CCVII. STUDY OF THE BLOOD-CALCIUM FOLLOWING THE INTRAVENOUS INJECTION OF CALCIUM SALTS.

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THROUGH the intravenous administration of calcium salts the blood suffers only a transitory increase in calcium concentration, for the blood-calcium sinks rapidly, returning usually within 2 hours of the injection to its original level. This has been observed by several workers in different animals, notably by Heubner and Rona [1919, 1] in cats, by Hetényi [1924] and Gerschmann [1930] in dogs, and by Clark [1920], Condorelli [1926, 1927] and Rothlin [1930] in rabbits. The last two authors and Taylor and Fine [1930] have commented on the remarkably large apparent loss of calcium from the blood-stream during the first 5 minutes after the injection. The object of the experiments described below was firstly to discover whether this apparent loss could be attributed in any measure to shortcomings of the analytical method, and secondly to study the kinetics of the fall of the blood-calcium.

Material.

In addition to calcium chloride, there was used in this work calcium levulinate, a very soluble calcium salt described by Conrad [1878], the value of which as an agent for calcium therapy by injection has recently been pointed out [Dodds and Greville, 1931; Bennett, 1931]. Pure calcium levulinate was kindly supplied by the Crookes Laboratories.

Adequacy of the analytical method.

In most of the researches on the behaviour of the serum-calcium following calcium administration, the calcium has been determined by direct precipitation as oxalate from diluted serum, usually by the technique of Kramer and Tisdall [1921] as modified by Clark and Collip [1925]. This much-used procedure of direct precipitation has recently been severely criticised. Incomplete calcium precipitation due to the presence of serum-proteins tends to lower the result [Van Slyke and Sendroy, 1929; Guillaumin, 1930], while at the same time entrainment of oxidisable foreign matter with the oxalate precipitate tends to raise it. Guillaumin [1930] concluded that with fresh human blood the second error preponderates. So that although direct precipitation has often been shown in animal serum to give the same results as

ashing [Loucks and Scott, 1929-30; Hendriks, 1929], it must be recognised that error may possibly accompany its use.

EXPERIMENTAL.

It was first shown that quite good recoveries were obtained by the Clark and Collip technique when large quantities of calcium were added to serum. To 2 cc. of horse serum in the precipitation-tubes measured volumes of standard calcium chloride solution were added. Analyses were performed in quadruplicate, the extreme error being less than 2 %. Recoveries of 98.4 % and 97.6 % were obtained with 0.577 mg. and 0.289 mg. of added calcium respectively.

The above results were obtained when serum and dilute calcium chloride solution were mixed in the precipitation-tube. If, on the other hand, a mixture was made by adding, with thorough shaking, 1 cc. of a stronger calcium solution to 49 cc. of serum in a graduated flask, 2 cc. portions of this mixture gave, when analysed by direct precipitation, low and erratic results. Thus with a mixture made as above from horse serum and calcium levulinate solution, with a total calcium content of 39.0 mg./100 cc., values ranging from 10 to 33 mg./100 cc. were obtained. Similar results were obtained with a mixture of calcium chloride solution and horse serum. The error was not due to insufficient mixing since consistent, if rather high, recoveries were obtained when the calcium was determined in the mixture following precipitation of the proteins by trichloroacetic acid. No systematic alteration occurred when determinations were made after leaving the mixture for many hours and centrifuging, or when the oxalate precipitation was allowed to proceed for 24 hours. The supernatant liquid after centrifuging the precipitate, and the washings, were observed to be cloudy; hence under these conditions the calcium oxalate is precipitated in a readily peptisable form, and variable amounts of colloidal calcium oxalate are decanted off in the supernatant fluid. It is interesting to note that Grollman [1927] found that, when calcium chloride was added to serum *in vitro* in amounts (up to a total concentration of 32 mg./100 cc.) which caused no precipitation or change in concentration of inorganic phosphate, the latter was rendered increasingly non-diffusible.

When strong calcium solutions are injected into the veins of living animals, the addition of calcium to serum takes place under circumstances similar to those in which the erratic results described above were obtained. It was therefore considered desirable to compare the serum-calcium value as determined by direct precipitation with that obtained by some reliable fundamental method, in blood abstracted from a living animal a short time after an intravenous injection of a strong calcium solution had been made. This experiment was of further interest in that Heubner [1925] found that after the injection into cats of calcium lactate, as well as of calcium hexosephosphate, only a part of the calcium could be precipitated directly as oxalate.

Accordingly (Exp. 1, Table I) a rabbit was put under open ether anaesthesia,

70 mg. of calcium as calcium levulinate in 5 cc. of solution were injected into the external jugular vein, and 5 minutes later about 30 cc. of blood were obtained rapidly from the carotid artery. In the heparin plasma obtained from the blood, the calcium was determined both by the Clark and Collip method, and also by the wet combustion method recently described by Widmark and Vahlquist [1931]. (The use of the filter-stick was replaced by the usual Clark and Collip technique, after precipitation in the cold and standing overnight.)

It will be seen (Table I) that the Clark and Collip method gave a value for the plasma-calcium 2.2 % higher than that obtained by the incineration method. We may therefore conclude that under these conditions the direct precipitation method is competent to give an accurate measurement of the plasma-calcium.

Table I. *Exp. 1.*

Weight of rabbit =	2.80 kg.	Ether anaesthesia.
Injection	= 70 mg. of calcium as calcium levulinate in 5 cc. solution.	
Blood taken	5 mins.	after injection.
Haematocrit reading:	(mean of 4) 30 %	corpuscles.
Plasma-calcium (Clark and Collip technique):	27.8, 27.5, 27.4; mean 27.6 mg./100 cc.	
Plasma-calcium (Widmark and Vahlquist ashing technique):	27.0, 27.1, 26.9; mean 27.0 mg./100 cc.	
Whole blood-calcium (Widmark and Vahlquist ashing technique):	20.0, 19.6, 19.1; mean 19.6 mg./100 cc.	
	70 % $\times$ 27.0 = 18.9 mg./100 cc.	
	Difference =	0.7 mg./100 cc.
(Blank tests: 0.0056 mg. Ca per cc. nitric acid: no allowance necessary for other reagents.)		

Two further ways in which calcium in the blood may escape determination by the serum-calcium analysis may be envisaged: it may pass into the corpuscles, or it may be present as a suspension of insoluble calcium salt which would be carried down with the corpuscles. To discover whether either of these events occurred, in the above experiment the calcium content of the whole blood was determined by the Widmark and Vahlquist [1931] method and haematocrit readings were taken. It will be seen (Table I) that the calcium in whole blood is greater than the amount calculated from the ashed plasma value by 0.7 mg./100 cc. or 3.6 %. This may well be within the limits of experimental error. Heubner and Rona [1919, 1] found quantities of calcium in the corpuscles of calcium-injected animals varying from 0 to 7 mg./100 cc.; but it may be mentioned that even with normal blood there is still controversy as to the calcium content of the corpuscles, two of the latest determinations giving zero [Leiboff, 1930] and varying quantities up to 2 mg./100 cc. [Guillaumin, 1930].

From Exp. 2 (Table II) more data may be obtained. Here the whole blood-calcium was 0.7 mg./100 cc. lower than the value calculated from the plasma content. The latter value however was determined by direct precipitation which we have seen gives slightly higher results. At any rate we may conclude from

Table II. *Exp. 2.*

Weight of rabbit = 3.12 kg. Ether anaesthesia.
 Injection as in Exp. 1, zero time to 0 min. 30 secs.
 Bleeding, 1 min. 15 secs. to 3 mins. 15 secs.
 Mean haematocrit reading = 73 %.
 Blood-calcium (ashing technique):
 21.9, 22.0, 22.2; mean 22.0 mg./100 cc.
 Plasma-calcium (Clark and Collip):
 15 mins. after drawing blood, 31.0, 31.0 mg./100 cc.
 3 hrs. 30 mins. after drawing blood, 31.0, 31.4 mg./100 cc.
 73 % of 31.1 mg./100 cc. = 22.7 mg./100 cc.

these experiments that the quantity of calcium which under the circumstances described is carried down with or inside the corpuscles on centrifuging is negligible.

Lastly, although we have no evidence of precipitation *in vivo* from the above experiments, it seemed desirable to discover whether calcium precipitated from the blood on standing *in vitro* at room temperature. Although Greenberg and Gunther [1930] had shown that with normal ox serum the serum-calcium value obtained is independent of the time (up to the 22 hours studied) during which the serum stands in contact with the clot, it did not follow that the serum of our experiments, so heavily charged with calcium, would be equally stable. The blood was heparinised, a portion rapidly centrifuged and the plasma-calcium determined as soon as possible. A second portion was kept, loosely corked, for 3½ hours, after which it was centrifuged at high speed for 15 minutes, and the plasma-calcium again determined. The value was found to be no lower, and to agree with the whole blood value on the assumption that there was no calcium in the corpuscles.

From the above experiments it may be concluded that the serum-calcium value as determined by the direct precipitation method gives an accurate measure of the blood-calcium after the intravenous injection of calcium levulinate.

Study of disappearance of calcium from the blood-stream.

Control experiments. In order satisfactorily to construct a curve showing the fall of serum-calcium after the injection, it was necessary to make sure that the serum-calcium returned finally to a constant level. In the curves constructed by previous authors, the serum-calcium is usually still falling when it reaches the initial level again. Accordingly attention was directed towards finding conditions under which, in the absence of injection, but with the degree of haemorrhage necessary for the withdrawal of the blood samples, the serum-calcium would stay constant for a length of time equal to the duration of the experiment.

Now the serum-calcium of the rabbit (which animal was used for these experiments as it was the most convenient) is by no means as constant as that of man or of other animals such as dogs. Thus Derevici [1929] gives the

serum-calcium of rabbits as 12–20 mg./100 cc. Most notable is the raising of the serum-calcium of the fasting rabbit by the ingestion of cabbage [Culhane, 1927, 1930]. Although this was disputed by Kapsinow and Underhill [1929], the writer has found a systematic difference between the serum-calcium levels of rabbits which have been continuously fed on cabbage and those of rabbits which have not. Thus the values for 23 cabbage-fed rabbits ranged from 14.7 to 16.9 (average 15.6), while the values for 18 rabbits of the second group, which had been fed on oats or on bran or which had taken no food since the previous evening, ranged from 8.2 to 14.9 (average 11.6) mg./100 cc. There is less variation in the cabbage-fed group. Rabbits kept continuously on this diet have a steady blood-calcium, as was shown by Davies, Dickens and Dodds [1926]. For the present purpose it was therefore decided to keep cabbage continuously in the cage both during the experiment and for at least 3 hours before. With these conditions, control experiments involving bleedings at the times to be used in the succeeding experiments showed a satisfactory steadiness of the serum-calcium, as in Exp. 3 (Table III).

Table III. *Exp. 3.*

		Rabbit, female, 2.50 kg.						
Time	...	0	5	20	40	60	120	180 mins.
Serum-calcium		15.0	14.6	14.5	14.2	14.1	14.4	14.5 mg./100 cc.

Technique.

The injection was made into the peripheral vein of the left ear. A puncture was made with a needle of triangular section in the peripheral vein of the right ear, and the blood allowed to flow into a centrifuge-tube where it clotted; the serum-calcium was then determined by the Clark and Collip technique. 5 cc. of blood were usually obtained in less than a minute, but in the later stages of the experiments a longer time was often consumed. The mid-time of the bleeding was noted. In these experiments no blood was taken before 5 minutes after the injection, as owing to the rapid fall of blood-calcium at this stage, the comparatively long times taken over the bleeding would introduce large inaccuracies.

Table IV. *Exp. 4.*

		Rabbit, female, 3.32 kg. Dose = 13.6 mg. Ca in 1 cc. per kg., as calcium levulinate.						
Time	...	(Initial)	0	5	20	43	65	125
Serum-calcium		14.7	(Injection)	22.9	19.6	17.0	15.9	14.9
Excess above final level	—	—	—	7.9	4.6	2.0	0.9	—
Log _e (excess)	—	—	—	2.07	1.53	0.69	−0.11	—
Antilog A	—	—	—	9.5	9.5	9.4	9.3	—

Table V. *Values of antilog A.*

Blood sample no.	...	1	2	3	4	5
Exp. 4		9.5	9.5	9.4	9.3	—
" 5		11.2	10.2	13.8	10.5	—
" 6		10.7	10.7	9.6	10.5	11.0
" 7		10.7	10.2	10.7	10.0	—

Table VI.

Exp. no.	Weight (kg.) and sex	Dose (mg. Ca per kg.) and salt	<i>k</i>	Approximate time to reach constant level (mins.)	Antilog <i>A</i>
4	3.32 (F)	13.6 (L)	0.037	110	9.5
5	3.18 (M)	16.0 (L)	0.036	120	11.2
6	3.18 (M)	13.6 (L)	0.021	210	10.3
7	3.36 (M)	14.5 (C)	0.037	150	10.6
8	3.04 (M)	16.0 (L)	(0.086)	40	(8.5)
9	3.10 (M)	14.5 (C)	(0.047)	80	(9.1)
10 Cat*	2.28 (M)	16.0 (L)	0.023	140	7.6

L = Calcium levulinate. C = Calcium chloride. * Under chloralose anaesthesia.

Table VII.

Exp. 11. Rabbit 3.0 kg. Injection 13.1 mg. Ca per kg. as 10 % levulinate.

Inorganic phosphate* in (citrated) plasma:

Before injection 4.0 mg. P/100 cc.

5-6 mins. after injection 4.7 "

Exp. 12. Rabbit 3.04 kg. Injection as in *Exp. 11.*

Inorganic phosphate in serum:

Before injection 4.5 mg. P/100 cc.

4-5 mins. after injection 4.9 "

* de Wesselow [1924].

Table VIII.

Exp. 13. Rabbit, 3.03 kg. Injection 31 mg. Ca per kg. as calcium chloride.

Haematocrit reading: 75 % plasma.

	Serum-calcium*	Haemoglobin†	Alkali reserve‡
Before injection	14.2	100 units	29
3-6 mins. after injection	32.0	92.6 units	25
Increase	17.8	-7.4 %	-4

mg./100 cc.

4 cc. CO₂ at N.T.P. = 3.6 mg. Ca

cc. CO₂/100 cc.

* Clark and Collip [1925].

† Myers [1924].

‡ Van Slyke [1917].

RESULTS.

The results are included in Tables IV-VI. A typical curve is shown in Fig. 1, in which the serum-calcium values found in *Exp. 4* are plotted against

Fig. 1.

the time after the injection. The curve is concave upwards, thus differing from the curves given by Hetényi [1924], but agreeing with those of Gersch-

mann [1930] and Rothlin [1930]. It will be observed that the final steady level differs little from the initial level: this was not so in all the experiments, the final level being sometimes higher and sometimes lower than the initial level. The greatest observed difference between the two was, however, only 1.4 mg./100 cc. There was one experiment (not included in the Tables) in which the serum-calcium did not return to a constant level, but fell steadily after reaching the initial value. In all the experiments doses of the same order of magnitude were given.

DISCUSSION.

It is of interest to see whether there is any approximate relation between the excess of the serum-calcium at any instant above the final constant level and the time to which it corresponds. In the third row of Table IV the values

Fig. 2.

of this excess (x) are given for each point of Exp. 4. In Fig. 2 $\log_e x$ is plotted against the time after the injection (t), and it will be seen that the points lie on a straight line. x and t are therefore connected by the relation

$$\log_e x = A - kt,$$

where k and A are constants for the particular experiment. Hence the rate of fall of calcium excess at any instant is directly proportional to the excess obtaining at that instant. A is the logarithm of the (theoretical) initial value of the excess, while k is the rate of fall per unit calcium excess (for Exp. 4, 0.037 mg./100 cc. per minute per mg./100 cc. excess).

In order to determine how closely the data fit this logarithmic law, we may insert in the above equation the values for x , t and k , and so calculate A for each point of the curve. From the constancy of A we may draw conclusions concerning the accuracy with which the law is obeyed. The corresponding values of antilog A for the points of Exp. 4 are given in the fifth

row of Table IV, and the constants for significant analyses in other rabbit experiments are given in Table V.

Out of the experiments performed, three satisfactory curves were obtained with calcium levulinate and one with calcium chloride. In addition there were some experiments in which the rate of fall of serum-calcium was so rapid that enough points on the significant part of the curve were not obtained to give logarithmic graphs of interest (data given in brackets in Table VI, in which the results of the important experiments are summarised).

It must be remembered, in interpreting the results obtained, that plotting data on a logarithmic scale tends to smooth out divergences; but an examination of Fig. 2 and Table V shows that the data are distributed evenly about the curve representing the logarithmic law, and do not show any systematically increasing divergence. It would of course be idle to suggest that the relationship is exactly accurate or to attach any physical significance to it, for we are dealing with the net result of several complicated processes and the return to a dynamic equilibrium. We can, however, say that the data are conveniently represented by this equation, and that the rate of disappearance of serum-calcium at any instant is approximately proportional to the excess above the steady value which obtains at that instant. Such a formulation has indeed previously been employed in the kinetics of blood chemistry: for example Fishberg [1930] found that the disappearance of foreign sugars from the blood-stream can be described by this (to use a chemical analogy) "unimolecular law."

It will be seen from Table VI, from the values of k and of the approximate time taken for the serum-calcium to return to a constant level, that the rate at which calcium disappears from the blood-stream varies greatly among the different animals. Exp. 7 with chloride yields results which fall in line with those with levulinate [*cf.* Rothlin, 1930]; there might well have been a difference since calcium chloride is said to disturb the acid-base equilibrium of the blood.

Interest attaches to the constant "antilog A ." This initial highest value of the serum-calcium is never of course realised in practice owing to disappearance of calcium from the blood-stream during the injection, and also because the injected calcium takes some time to become completely mixed with the circulating blood [*v.* Erlanger, 1921]. But it gives an approximation to the amount of serum-calcium which would disappear during the course of the experiment if the fall of serum-calcium during the first 5 minutes were to exhibit the same relationships as it does during the later part of the experiment. It is found that the loss calculated for the whole experiment is quite inadequate to account for the total disappearance. Thus in Exp. 4, 13.6 mg. of calcium per kg. are injected. Now from the table given by Erlanger [1921] it appears that 50 cc. is a reasonable figure to take for the blood-volume per kg. of rabbit, and as 70 % is a usual haematocrit reading for rabbit's blood we may suppose that there are 35 cc. of serum per kg. The potential increase

in serum-calcium is therefore $\frac{13.6 \times 100}{35} = 39$ mg./100 cc. But antilog A is only 9.5. Hence about 30 mg./100 cc. have not been accounted for. Even if we take the improbable figure of 9 % for the blood-volume, the unexplained disappearance amounts to 12 mg./100 cc., a quantity greater than the total loss calculated by extrapolation from the curve. Obviously, in addition to the disappearance of serum-calcium at a rate expressed approximately by the logarithmic relationship, there is a rapid disappearance immediately after the injection. This idea is borne out by a curve given by Rothlin [1930]; following injection of 29 mg. Ca per kg. the rate of fall during the first 20 minutes is relatively very much greater than that during the succeeding 100 minutes. It is reasonable to suppose that two different processes take place.

The analyses of Heubner and Rona [1919, 2; 1923], Jungmann and Samter [1924], Hecht [1924] and Heubner [1925] showed that, after toxic doses, the only significant rise in the calcium content of the tissues occurred in the kidneys. This, together with the calcium in urine and intestinal contents, did not account, a few hours after the injection, for the large disappearance of injected calcium from the blood-stream: deposition of calcium in the bones seemed the only explanation. Hetényi [1924], in an animal experiment, recovered after 3 hours only 15.6 % of the injected calcium from urine and faeces. In the same animal, before and after injecting a lethal dose (294 mg. per kg.) of calcium, he obtained a 2 % increase in the calcium concentration of the bone, which may be significant.

Dadlez [1926] found that the urinary calcium excretion was abnormally high at the end of 6 hours after injection. Brull [1930] on the other hand [*cf.* also Proverman and Brull, 1930] considers that the renal calcium excretion following intravenous calcium chloride injection is dependent entirely on the rate of flow of urine, thereby differing from Hetényi and Nógrádi [1925], who found in normal human beings a trebled calcium concentration in the urine during the first 30 minutes after injection. Recently Taylor and Fine [1930], by washing out loops of isolated intestine [*cf.* Stewart and Percival, 1927], found that the intestinal calcium excretion does not rise above the small normal value during the first 3 hours. They confirmed the idea that the calcium does not leave the body at first by observing that in eviscerated and nephrectomised animals the fall of blood-calcium was just "as abrupt and profound" as in normal animals.

It thus appears probable that during the first 3 hours most of the injected calcium is deposited on the bones, but we have yet to explain the large disappearance in the first few minutes.

Precipitation as phosphate following toxic doses is envisaged by Heubner [1923, 1931]. On the other hand, while injection of phosphate causes a hypocalcaemia [Binger, 1917], large injections of calcium actually cause a rise in inorganic phosphate [Salvesen, Hastings and MacIntosh, 1924; Collip, 1926]. Brull [1930] added calcium chloride to serum circulating in a Starling

heart-lung preparation: there was no precipitation or alteration of the inorganic phosphate; some of the added calcium became non-diffusible. The writer has shown *in vivo* (Table VII) that a few minutes after the injection of calcium levulinate the inorganic phosphate is not lowered. Earlier in the paper it was shown that there was no calcium in the form of a suspension, which can be centrifuged. There seems, therefore, no evidence of the immediate precipitation of calcium phosphate.

The immediate precipitation of injected calcium chloride as carbonate according to the equation

would of course cause a decrease of alkali reserve. Acidosis is said to be caused by injection of calcium chloride owing to excretion of calcium through the intestine [Mainzer, 1927]. In Exp. 13 (Table VIII) about 5 minutes after a large injection of calcium chloride the alkali reserve had diminished by 14 %, corresponding to a precipitation of 3.6 mg. Ca/100 cc. serum. This figure is however too high, as a dilution of the blood of at least 7.4 % had occurred (haemoglobin comparison). Using the high figure of 9 % for the blood-volume, 28 mg. Ca/100 cc. had disappeared in the 5 minutes. There is clearly little precipitation as carbonate.

There remains the possibility of immediate rapid diffusion into the tissue spaces. Following the work of Chiari and Januschke [1910] and Meyer [1910], it was believed that calcium decreased the permeability of the capillary walls; that such is not the case to any extent has been shown by Yanagawa [1916], Bayliss [1918], and Smith and Mendel [1920]. That calcium can readily penetrate the capillary walls is shown by the appearance of calcium in oedema fluid and serous transudates; while Arnold and Mendel [1927] concluded that calcium passes easily and rapidly at all times between blood, lymph and tissues.

We are thus led to picture the following events: immediately following the injection of hypertonic calcium salt solutions into the blood-stream, calcium diffuses into the tissue spaces until there is some form of equilibrium between the calcium in these spaces and the raised blood-calcium. The added calcium now disappears from the tissues and from the blood-stream, the rate of disappearance from the latter being approximately proportional at any instant to the excess of blood-calcium above the normal value, until at the end of 2-3 hours (when the tissue analyses of Heubner and Rona, *etc.* were made) the calcium content of tissues and blood has returned to normal. The calcium appears for the most part to be deposited in the bones, but a smaller quantity is probably simultaneously excreted.

There is some doubt concerning the fate of the injected calcium after this, for varying results have been obtained. Dadlez [1926] claimed that the retention of calcium in tissues or bones immediately following the injection is only temporary, a complete excretion of the injected calcium being achieved

in 24 hours. On the other hand, Greenwald and Gross [1925] recovered in 6 days only 0.40 g. of the 0.76 g. which was injected, while Salvesen, Hastings and MacIntosh [1924] even state that the faecal excretion of calcium during the 6 days following a large injection of calcium chloride is lower than that before the injection. Presumably, the relation of the amount of calcium in the diet to the demands of the animal is a factor which has a profound influence in this matter.

SUMMARY.

1. The serum-calcium as determined by direct precipitation can give an accurate measure of the calcium content of the blood following the intravenous injection of calcium levulinate.

2. Following the intravenous injection of calcium chloride and of calcium levulinate into cabbage-fed rabbits, it was found that the rate of fall of serum-calcium at any time greater than 5 minutes after the injection was approximately proportional to the excess at that time of the serum-calcium above the final constant level. In addition, there was a large disappearance of serum-calcium during the first 5 minutes not accounted for by this relationship.

3. The bearing of the experimental results upon our knowledge of the fate of intravenously injected calcium is discussed, and a description is given of the events which probably occur.

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