CXXII. FURTHER INVESTIGATIONS INTO THE FUNDAMENTAL NATURE OF VITAMIN D ACTION.

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IN a preliminary paper [McGowan, Cunningham and Auchinachie, 1931] certain tentative conclusions were drawn, on the basis of incomplete balance experiments, regarding the intrinsic and essential nature of vitamin D action. A series of complete calcium and phosphorus balance experiments has since been carried out, and these are dealt with in this paper.

These experiments were conducted in the customary way on rabbits in metabolism cages, the usual precautions being taken with regard to feeding and collection of urine and faeces. As the animals, in eating their ration, selected certain parts of it, care was taken to recover all the non-eaten portion, and the calcium and phosphorus intake was determined from its analysis. Water was freely available. For roughage, 100 g. turnip was given daily. This was not taken account of in the eventual balance. As a general rule, the faeces and urine were collected daily and were analysed, as far as possible, in 2-day periods.

The basis of the ration fed was 8 parts each of "Porage" oats, white sharps and bran, together with 1 part of fish meal. In some cases, the fish meal was withdrawn, while, in others, CaCO_3 was added. The composition of the various rations fed as regards CaO and P_2O_5 was as follows:

Table I.

	CaO %	P_2O_5 %
1. Rabbit A	4.5589	1.6193
2. " B	4.5589	1.6193
3. " C (stock)	0.4200	1.7883
4. " C (CaCO_3)	4.5589	1.6193
5. " D (stock)	0.4200	1.7883
6. " D (minus fish meal)	0.0970	1.4848
7. " E (CaCO_3)	1.1512	1.8476
8. " H (CaCO_3)	4.5589	1.6193
9. " H ¹ (minus fish meal)	0.0970	1.4848

Rabbit A, buck, weight 2130 g.; ration fed consisted of stock ration as above, to 25 lbs. of which were added 2 lbs. CaCO_3 (Table I, 1). The experiment lasted from 8 to 12. vii. 31 (pre-period). It received 4 cc. of a solution of irradiated ergosterol (1,000,000 rat units per cc.), on 12. vii. 31, and the same dose on 13 and 14. vii. 31. It was killed on 15. vii. 31. The results of the balance are given in Table II.

The points to be noted in Table II are the very marked negative P_2O_5 and CaO balances and the shift of the excretion of P_2O_5 from the faeces to the urine in the experimental period. In the pre-period, the urine, as voided, was very alkaline and contained a large deposit of CaCO_3 crystals: while in the experimental period the urine was acid and clear, becoming turbid on cooling owing to separation of phosphates.

Table II*.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
<i>A</i>			P_2O_5				
Pre-period	8-12. vii. 31 (5 days)	0.8090	0.0472	7.15	0.6128	92.85	+0.1480
Exp. period	13-15. vii. 31 (3 days)	0.1212	0.3223	30.2	0.7440	69.8	-0.9451
			CaO				
Pre-period	8-12. vii. 31 (5 days)	1.6159	0.7976	63.2	0.4645	36.8	+0.3738
Exp. period	13-15. vii. 31 (3 days)	0.261	0.4155	51.0	0.3993	49.0	-0.5538

* All figures are average daily figures.

Rabbit B, buck, weight 2365 g., fed on same ration as rabbit A (Table I, 2); pre-period lasted from 7 to 12. vii. 31; experimental period from 13 to 16. vii. 31. On 12. vii. 31, it received, subcutaneously, at 10.45 a.m., 2 cc. $CHCl_3$. The results of the balance are given in Table III.

Table III.

Rabbit and period <i>B</i>	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
			P_2O_5				
Pre-period	7-12. vii. 31 (6 days)	0.9684	0.0088	1.3	0.6584	98.7	+0.3012
Exp. period	13-16. vii. 31 (4 days)	0.2653	0.1425	12.8	0.9743	87.2	-0.8495
CaO							
Pre-period	7-12. vii. 31 (6 days)	2.2889	0.4040	35.8	0.7254	64.2	+1.1595
Exp. period	13-16. vii. 31 (4 days)	0.6387	1.0781	55.3	0.8726	44.7	-1.3121

The same points in regard to the balance, shift of P_2O_5 from faeces to urine and urinary deposit, as noted in regard to rabbit A, are in evidence here. There is also a shift of the CaO excretion from the faeces to the urine, not observed in rabbit A.

Rabbit C, buck, weight 2855 g. This rabbit has two pre-periods—the first, from 30. ix. 31 to 12. x. 31, being on the stock ration (Table I, 3); and the second, from 13 to 26. x. 31, on the stock ration *plus* $CaCO_3$ (Table I, 4). The experimental period lasted from 27 to 30. x. 31. 50,000 rat units of irradiated ergosterol were given on 26. x. 31; 100,000 on 27 and 28. x. 31; 50,000 on 29. x. 31; and 50,000 on 30. x. 31, when the rabbit was killed. The results of the balance are given in Table IV.

In comparing the second pre-period and the experimental period, the same points, in general, are to be noted as in the two previous cases. There is here also a shift of CaO excretion from the faeces to the urine, a condition present in rabbit B but not in rabbit A.

Table IV.

Rabbit and period <i>C</i>	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
			P ₂ O ₅				
Stock ration	30. ix. 31– 12. x. 31 (13 days)	0.8368	0.3767	47.5	0.4160	52.5	+0.0441
Stock ration +CaCO ₃	13–26. x. 31 (14 days)	0.8535	0.0398	4.5	0.8347	95.5	–0.0210
Exp. period	27–30. x. 31 (4 days)	0.3734	0.2935	22.5	1.0104	77.5	–0.9305
			CaO				
Stock ration	30. ix. 31– 12. x. 31 (13 days)	0.1660	0.0115	6.6	0.1620	93.4	–0.0075
Stock ration +CaCO ₃	13–26. x. 31 (14 days)	1.9434	0.5421	44.5	0.6752	55.5	+0.7261
Exp. period	27–30. x. 31 (4 days)	0.7984	1.7227	69.2	0.7646	30.8	–1.6889

When the animal was killed, the p_H of the blood was found to be somewhat lowered at 7.3; its alkali reserve was lower also, corresponding to 1.68 cc. $N/100$ NaOH per 1 cc. (normal = 2.8 to 3 cc.); blood-P was 6.75 mg./100 cc.; Ca, 14.3 mg./100 cc.

Rabbit D, buck, weight 1900 g. There were two pre-periods here also, the first, from 30. ix. 31 to 12. x. 31, on the stock ration (Table I, 5), the second, from 13 to 21. x. 31, on this stock ration, *minus* fish meal (Table I, 6). The experimental period, when vitamin D was given, was on the latter ration and lasted from 22 to 26. x. 31. On 21. x. 31, it received 1,000,000 rat units of irradiated ergosterol and the same dose on 22. x. 31 and 23. x. 31; on 24. x. 31, 500,000 rat units and none on 25. x. 31. The results of the balance are given in Table V.

Table V.

Rabbit and period <i>D</i>	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
			P ₂ O ₅				
Stock ration	30. ix. 31– 12. x. 31 (13 days)	1.2849	0.2212	24.9	0.6656	75.1	+ 0.3981
Stock ration less fish meal	13–21. x. 31 (9 days)	1.0856	0.2626	22.1	0.9240	77.9	– 0.1010
Exp. period	22–26. x. 31 (5 days)	0.1910	0.5158	62.0	0.3157	38.0	– 0.6405
			CaO				
Stock ration	30. ix. 31– 12. x. 31 (13 days)	0.2280	0.0041	1.2	0.3512	98.8	– 0.1273
Stock ration less fish meal	13–21. x. 31 (9 days)	0.0694	0.0018	0.6	0.2942	99.4	– 0.2266
Exp. period	22–26. x. 31 (5 days)	0.0125	0.0323	23.6	0.1043	76.4	– 0.1241

Here again there is a marked negative balance and a shift of the excretion of the P_2O_5 and CaO from the faeces to the urine. The urine, which was clear and acid in the pre-periods, became very turbid with phosphates in the experimental period. The p_H of the blood, when the animal was killed on 26. x. 31, was 7.3, the alkali reserve, expressed as previously, gave a figure of 2.58 cc. $N/100$ NaOH per cc.; the blood-P was 12.99 mg./100 cc. and Ca 11.7 mg./100 cc.

Rabbit E, buck, weight 2310 g. After it had been kept on the ordinary stock ration for some time, the pre-period, which lasted from 28. x. 31 to 9. xi. 31, was conducted on a ration in which the P_2O_5 was roughly balanced in regard to the CaO by the addition of $CaCO_3$ (Table I, 7) (by adding 4.8 oz. $CaCO_3$ to 25 lbs. of the stock ration). The experimental period lasted from 10 to 16. xi. 31. 50,000 rat units of irradiated ergosterol were given on 9, 10, 11, 12, 13, 14, 15 and 16. xi. 31. The results of the balance are given in Table VI.

Table VI.

Rabbit and period <i>E</i>	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
			<i>P₂O₅</i>				
Pre-period	28. x. 31– 9. xi. 31 (13 days)	1.3622	0.2359	18.9	1.0092	81.1	+0.1171
Exp. period	10–16. xi. 31 (7 days)	0.8524	0.5146	55.2	0.4184	44.8	–0.0806
<i>CaO</i>							
Pre-period	28. x. 31– 9. xi. 31 (13 days)	0.7033	0.0274	4.8	0.5458	95.2	+0.1301
Exp. period	10–16. xi. 31 (7 days)	0.4678	0.1548	41.5	0.2181	58.5	+0.0949

In this case there is a lowering of the CaO and P_2O_5 balances and a shifting of the excretion of the CaO and P_2O_5 from the faeces to the urine. The effects here, however, are much less than in the more or less comparable (as regards dosage of vitamin D) rabbit C, for which the proportion of $CaCO_3$ in the diet is much greater, an occurrence which appears to be the only outstanding difference between the two cases in experimental conditions. The urine, during the experimental period, was very turbid with phosphates.

When the animal was killed on 16. xi. 31, the p_H of the blood was 7.3; alkali reserve = 1.35 cc. $N/100$ NaOH per 1 cc.; blood-P = 7.5 mg./100 cc. and Ca = 15.95 mg./100 cc.

Rabbit H, buck, weight 2320 g. As one of the major effects of the administration of large doses of vitamin D to the rabbit is to produce a loss of appetite and a diminution in the intake of food, it became necessary to try to elucidate the effect of starvation alone on the excretion of P_2O_5 in the rabbit. This was carried out in the present experiment. The rabbit was fed on the calcium-rich diet (Table I, 8); and, as complete starvation had never occurred in any of the rabbits poisoned with vitamin D, a certain very limited amount of food, comparable with the amount taken in these latter cases, was allowed. The pre-period lasted from 29. xi. 31 to 11. xii. 31, and the experimental period from 12. xii. 31 to 17. xii. 31. The results are given in Table VII.

The main point to notice in this case is not so much the balances or the shift of excretion of P_2O_5 and CaO from the faeces to the urine but the very small

Table VII.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
			<div> <div>P₂O₅</div> <div>CaO</div> </div>				
Pre-period	29. xi. 31– 11. xii. 31 (13 days)	1.0883	0.0346	2.8	1.1810	97.2	–0.1273
Starvation	12–17. xii. 31 (6 days)	0.2933	0.0938	14.8	0.5381	85.2	–0.3386
Pre-period	29. xi. 31– 11. xii. 31 (13 days)	2.3557	0.5679	36.1	1.0069	63.9	+0.7809
Starvation	12–17. xii. 31 (6 days)	0.5933	0.3664	48.3	0.3925	51.7	–0.1656

absolute amount of P_2O_5 that is excreted in the urine daily during the starvation period. This should be compared with the large absolute amount present in the urine in the vitamin D cases. These occurrences are indicative of an endogenous source for the large excess present in the latter cases. It should be noted also that a large amount of the calcium, absorbed in the pre-period, is excreted in the urine in the starvation period, as carbonate, however, and not as phosphate as in the vitamin D cases.

Rabbit H¹, buck, weight 2100 g. This experiment was undertaken to test the effect of irradiation, as an alternative to irradiated ergosterol administration, on the Ca and P metabolism. Rabbit H was again used, after a considerable rest. The ration consisted of the stock ration, less fish meal (Table I, 9). The hair on the back, over an area of about 12 square inches, was kept closely shaved and the skin, thus exposed, irradiated daily for a period of 20 minutes at a distance of $2\frac{1}{2}$ feet from a powerful "Hanovia" mercury vapour lamp. The pre-period lasted from 10 to 21. i. 32 and the irradiation period from 22. i. 32 to 9. ii. 32. The results are given in Table VIII.

Table VIII.

Rabbit and period <i>H</i> ¹	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
Pre-period	10-21. i. 32 (12 days)	1.3681	P ₂ O ₅ 0.4989	38.0	0.8125	62.0	+0.0567
Irradiation period	22. i. 32- 9. ii. 32 (19 days)	0.9309	0.4850	52.6	0.4364	47.4	+0.0095
			CaO				
Pre-period	10-21. i. 32 (12 days)	0.0564	0.0024	1.1	0.2234	98.9	-0.1694
Irradiation period	22. i. 32- 9. ii. 32 (19 days)	0.0392	0.0023	2.2	0.1033	97.8	-0.0664

The results obtained here are not so striking quantitatively as in the majority of the vitamin D poisoning cases. This is natural as the treatment has been much less drastic. Leaving out of consideration the calcium results on account of the

smallness of the amount in the diet and in the excreta, especially the urine, the results are of the same order as those obtained in vitamin D poisoning. There is less food eaten in the experimental period than in the pre-period; but, in spite of this, there is the same amount of P_2O_5 excreted in the urine as in the pre-period, there is a shift of excretion of P_2O_5 from the faeces to the urine and the P_2O_5 balance is diminished.

The experiments have been carried out according to the accustomed routine, and, in the tables as submitted, the data obtained are arranged in the traditional fashion to show the state of the balance, the shift of the phosphorus from the faeces to the urine *etc.* The results, however, must be interpreted with care, as the designation of some of the experiments as "balance" experiments would appear to be a misnomer.

In many of the experiments, from the very outset after the administration of the vitamin D, the taking of food by the animal practically ceased; the gastro-intestinal functions were completely upset; the food, consumed on the last day of the pre-period, remained undigested and unabsorbed in the intestine; there was no evacuation of faeces¹: while the large amount of $CaCO_3$, present in the diet in some cases, would lead to difficulties by neutralising the HCl of the gastric juice, and it would also tend to precipitate and retain in the intestine the phosphates derived from the food. These occurrences would all tend to obviate a real balance being struck; while, from the practical aspect, it needs to be mentioned that the "faeces," in most of the cases in the experimental period, had to be obtained from the dead animal by scraping out the contents of the intestines, those of the stomach, however, not being included.

Any attempt, therefore, to draw conclusions, under the circumstances, from the state of the "balance" as given in the Tables would (in most of the cases) be out of place. At the same time attention should be directed to the high degree of negativity, as regards CaO and P_2O_5 , that did occur in these cases, a circumstance to which significance is added by the fact that those rabbits (*D*, *E*, and *H*¹), which were subjected to less drastic treatment (less CaO in food or less vitamin D administered), and which consequently lived for a longer period and were more normal in their excretory activities, exhibited a similar, if less marked, negativity in their CaO and P_2O_5 balances.

The shift of the P_2O_5 excretion from the faeces to the urine, which occurred in every case, has a very definite significance as will be seen from the following considerations. One recognises the fallacy underlying any attempt, under the circumstances of the stagnation of the intestinal contents *etc.*, to place reliance on this ground alone on the percentage figures of "shift" as given in the Tables; but if one compares the absolute amounts of P_2O_5 in the urine of the animals in the experimental periods with that in their urine in the pre-period and with that in the urine of a healthy rabbit in a similar grade of starvation, one is on much firmer ground, and the figures for the "shift" become significant. In the first case, when the pre-period and experimental period are compared, in spite of the fact that practically no food is being taken in the experimental period, the absolute amount of P_2O_5 in the urine is greatly, sometimes enormously, increased; while, in the second case of equal degree of starvation (though not necessarily of equal degree of want of absorption from the intestines in the case of the "healthy" rabbit), the amount of P_2O_5 excreted in the urine of the "healthy" starved rabbit is only a small fraction of that excreted by those poisoned with vitamin D. Only one conclusion would seem to be valid under the circumstances,

¹ The difficulties in regard to possible excretion of Ca and P into the intestine are appreciated. In the present instance, these are of no moment as subsequent discussion will show.

namely that the excess P_2O_5 in the animals poisoned with vitamin D must be of endogenous origin, and it has already been suggested [McGowan *et al.*, 1931] that its source is the lipins of the body which are broken down under the influence of the vitamin D. It should be noted also that the amount of P_2O_5 actually passing by the urine does not give an exact criterion of the extent of this splitting up, for a considerable amount of the phosphorus, so formed, is fixed in the tissues of the body in an insoluble form in the various "calcifications."

The results obtained in the rabbit poisoned with $CHCl_3$ (rabbit *B*) and in the one treated by irradiation (rabbit *H*¹) are the same in type as those just discussed and require no special discussion.

The peculiar circumstances attending the performance of such experiments in regard to the starvation during the experimental period, as has just been discussed, make it possible to derive the same conclusions, as have been arrived at in the present instance, also from the results obtained in the preliminary investigation [McGowan *et al.*, 1931], where urinary samples alone were analysed.

The behaviour of calcium in the present investigation, being of less importance, is not discussed at length. It plays a secondary rôle to the phosphorus, being drawn from the intestines or endogenous sources to neutralise the phosphoric acid set free by the action of vitamin D. Where $CaCO_3$ has been fed in large amounts in the pre-period, the large positive balance of loosely bound calcium forms an easily available endogenous source. Attention may also be drawn to the fact that, while rabbits on a $CaCO_3$ -rich diet excrete a large part of the Ca as $CaCO_3$ in the urine, when poisoned with vitamin D the calcium appears in the urine as calcium phosphate. As the animals during this latter period are in a condition of starvation, this may be accepted as further evidence of vitamin D producing the phosphoric acid from an endogenous source and of calcium acting as an anti-acid as already indicated.

In the course of the investigation some observations were made on the calcium and phosphorus contents of the blood. The results are given in Table IX.

Table IX.

Rabbit	Date	Ration	Experimental treatment	Ca mg./100 cc.	P mg./100 cc.
<i>C</i>	7. x. 31	Stock	None	15.6	3.38
	16. x. 31	Stock + $CaCO_3$	None	16.7	2.81
	29. x. 31	Stock + $CaCO_3$	Vitamin D	15.3	6.01
	30. x. 31	Stock + $CaCO_3$	Vitamin D	14.3	6.75
<i>D</i>	7. x. 31	Stock	None	13.4	4.07
	16. x. 31	Stock <i>minus</i> fish meal	None	13.1	4.22
	26. x. 31	Stock <i>minus</i> fish meal	Vitamin D	11.7	12.99
<i>E</i>	5. xi. 31	Stock + $CaCO_3$	None	13.7	4.0
	13. xi. 31	Stock + $CaCO_3$	Vitamin D	13.0	5.72
	16. xi. 31	Stock + $CaCO_3$	Vitamin D	15.95	7.5
<i>H</i> ¹	18. i. 32	Stock <i>minus</i> fish meal	None	14.6	3.54
	22. i. 32	Stock <i>minus</i> fish meal	Irradiation	15.9	4.19
	26. i. 32	Stock <i>minus</i> fish meal	Irradiation	15.9	4.21
	1. ii. 32	Stock <i>minus</i> fish meal	Irradiation	15.7	4.25
	8. ii. 32	Stock <i>minus</i> fish meal	Irradiation	15.9	4.63

The only significant feature in this table is a definite and consistent rise of the blood-P, following the administration of vitamin D. Because this rise has taken place in spite of the lack of absorption and of the greatly increased excretion of P by the urine the result has significance and supports the main thesis. Too much emphasis however should not be laid on such increases, for they constitute the resultant of several processes, absorption and excretion, together with

endogenous fixation and formation; and it is quite possible, to give an illustration, that in the present instance, with a different relative participation of the different factors, a lowering of P concentration in the blood might have been obtained, even although there was no abatement of endogenous formation.

The figures for P and Ca concentration in the blood indicate an activity somewhere, but, being of the nature of a resultant, give no information as to its essential *locus* or nature, unless the factors producing this resultant are known and to each is apportioned its due meed. The figures in Table IX show no consistent rise in the calcium content of the blood although others, *e.g.* Ashford [1930], Warkany [1930], Naser [1930], *etc.*, have found a rise in the blood-calcium following the administration of vitamin D. Such findings, however, are not necessarily contradictory; for vitamin D may be producing its essential action without, for the reasons mentioned above, the index appearing in the blood, a view opposed to the narrow one fairly commonly held, that vitamin D affects the blood directly and has as chief function the regulation of its Ca and P content, as an end in itself.

SUMMARY.

The results obtained in this extended investigation confirm those found in the preliminary inquiry and go to show that vitamin D produces its action by setting free inorganic phosphate endogenously and in all probability from the lipins of the body, for which evidence was supplied previously.

I wish to record my great obligation to Mr William Godden in connection with this investigation. He, with his assistants Mr W. H. Williams and Miss B. W. Simpson, carried out the analysis of the foods, faeces and urine in the balance experiments. My thanks are also due to Dr D. W. Auchinachie and Mr A. R. G. Emslie for the blood analyses. I am indebted to Mr A. L. Bacharach, Glaxo Laboratories, for supplying the solution of vitamin D.

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