THE EFFECT ON BLOOD CALCIUM AND PHOSPHORUS OF FRACTIONS OBTAINED FROM CRUDE OVARIAN EXTRACTS

by

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INTRODUCTION

It is a recognized fact that the ovary is concerned in reproduction and that there is a definite relationship between ovarian function and skeletal growth, for at onset of puberty when the ovarian harmone becomes active there is a beginning of the closure of the growth zones which, when completely closed, cause growth of the long bones to cease; failure of this hormonal function results in abnormal growth. It is considered that calcium no longer needed for growth goes to foetal or lactal requirements; lacking this, it is excreted in menstruation.

In this experiment I have undertaken to find the effect upon serum calcium and inorganic phosphorus blood levels of normal rabbits 24 hours after the injection of three impure fractions, eliminated in the process of purification of the whole ovary, the fractions designated as M_1 , M_2 , M_3 , the pure extract and a combination of the four.

REVIEW OF LITERATURE

A number of investigations have been made on the constituents of the blood, namely, calcium and inorganic phosphorus, and it has been found that the concentrations of these minerals undergo wide variations. Factors which may be responsible for these fluctuations are environment, nu-

trition and hormonal influence.

Mirvish and Bosman[13] of the University of Capetown, South Africa, have carried out an experiment in an attempt to find the relationship between the hormone function of the ovary and the calcium metabolism of the body. The nature of this experiment corresponds more closely with my work than does any other; it deals with the effect of ovarian extracts upon the calcium blood level of rabbits and man. Hence it will be reviewed in some detail.

They quote the calcium blood level of rabbits as remarkably constant under normal conditions, and therefore large doses of extract were necessary to upset the mineral balance. From the liquor folliculi, residual ovary and corpus luteum they obtained a brown gummy residue called "crude extract." In some cases this was purified by Dickens, Dodd and Wright[7] method and the pure form used.

Of 106 blood calcium determinations run on 55 normal rabbits of both sexes, the average was 15.17 mg. per 100 cc. serum. This average was high compared to other investigators. Neither sex nor repeated withdrawal of blood caused any difference in calcium. The animals for this experiment were kept under the same food and environment conditions.

The crude extract often produced a swelling of chronic nature at site of injection, probably due to cholesterol content. and repeated estimations proved this did not affect

amounts of calcium. The injection of pure extract produced only a local reaction.

The calcium determinations were made by Clark of Collip's modification of Kramer and Tisdall method. The blood was drawn from an ear vein.

Results of the injections were as follows:

1. Liquor folliculi - the most potent extract - gave a two mg. per cent drop in 24 hours.
2. Residual ovary - 12 of 13 rabbits gave a drop in calcium in 24 hours provided the dose was large enough.
3. Corpus luteum crude extract injected into four rabbits gave a drop that was usually maintained over a longer period than residual ovary.

The extent of the drop in blood calcium varied with the dose and potency of extract. In typical cases there was a drop of 30 to 35 per cent of the normal level in 24 hours. No further drop could be produced either by increased size of dose or by frequent injections. The calcium could not be dropped low enough to produce tetany.

A dosage equivalent to 25 gm. of fresh tissue was always sufficient to give the typical fall of 30 per cent below normal in 24 hours. A 15 gm. dosage usually gave positive results.

The extract was usually administered subcutaneously in an emulsion with olive oil or a one per cent sodium bicar-

bonate solution - both gave positive results. However, the alcohol extracts of liquor folliculi emulsified in water gave no fall in calcium. An oral or intraperitoneal dose to be effective had to be four to five times the size of a subcutaneous dose.

Ovarian extract was administered to two ovariectomized rabbits 18 days after operation and the blood calcium gave the typical fall. This fall in blood was produced in young, old, male, female, pregnant, non-pregnant and ovariectomized animals.

Mirvish and Bosman carried out another experiment on eight persons, four males and four females. Each was injected in the arm with pure ovarian extract and all except one gave a 35 per cent fall below normal calcium level in 24 hours; they were back to normal in 48 hours. In comparison to rabbits the dose for man is smaller. They conclude that from a therapeutic point of view, ovarian extract is an agent which, irrespective of sex, lowers blood calcium and is safe to deliver, therefore it may be very valuable in clinical cases.

An extract from the suprarenal cortex, one injection into each of 14 rabbits lowers blood calcium 30 per cent in 24 hours; determinations were made before injection and at 12, 24 and 48 hour periods after injections.[12] This extract resembles in physical characteristics and effects on blood

calcium that of ovary and corpus lutea, therefore justifying a possible definite relationship between suprarenal cortex and ovary.[12]

A dose of crude testicular extract equivalent to 200 gm. of fresh tissue caused a 30 to 35 per cent calcium drop in 75 per cent of the animals; injections were on same plan as suprarenal extract. The pure testicular product did not cause a fall.

Factors that may have an effect on blood calcium have been advanced by other investigators in this field. Dickens, Dodd and Wright claimed a rise in calcium after injection of commercial insulin; Culhane[5] disagrees with this and found that feeding cabbage causes a rise. Kapsinow and Underhill[10] restudied the feeding of cabbage and found it could not be said definitely to cause an increase in the concentration of this mineral.

Brown[3] states that neither calcium nor inorganic phosphorus can be considered as a constant and that clearly defined periodic variations occurred in all classes of animals.

Bourne and Campbell[1] suggest that diet may play a very important part in the concentrations of calcium and inorganic phosphorus in the blood.

METHODS AND DISCUSSION

Quantitative determinations were made for the concentra-

tions of calcium by the Roe and Kahn[15] modification of Fiske and Subbarow and inorganic phosphorus by the modified Fiske and Subbarow[8]. These methods allowed duplicate checks to be made of each from one cc. of serum. This method for calcium gives results about two mg. calcium per 100 cc. serum higher than the Kramer and Tisdall method.

The blood was drawn from a marginal ear vein and throughout the experiment the animals remained in good health. The samples were taken at regular hours each Monday, Wednesday and Friday. This blood was handled carefully to avoid experimental error as much as possible. The animals used were approximately the same age, with one female and two males in each the experimental and control group. They were kept under identical food and environment conditions; the diet consisted of oats, carrots and alfalfa hay.

The material which I used for injections was obtained from Dr. H. W. Marlow, preparations made at the University of Chicago in 1931. The extracts are very stable. Dr. Marlow made the pure extracts (according to Dickens, Dodd and Wright) from minced whole ovary (M O), residual ovary (R O), follicular fluid (F O), corpus luteum (C O).

The first impurity in purification of M O (M_1) was composed largely of neutral fats and some inert matter, the second impurity (M_2) largely phosfolipins and the third impurity (M_3) largely cholesterol with some phosfolipin and fat.

Mirvish and Bosman[12, 13] report that both impure extract and the purified material from both whole ovary and residual ovary gave a characteristic drop of 30 to 35 per cent in blood calcium. Crude testicular and suprarenal cortex material also gave this drop. The pure extract of suprarenals lowers calcium but pure testicular does not. They therefore concluded that the pure material was not soluble in ether.

As Dr. Marlow did not get this drop with M O on fowls, rats, rabbits, young or old, castrates or normals, it was decided to test each of the above named fractions, the pure product and a combination of the four, combined in as near the original composition as possible, in an attempt to see if the crude extract had in it anything that would cause a definite change in blood serum calcium.

In order to get this material in a form to inject I used ether as a solvent and olive oil as a medium for solution. In every case ether dissolved but upon evaporation of the ether, M_1, M_2, and M_3 formed a suspension rather than a true emulsion or solution as did the pure extract. Olive oil was used as the control substance.

The animals were bled 24 hours after injection in order to catch the typical drop recorded by Mirvish and Bosman. The pure extract and olive oil were absorbed very readily; occasionally a soft lump remained for a very short time; they caused no irritation.

On the other hand, the impure fractions M_1, M_2 and M_3 were somewhat painful and caused hard swellings of a chronic nature; some, however, could be removed in a few weeks and healing take place. Mirvish and Bosman also found that the crude extract caused swellings of the same nature.

My injections were made subcutaneously in dosages in which one cc. was equivalent to 25 gm. of original fresh tissue; this dosage coincides with that used by Mirvish and Bosman. I injected one and sometimes two cc.; however, the size of the dose made little difference.

From 146 blood calcium determinations I report an average of 17.94 mg. of calcium per 100 cc. serum as compared to that of Mirvish and Bosman, 15.17 mg. The difference may be due to the method of analysis used. My phosphorus average is 3.58 mg. per 100 cc. serum.

The following tables show the results of each day's bleeding upon each rabbit. The experimental animals are listed as:

Ex 1 - female

Ex 2 - male

Ex 3 - male

The control animals:

C 4 - female

C 5 - male

C 6 - male

In spite of the fact that blood calcium has been quoted to be remarkably constant under normal conditions, I failed to find an absolute constant in five determinations upon each rabbit for normal range prior to the experimental work.

Table I. Determinations made for normal calcium and phosphorus levels.

Date	Treat.	Dosage	Animal	P	Ave.	Ca	Ave.
				Mg. per 100 cc. Blood Serum			
Mar. 6	Normal		Ex 1	3.59		16.57	
			" 2	3.33		16.10	
			" 3	3.65	3.52	15.59	16.05
			C 4	3.77		16.16	
			" 5	3.24		16.03	
			" 6	2.81	3.27	14.00	15.39
Mar.10	Normal		Ex 1	3.40		16.47	
			" 2	3.66		15.40	
			" 3	3.86	3.64	14.70	15.52
			C 4	4.52		14.70	
			" 5	4.69		14.91	
			" 6	3.47	4.23	16.00	15.20
Mar.13	Normal		Ex 1	4.00		18.30	
			" 2	3.33		16.70	
			" 3	3.59	3.64	17.20	17.40
			C 4	4.22		18.40	
			" 5	2.86		18.90	
			" 6	2.94	3.34	15.95	17.60
Mar.15	Normal		Ex 1	3.50		18.20	
			" 2	3.40		18.35	
			" 3	2.97	3.29	19.13	18.56
			C 4	4.00		17.27	
			" 5	3.21		18.18	
			" 6	2.99	3.40	17.60	17.68
Mar.17	Normal		Ex 1	3.60		18.02	
			" 2	3.26		17.98	
			" 3	2.80	3.20	19.95	18.32
			C 4	3.89		18.14	
			" 5	3.54		17.70	
			" 6	3.67	3.70	18.28	18.04

Table II. Showing effect of M_2 on calcium and phosphorus.

Date	Treat.	Dosage	Animal	P	Ave.	Ca	Ave.
				Mg. per 100 cc. Blood			Serum
Mar.20	M_2	1 cc	Ex 1	3.40		19.04	
		"	" 2	3.27		17.27	
		"	" 3	3.83	3.50	16.74	17.68
	Olive	1 cc	C 4	3.56		16.60	
	oil	"	" 5	3.32		17.77	
		"	" 6	3.35	3.41	17.38	17.25
Mar.22	M_2	2 cc	Ex 1	3.32		19.56	
		"	" 2	3.08		18.06	
		"	" 3	4.41	3.51	17.45	18.36
	Olive	2 cc	C 4	4.34		18.26	
	oil	"	" 5	4.06		18.36	
		"	" 6	3.47	3.62	18.51	18.37
Mar.24	M_2	2 cc	Ex 1	3.74		19.70	
		"	" 2	3.36		18.05	
		"	" 3	3.35	3.48	17.75	18.50
	Olive	2 cc	C 4	4.00		17.70	
	oil	"	" 5	3.49		18.77	
		"	" 6	2.83	3.44	19.18	18.53

1 cc. extract equivalent to 25 gm. fresh tissue.

This impure fraction designated as M_2 was very irritating to the animals upon injection. The solution was filled with suspended particles which were not absorbed, for the injection was followed by the formation of large lumps under the skin.

Table III. Showing effect of M_1 on calcium and phosphorus.

Date	Treat.	Dosage	Animal	P	Ave.	Ca	Ave.
				Mg. per 100 cc. Blood Serum			
Mar.31	M_1	2 cc	Ex 1	3.35		18.64	
		"	" 2	3.33		19.98	
		"	" 3	3.44	3.37	19.97	19.20
	Olive	2 cc	C 4	4.74		17.62	
	oil	"	" 5	3.66		19.04	
		"	" 6	3.07	3.82	18.06	18.24
Apr. 3	M_1	2 cc	Ex 1	3.36		18.26	
		"	" 2	3.72		15.81	
		"	" 3	3.57	3.55	16.82	16.96
	Olive	2 cc	C 4	3.77		17.13	
	oil	"	" 5	4.01		17.44	
		"	" 6	3.70	3.83	18.41	17.66
Apr. 5	M_1	2 cc	Ex 1	3.71		19.27	
		"	" 2	3.62		18.14	
		"	" 3	3.29	3.54	18.31	18.57
	Olive	2 cc	C 4	3.79		19.80	
	oil	"	" 5	4.09		19.51	
		"	" 6	3.15	3.68	19.66	19.66

1 cc. extract equivalent to 25 gm. fresh tissue.

M_1 formed a satisfactory emulsion for injection with scarcely noticeable after effects.

I followed this series of determinations with two normal bleedings to give the animals a rest before the pure extract M 0.

Table IV. Determinations of calcium and phosphorus with no injection of extract.

Date	Treat.	Dosage	Animal	P	Ave.	Ca	Ave.
				Mg. per 100 cc. Blood Serum			
Apr. 7	Normal		Ex 1	3.63		19.14	
			" 2	3.96		17.43	
			" 3	3.24	3.61	18.91	18.49
			C 4	3.20		19.42	
			" 5	3.71		19.49	
			" 6	3.38	3.43	19.19	19.33
Apr.10	Normal		Ex 1	3.56		19.41	
			" 2	3.60		18.77	
			" 3	4.46	3.87	18.10	18.76
			C 4	3.46		18.26	
			" 5	3.43		19.18	
			" 6	3.14	3.34	19.43	18.96

Table V. The effect of pure ovarian extract on calcium and phosphorus.

Apr.12	M 0	1 cc	Ex 1	4.18		18.43	
		"	" 2	3.98		18.91	
		"	" 3	3.16	3.77	17.78	18.37
	Olive oil	1 cc	C 4	3.15		17.94	
		"	" 5	3.99		19.09	
		"	" 6	3.22	3.45	19.28	18.77
Apr.14	M 0	1 cc	Ex 1	3.27		18.02	
		"	" 2	3.88		16.66	
		"	" 3	3.04	3.40	16.95	17.21
	Olive oil	1 cc	C 4	3.33		17.46	
		"	" 5	3.85		18.43	
		"	" 6	3.75	3.64	18.26	18.05
Apr.17	M 0	2 cc	Ex 1	3.48		18.95	
		1 "	" 2	3.84		16.06	
		1 "	" 3	4.41	3.91	16.82	17.28
	Olive oil	1 "	C 4	3.37		17.16	
		1 "	" 5	4.22		16.88	
		2 "	" 6	3.20	3.60	19.28	17.77

The pure extract M 0 went into solution in olive oil and was readily absorbed.

One cc. extract is equivalent to 25 gm. fresh tissue.

Table VI. The effect of M_3 on calcium and phosphorus.

Date	Treat.	Dosage	Animal	P	Ave.	Ca	Ave.
				Mg. per 100 cc. Blood Serum			
Apr.19	M_3	2 cc	Ex 1	3.84		17.86	
		"	" 2	3.52		15.20	
		"	" 3	2.93	3.43	16.26	16.44
	Olive oil	2 cc	C 4	2.94		17.16	
		"	" 5	4.00		16.36	
		"	" 6	4.25	3.73	16.32	16.61
Apr.21	M_3	2 cc	Ex 1	3.01		19.51	
		"	" 2	3.61		17.82	
		"	" 3	3.64	3.42	18.64	18.66
	Olive oil	2 cc	C 4	2.11		19.96	
		"	" 5	3.58		19.86	
		"	" 6	2.68	2.79	18.95	19.59
Apr.24	M_3	2 cc	Ex 1	3.89		18.10	
		"	" 2	3.23		17.38	
		"	" 3	4.37	3.83	17.43	17.64
	Olive oil	2 cc	C 4	3.37		16.91	
		"	" 5	3.84		18.43	
			" 6	3.44	3.55	17.36	17.60

M_3 dissolved readily in ether and olive oil but upon evaporation of ether a heavy gummy precipitate formed that settled to the bottom. I injected the brown oil of the upper layer. M_3 contains cholesterol and this solution caused more distress and larger swellings than the other materials injected. The results of M_3 are similar to those obtained by Mirvish and Bosman.

One cc. equivalent to 25 gm. fresh tissue.

Table VII. Determinations run for normal levels before injection of the combined extracts.

Date	Treat.	Dosage	Animal	P	Ave.	Ca	Ave.
				Mg. per 100 cc. Blood Serum			
Apr.26	Normal		Ex 1	4.03		18.06	
			" 2	4.09		18.14	
			" 3	4.87	4.33	18.31	18.17
			C 4	3.98		17.78	
			" 5	4.40		16.84	
			" 6	3.88	4.09	19.13	17.92
Apr.28	Normal		Ex 1	4.10		18.26	
			" 2	3.48		17.54	
			" 3	3.63	3.73	17.91	17.90
			C 4	4.00		18.47	
			" 5	4.39		18.11	
			" 6	3.75	4.04	19.48	18.69
May 1	Normal		Ex 1	3.08		18.30	
			" 2	3.16		18.60	
			" 3	3.80	3.35	18.02	18.31
			C 4	2.95		18.86	
			" 5	3.12		18.68	
			" 6		3.03		18.77

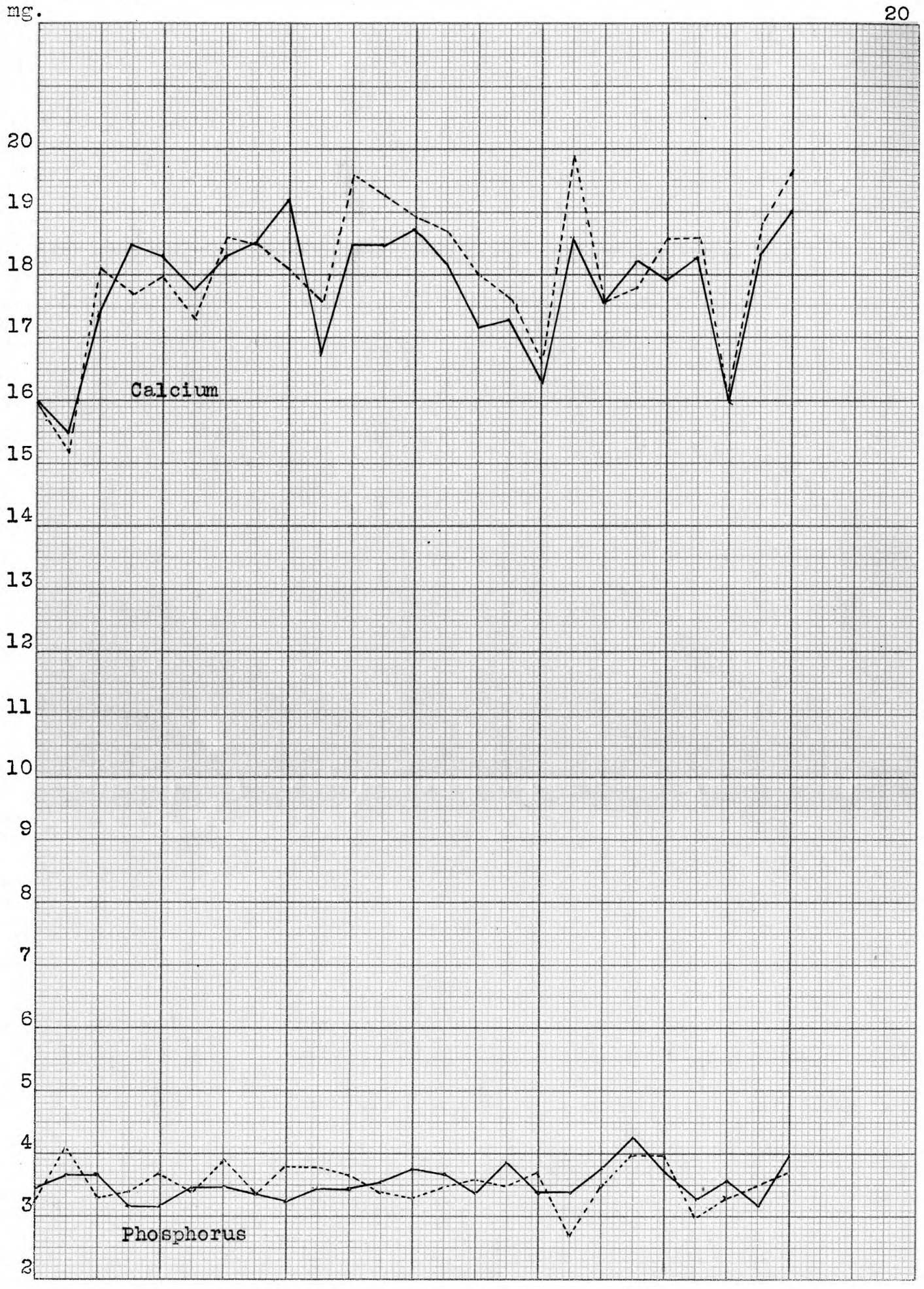
Table VIII. The effect of a combination of the impure fractions and pure ovarian extract.

Date	Treat.	Dosage	Animal	P Mg. per 100 cc. Blood Serum	Ave.	Ca Mg. per 100 cc. Blood Serum	Ave.
May 3	Mixture	2 cc	Ex 1	3.35		16.06	
		"	" 2	3.25		16.00	
		"	" 3	4.32	3.64	16.06	16.04
	Olive oil	2 cc	C 4	3.13		15.78	
		"	" 5	3.56		16.96	
		"	" 6		3.34		16.37
May 5	Mixture	2 cc	Ex 1	2.83		18.95	
		"	" 2	2.88		17.35	
		"	" 3	3.86	3.19	18.34	18.31
	Olive oil	2 cc	C 4	3.69		17.86	
		"	" 5	3.36		19.00	
		"	" 6		3.52		18.43
May 8	Mixture	2 cc	Ex 1	4.15		19.90	
		"	" 2	3.91		18.35	
		"	" 3	4.09	4.04	19.28	19.18
	Olive oil	2 cc	C 4	2.85		19.52	
		"	" 5	4.72		20.20	
			" 6		3.79		19.86

This represents a mixture of equal weights M_1, M_2, M_3 and pure extracts. Following injection large lumps appeared.

One cc. is equivalent to 25 gm. fresh tissue.

-------Controls
———————Exper.



OBSERVATIONS

1. The serum calcium and inorganic phosphorus blood levels of rabbits are not constant but fluctuate from day to day.

2. The pure extract is as readily absorbed as olive oil and causes no irritation, whereas the impure fractions M_1, M_2 and M_3 are irritating and are not readily absorbed.

3. Control animals receiving olive oil show as great variation in calcium and phosphorus values as the experimental animals on the same day.

4. It may therefore be concluded that no fraction eliminated during the purification of M O, the pure extract or a combination of these causes a definite change in blood calcium or phosphorus of rabbits.

REFERENCES

1 Bourne, M. C., and Campbell, D. A.
Variations of serum calcium in rabbits.
Biochem. Jour. 26:183-195. 1932.

2 Brookfield, R. W.
Variations in concentrations of magnesium, calcium and phosphorus in rabbit serum. Biochem. Jour. 27:173-182. 1933.

3 Brown, W. H.
Inorganic phosphorus and calcium in rabbits; periodic and progressive variations in normal rabbits. J. Exper. Med. 47:637-662. May 1928.

4 Brown, W. H.
Inorganic phosphorus and calcium in blood of rabbits; influence of light environment on normal rabbits. J. Exper. Med. 49:103-126. Jan. 1929.

5 Culhane, K.
Variations in serum calcium of rabbits. Biochem. J. 21:1015-23. 1927.

6 Culhane, K.
Does cabbage fed to rabbits increase serum calcium? J. Biol. Chem. 86:113-116. March 1930.

7 Dickens, F., Dodds, E. C., and Wright, S.
Observations upon the preparation and standardization of the ovarian hormone. Biochem. Jour. 19:853-859. 1925.

8 Fiske, C. H., and Subbarow, Y.
The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400. 1925.

9 Harnes, A. R.
Biometry of calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus in normal rabbits from recently acquired stock. J. Exper. Med. 49:287-300. Oct. 1928.

10 Kapsinow, R., and Underhill, F. P.
Does cabbage fed to rabbits increase serum calcium? J. Biol. Chem. 82:377-384. May 1929.

11 Leiboff, S. L.
Absence of calcium in human red blood cells. J. Biol. Chem. 85:759-764. 1930.

12 Mirvish, L., and Bosman, L. P.
Influence of testicular and suparenal extract on blood calcium. British J. Exper. Biol. 6:350-359. 1929.

13 Mirvish, L., and Bosman, L. P.
Influence of internal secretions of ovary on calcium blood level and on calcium metabolism. Quart. J. Exper. Physiol. 18:11-32. July 1927.

14 Riddle, O., and Rhinehart, W. H.
Studies on physiology of reproduction in birds; blood calcium changes in reproductive cycle.
Am. J. Physiol. 76:660-676. May 1926.

15 Roe, J. H., and Kahn, B. S.
The colorimetric determination of blood calcium.
J. Biol. Chem. 81:1-8. Nov. 1928.