CXXXV. VARIATIONS IN THE SERUM-CALCIUM OF RABBITS.

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IN a comparison of the effect on rabbits of the parathyroid hormone with that of insulin and various other substances, Davies, Dickens and Dodds [1926] showed that the serum-calcium could be raised by the injection of insulin at regular intervals of time. The curve that they obtained for the serum-calcium resembled those published by Clark and Collip [1926, 2] showing the results of similar repeated injections of parathyroid extract into dogs. Further, by following Clark and Collip's [1926, 1] method of extraction of the parathyroid hormone, they obtained a product from pancreas which, when injected into rabbits, caused an increase in the serum-calcium; but both with this extract and that of the actual parathyroid glands a rise was only obtained when very large doses were used. The authors suggested that the parathyroid hormone, like insulin, might be fairly widely distributed in the body. If these two substances occur together in the pancreas, owing to the marked similarity between their properties they might be extracted together and some of the active principle of the parathyroid would then remain as an impurity in the final product of insulin. Hence, the effect observed on the serum-calcium of the rabbit might be due, not to some additional property of insulin, but to its association with this impurity.

Should this hypothesis prove to be correct, a number of practical results would follow, and it appeared worth testing with regard to the possibility of obtaining (a) a new criterion for the purity of insulin—that it should have no effect on the serum-calcium; (b) a use for apparently worthless by-products of insulin manufacture; and (c) an explanation of the frequent very erratic response of rabbits to insulin. While the hypoglycaemic effect is known to last for only a few hours, that on the calcium might be much more prolonged and might account both for the "bulk variation" of groups of animals in their reaction to insulin, which has been repeatedly observed by workers engaged in its physiological assay, and also for the individual variations of particular animals.

Method.

A group of ten young rabbits was selected for experiment. Four acted as controls throughout, two receiving doses of saline, two having no injections at all.

All doses were calculated on the weight of the animal, and dilutions were made such that a volume of 0.25 cc. per kg. was always injected. The animals

were fed throughout unless otherwise stated, their diet consisting of hay, oats and cabbage.

Serum-calcium determinations were carried out by the method of Kramer and Tisdall [1921] modified by Clark and Collip [1926, 3].

To test the method, seven separate estimations of serum-calcium were made on the same blood and gave the following values in mg. per 100 cc. of serum: 15.37, 15.27, 15.18, 15.18, 15.27, 14.88, 15.08. Hence, a difference of 0.5 mg. per 100 cc. can be regarded as outside the range of experimental error.

THE EFFECT OF INSULIN ON THE SERUM-CALCIUM.

Since Davies, Dickens and Dodds observed a significant rise four hours after the injection of what they describe as a sample of "commercial insulin," a number of preliminary experiments were made in which blood samples were taken before and four hours after injection; but neither with the purest nor with very impure samples of insulin was any rise obtained, and both the rabbits receiving insulin and the controls which received none gave similar results.

The following is a typical experiment.

The rabbits were given a dose of one unit per kg. of a very impure preparation of which one unit was contained in 8 mg. They had received their morning ration of cabbage about half an hour before the experiment was started, and hay and oats were present in their cages throughout. The results are shown in Table I, and, for the sake of comparison, figures are also given for an experiment using the same animals in which precisely similar conditions were observed but no injections were given to any of them.

Table I.

TABLE I.

		(1) Nov. 10th			(2) Nov. 17th	
		Serum-calcium mg. per 100 cc.			Serum-calcium mg. per 100 cc.	
Rabbit no.	Dose	Initial	4 hours	Dose per kg.	Initial	4 hours
N 1	None	14.50	13.82	1 u.	15.09	13.44
N 2	"	14.31	13.44	"	14.31	12.96
N 3	"	17.12	14.60	"	14.89	13.54
N 4	"	14.89	13.73	"	14.41	14.41
N 6	"	15.57	14.21	"	14.50	14.02
O 6	"	16.44	13.85	"	13.82	12.47
Average ...	...	15.47	13.94	—	14.50	13.47
Change from initial...		—	-1.53	—	—	-1.03
<i>Control 1. No insulin. Saline.</i>						
O 1	None	13.82	13.35	0.25 cc.	14.01	11.70
O 10	"	14.79	13.24	"	13.93	12.86
Average ...	...	14.31	13.30	—	13.97	12.28
Change from initial...		—	-1.01	—	—	-1.69
<i>Control 2. No insulin. No saline.</i>						
P 9	None	15.09	13.93	None	14.12	13.56
P 10	"	15.18	13.68	"	13.73	12.67
Average ...	...	15.14	13.81	—	13.93	13.12
Change from initial...		—	-1.33	—	—	-0.81

It will be seen that in both cases a fall was obtained. Work by Clark [1920], Moritz [1925] and Stewart and Percival [1927] suggests that this is the usual effect of haemorrhage on rabbits and a number of results obtained in the course of this investigation lead to the same conclusion. Haemoglobin estimations indicate a small but progressive dilution of the blood with respect to this constituent when rabbits are bled at frequent intervals of time. The two observations may possibly be correlated. In the above experiments, however, the animals were fed, and, as will be shown later, this may have a considerable effect: it is possible that with careful control of the diet the rabbit might show the same constancy with regard to the calcium content of its serum as has been demonstrated in the case of the cat (Stewart and Percival).

There appeared to be two possible explanations of these negative results:

(a) that the particular insulin samples used would cause a rise in serum-calcium at some other interval of time than that investigated in these preliminary experiments; or

(b) that only certain rabbits show this effect.

A. (i) *The effect of repeated small doses of insulin when rabbits are fed with hay, oats and cabbage.*

A number of experiments were performed in which the rabbits were given injections of 0.5 or 1 unit per kg. every two hours for six hours, blood samples being taken either at the 4th or 5th and 20th hours, but in no case was any rise in the serum-calcium obtained. Finally the curve for the whole 24 hours was followed, injections being made every four hours, and the conditions described by Davies, Dickens and Dodds being as far as possible observed.

According to the usual routine of this laboratory the rabbits were given hay, oats and cabbage at 9 a.m. and 5 p.m., and in addition during the night cabbage and oats were given at 3 a.m.

The results are shown in Fig. 1.

Fig. 1. A. Average curve for six rabbits receiving doses of 0.5 unit of insulin per kg. every four hours.

B. Average curve for two rabbits receiving doses of 0.25 cc. per kg. of normal saline solution every four hours.

C. Average curve for two rabbits having no injections.

Both the controls and the rabbits receiving insulin showed a preliminary fall followed by a rise to a high value. This rise cannot be due to insulin because the animals that received none reacted similarly. It is a significant fact that on every occasion on which cabbage was given there was a considerable increase. An apparent exception to this is seen in the rise at 2 a.m., but a probable explanation lies in the fact that the cabbage given at 5 p.m. was not all consumed until much later; thus, it was observed at 10 p.m. that all the rabbits on insulin and one of the controls still had cabbage in their cages left over from the 5 o'clock meal. Unfortunately no note was made of this at the time nor observation made on the other three controls, because the importance of these details of feeding was not then recognised. During the next four hours all the cabbage was eaten, since none remained in the cages at 2 a.m. Throughout the whole period oats and hay were present in all the cages, only the cabbage varied.

One rabbit went into convulsions at 9 o'clock and was given a large quantity of cabbage in the hope that it might recover without the necessity of an injection of glucose. It recovered without sugar and showed the greatest rise in serum-calcium.

A. (ii) *The effect of repeated small doses of insulin when rabbits are fed with hay and oats only.*

Fig. 2 shows the results of four-hourly doses of 0.5 unit per kg. of the same sample of insulin as was used in the above experiment, the rabbits receiving their usual diet but with the cabbage omitted.

Fig. 2. A. Average curve for six rabbits receiving doses of 0.5 unit of insulin per kg. every four hours when cabbage was withheld.

B. Average curve for two rabbits receiving no injections but to whom cabbage was given twice during the experiment.

C. Average curve for two rabbits who received no insulin and no cabbage.

Cabbage was withheld from 12 noon on the day preceding experiment and water given instead until 5 o'clock in the evening in order to avoid any

possible complications from undue thirst, cabbage being normally the chief source from which these animals obtain water.

Of the four controls which received no insulin, two were given cabbage twice during the course of the experiment, at 5 p.m. and at 4 a.m. Both showed a rise in the serum-calcium at 6 p.m. and 6 a.m. It will be seen that the largest deviations from the initial values are shown by these two and that no changes in the serum-calcium of the animals that were injected can be ascribed to their doses of insulin.

B. *The effect of insulin convulsions.*

It is noticeable in the experiment reported by Davies, Dickens and Dodds in which they gave about 0.5 unit per kg. of insulin that the rabbit went into convulsions twice during the 24 hours in spite of the fact that it was fed throughout on cabbage and bran-mash. This led to the consideration that possibly only very sensitive rabbits show a rise in serum-calcium following insulin injection, and that actual convulsions might even be the cause. Hence, three very sensitive rabbits were selected which had been used for some time for insulin testing and were known to convulse on a dose of 0.5 unit per kg. when starved, and a series of experiments was performed with these animals (Table II). Again the results point to the conclusion that when a rise was observed it was caused by cabbage.

Table II.

Date	Conditions of experiment	Dose of insulin in units per kg.	Serum-calcium. Average change in 4 hrs after injection, in mg. per 100 cc.
Nov. 3, 1926	Fed at 9 a.m. with oats, hay and cabbage. Injected at 10 a.m.	0.5	- 1.11
Nov. 8	Fed at 9 a.m. with oats, hay and cabbage. Injected at 10 a.m.	1.0	- 0.56
Nov. 24	Starved for 22 hours. Injected at 10 a.m. All convulsed within 2 hours and were given sugar and cabbage	1.0	+ 1.32
Dec. 1	Starved for 22 hours. Injected at 10 a.m. All convulsed within 2 hours and were left in convulsive state for 1½ hours before being given sugar and cabbage	1.0	+ 1.26
Dec. 8	Starved for 22 hours. Injected at 10 a.m. and fed with hay, oats and cabbage at 10.30 a.m. in order to avoid convulsions	1.0	+ 1.10
Dec. 15	Ditto. (N.B. One convulsed in spite of feeding and was given sugar)	2.0	+ 1.26
Dec. 29	Starved for 22 hours. Initial blood samples taken at 10 a.m. Given oats, hay and cabbage at 10.30 a.m.	None	+ 1.26
Jan. 5, 1927	Starved for 22 hours and throughout experiment	None	- 0.03
Jan. 12	Starved for 22 hours. Initial blood samples taken at 10 a.m. 6 cc. 20 % glucose injected subcutaneously at 1 p.m.	None	+ 0.52

0.5 and 1 unit per kg. failed to effect any increase in serum-calcium or to produce convulsions when the rabbits were fed. After starvation for 22 hours

(according to the usual routine for insulin testing) a rise was obtained with 1 unit per kg., both after convulsions when sugar and cabbage were given and also when convulsions were avoided by feeding the animals with hay, oats and cabbage half an hour after injection. The rise obtained could not be increased by withholding food and leaving the animals in convulsions for an hour and a half nor by doubling the dose. A control experiment in which the rabbits were fed with hay, oats and cabbage half an hour after the initial blood sampling but were given no injections confirmed the suspicion that insulin was not the cause of the rise obtained in the earlier experiments.

It is a routine in this laboratory that when glucose is used as an antidote to insulin convulsions the rabbit is also given a few cabbage leaves, the consumption of which is taken as a sign of recovery. Thus, on every occasion when the injection of sugar was necessary, the animals were also fed with cabbage.

An examination of the results shows that a rise was obtained after starvation in the following circumstances:

1. On feeding with cabbage and oats.
2. On feeding with cabbage and oats after the injection of insulin.
3. On feeding with cabbage and injecting sugar after the onset of insulin convulsions. (Sugar itself has not been shown to have any effect on the serum-calcium, either by us or by Davies, Dickens and Dodds.)

The only factor that did not vary is the consumption of cabbage. On no occasion was a significant rise obtained when cabbage was not given.

THE EFFECT OF CABBAGE ON THE SERUM-CALCIUM.

The conclusion having been reached by two separate series of experiments that any rise in serum-calcium following insulin injection is caused by cabbage and not by the insulin, the effect of cabbage alone was then tested.

The following procedure was followed.

At 12 noon on the day preceding experiment cabbage was removed from the cages and the animals were given water until 5 p.m. In order to control any possible effect of the water two of the controls were given none. Samples of blood were taken at 10 a.m., and six rabbits were then given a plentiful supply of cabbage which was maintained for 2 hours and then removed. The last four rabbits as usual served as controls and received no cabbage. All were kept supplied throughout with oats and hay, and serum-calcium determinations were made at two-hourly intervals. The results are shown in Fig. 3.

The only rabbit receiving cabbage which failed to show an increased serum-calcium ate none (*N* 6). For the purpose of averages it is therefore included among the controls. Those animals that were given no cabbage showed a slight fall.

Table III.

<i>Exp 1.</i> Feb. 9th. Cabbage at 10 a.m. Serum-calcium mg. per 100 cc.					<i>Exp 3.</i> April 6th. Cabbage at 11.30 a.m. Serum-calcium mg. per 100 cc.				
Rabbit no.	10 a.m.	12 noon	2 p.m.	4 p.m.	Rabbit no.	9.30 a.m.	11.30 a.m.	1.30 p.m.	3.30 p.m.
<i>N 1</i>	13.44	15.38	14.31	14.12	<i>U 1</i>	13.24	13.04	15.53	14.64
<i>N 2</i>	13.93	15.38	14.03	13.05	<i>U 2</i>	14.34	13.14	15.24	14.24
<i>N 3</i>	14.03	14.79	13.93	14.31	<i>U 3</i>	14.44	13.84	15.93	14.34
<i>N 4</i>	13.54	14.31	13.35	13.24	<i>U 4</i>	14.64	13.94	15.24	13.94
* <i>N 6</i>	(13.24	12.76	12.76	12.38)	<i>U 5</i>	13.64	11.05	14.94	13.54
<i>O 6</i>	11.99	12.57	12.09	10.47	<i>U 6</i>	14.44	13.84	15.14	14.04
Average	13.39	14.49	13.54	13.04		14.12	13.14	15.34	14.12
Change from initial		+1.10	+0.15	-0.35		—	-0.98	+1.22	—
<i>Control 1.</i> No cabbage. Water given on the day preceding experiment.									
<i>O 1</i>	12.57	12.38	11.70	11.21	<i>U 7</i>	13.04	12.94	13.04	12.05
<i>O 10</i>	12.47	12.47	11.99	11.90	<i>U 8</i>	13.54	12.15	13.04	12.35
Average including <i>N 6</i>	12.76	12.54	12.15	11.83		13.29	12.55	13.04	12.20
Change from initial		-0.22	-0.61	-0.93		—	-0.74	-0.25	-1.09
<i>Control 2.</i> No cabbage. No water.									
<i>P 9</i>	13.15	12.86	12.18	11.12	<i>U 9</i>	14.24	13.84	12.84	13.04
<i>P 10</i>	12.47	13.35	11.99	11.21	<i>U 10</i>	13.14	13.14	11.65	11.55
Average	12.81	13.11	12.09	11.17		13.67	13.49	12.25	12.30
Change from initial		+0.30	-0.72	-1.64		—	-0.20	-1.44	-1.39

* *N 6* ate no cabbage and is therefore omitted from the average and included in Control 1.

This experiment (*Exp. 1*, Table III) appeared to prove definitely that the previous conclusion as to the effect of cabbage was correct, and further confirmation was obtained by an experiment on the three abnormally sensitive rabbits which gave the same result.

Fig. 3. A. Average curve for five rabbits fed with cabbage between 10 a.m. and 12 noon.
B. Average curve for five rabbits who were given no cabbage.

The next step attempted was to determine what constituent of cabbage has the property of raising the serum-calcium. For the purpose of making a systematic investigation of this problem it was thought advisable to start off with an entirely new set of rabbits. As a preliminary experiment, therefore,

the above test was repeated on these young rabbits. The results were entirely negative, the experimental animals reacting in the same way as the controls.

It is a well-known fact reported by many different observers that when the serum-calcium is high it is not easily changed, whereas when it is low it may be increased by a variety of means. Also it has been stated by Grant and Gates [1924], and confirmed by this work, that rabbits show a seasonal variation in serum-calcium, giving minimum values in January and high values in May and November. This is shown in Fig. 4, which gives a curve for the average of the initial values for ten rabbits during 3 months. Hence, the possible explanation of the different response to cabbage of the young rabbits tested in the middle of March from that of the group tested 5 weeks earlier is that the new ones were very near their maximum at the beginning of the experiment. Therefore, when it was repeated again on the same young animals 3 weeks later, instead of cabbage being given immediately after the initial blood samples were taken, it was given 2 hours later when the calcium content of the serum had already fallen. This time all the rabbits receiving cabbage showed a considerable rise (Fig. 5). The results are shown in Exp. 3, Table III.

Fig. 4. Curve showing the average "seasonal variation" of the initial serum-calcium values for ten rabbits over a period of three months.

Fig. 5. A. Average curve for six rabbits fed with cabbage between 12 and 2 p.m.
B. Average curve for four rabbits who were given no cabbage.

CONCLUSIONS.

1. Insulin causes no rise in the serum-calcium of rabbits.
2. Insulin convulsions cause no rise in the serum-calcium of rabbits.
3. An immediate rise in the serum-calcium of rabbits occurs on feeding with cabbage, except when the initial value may be regarded as exceptionally high. The cause of this effect remains to be found.

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REFERENCES.

- Clark (1920). *J. Biol. Chem.* **43**, 89.
Clark and Collip (1926, 1). *J. Biol. Chem.* **63**, 395.
—— — (1926, 2). *J. Biol. Chem.* **63**, 439.
—— — (1926, 3). *J. Biol. Chem.* **63**, 461.
Davies, Dickens and Dodds (1926). *Biochem. J.* **20**, 695.
Grant and Gates (1924). *Proc. Soc. Exp. Biol. Med.* **22**, 315.
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.
Moritz (1925). *J. Biol. Chem.* **66**, 343.
Stewart and Percival (1927). *Biochem. J.* **21**, 301.