

LX. THE INFLUENCE OF OVARIAN AND ANTERIOR PITUITARY HORMONES ON CALCIUM METABOLISM.

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THE belief that internal secretions associated with the reproductive organs have some influence on the calcium metabolism of the body is widely held. In particular the internal secretions of the ovary and of the anterior pituitary have been stated to have such an effect. Much of the evidence put forward is unsatisfactory.

Bell [1908], using methods of doubtful accuracy, reported a fall in the calcium content of human serum just before menstruation. Widdows [1922], on the other hand, could find no significant alteration in serum-calcium at any time during the menstrual cycle, while Sherman, Gillett and Pope [1918] and Bogert and McKittrick [1923] could find no evidence of change in the rate of calcium excretion during menstruation. More recently Frei and Emmerson [1930] have shown that there is no appreciable difference between the calcium contents of sera of cows at oestrus and dioestrus, although they hold the view that the chemical composition of serum is in some way connected with oestrus.

The results bearing on the influence of ovariectomy on the blood-calcium are entirely inconclusive. Blanchetière [1925] found an increased amount of calcium in the sera of women after ovariectomy and at the menopause. On the other hand Dalsace and Guillaumin [1925], also working with women, observed a fall in blood-calcium after ovariectomy. More recently Hogben and Charles [1932] have stated that ovariectomy in rabbits causes no change in serum-calcium.

Many workers have noted a fall in the serum-calcium of women during pregnancy [*cf.* Widdows, 1923; Mazocco and Moron, 1921; Plass, 1923]. This fall is probably due to the drain on the maternal tissues by the foetus which may demand as much as 0.6 g. calcium daily towards the end of gestation. It might be supposed of course that this mobilisation of maternal calcium is under control of a hormone, *e.g.* that of the corpus luteum. There is, however, no evidence that this is the case.

Luckhardt and Goldberg [1923] showed that parathyroidectomised dogs exhibited a recurrence of the symptoms of tetany at oestrus. This fact might suggest that the hormones concerned in the production of oestrus (one of the anterior pituitary hormones and oestrin) cause a lowering of the serum-calcium.

Mirvish and Bosman [1928] were among the first to try the effect of injection of extracts of the reproductive organs on the blood-calcium in experimental animals and in man. They claimed that the injection of oily solutions of extracts of liquor folliculi, ovary, corpus luteum and placenta, made by methods based

on those introduced by Herrmann and Fränkel [1915] for oestrus-producing extracts, caused a lowering of the blood-calcium of rabbits. Since the effect was the same whether males, normal, pregnant or ovariectomised females were used, they were inclined to the view that the oestrus-producing hormone was not the agent responsible, but rather, a different hormone present in the extract. Subsequently they stated that similar effects could be observed on injecting the extracts into human males and females. Reiss and Marx [1928] have reported similar lowerings of the blood-calcium of rabbits as a result of injection of a commercial ovarian extract ("homovar"). Later work by Frei and Emmerson [1930], in which they injected a commercial oestrin preparation, "progynon," into cows, showed that there was a slight fall in serum-calcium (0.5 mg./100 cc.). Mirvish and Bosman, in later work [1929], showed that extracts of testis and adrenal cortex also possessed this calcium-lowering potency.

A fresh aspect of the problem has been opened up by the recent work of Hogben and Charles [1932]. These workers have shown that the injection of saline extracts of the anterior lobes of ox pituitaries produced a marked fall in serum-calcium when injected into normal female rabbits. That the effect was a direct one of the anterior lobe, and not an effect due to the stimulation of the ovary by the extract, was clear from the fact that similar falls were observed in ovariectomised rabbits. It was also shown that a few hours after coitus there was a fall in the serum-calcium of female rabbits which was followed by a rise coincident with the time of ovulation and a second fall which subsided by the time the corpus luteum was fully formed. These latter effects appear possibly to be due to the action of the anterior pituitary also. Frei and Emmerson [1930] have shown that there is a slight rise in serum-calcium 8 hours after the injection of anterior pituitary extract into cows.

Cannavo [1932], on the other hand, has shown that injections of active anterior pituitary extracts into man, the dog and the rabbit cause little change in the calcium content of the serum.

The present work, following on that of Mirvish and Bosman and of Reiss and Marx was an attempt to find out if the alleged lowerings of serum-calcium found by these workers on injection of ovarian, corpus luteum and follicular extracts into rabbits were due to oestrin or other substances present in the ovary. If they were due to oestrin it was thought that this might provide a useful method for the assay of the latter.

EXPERIMENTAL.

The first step, then, before injection of crystalline oestrin was the repetition of the above work with ovarian extracts. Extracts of whole ovary, residual ovary and corpus luteum were accordingly made by repeatedly boiling the minced tissues with alcohol, evaporating and extracting the residue with methyl alcohol. The methyl alcohol fraction on evaporating to dryness and dissolving in olive oil was used for injection without further purification.

Serum-calcium determinations were made by the Trevan-Bainbridge [1926] method and inorganic phosphorus by the Fiske-Subbarow [1925] method. All determinations were done in duplicate.

The animals first used for the tests were normal buck and ovariectomised doe rabbits previously kept on a diet of cabbage and oats for several weeks. The animals were bled on alternate days for a week or so previous to injections to find out if a reasonable constancy of serum-calcium from day to day could be expected. The results of these determinations before and after injection of

the extracts are shown in Fig. 1, and are unsatisfactory for they do not show any great difference in serum-calcium after injection nor is the serum-calcium very constant from day to day but appears to depend to a large extent on diet, cabbage alone causing it to increase.

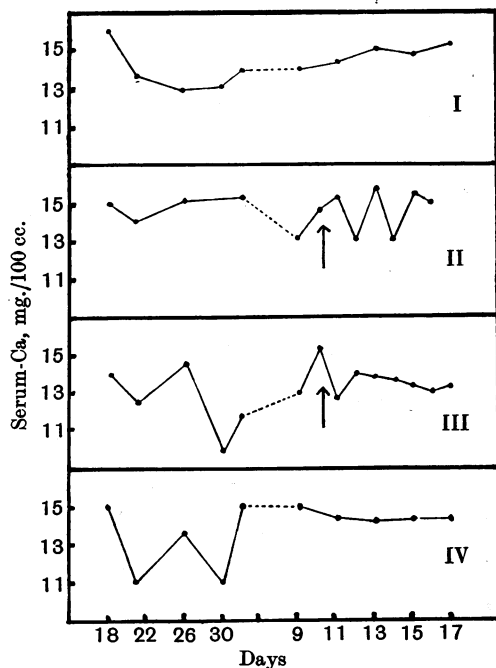


Fig. 1. Ovarian extract injected into rabbits II and III=300 g. fresh substance; blood samples taken 12 and 24 hours after injection. I and IV controls. Rabbits I and II bucks; III and IV does.

Since these experiments, two papers dealing with the influence of diet on serum-calcium have appeared [Dupré and Semeonoff, 1931; Bourne and Campbell, 1932]. These authors show conclusively that feeding cabbage causes the serum-calcium level to rise and that of phosphorus to fall; whereas feeding oats causes the calcium content to decrease and that of phosphorus to rise. Since it is impossible to make a rabbit eat the same proportions of cabbage and oats per day, the fluctuations in serum-calcium observed by us and ascribed by other authors to the effects of injections, bleeding *etc.* are readily explained. That rabbit serum-calcium can vary from 10 to 18 mg./100 cc. under the influence of diet shows that unless rigid precautions are taken with regard to feeding the interpretation of changes in serum-calcium may be misleading. It has been suggested by Dupré and Semeonoff that calcium determinations on rabbit serum should be done after an 18 hours' fast, when some kind of equilibrium between the calcium and phosphorus has been attained.

Some authors report changes in rabbit serum-calcium when blood is taken on successive days. Charles [1931] reports a rise, Clark [1921] and Stewart and Percival [1928] report a fall, whereas Mirvish and Bosman [1928] state that repeated bleeding has no effect on serum-calcium in rabbits. As shown above no deductions about this can be drawn unless diet is rigidly controlled. Hogben and

Charles [1932] suggest the use of numbers of parallel control and experimental groups of animals, the blood of control and experimental groups being taken at the same time to obviate the complication due to successive bleedings. There is no doubt that this is an improvement over the use of single experimental animals. But, as is shown in Table I, the serum-calcium of 10 normal rabbits previously

Table I. Rabbits. Serum-calcium of 10 normal doe rabbits kept under standard conditions for 3 weeks. Cabbage and oats in morning, 12 noon, bleeding from 3 to 5 p.m.

31. i. 32	Rabbit No.	Calcium mg./100 cc.	Rabbit No.	Calcium mg./100 cc.
	1	14.22	7	14.77
	2	15.99	8	15.65
	4	16.65	9	15.00
	5	17.05	10	13.93
	6	16.15	15	16.22

kept on a standard diet for 3 weeks, all bled at the same time, varies from 13 to 17 mg./100 cc. Thus unless very large numbers of animals in each group are used, no values of serum-calcium, unless outside the limits of normal variation, can be taken to prove that any particular condition is responsible for a difference in the serum-calcium level.

Some other animal having a more constant serum-calcium was sought for, and preliminary experiments on dogs showed that they were far less subject to daily and dietary variations. Extracts of residual ovary and corpus luteum were accordingly made and injected subcutaneously into dogs, blood samples being taken from leg veins at various intervals after injection. As the results in Figs. 2-4 show there is no appreciable change in serum-calcium after injection.

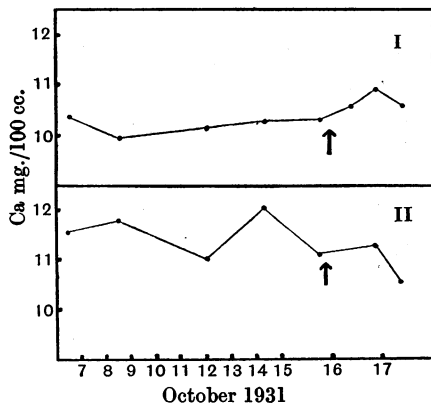


Fig. 2.

Fig. 2. Ovarian extract and dog serum-Ca. I injected; II control. Injection=400 g. fresh substance. Normal bitches.

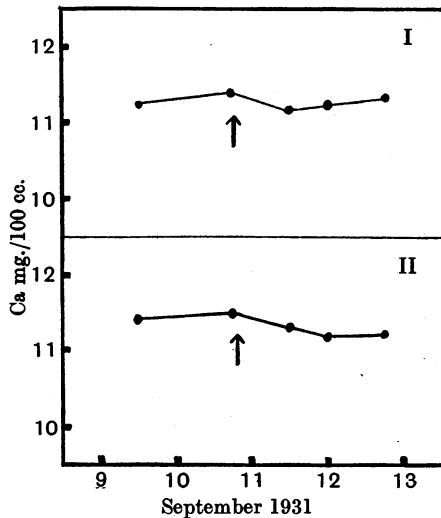


Fig. 3.

Fig. 3. Injections of ovarian extract into 2 ovariectomised bitches. I injected extract equal to 300 g. fresh substance; II injected olive oil only.

It was then thought that if an animal having a regular oestrous cycle was used it might be possible to demonstrate changes in calcium metabolism during the cycle due to intermittent secretion of oestrin by the ovary. Thus it was decided to carry out experiments on rats, since these animals have a regular

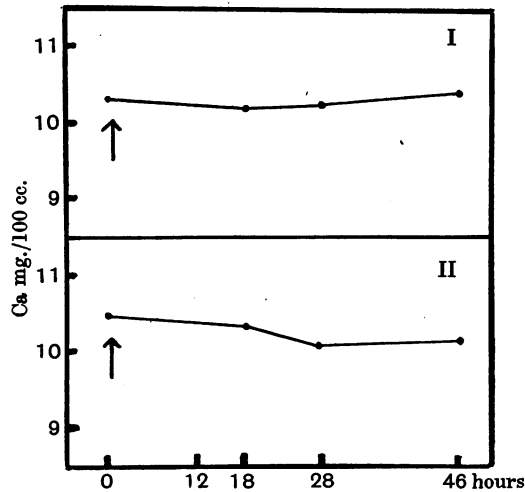


Fig. 4. Effect of injection of corpus luteum extract equal to 300 g. fresh substance into ovariectomised bitch I. II was an ovariectomised control injected with olive oil only.

oestrous cycle every 5 days which can be followed by means of the vaginal smear technique. Batches of rats kept on standard diet were killed off at various stages of their oestrous cycle and their blood drawn by cardiac puncture. Calcium determinations at oestrus when uterine distension is at a maximum and at dioestrus show no appreciable difference.

Table II. *Serum-calcium of normal rats.*

Oestrus					Dioestrus				
Date	No.	Serum-Ca	How killed	Diet	Date	No.	Serum-Ca	How killed	Diet
30. xi. 31	16	13.9	CHCl ₃	Stock	30. xi. 31	19	13.6	CHCl ₃	Stock
30. xi. 31	11	13.3	"	"	30. xi. 31	18	13.85	"	"
6. xi. 31	38	11.7	Ether	"	6. xi. 31	32	11.45	Ether	"
13. i. 32	200	12.2*	"	"	8. xi. 31	36	13.95	"	"
18. i. 32	267	17.0†	"	"	8. iv. 32	397	10.75	Chloralose	"
18. i. 32	268	17.8‡	"	"	8. iv. 32	402	10.9	"	"
4. ii. 32	242	13.2	"	"	13. iv. 32	454	11.15	"	α
7. iv. 32	257	10.7	Chloralose	"	27. iv. 32	478	10.6	Amytal	α
8. iv. 32	397	—	—	—					
3. v. 32	476	11.0	Amytal	α					
2. iv. 32	474	11.0	"	—					

The stock diet contained 1.2% calcium 0.3% phosphorus.

The stock diet α contained 1.2% calcium 0.8% phosphorus.

* Injected with 6 units parathormone 9 hours.

† Injected with 20 units parathormone 6 hours.

‡ Injected with 20 units parathormone 12 hours.

A group of rats was then injected with extract of bovine anterior pituitary, injections being made daily for 5 days. The animals were killed 7 days after the first injection when the corpora lutea were fully grown as was found on dissection

of the ovaries. The blood-Ca exhibited no appreciable difference from that of controls killed at the same time. The condition of the ovaries of these animals was similar to that in pseudopregnancy which occurs after copulation with a sterile buck.

Table III. *Injection of anterior pituitary extract into rats.*

No.	Ca mg./100 cc.	Control	
		No.	Ca mg.
401	11.9	435	11.85 (oestrus)
405	—		
406	10.65		
431	12.1		

Rats Nos. 401, 405, 406 and 451 on dissection showed ovaries to be full of corpora lutea.

Injections were carried out on 5 consecutive days, rats being killed on 7th day. The rats were smeared every day and it was noted that in each case the oestrous cycle was inhibited after the 2nd day.

It was then decided to study the effect of pregnancy and pseudopregnancy on the serum-calcium and phosphate levels in rabbits using the technique of 18 hours' starvation before taking blood samples. Table IV shows that a group of 10 doe rabbits, some in oestrus and some in resting condition exhibit a very small range of variation in serum-calcium. Of these rabbits previously kept caged on a standard diet for 3 weeks, 4 copulated twice with a vasectomised buck and 2 with a normal buck, the remainder being used as controls. Blood samples were taken from these animals 8 days after copulation, when the corpus luteum was assumed to have reached a reasonable degree of activity and growth, together with blood samples from controls. As Table V shows, the serum-calcium values are not materially different from each other or from those in Table IV which were values for normal females.

Table IV. *Serum-calcium of 10 normal doe rabbits kept under standard conditions for 4 weeks and starved 18 hours before bleeding.*

11. iii. 32	Rabbit No.	Ca	Rabbit No.	Ca
	1	14.1	7	13.5
	3	13.6	8	14.5
	4	14.0	9	14.0
	5	13.6	11	14.1
	6	13.5	12	14.2

This shows the effect of 18 hours' starvation on the reduction of variation of the serum-calcium.

Table V. *Serum-calcium and phosphorus of 4 pseudopregnant, 2 pregnant and 3 normal immature doe rabbits.*

3. iii. 32	Rabbit No.	Ca	P
	8 pseudopregnant	13.7	4.2
	P ₁ "	13.4	4.3
	P ₂ "	13.5	4.0
	P ₃ "	13.35	3.9
	P ₄ 9 days pregnant	13.9	4.3
	P ₅ "	12.3	4.4
	6 immature control	12.8	4.5
	12 "	13.5	4.1
	8 "	13.7	4.0

The observation by Luckhardt and Goldberg [1923] that oestrus brings about recurrence of tetany in parathyroidectomised dogs suggested that the parathyroids might mask or compensate for changes in serum-calcium due to secretion of oestrin. Experiments were therefore carried out on rats which had been parathyroidectomised. A group of these animals all receiving the same diet were killed at stages of oestrus and dioestrus and their blood drawn and analysed for calcium and phosphate. The serum-calcium values for the oestrus rats ranged from 5.4 to 8.4 mg./100 cm. with an average of 6.71 and that of the dioestrus ones from 6.0 to 8.4 mg./100 cc. with an average of 8.0 mg. This difference in view of the range of variation does not appear to be significant.

Table VI.

Oestrus					Dioestrus				
No.	Date of killing	Ca	P	Days after operation	No.	Date of killing	Ca	P	Days after operation
442 C	12. iv. 32	5.4	14.4	21	444 C	12. iv. 32	6.0	10.8	21
445 E	12. iv. 32	7.45	10.9	21	441 E	12. iv. 32	8.40	7.8	21
450 C	18. iv. 32	8.1	11.6	25	453 C	18. iv. 32	8.3	8.3	25
440 A	18. iv. 32	7.28	16.0	25					
468 C	19. iv. 32	8.5	11.6	25	467 C	19. iv. 32	8.8	8.5	25
443 A	25. iv. 32	7.27	11.9	34	446 A	25. iv. 32	8.43	10.1	34
449 A	25. iv. 32	6.50	14.2	34	456 A	25. iv. 32	7.50	12.9	34
479 A	27. iv. 32	6.7	—	27	478 A	—	—	—	27
473 A	3. v. 32	7.5	—	32	462 A	2. v. 32	8.4	—	37
					460 A	2. v. 32	7.8	—	37
471 A	3. v. 32	6.75	—	38	465 A	3. v. 32	7.6	—	38
470 A	6. v. 32	8.4	13.5	41	466 A	6. v. 32	8.85	11.5	41
	Mean	6.71	12.9				8.0	9.9	

Serum-calcium of parathyroidectomised rats:

A = killed amytal anaesthesia intraperitoneally 0.02 g.

C = killed chloralose anaesthesia intraperitoneally 0.05 g.

E = killed ether.

Another group of rats was ovariectomised at about the beginning of maturity and a week later parathyroidectomised. None of these animals died from tetany during the following week. Half of them were then injected with approximately 6000 m.u. of cryst. trihydroxyoestrin in 4 doses at 12-hour intervals and all

Table VII. *Effect of trihydroxyoestrin injections on ovariectomised parathyroidectomised rats.*

20. v. 32	No.	Ca mg./100 cc.	Condition of uterus
	714	5.3	Well distended
	715	6.15	"
	719	6.8	Moderately distended
	721	6.4	Well distended
	722	6.0	Moderately distended
Controls:	724	6.2	Undistended
	725	6.25	"
	726	4.60	"
	727	5.8	"

Animals were ovariectomised 5. v. 32 and parathyroidectomised 11. v. 32.

Oestrin was given in 4 equal doses of 1500 m.u. totalling 6000 m.u.

They were killed by intraperitoneal injection of 0.01 g. amytal. Their diet up to 6 hours before death when they were starved contained 1.2% Ca, 0.86 P.

Blood taken by cardiac puncture about 10–15 minutes after injecting anaesthetic.

were killed 30 hours after the last injection, at which time the vaginal smears of those injected with oestrin were cornified and the uteri on dissection well distended. The serum-calcium of the oestrin-injected varied from 5.3 to 6.8 mg., average 6.13 mg./100 cc. and of the ovariectomised parathyroidectomised controls from 4.6 to 6.2 mg./100 cc., average 5.7 mg. This difference is also not significant. The wide range of variation of serum-calcium in parathyroidectomised rats may be accounted for possibly by incomplete removal of parathyroids.

DISCUSSION.

As already pointed out by Bourne and Campbell and Dupré and Semeonoff the serum-calcium of rabbits is very variable under normal conditions of diet and estimations must be interpreted with caution, unless the food intake is controlled. It is not sufficient to give the same amount of green stuff and oats or bran daily or either of these foodstuffs singly, as variations in serum-calcium may still exist. The only satisfactory way is to withhold food for 18 hours before the withdrawal of blood.

In dogs variations in serum-Ca due to variation in diet are small. In rats, as Howland and Kramer [1932] have shown, the percentage of calcium and phosphate in the food influences the amounts of calcium and phosphorus in the serum, but the latter are moderately constant for the same diet.

From the above experimental results it can only be concluded that oestrin and corpus luteum hormone cause no gross change in serum-calcium in rabbits, dogs and rats.

SUMMARY.

1. Ovarian and corpus luteum extracts injected into rabbits and dogs produce no significant change in their serum-calcium levels.
2. In rabbits pregnancy and pseudopregnancy produce no demonstrable change in serum-calcium after 8 days.
3. Serum-calcium levels of rats in oestrus and dioestrus are not appreciably different.
4. Injections of anterior pituitary extract into rats sufficient to cause luteinisation of ovaries caused no change in their serum-calcium.
5. The serum-calcium values in oestrus and dioestrus of parathyroidectomised rats are very variable and no significant difference can be observed.
6. Injections of crystalline trihydroxyoestrin sufficient to produce uterine distension and vaginal cornification in ovariectomised parathyroidectomised rats do not produce any marked difference in serum-calcium from controls.

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