CXLII. ON THE FUNDAMENTAL NATURE OF VITAMIN D ACTION.

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(Preliminary Communication.)

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(*Received July 4th, 1931.*)

IN a previous paper [McGowan, 1930] it was shown that, in chloroform poisoning in the rabbit, there is an accumulation of fatty substances together with calcium in certain of the tissues, especially the liver. This was interpreted as meaning that chloroform attacked and broke up the lipins, thereby setting phosphoric acid free to be neutralised *in situ* by calcium to form insoluble calcium phosphate. A similar deposition of calcium salts in certain of the tissues, following on a preliminary degeneration, has been recorded as the main feature of vitamin D poisoning [Duguid, 1930; and others]. Experiments were therefore undertaken to see if this hypervitaminosis D was explicable on a similar basis.

Rabbits were employed. These, for several weeks prior to the experiment, had been kept on the experimental ration, which was a modification of the stock ration in the direction of a considerable increase of its calcium carbonate content. The stock ration consisted of bran 8 parts, white sharps 8, bruised oats 8, and fish meal 1. To this was added, to form the experimental ration, 2 parts calcium carbonate, making thus roughly 8 %.

The rations were fed *ad lib.* and, in every case, in addition there was an abundant supply of freshly cut clover-rich grass. Water, for drinking purposes, was always available.

The vitamin D employed was in the form of a solution of irradiated ergosterol in olive oil, of a strength of 1,000,000 rat units per cc. For a supply of this we are greatly indebted to Mr A. L. Bacharach of the Glaxo Research Laboratories. It was administered by mouth by means of a pipette twice daily. So far the dosage employed has been 4–8 million rat units per day.

In Table I are summarised some results obtained in illustrative cases.

Table I deals with results obtained from a rabbit (No. 2) poisoned with vitamin D; a rabbit (No. 4) poisoned with chloroform; normal rabbits (Nos. 5, 10 and 11), fed on the stock ration for the composition of normal urine for comparison purposes; and two normal rabbits for the calcium content of the liver.

Table I.

Rabbit and dose	Diet	Date	Weight g. A	Cc. urine in 24 hrs. B	Nature of deposit, *etc.* Crystals C	Gas with acid D	Urine reaction C	Total CaO as carbonate % E	Total CaO % F	Total P_2O_5 % G	CaO in 24 hrs. g. H	P_2O_5 in 24 hrs. g. I	Ca in blood mg./100 cc. J	P in blood mg./100 cc. K	Ca % moist weight liver L	Ca % moist weight kidney M
No. 2. Irradiated ergosterol, 4 million rat units daily commencing 11. vi. 31	Stock ration +8 % $CaCO_3$ +grass, all *ad lib.*	10. vi. 31	2020	72	Almost pure $CaCO_3$	+ + + + + +	Alk.	—	0·36	0·01	0·259	0·007	17·5	3·03	—	—
		11. vi. 31	2030	150	do.	+ + + + + +	do.	—	0·44	0·01	0·660	0·015	—	—	—	—
		12. vi. 31	1950	121	Almost pure amorphous phosphates	+	do.	—	0·64	0·13	0·774	0·157	—	—	—	—
		13. vi. 31	1950	65	do.	+	do.	—	0·57	0·46	0·370	0·299	—	—	—	—
		14. vi. 31	1840	72	do.	+	do.	0·13	0·42	0·47	0·302	0·338	—	—	—	—
		15. vi. 31	1795	85	do.	+	do.	0·04	0·50	0·46	0·425	0·391	—	—	—	—
		16. vi. 31	1750	*											0·0076	0·23
		16. vi. 31	Died	22·5†	do.	+	do.	0·17	0·64	0·29	—	—	—	—	—	—
No. 4. 1·0 cc. $CHCl_3$ on 17. vi. 31 subcutaneously	Stock ration +8 % $CaCO_3$ +grass, all *ad lib.*	15. vi. 31	1590	—	—	—	—	—	—	—	—	—	—	—	—	—
		16. vi. 31	1650	274	Almost pure $CaCO_3$	+ + + + + +	Alk.	0·60	0·61	0·02	1·671	0·055	15·3	5·3	—	—
		17. vi. 31	1640	145	do.	+ + + + + +	do.	0·95	1·17	0·03	1·697	0·044	—	—	—	—
		18. vi. 31	1600	82	Almost pure amorphous phosphates	+ ?	Neutral	0·00	0·33	0·43	0·271	0·353	13·8	3·2	—	—
		19. vi. 31	1550	144	do.	+	Alk.	0·03	0·15	0·08	0·216	0·115	—	—	—	—
		19. vi. 31	Killed	25†	do.	+	do.	0·05	0·14	0·05	—	—	—	—	0·0078	0·028
Normal. No. 5	Stock ration alone + grass	20. vi. 31	1800	130	None	None	Acid	None	0·04	0·26	0·052	0·338	14·8	3·2	—	—
No. 10		20. vi. 31	2350	115	do.	do.	do.	do.	0·009	0·46	0·010	0·520	—	—	—	—
No. 11		20. vi. 31	1900	50	do.	do.	do.	do.	0·039	0·18	0·078	0·360	—	—	—	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·0065	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·0066	—

* Urine lost by being passed into food dish. † In bladder.

Column A shows the loss of weight from vitamin D and chloroform poisoning. Rabbit 2 ceased to eat on the 14th, two days after vitamin D administration commenced, whereas rabbit 4 stopped on the 17th, as soon as the chloroform had been administered. These occurrences have to be considered in relation to the results obtained below.

Column B gives the amount of urine passed in 24 hours. When the animal died or was killed, the urine present in the bladder was also recovered.

Column C deals with the reaction and the nature of the deposit present in the urine as observed microscopically. In the rabbits on high-calcium diet, the urine was alkaline; on stock ration, acid. In rabbits 2 and 4 on high-calcium diet, the deposit prior to poisoning consisted practically entirely of calcium carbonate crystals. Immediately on poisoning, the calcium carbonate was replaced by amorphous phosphates. There was no deposit present in the normal rabbits on stock ration.

In Column D is given the effect of adding acid to the microscopical preparations. For comparative purposes, a record in arbitrary symbols is given of the amount of gas (CO_2) evolved. It will be seen that this supports the findings in Column C.

In Column E is recorded, as CaO % in the urine, the amount of calcium present as carbonate, as calculated from the amount of gas collected on making the urine distinctly acid. This line of observation was not adopted till 14. vi. 31, hence the results for rabbit 2 prior to this date are lacking. From those obtained in Column D, however, they are undoubtedly quantitatively much the same as in rabbit 4 for the pre-poisoning period. The great diminution of the calcium in the form of carbonate in the poisoning period is again evident, while the very great excess of calcium as carbonate or phosphate in the urine of the experimental animals over that in the urine of the normal rabbits on the stock ration is demonstrated.

Columns F and G give respectively the percentage total CaO and P_2O_5 in the urines. These results, however, can only be discussed in relation to Columns H and I.

In regard to Column H, it will be seen that in rabbit 2 the calcium in the poisoning period remains more or less at the level it was in the pre-poisoning period, with the exception of a possible fall at the very end (see Column F), whilst in rabbit 4 there is a very marked drop in the poisoning period. The result in rabbit 4 may be connected with the diminished intake of food, with its high calcium content, which occurred during this period.

In Column I is demonstrated the very marked increase in the poisoning period of the P_2O_5, more gradual in the case of rabbit 2, with a definite falling off in rabbit 4 and a possible one also in rabbit 2 towards the end of the period. This terminal fall may possibly be attributed to metabolism as a whole having more or less ceased, owing to the animal being *in extremis*, or to the supply of readily available lipins, from which phosphorus could be split off, being used up. The results, therefore, as far as regards the phosphorus, indicate a greatly

increased excretion of phosphorus, which agrees with Duguid's summing up of the situation [1930], where he states that "there is a very greatly increased excretion of phosphorus especially by way of the kidneys and this, occurring when the food consumption is diminishing as is usual in hypervitaminosis D, involves a reduction in the phosphorus retention in the body." Duguid [1930] also states that an increased excretion of calcium in the urine is the rule in such cases. The different result obtained in the present instance may have reference to the very high calcium content of the food and the high level of calcium excretion in the urine to begin with.

The results in Columns E, H and I go to show that the calcium, formerly excreted as carbonate, is got rid of in the poisoning period in the form of phosphate.

The bones of the rabbits were ivory hard and showed no trace of thinning of the cortical bone or of any demineralisation process.

In Columns J and K a few results are given of the estimation of Ca and P in the circulating blood. This mode of observation, however, was not pressed. It was recognised that the withdrawal of 10 cc. of blood from a rabbit at frequent intervals would jeopardise more important results, while it was also borne in mind that, in the poisoning stage, owing to the depressed condition of the circulation, it is often impossible to obtain a sample of blood. More important still, it was realised that, in such procedures, one is estimating the Ca and P in a sample of blood taken in its passage from an irregular and varying "source" or "sources" to an irregular and varying "distribution," two variables regarding the essential activity of either of which, at the time the blood sample was being taken, there is and can be, under present circumstances, no real knowledge. Thus, while a high Ca % in the blood might mean a high intake from the "sources," it might equally well denote a diminished "distribution." A similar state of matters exists in regard to phosphorus. There is also possible a variety of further permutations and combinations of "source" and "distribution" with an effect on the blood composition.

In a previous communication [McGowan, 1930, Table, p. 288], it was shown that in chloroform poisoning there was a great increase in the amount of fat and calcium salts in the central two-thirds of the liver lobules. The amounts present of these two substances ran more or less parallel. Where relatively small amounts of each existed, the histological picture showed that these were remnants, left from large amounts previously present, which, in the meantime, had been removed by the circulation. This process of removal is a rapid one, for the experiments lasted in most cases for a few days only. In these experiments also, the fatty material was present not only as neutral fat but to a large extent as calcium soap. For comparison with the figures obtained in the present investigation, it may be stated that, in one of these cases of chloroform poisoning in the rabbit, the calcium as metal present in the liver was as much as 2·66 % of the moist weight. On the other hand, figures as low as 0·025 % were obtained.

In the present case of chloroform poisoning (rabbit 4) the findings were quite different from those just stated. The inner two-thirds of each lobule was indeed crammed with lipoid material, which, on staining with Nile blue sulphate, was found to be neutral fat without any fatty acids. Von Kossa's silver nitrate test for calcium phosphate gave entirely negative results, while the amount of calcium present was only 0·0078 % of the moist weight, a figure differing but little from the normal (*vide* bottom of column L). It is difficult to explain the anomalous condition present here unless one assumes that the large amount of $CaCO_3$ in the diet had prevented the absorption of inorganic phosphates from the food, thus rendering the blood relatively deficient in, and avid for, inorganic phosphate and so withdrawing the latter from the tissues whenever and as soon as it was formed [*cf.* Cushny, 1918].

If one now turns to consider the appearances present in the livers of the rabbits poisoned with vitamin D, frozen sections stained with Sudan III gave no evidence of the presence of fatty material in any considerable amount. In this connection, however, it should be noted that Haendel and Malet [1930] have found large quantities of fat in the liver, kidney, heart and spleen of rabbits poisoned with vitamin D. Von Kossa's stain also gave a negative result with regard to the presence of calcium phosphate. Fat, nevertheless, may have been present in the cells of the present cases in globules too minute to be detected by the coarse technique just alluded to. Osmic acid preparations have not as yet been made, but ordinary paraffin sections show a very advanced stage of cloudy swelling, with granular degeneration and a basophil reaction of the cytoplasm, conditions found present also in the liver cells at the periphery of the liver lobules in chloroform poisoning. This latter appearance may indicate a reaction to the acid side, which may possibly be due to acid phosphates, derived from a breaking down of the lipoids. In any case, cloudy swelling is the well-known precursor of the ordinary easily recognisable fatty degeneration. The calcium present varied little from normal, being 0·0076 % of the moist weight.

The histological changes in the various organs and tissues of the rabbits poisoned with vitamin D may now be discussed. They were practically those found by previous observers. The following tissues were examined: liver, spleen, kidney, testicle, suprarenal, heart muscle, wall of aorta, lung, skeletal muscle, stomach wall, small intestine, colon and bone marrow. Briefly put, the changes observed were, in the first place, cloudy swelling and granular degeneration, especially present in the liver cells, secretory tubule cells of the kidney and in the muscle of the heart, arteries and stomach wall. Deposition of calcium salts was found present in the media of the arterioles of the cortex of the kidney and of the lung and in the media of the aorta. Specially, there was no deposition in the heart muscle or in the arterioles of the heart. There were large patches of precipitation in the muscular wall of the stomach of such size that they could be seen by the naked eye as white striations. A few small spots were also present in the gastric mucosa. As already suggested in relation

to rabbit 4, the high calcium diet may have interfered somewhat with calcium deposition. The kidney showed the presence of calcium in its tubules. Doubtless this is to be explained by the high excretion of calcium in the urine and is not necessarily indicative of a degenerative change in the epithelium. The relatively high calcium in the kidney (*vide* Column M) seems also explicable on this basis, the higher figure for rabbit 2 being an index of the increased calcium in the walls of the arterioles.

The suprarenals in these cases were much enlarged, the diameter being about five times that of the normal gland. The enlargement was mainly in the cortex at the expense of the medulla. The cortex was pure white in colour and gave a marked Sudan III reaction, while in paraffin sections the cytoplasm of the cells stained deeply basophil. The significance of these findings awaits elucidation.

Contrasting the condition found in these cases with that present in chloroform poisoning, it would seem that, in both instances, there is an attack on the protoplasm of the cell. In chloroform poisoning, however, there is a much larger amount of the poison active at any one time, the condition being, in every respect, a more fulminating one. The poisoning produces its maximum effect almost at once, to be followed almost immediately by phenomena of recovery. Hence the lipins are broken up rapidly, fatty bodies and calcium phosphate being produced in large abundance *in situ* without time being given for their removal into the circulation. Thus, within the time employed for such investigations, in most instances they remain to some degree in the tissues and are observed on histological examination. Evidence has, however, already been adduced to show that they can be, and are at times, fairly rapidly removed [McGowan, 1930, Table, p. 288].

In the case of vitamin D poisoning, however, the amount of poison active at any one time is very small, the attack on the cell and its lipins is not so drastic, and ample time is given for the removal of by-products. Hence, on histological examination, the appearances found are much milder in type and calcium is found only in those situations, the media of the arterioles, *etc.*, where, once having been deposited, it cannot so readily be removed as it can in the case of parenchymatous cells of actively metabolising glands, like the kidney and liver, which abut on a rich blood supply. This view is supported by the results obtained by Hertzenberger [1929], who found evidence of the healing of the lesions and disappearance of the calcium deposits after cessation of administration of vitamin D and by those of Schiff [1930], who found no evidence of resorption of the calcium from the arterial lesions after poisoning was stopped.

The rather insignificant pathological findings just described have been obtained in the case of an animal which actually died within 5 days from the effects of the poisoning. It is a question therefore whether changes of this degree are not the essential ones and whether the more advanced changes, involving parenchymatous cells of various organs in fatty change and calcium

deposition, which have been described, may not have to be attributed to an anoxaemia brought about by the alterations in the vascular walls.

The broad aspects of the subject in hand may be discussed and the position summed up by a consideration of some points raised by Cushny [1918] relative to phosphorus and calcium metabolism. On p. 108, he makes the statement that in the herbivora phosphates are excreted exclusively by the bowel wall. Obviously, this generalisation is incorrect, for, in Table I, it will be seen that rabbits 5, 10 and 11, on the stock ration, pass considerable quantities of phosphates in the urine. Dealing with the subject of calcium metabolism at p. 560, Cushny states that calcium is excreted in part by the urine but for the most part through the epithelium of the large intestine. The relative amounts excreted by the kidney and bowel seem to be determined by the quantity of available phosphates among other factors. If these are present in large quantities in the blood, the calcium is excreted mainly in the bowel in the form of calcium phosphate. The elimination of calcium thus appears to vary with the character of the combinations which it can form. If these are soluble they appear in the urine, while the insoluble ones tend to pass into the stools. Calcium lessens the phosphates of the urine by forming insoluble phosphates in the bowel and thus preventing the absorption of the phosphates of the food.

It seems possible to harmonise these views of Cushny with the results obtained in our experiments. It may be supposed that, in the rabbits fed on the stock ration, there is not enough calcium to join up with the phosphate and carry it in the insoluble form of calcium phosphate to the bowel for elimination. There is an over-plus of phosphate which appears in the urine as alkaline phosphates. With more calcium in the food, it could be supposed that practically all the phosphate goes as calcium phosphate to the bowel, leaving only a trace to appear in the urine. *A fortiori*, this holds for conditions where there is great excess of calcium in the diet, with the further significant occurrence that the excess calcium appears in the urine as the carbonate conveying CO_2 out of the body, a function more efficiently performed by the lungs. In conditions of vitamin D and chloroform poisoning in animals fed excess of calcium carbonate, there is great excess of phosphate produced, more than can be got rid of as calcium phosphate by the intestine. The excess calcium phosphate produced now goes by the kidney, the calcium formerly excreted as carbonate being utilised for this purpose. One slight blemish—it is, however, a slight and unimportant one from the present point of view—appears at first sight to mar the complete correlation. Cushny states that the insoluble calcium compounds pass by the bowel and apparently by the bowel only. Yet, it is indubitable that, in the present experiments, not only has calcium phosphate passed by the kidney (for this of course an explanation has been offered), but also such an insoluble substance as calcium carbonate. Doubtless, however, Cushny's statement will hold if the food supplies no more calcium than can be dealt with by the phosphate in the blood and so be removed as insoluble calcium phosphate by the intestine.

It seems possible that the findings of Harris and Innes [1931] that vitamin D poisoning is accentuated by increase of calcium in the diet, but that vitamin D, even in large doses over a long period, becomes relatively harmless when calcium is balanced by the addition of phosphate or when calcium is omitted and phosphate remains high, may derive some explanation from what has just been discussed. For one thing, there will be less calcium present to form deposits and, for another, the less speedy "insoluble" method of elimination by the bowel in the form of calcium phosphate may be supplanted by the more speedy "soluble" method of elimination by means of the kidney in the form of acid phosphates of the alkali metals.

The subject remains for discussion as to the relation of the present findings to the question of the nature of rickets and of the action of vitamin D in this disease. Elsewhere [McGowan, 1926], it has been pointed out (and the implications emphasised) that rickets begins very early during the suckling stage of the animal. Evidence was also adduced to show that the appearance of rickets depends on a deficiency of consolidating materials in the milk relative to the growth-promoting ones and to the growth potential of the young animal. Further, it has been shown that the administration of inorganic phosphate, in the form of sodium phosphate, can prevent and cure rickets; in the fowl, by Bethke, Kennard, Kick and Zinzalian [1927–28] and in the rat by Karelitz and Shohl [1927] and Kramer, Shear and Siegel [1931]. From our previous discussion and these considerations, we draw the conclusion that there is more to be said for a relative deficiency of inorganic phosphate being the actual *causa causans* of rickets than for the lack of any other substance hitherto alleged to be operative[1].

As to the possible mode of action of vitamin D in combating the disease, apart from what has already been adumbrated above in this direction, an insight may be gained by a consideration of the significance of the persistent low blood phosphorus that occurs in rickets. This low phosphorus may be attributed, on the one hand, to the constant removal, for constructional purposes, from the blood of inorganic phosphorus by the avid bony tissues and, on the other hand, to a low supply to the blood from the diet which is deficient in inorganic phosphorus. This can be rectified, directly, by supplying additional inorganic phosphorus in the diet or, indirectly, by supplying a substance—vitamin D—which will make available to the blood the inorganic phosphorus stored in the body lipins. Further, it seems obvious that the supply of great excess of inorganic calcium in the diet, by its action in rendering the inorganic phosphates of the diet insoluble and unabsorbable, will predispose to the production of rickets.

[1] A calcium-deficient ration produces osteoporosis, not rickets. At the same time, it should be noted that rickets is not, in our opinion, due essentially and primarily to a lack of vitamin D.

SUMMARY.

Evidence has been adduced to show that the probable mode of action of vitamin D in the cure and prevention of rickets is by the setting free from the lipins of the body of inorganic phosphate, the relative deficiency of which is regarded as the essential cause of rickets.

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