Rise in net absorption at beginning of hypervitaminosis, complicating influence of starvation, and fall in cure.
– – – Starvation control without hypervitaminosis.

a rational interpretation of the underlying causes of the "even graver disturbances in absorption and retention during the recovery period," to which attention was first drawn by Watchorn [1930, 1].

We have therefore the following evidence pointing to increased net absorption under the influence of vitamin D:

(1) the dependence of the hypercalcaemia and calcareous deposition in hypervitaminosis D upon the Ca intake;

(2) the occurrence of a greatly increased faecal excretion when the hypervitaminosis is terminated;

(3) direct calculations upon data for intakes and faecal outputs, including the earlier uncomplicated stages of hypervitaminosis;

(4) measurements by earlier workers upon clinical and experimental hypovitaminosis and its cure.

Raised blood-Ca and/or -phosphate.

The production of hypercalcaemia and/or hyperphosphataemia in rabbits or rats by the action of excessive doses of vitamin D, first reported by Harris and Stewart [1929], and repeated under a wider variety of conditions [Harris, 1930], has been repeatedly confirmed, *e.g.* by Ashford [1930] who showed that there was no concurrent increase in the organic acid-soluble phosphate, and the observation has been extended to other species including dogs [Jones, Rapoport and Hodes, 1930, 1], chickens [Massengale and Nussmeier, 1930], and cows [Greig, 1930]. But certain workers have obtained less consistent or even negative results, the reasons for which demand consideration here.

In the first place the important consideration must be borne in mind, as originally indicated [Harris and Stewart, 1929] and again confirmed in our present results, that the severity of the condition is largely governed by the amounts of calcium or phosphate ingested. The influence of this factor is clearly apparent also in the recent result of Warkany [1928], who showed that the rise in blood-phosphate following a meal of inorganic phosphate is greatly increased by the administration of irradiated ergosterol. In the second place it must be realised that an increase in both Ca and P simultaneously is not necessarily seen; sometimes one is affected first, sometimes the other. The influence of calcium equilibrium upon phosphate and *vice versa* is apparent here. Next, the hyperphosphataemia or hypercalcaemia, varying as it does with the diet, is to be understood in a relative rather than an absolute sense; for example, the figures of Shohl, Goldblatt and Brown [1930] show that in rats fed on high-phosphate or high-calcium diets with excess vitamin D, the blood-Ca or -P is high compared with the corresponding controls, although not necessarily of course compared with the normal rat. Finally, negative results have sometimes undoubtedly resulted from failure to give sufficiently large excess of the vitamin. It was earlier suggested [Harris and Moore, 1929, 1, p. 272] that such an explanation might account for the observations of Havard and Hoyle [1928], who, working on man, were unable to obtain any rise in inorganic P or serum-Ca above the low winter level. Confirmation for this view appears to be found in more recently published work on human beings, by various authors, in which very high levels of vitamin D have been fed, and one or other of the following typical abnormalities reported, *viz.* hypercalcaemia, or hyperphosphataemia, or increased renal excretion of P or Ca, or overcalcification of bone-endings, and, even, calcareous deposits [Hess, Lewis and Rivkin, 1928; György, 1929; Puschler, 1929; Hughes *et al.*, 1929; Hottinger, 1929; Kroetz, 1927; Lasch, 1928; Ghirardi, 1929].

Withdrawal of calcium from bone.

Our results show that with diets very rich in calcium a relatively small excess of vitamin D is sufficient to cause hypercalcaemia (or hyperphosphataemia) and the appearance of calcareous deposits. Under these circum-

stances there is, of course, little evidence of osteoporosis. Similarly, chemical analysis showed that there was no appreciable change in the mineral content of the bone. These results were published in a preliminary communication which stressed the importance of the Ca intake as a determining factor in hypervitaminosis; but unfortunately the following statement, couched in far too sweeping terms, was made, "we find by chemical analysis that there is no loss of Ca or P from the bones to account for the rise in the blood figure" [Harris, 1930]. It should have been made clear that this related only to these particular diets. With very high doses of vitamin D, particularly on Ca-deficient diets, the withdrawal of mineral from the bone becomes the outstanding feature. (This conclusion was also reached indirectly [Watchorn, 1930, 2] from analyses of faeces and urine for the animals shown in Fig. 8.) The evidence of a similar withdrawal and re-deposition in the cure of rickets has already been alluded to.

Abnormalities in bone structure in hypervitaminosis.

In marked contrast with our evidence of immense stimulation of spongiosa formation and resorption of compact bone, Shohl, Goldblatt and Brown [1930] found "no striking abnormalities" histologically in the ribs in hypervitaminosis. On the other hand in the recent independent work of several other investigators [Kreitmair and Hintzelmann, 1928; Baumgartner, King and Page, 1929; Weinmann, 1929; Collazo, Varela and Rubino, 1928, 1929, 1, 2, 3] many conclusions may be found in common with our own. Hypercalcification of the growing end of the bone was also clearly shown in radiological observations on children by Hess, Lewis and Rivkin [1928], and on rats by Shohl, Goldblatt and Brown [1930], and by György [1929].

Urinary calcium (or phosphate) excretion.

Harris and Stewart [1929] found that in hypervitaminosis D the renal excretion of calcium was so much increased that the urine became cloudy with calcium salts. They interpreted this to mean merely that the organism was endeavouring to dispose of the excessive calcium carried into the blood stream. More recent quantitative tests have abundantly confirmed the existence of the remarkably high urinary output either of phosphate or of calcium or both [*e.g.* Ashford, 1930; Watchorn, 1930, 1, 2; Brown and Shohl, 1930] and the negative result of Hoyle and Buckland [1929] must presumably be ascribed to the use of insufficient excess of vitamin.

Summary.

A study has been made of the influence of variations in the calcium and phosphate intake upon the abnormalities resulting from overdoses of irradiated ergosterol. An increase in the calcium content of a diet (or in the Ca/P ratio) intensifies the severity of the hypervitaminosis and gives rise to

an increased formation of the calcareous deposits, at a given level of vitamin D excess. With diets virtually devoid of calcium and phosphate, on the other hand, a hypervitaminosis of a distinctive character can still be produced provided now that the level of vitamin D excess is sufficiently raised; under these conditions calcareous deposits are not in evidence but there is a greatly increased resorption of bone substance.

X-ray and histological examinations of the bones have shown that large doses of vitamin D (with normal diets) stimulate osteogenesis, and a densely calcified overgrowth appears at the growing end of the bone (in contrast with rickets); while in the advanced degrees of the hypervitaminosis resorption is extensive, and the cortex of the shaft and other "compact" bone becomes spongy. Vitamin D excess also gives rise to a remarkable overgrowth of cement in the growing animal.

In explanation of these and earlier results it is shown that feeding of excess of vitamin D (in the various types of hypervitaminosis examined) gives rise to a raised blood-calcium or -phosphate or both, with a tendency to deposition of calcium in certain sites, just as vitamin D deficiency (*e.g.* in rickets) gives rise to lowered blood-phosphate or -calcium or both and accompanying inadequate calcification. The extra calcium and phosphate may be derived in two ways: (1) increased net absorption from the gut; and (2) increased withdrawal from bony stores, vitamin D therefore having a distributive action. In the hypervitaminosis produced on diets rich in calcium with moderate overdoses of vitamin D the first of these factors is of special consequence, while with Ca-deficient diets and with larger excesses of the vitamin, withdrawal from the bone shaft is the main source.

That vitamin D increases absorption of Ca (or P) from the gut (or decreases excretion into the gut) is shown: (1) from the effects of increased Ca intake in hypervitaminosis, (2) from the observation that withdrawal of vitamin excess causes greatly increased faecal excretion of Ca (or P), (3) from recalculations of available data for intake and faecal output, it being shown that each addition of vitamin D to a diet causes an increased net absorption, until ultimately with highly toxic levels a generalised failure complicates the result, and (4) from evidence from rickets and its cure. The mode of action of vitamin D in this respect stands in contrast with that of the parathyroid hormone.

Thus while addition of vitamin D to the diet tends to raise the blood-Ca (or -P, since one influences the other) the actual level so attained is the resultant of several factors: operating in one direction is the increased net absorption and dissolution from certain sites, and, in the opposite, the deposition in other sites, and (when high levels are reached) an increased urinary excretion. With increasing doses of vitamin D the retention by the animal as a whole first rises but ultimately falls, the kidney excretion overtaking the gut absorption.

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DESCRIPTION OF FIGURES IN PLATES I AND II

PLATE I. Comparative X-ray photographs showing end of long bone (knee joint), (1) normal, (2) in rickets, and (3) in hypervitaminosis.

C = epiphyseal cartilage. *E* = epiphyseal end of diaphysis.

1. Normal.

2. Rickets: widening of epiphyseal cartilage and deficient calcification of epiphyseal end of diaphysis.

3. Early hypervitaminosis, 8 days: abnormally narrow epiphyseal cartilage; and broad dense band of excessive calcification at epiphyseal end of diaphysis, extending into marrow cavity.

4. Later stage, 14 days (complicated by cessation of growth and osteogenesis): appearance of a gap between the dense overcalcified area and the epiphyseal cartilage.

5. Same, more advanced, 29 days, with calcification at epiphyseal cartilage *C*.

6. Cure of early hypervitaminosis: (*a*) partial loss of excessive calcium, (*b*) cure complete, bone structure returned to normal.

7. Hypervitaminosis with same level of vitamin D excess but on Steenbock's rachitogenic diet: bone structure almost normal.

PLATE II. Bones and teeth in hypervitaminosis D (rat).

A. TEETH.

1. *Normal control.* Note uniform normal dentine (D), cement (C) and jawbone (J). (Transverse section of incisor. Haematoxylin and eosin. × 160/3.)

2. *Hypervitaminosis in young rat.* Enormous overgrowth of cement (C). Formation of true bone (b) near dentinal junction. Osteoporosis of jawbone (J). Abnormal secondary dentine (D). (× 160/3.)

B. LONG BONE.

1. *Normal control.* Note features of normal osteogenesis at epiphysis: regular intermediate growth-cartilage (C), showing normal columns of cartilage cells and trabecular formation (T). Normal compact cortex of shaft (S). (Distal end of femur. × 50/3.)

2. *Early degree of hypervitaminosis* before much resorption had occurred from cortex of shaft (no severe calcification of kidney or aorta was yet apparent at *post mortem*). Decrease in thickness of intermediate growth-cartilage (C). Increased new bone formation: trabeculae covered with thick lamellae of bone (T). (Proximal end of tibia. × 50/3.)

3. *Later stage.* Thin intermediate growth-cartilage (C). Further great increase in subepiphyseal spongiosa (T) extending far into marrow cavity. Thin osteoporosed cortex of shaft (S). Slight resorption (R) of spongiosa nearest growth cartilage. (Distal end of femur. × 50/3.)

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Page 390. Lettering on Plate II, A, in figs. 1 and 2:
"C" should be raised $\frac{1}{8}''$ and $\frac{1}{2}''$ respectively.

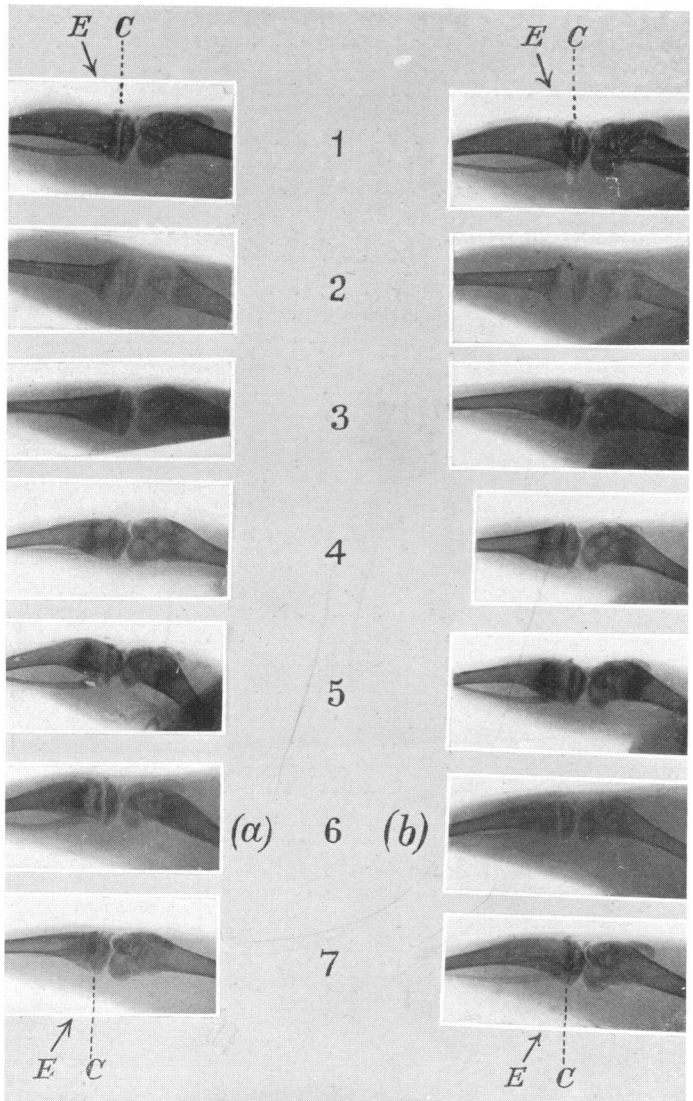
Plate II, description of A, fig. 2:

for Enormous overgrowth of cement (C). Formation of true bone (b)

read Enormous overgrowth of cement (C), with formation resembling true bone ("adventitious cement") (b)

Page 492, 10th line from bottom:

for vitamin B deficiency *read* vitamin C deficiency


E
C
E
C
1
2
3
4
5
(a)
6
(b)
7
E
C
E
C

A
D
C
EJ
1
D
b
EJ
C
2
B
S
T
C
1
T
C
2
S
T
C
3