CCXIX. STUDIES ON THE PARTITION OF SERUM CALCIUM.

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IN a previous communication [Godden & Duckworth, 1935] we recorded certain data as to the partition of serum-Ca at or about the time of calving in the case of normal cows and of cows suffering from milk fever. Apart from these results no record appears to exist in the literature of systematic studies on the serum-Ca partition of individual animals throughout a reasonable period of their life span.

Since Benjamin & Hess [1933, 1] and Benjamin [1933] first presented their barium sulphate adsorption method of serum-Ca fractionation several writers have expressed views contrary to those of these two workers. We wish therefore to give the results which we have obtained as part of an investigation on the effect of certain food constituents on the growth and health of sheep, together with some physico-chemical studies of these data, in the hope that these new results may help to elucidate the problem.

EXPERIMENTAL.

Six half-bred wether lambs were divided into two groups. Nos. 25 and 26 constituted the control group and Nos. 21, 22, 23 and 24 the starch-fed group. The animals were kept indoors throughout the experiment in concrete pens bedded with sawdust. Both groups received the same basal ration consisting of turnips 3 lb., linseed cake meal 6 oz., chopped straw 8 oz., these amounts being just sufficient, when fed daily, to maintain the control group in practically steady body-weight. The starch-fed group received, in addition to this basal ration, a gradually increasing amount of maize starch, which was adjusted in amount to the appetite of the group. For the control group the daily intake of Ca was 1.84 g. and of P 1.92 g. For the starch-fed group, owing to a trace of P in the starch, the intake of P was slightly higher reaching a maximum of 2.04 g. Thus the respective Ca:P ratios were 1:1.04 and 1:1.11, a negligible difference. The vitamin D content of the diet was low, no cod-liver oil being added.

The experiment lasted approximately 6 months, the animals being weighed individually at fortnightly intervals. Blood samples were drawn at approximately three-weekly intervals. The blood was drawn from the jugular vein, employing partial stasis and the following determinations were made:

- (1) Total serum-Ca. The method of Clark & Collip [1925].
- (2) Serum-Ca partition. The method of Benjamin & Hess [1933, 1, 2]. The membranes were prepared by the method previously described by one of us [Duckworth, 1935].
- (3) Serum inorganic P. The method of Bodansky [1932-33].
- (4) Serum protein. Kjeldahl method.

Table I. *Serum analyses, including Ca partition. All results expressed in mg. per 100 ml.*

Sheep no.	Dates of sampling									
	14. xii. 34	23. i. 35	12. ii. 35	25. ii. 35	13. iii. 35	1. iv. 35	15. iv. 35	23. iv. 35	18. v. 35	4. vi. 35
Total Ca.										
21	9.3	8.2	6.2	6.0	5.5	6.0	6.0	6.5	7.5	7.3
22	9.5	8.8	7.6	6.6	5.7	7.2	7.1	6.5	7.4	7.3
23	9.6	8.7	8.0	5.8	6.7	7.1	5.6	5.4	7.6	7.0
24	9.7	8.9	9.1	8.0	7.3	8.4	7.7	7.8	8.0	7.6
25*	8.7	9.9	8.8	9.2	9.4	10.6	10.1	10.1	10.4	10.6
26*	10.6	10.8	10.2	10.0	10.6	11.1	10.3	11.1	11.0	10.3
Ca ⁺⁺ .										
21	1.4	0.9	0.6	0.5	0.6	0.6	0.6	0.8	1.1	0.7
22	1.4	1.2	0.9	1.0	0.6	0.8	1.1	0.7	1.2	1.1
23	1.7	1.1	1.1	0.5	0.8	0.6	0.5	0.5	1.1	0.8
24	1.5	1.2	1.0	1.0	0.7	1.0	0.8	1.1	1.5	1.1
25*	1.5	1.5	1.0	1.2	1.2	—	1.2	1.2	1.6	1.1
26*	2.1	2.1	1.6	1.8	1.8	2.1	1.3	2.1	2.4	1.3
Ultrafiltrable Ca complex.										
21	3.4	4.2	3.0	3.0	2.5	1.7	2.5	3.0	3.3	3.6
22	3.9	4.5	3.2	2.8	2.2	2.4	3.0	2.2	3.2	2.7
23	3.5	4.1	3.6	3.0	2.6	2.6	2.7	2.6	3.5	3.4
24	3.4	4.4	4.4	4.1	3.3	3.2	3.5	3.1	3.2	3.3
25*	3.3	4.4	4.2	4.4	3.9	—	4.7	4.6	4.6	4.7
26*	3.9	5.0	4.5	4.8	4.5	4.8	5.1	4.5	4.2	4.9
Non-ultrafiltrable Ca complex.										
21	1.6	1.2	1.3	1.3	1.5	2.2	1.9	1.5	1.5	0.5
22	1.4	1.0	1.1	1.5	1.9	2.5	1.2	2.3	1.1	0.9
23	1.9	1.5	1.0	1.3	2.1	2.5	1.5	1.7	1.3	0.2
24	2.4	1.4	1.3	1.3	2.2	2.4	2.0	2.4	1.4	1.4
25*	1.0	1.9	1.6	1.9	2.5	—	2.3	2.6	1.9	2.0
26*	2.0	1.0	1.6	1.7	2.5	2.1	2.0	2.0	1.8	2.1
Protein-bound Ca.										
21	2.9	1.9	1.3	1.2	0.9	1.5	1.0	1.2	1.6	2.5
22	2.8	2.1	2.4	1.3	1.0	1.5	1.8	1.3	1.9	2.6
23	2.5	2.0	2.3	1.0	1.2	1.4	0.9	0.6	1.7	2.6
24	2.4	1.9	2.4	1.6	1.1	1.8	1.4	1.2	1.9	1.8
25*	2.9	2.1	2.0	1.7	1.8	—	1.9	1.7	2.3	2.8
26*	2.6	2.7	2.5	1.7	1.8	2.1	1.9	2.5	2.6	2.0
Serum inorganic P.										
21	6.48	6.74	8.00	8.24	6.80	9.24	6.06	7.84	6.56	7.84
22	6.31	5.28	4.64	5.46	5.74	5.08	3.32	5.38	4.50	3.42
23	4.59	6.06	4.74	9.24	5.88	6.64	7.10	10.60	5.64	4.38
24	6.48	6.68	7.36	7.36	8.68	5.96	6.78	7.32	5.54	4.76
25*	5.81	7.04	7.16	7.62	7.36	6.16	4.66	8.56	7.22	7.70
26*	5.52	5.34	5.72	6.40	6.16	5.06	4.86	5.02	3.92	4.96
Serum protein.										
21	—	—	5.09	4.94	5.67	5.69	5.85	5.64	—	—
22	—	—	5.85	5.48	5.54	5.86	5.87	6.06	—	—
23	—	—	5.85	5.50	5.40	5.78	5.96	5.38	—	—
24	—	—	5.38	5.09	5.37	5.99	5.56	6.06	—	—
25*	—	—	4.79	5.27	5.19	5.95	5.62	5.31	—	—
26*	—	—	5.29	5.34	5.39	5.73	5.56	5.62	—	—
pK Ca proteinate.										
21	—	—	2.09	2.10	2.06	2.41†	2.13	2.03	—	—
22	—	—	2.11	2.06	2.21	2.28	2.04	2.27	—	—
23	—	—	2.04	2.01	2.21	2.27	2.02	2.25	—	—
24	—	—	2.07	1.99	2.15	2.18	2.11	2.10	—	—
25*	—	—	2.13	2.05	2.18	—	2.07	2.11	—	—
26*	—	—	2.07	1.95	2.07	1.99	2.00	2.06	—	—

* Denotes control animals.

† Figure probably erroneous and not included in calculations.

For the sake of brevity the following abbreviations for the four serum Ca fractions are used throughout this paper.

- Ca⁺⁺ = Ca ion.
 U.Ca.C. = ultrafiltrable Ca complex.
 N.U.Ca.C. = non-ultrafiltrable Ca complex.
 P.B.Ca. = Protein-bound Ca.

The analytical results for all the samples are shown in Table I.

It is of considerable interest that the animals tolerated such reduced Ca⁺⁺ levels with no apparent symptoms of heart disorder. Stewart & Percival [1928] have claimed that Ca in complex form is capable of furnishing Ca⁺⁺ at the surface of the heart cell. Further reference to the data of this experiment will be made in the discussion.

Results.

It is not proposed, in this paper, to discuss the clinical observations or the practical significance of these data in relation to animal nutrition. It should be noted, however, that at the conclusion of the experiment, the "knock-kneed" condition, generally supposed to be rickets, had developed in sheep Nos. 21, 22 and 23, while No. 24 appeared to be normal. At the sampling on 4. vi. 35 this condition had become severe. It is remarkable that it was only when both complexes showed *simultaneous* low values that severe leg-weakness occurred. Of the starch-fed group only No. 24 appeared normal and in this case the N.U.Ca.C. was only slightly depressed.

Effect of depression of the Ca⁺⁺ upon serum-Ca partition. (In vivo and in vitro experiments.)

(a) *In vivo depression of the Ca⁺⁺.* In this experiment five successive intra-jugular injections of 100 ml. each of a warm 20% solution of sodium citrate (Na₃C₆H₅O₇·2H₂O) were given to a normal, mature Ayrshire cow. The injections were made subject to changes in the condition of the animal rather than on a regular time interval basis. The blood samples were drawn from the jugular vein on the opposite side to that in which the injections were made. All analyses were made on serum.

Analytical methods.

- (1) Total serum Ca and Ca partition as in the sheep experiment.
- (2) Serum Mg. On the supernatant fluid from the serum Ca estimation, by precipitation as MgNH₄PO₄ and colorimetric estimation by the method of Fiske & Subbarow [1926].
- (3) Inorganic P. Method of Fiske and Subbarow.

Table II. *Injection of sodium citrate into the jugular vein of a normal cow.*

Values in mg. per 100 ml. of serum.

Sample no.	Time of sampling a.m.	Time of injection a.m.	Inorg. P	Mg	Total Ca	Ca ⁺⁺ *	U.Ca.C.	N.U.Ca.C.	P.B.Ca.
1	10-01	10-03	5-13	3-00	11-5	2-3	4-0	0-6	4-6
2	10-06	10-11	5-00	3-00	11-4	3-0	4-0	0-6	3-8
3	10-16	10-20	3-24	3-16	12-0	4-7	3-1	1-0	3-2
4	10-25	10-29	4-91	2-94	11-2	4-5	3-6	0-5	2-6
5	10-35	10-40	4-91	2-83	11-2	4-5	3-3	0-5	2-9
6	10-44		5-59	2-63	10-4	4-5	3-3	0-5	2-1

* Under Ca⁺⁺ is also included the Ca bound as citrate.

Results.

Immediately following each injection there was a response followed by a recovery period. With each successive injection the magnitude of the response increased and the animal became unconscious after the fifth injection.

The final effect of repeated citrate injections was a slight depression of the total serum Ca. The apparent rise and final high values of Ca^{++} are fictitious. Ca citrate is not adsorbed by BaSO_4 and therefore, under the system of analysis, appears in the Ca^{++} fraction. The values for Ca^{++} really represent mixtures of Ca^{++} and Ca citrate. There appears to be little doubt that the concentration of Ca^{++} was gradually decreasing if the increase in severity of the clinical symptoms can be taken as indicative. Further, the gradual and almost regular fall in P.B.Ca., ending in a final value of less than half the pre-experimental level, shows clearly the mobilization of P.B.Ca. in an endeavour to remedy the Ca^{++} deficit caused by the binding action of the citrate ions.

The two complexes do not show corresponding mobilizations. In fact it might appear that mobilization from the N.U.Ca.C. did not occur. In this connection it should be noted, however, that the animal was showing, at the pre-injection sampling, a lower value for this fraction than we have ever found for a normal animal of any species. On the other hand, the U.Ca.C. did show evidence of some slight mobilization, there being a final drop of about 15%. From our observations we would say that, whilst some of the clinical symptoms of this animal were similar to those occurring in milk fever, the complete picture did not parallel what we have noted in such cases. We have previously pointed out [1935] that, in cases of true milk fever, there are *simultaneous* low levels for both adsorbable and non-adsorbable Ca. In this experiment there was a definite fall in the non-adsorbable Ca (i.e. Ca^{++} + P.B.Ca.) but the adsorbable Ca (i.e. U.Ca.C. + N.U.Ca.C.) showed relatively little change (maximum fall 18%) and the final values were markedly above those which we cited for cases of milk fever.

The other values do not call for particular comment.

(b) *In vitro depression of the Ca^{++} in serum.* 30 ml. aliquots of a bulk serum were added rapidly with shaking to increasing amounts of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) dissolved in 1 ml. of water and left overnight. The Ca fractionation was performed the next morning. The results of one of a series of such experiments are shown in Table III.

Table III.

All results in mg. per 100 ml.

Sample no.	Na citrate in mg. per 100 ml. of serum	Total Ca	Ca^{++}	U.Ca.C.	N.U.Ca.C.	P.B.Ca.
1	0	10.8	2.4	4.3	0.7	3.4
2	47	10.8	3.4	4.0	0.8	2.6
3	93	10.8	4.2	4.0	0.6	2.0
4	143	10.8	5.2	3.6	0.6	1.4
5	190	10.8	5.9	3.2	0.6	1.1
6	240	10.8	6.6	2.9	0.5	0.8
7	287	10.8	7.3	2.9	0.2	0.4

The progressive increase in the Ca^{++} values does not indicate a real increase in Ca^{++} because, as before mentioned, the method employed includes Ca citrate with Ca^{++} ; in presence of citrate ion true Ca^{++} would undoubtedly be diminished. The steady mobilization of P.B.Ca. is obvious and this fraction has practically

disappeared with the highest citrate concentration. Of greatest interest is the slow mobilization of the Ca in complex form, both complexes gradually liberating Ca^{++} but not to such an extent as the P.B.Ca.

Stewart & Percival [1928] studied the effect of sodium citrate on the diffusibility of serum Ca in relation to the time of coagulation, but do not state clearly which hydrated salt they used. Our results confirm theirs in that we found, with increasing concentrations of citrate, that the serum Ca gradually becomes more and more diffusible. They further report that the time required for blood to clot, when gradually increasing amounts of citrate were added, decreased at first and then increased until no coagulation occurred. The failure to coagulate preceded somewhat the rendering of the Ca almost completely diffusible and this they interpreted as evidence of a non-diffusible Ca complex essential for the normal coagulation of blood and evidence further that the Ca^{++} had no effect on the process of blood clotting. We suggest that a more probable explanation is that the P.B.Ca., which acts as a circulatory reserve of readily available Ca^{++} , had been completely mobilized and was therefore incapable of liberating more ions to remedy the deficit caused by citration. It may be noted that these authors found that inhibition of coagulation occurred when the citrate concentration, calculated on the serum, was between 250 and 330 mg. per 100 ml., a result which, assuming that they used the dihydrate, coincides with our value, 287 mg., for almost complete mobilization of P.B.Ca.

DISCUSSION.

Theories as to the physico-chemical state of Ca in body fluids may be divided into two groups: (a) that all the Ca not combined with protein is ionic, (b) that the Ca not combined with protein is partly in non-ionic forms. Our results lead us to support the second alternative.

Maclean & Hastings [1934, 1, 2; 1935, 1, 2] and Maclean *et al.* [1934] have published comprehensive studies on Ca partition. They believe that the amplitude of the contraction of the ventricle of an isolated frog heart preparation is proportional to the Ca^{++} concentration of the perfusing fluid. They maintain that practically all the serum Ca not in combination with serum proteins is ionic. They then calculate the constant, K , from the mass law equation for the dissociation of Ca proteinate, assuming that the protein molecule behaves as though it were composed of a series of negatively charged divalent ions, i.e.

$$\frac{[\text{Ca}^{++}][\text{Protein}^-]}{[\text{Ca proteinate}]} = K.$$

The concentrations are in mols.

We have tested the validity of this expression as applied to our own results. We assumed, as did Maclean & Hastings, that grams of protein can be converted into milliequivalents by the factor for serum proteins, 0.243, found by Van Slyke *et al.* [1928]. In converting milliequivalents into millimols we have accepted their hypothesis that serum proteins act as divalent anions towards Ca. We have combined the values for Ca^{++} and U.Ca.C. as if all the ultrafiltrable Ca were ionic, and combined the N.U.Ca.C. and P.B.Ca. as non-ultrafiltrable Ca.

Our expression then becomes

$$\frac{[\text{U.Ca.}][\text{Protein}^-]}{[\text{N.U.Ca.}]} = K.$$

The $[\text{Protein}^-]$ was obtained by difference between $[\text{Total protein}]$ and $[\text{N.U.Ca.}]$. The results, expressed as the negative logarithms (pK), are given in Table I.

Statistically considered we find that $pK=2.10$ with an s.d. = ± 0.08 . Our actual figures show a pK range of 1.95 to 2.28 and hence a K range of 0.01131 to 0.00533. Taking only the s.d. the corresponding ranges are pK 2.02–2.18 and K 0.00966–0.00664. Maclean & Hastings give a mean pK of $2.22 \pm \text{s.d. } 0.07$, neither figure being far removed from ours. These values would give a K range of 0.00708–0.00513, while their complete K range would be correspondingly greater. We feel that dissociation constants with such ranges as are shown in both these sets of figures are of doubtful value and that the presence of such variability is, in itself, evidence of other influencing factors, which are not considered in the expression. We do not deny, but rather affirm, that an equilibrium between ultrafiltrable Ca and at least a part of the non-ultrafiltrable Ca does exist but we would add that the phosphate and bicarbonate ions play a rôle in this equilibrium.

Maclean & Hastings have constructed a nomogram which gives the Ca^{++} concentration of serum from the serum proteins and total serum Ca. Andersch & Oberst [1936] found that, in the case of the new-born child the ultrafiltrable Ca values as determined were about 20% lower than the Ca^{++} values for the serum (as determined by the nomogram). The agreement was closer for the serum of non-pregnant, pregnant and parturient women. Further, Benjamin & Hess [1933, 2] have recorded figures in the case of hypercalcaemia of egg-laying in fowls from which the pK , when calculated, shows a value of 3.04 with a corresponding value for K of 0.00091.

The values found for the Ca^{++} concentration of serum by Brinkman and van Dam [1920] using the solubility product method, by Neuhausen and Marshall [1922] using the flowing Ca electrode, and by the BaSO_4 adsorption method are in general agreement. The value obtained is, in all cases of the order of 2 mg. per 100 ml. Further, our own studies on Ca partition in pathological cases, the results of which will be published elsewhere, show a correlation between the clinical symptoms and the Ca partition data, which we find impossible to regard as fortuitous. Consequently we conclude that the four fractions of Ca found by the BaSO_4 adsorption and ultrafiltration methods represent real entities.

SUMMARY.

1. The results of studies in the Ca partition of sheep on experimental rations are recorded along with some results of physico-chemical investigations of the blood of cattle following both *in vitro* and *in vivo* addition of sodium citrate.
2. The results are considered as demonstrating that the so-called dissociation constant of Maclean and co-workers for Ca proteinate in serum is too variable to be regarded as an aid to the study of Ca partition.
3. It is concluded that the four Ca fractions found in serum by the barium sulphate method of adsorption coupled with ultrafiltration are real entities.

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