Biochemistry. — *Active and inactive calcium in the animal organism. Importance as to diagnostics and therapeutics.* *) By L. SEEKLES and E. HAVINGA. (The Laboratory for Veterinary Biochemistry, State University of Utrecht, Netherlands.) (Communicated by Prof. G. KREDIET.)

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On account of the research work performed of late years in this laboratory and in other institutes, concerning deviations of the calcium content of body fluids and tissues in man and different animals, we made a classification into two groups of a number of the principal forms of these disorders.

First group.

1. Man and different experimental animals: condition after removal of the parathyroid glands.
2. Cow: milkfever, grass tetany.
3. Man: the syndrome, characterized by tetany, epilepsy and turbidity of the eye lens.
4. Dog: canine hysteria (?).
5. Man: certain forms of tetany, for example in children.
6. Man and different animals: certain forms of rickets.
7. Pig: epileptic attacks in young pigs, with slight symptoms of rickets, observed during the period of dentition, at the age of about 9 weeks. (?)

Second group.

1. Man, dog (and cat?): condition in case of adenomes of the parathyroid glands (ostitis deformans Recklinghausen = osteitis fibrosa generalisata).
2. Cow: condition, in which the colloids of milk flocculate spontaneously, or on boiling, or after the addition of an equal volume of 70 per cent alcohol, the acid degree being unchanged.

It may be assumed that in the first group of deviations the activity of the calcium ions in the body liquids is lowered, whereas in the second group it may be raised.

In connection with this *provisional and by no means complete* classification [1]) we want to point out, that in the cases mentioned the modified

*) Read in June 1942 during "The Veterinary Week, Utrecht"; published in dutch in 1944 by the "Maatschappij voor Diergeneeskunde" (J. van Boekhoven, Utrecht).

[1]) In case of doubt as to the correctness of this classification, we put a question mark.

activity of the calcium ions is not the only deviation in the chemical constellation of body liquids (and tissues), nor need be so. Our results up to now rather support the opinion that in several of the deviations mentioned before, great importance — and may be sometimes a greater importance — must be attached to other chemical disease symptoms, c.q. to changes in the activity of other ions. We leave this problem, however, out of discussion and rather concentrate our attention in the first place on the problems mentioned in the title of this treatise.

Activity of the calcium ions.

Bloodserum of normal cows contains about 10 mg of calcium in 100 cm^3. It appears that 40—50 % is bound to the colloids of the serum, chiefly to protein.

The rest can move freely through ultrafilters, contrary to the calcium bound to protein.

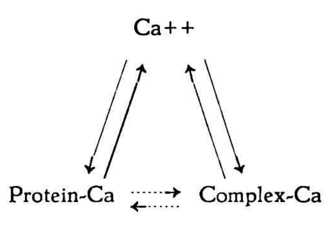
The "free" calcium is found in at least two forms, namely as calcium ions and as complexes in which calcium is bound to certain organic componds e.g. citric acid.

So we distinguish for instance:

Calcium, total	10 mg %
Calcium, bound to protein	4 „
Calcium "free" (ultrafiltrable)	6 „
Calcium ions	2 „
Calcium, in complex compounds	4 „

It is generally assumed, that in the first place it is the calcium ions which cause the physiological activities of "the Calcium" and therefore are "physiologically active". The calcium bound to protein and the calcium in complex compounds form so to say a stock out of which possible replenishment of the calcium ions takes place or to which a possible surplus of calcium ions runs back according to the need of the normal organism. On the other hand it goes without saying, that to exert their physiological activity the calcium ions most probably have to unite with some constituent of the organism, e.g. building up a membrane or an enzyme.

So in the blood serum an equilibrium exists between the three abovementioned forms in which calcium is found [2]).



[2]) As it is not an established fact that a direct transition — without mediation of the calcium ions — takes place between protein-Ca and complex-Ca, the arrows between fractions last mentioned are dotted.

The state of this equilibrium — expressed by the percentage of each of the three compounds — depends on the temperature and the chemical condition of the serum. So, by supply of hydrogen-ions, sodium ions etc., part of the calcium bound to protein gets displaced and transformed into diffusible calcium. A supply of certain other compounds, e.g. citric acid, causes forming of a higher percentage of complex bound calcium at the cost of the other two fractions [3]). How great the latter influence can be, follows from a measurement we performed some time ago.

When, all other conditions remaining unchanged, 0.01 grammol. of sodium citrate per litre is added to a liquid containing 4 mg % of calcium ions — which is a higher content than that of normal bloodserum — the calcium ions content of this liquid falls to about 0.2 mg %. (A quantity of 0.01 grammol. of sodium citrate added to 1 litre of blood prevents its coagulation.)

So the activity of the calcium ions falls to about 5 % of the original value by adding the quantity of citrate mentioned, which is of the same order of size as the citrate content of normal milk. It is easy to understand that by such a change of the calcium ions activity the physiological properties of the liquid undergo a very considerable change, remembering what we communicated before as to the physiological properties of the calcium ions.

The importance of our knowledge of the activity of the calcium ions with respect to diagnostics.

In connection with the importance already mentioned of the calcium ions as bearers of the physiological activity of calcium, it is of great importance to diagnostics to know the activity of the calcium ions in the body-liquids. A parallelism between the chemical activity of the calcium ions and the physiological activity is stated to exist. A striking practical example may illustrate the correctness of this opinion. Patient in point is a very old Frisian cow, showing some days after parturition symptoms of non-typical milkfever (paresis without sopor, decreasing appetite), as had been the case for several years after each parturition. The animal's condition having remained fairly stationary for several days, the calcium content of the blood appears to amount to 6.8 mg %. So we state a moderate hypocalcaemia: the calcium content has fallen to about 2/3 of the normal level. The concentration of calcium ions amounts here to 0.50 mg %, whereas the calcium ions-content of normal cow serum was fixed on an average of 1.60 mg % [4]).

This explains atony of the muscles (paresis), although the total calcium content has fallen only in a relatively slight degree. Now para-

[3]) L. SEEKLES. Arch. néerl. de Physiol. **21**, 526 (1936); **22**, 93 (1937).

[4]) The method applied formerly for the determination of calcium ions is not very exact. It yields rather low values for the concentration of calcium ions, but this is not in the way of a comparison of values obtained according to this method.

thyroid extract is injected, which is known to possess the ability of mobilising and removing the calcium found in the depots. After an hour and a half the paresis disappears. An examination of the blood, made immediately after this, yields a value of 6.3 mg % for the calcium content of the bloodserum; so this means a slight fall compared to the concentration originally determined (6.8 mg %). The content of calcium ions, now, however, comes to 1.04 %, that means to say *the chemical "activity of calcium" has risen to double the value and this was attended with a parallel rise of the physiological activity of the calcium leading into the disappearing of the atony of the muscles (paresis).*

From the state of equilibrium mentioned before between calcium ions on the one hand and the calcium store — the calcium bound to protein and the calcium bound as complex — on the other hand, other facts can also be explained in an unrestrained manner.

From information, given by veterinary practitioners, it appeared to us to have been stated tens of years ago, that the symptoms of milkfever can be caused to disappear by injection of large quantities (1—2 litres) of a physiological solution of sodium chloride (0.9 % NaCl). This fact can be explained by the ability of the sodium ions (brought into the body in large quantities), of eliminating part of the calcium bound to protein. By this the liberated calcium comes into circulation as calcium ions and so raises the abnormally low activity of the calcium ions adherent to milk fever. By this apparently we can reach the physiological threshold-value, essential to cause the milkfever symptoms to disappear.

In the course of years we repeatedly found samples of bloodserum in cases of milkfever, the calcium-content of which was shown not to be lowered. Nevertheless these patients reacted normally upon an intravenous administration of calcium chloride. The next case is a striking example of this.

The bloodserum of a cow, showing typical symptoms of milkfever during the first 24 hours after parturition and getting cured instantly after an intravenous injection of calcium chloride, appears to contain 5,8 mg % of Ca, 3,0 mg % of inorg. P and 1,9 mg % of Mg. So we find a very distinct hypocalcaemia and hypophosphataemia. After 10 hours the animal relapses, exactly the same clinical picture developing again. Once more the patient recovers as readily as the first time after an intravenous administration of calcium chloride. However, the blood taken before the second injection was shown to contain 10,2 mg % of Ca, 4,1 mg % of inorg. P and 1,5 mg % of Mg, that is to say the mineral content of the bloodserum was almost normal and particularly the symptom of hypocalcaemie was missing here.

From the example given it may be concluded that the total calcium content of a patient's bloodserum not always produces an indication for applying the calcium therapy. In the meantime it is quite possible, that in this case the equilibrium between the three calcium fractions of the blood had been shifted, at the cost of the calcium ions. The therapeutic effect of the injection of calcium chloride may be ascribed to a rise of the activity of the calcium ions caused by the liquid injected. We can consider as a second possibility that the activity of the calcium ions of the blood was normal in the case taken as an example. The symptoms of milkfever may have been caused by a diminised irritability of the neuromuscular apparatus and of the other organs, the disfunction of which contributes to causing symptoms of milkfever [5]).

Incidentally it may be remarked, that lately we succeeded in showing that in certain cases of excitation and tetany in cows during the stall period conditions according to magnesium may be found in the bloodserum, as are sometimes found in cases of milkfever with regard to the calcium element.

So we found for instance Ca 10,7 mg %; P 7,0 mg %; Mg 3,0 mg %, Na 336 mg %, Cl 348 mg %, K 24 mg % in the bloodserum of such a patient, therefore a magnesium-content not lowered — as in case of typical grass tetany — but raised. Nevertheless these patients react favourably upon a subcutaneous injection of for instance 30 g of crystallised magnesium chloride ($Mg\,Cl_2 \cdot 6\,H_2O$) in 300 cm^3 of water, by which treatment the symptoms of tetany and excitation disappear [6]).

From the examples mentioned it may have become clear that, from a diagnostic point of view, it must be considered to be very important to have a method at our disposal enabling us to determine the activity of the calcium ions. This being attained, it will be possible to gain an insight into the nature and by this into the therapy of the non-typical cases of paresis and tetany in cows and other animals, as well as of a number of disorders, some principal forms of which have been mentioned in groups 1 and 2 at the beginning of this paper.

The importance of the activity of calcium ions of injection liquids with respect to therapeutics.

Some time ago we called attention to the fact that from experiments formerly made in this laboratory [7]) it appeared that solutions of calcium chloride and of calcium gluconate cause a very different local effect when subcutaneously administered.

A solution of calcium chloride, subcutaneously injected with all usual precautions, causes a painful swelling and in the end extensive phlegmones, even if the osmotic concentration of the solution does not exceed that of a physiological solution of sodium chloride. Necrosis and sloughing of the skin over an extensive region may occur which can cause serious complications. Contrary to this a solution of calcium gluconate administered in the same way causes only a painful swelling as a rule, which disappears in the course of some days to a week, without any injurious consequences. A solution of calcium lactate ranges, as far as the irritating effect on the subcutis is concerned, between the solutions of calcium chloride and of calcium gluconate.

Some time ago we succeeded in making it probable that the difference in irritating effect between equivalent solutions of calcium chloride, calcium lactate and calcium gluconate gets determined by the difference in con-

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[5]) L. SEEKLES. Tijdschrift v. Diergeneesk. **67**, N°. 2 (1940).

[6]) We hope to communicate further particulars as to these special cases of tetany.

[7]) B. SJOLLEMA, L. SEEKLES, F. C. VAN DER KAAY, Tijdschrift voor Diergeneesk. **58**, 254 (1931).

centration of the calcium ions in the liquids mentioned. In solutions of simple compounds an impression of the concentration of the ions can be obtained by measuring the electric conductivity of these liquids. In figure 1 we see the equivalent conductivity: $\left(\frac{\Lambda_c}{\Lambda_0}\right)$ plotted against the concentrations as we determined for some salts.

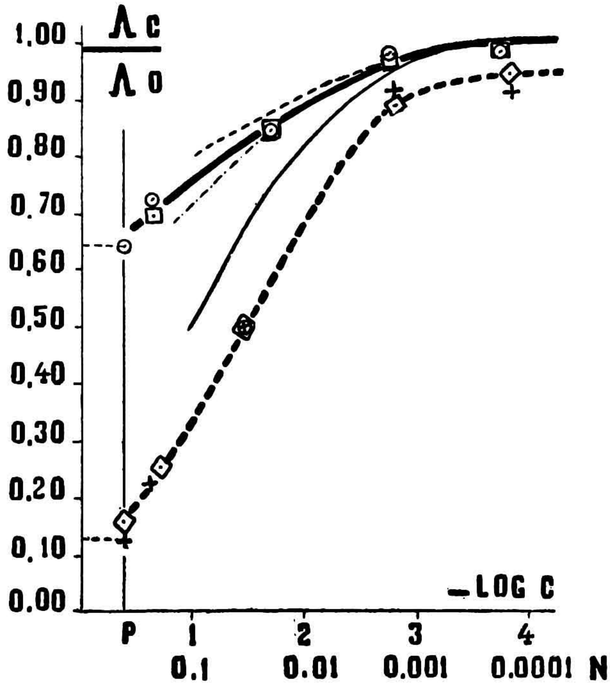

Fig. 1. Equivalent conductivity of salt solutions at different concentrations, temperature 20° and 40° C (calcium chloride, calcium gluconate and calcium lactate, in comparison with sodium gluconate and sodium lactate).

—·—·—·—·— sodium lactate
▬▬▬▬▬ calcium chloride
- - - - - - - - sodium gluconate
———— calcium lactate
■■■■■■■ calcium gluconate
P: concentration for subcutaneous injection.

With respect to calcium chloride the equivalent conductivity decreases very slowly only in proportion as the concentration increases. So the splitting into ions remains very considerable even in concentrated solutions. Calcium gluconate, however, behaves in a quite different way: the equivalent conductivity decreases strongly in proportion as the concentration of the solution increases. So we see from figure 1 that in the case of a 10 % solution of calcium gluconate as used for injection (marked by point P) $\frac{\Lambda_c}{\Lambda_0} = 0.15$. For an equivalent solution of $CaCl_2$ (about 5 %, also marked by point P) this value amounts to 0.65.

The most plausible explanation is, that the splitting into ions for calcium gluconate in more concentrated solution is far less than in the case of calcium chloride.

We may assume that the splitting of calcium gluconate molecules into ions takes its course in two consecutive steps.

1) Ca (gluconate)$_2$ $\rightleftarrows$ Ca gluconate+ + gluconate⁻

2) Ca gluconate+ $\rightleftarrows$ Ca++ + gluconate⁻

Especially by the second step, yielding the calcium ions, the dissociation-equilibrium in concentrated solution is moved up to the left. The relatively slight conductivity of a solution of calcium gluconate as it is used for injections therefore points in this train of thought to a small content of calcium ions.

Calcium lactate too — although perhaps a little better split into ions than calcium gluconate — forms concentrated solutions of a considerable less molecular conductivity than $CaCl_2$. In this case the concentration of calcium ions evidently also is slight. Finally from measurements of the conductivity at 20° and 40° the influence of the temperature on the splitting of the salts appears to be slight.

The results of the chemical examination of the solutions of calcium chloride, calcium lactate and calcium gluconate are in agreement with the results of the injection-experiments mentioned before, with respect to the physiological activity — irritation of the subcutis — exerted by these salts.

Finally it may be remembered that by building on systematically on the basis as stated above, we succeeded lately in preparing a calcium containing injection liquid possessing the advantages of the calcium gluconate-solution without certain disadvantages: the solution of sodium-calcium borogluconate [8]).

Determination of the calcium ions activity by means of the spreading method [9]).

It is not possible to estimate the concentration of the calcium ions from conductivity measurements in biological liquids such as blood serum, milk, etc. containing, apart from calcium ions, several other ions in large quantities. Neither can other electrometrical methods be applied for the determination of the activity of calcium ions. During the last few years we succeeded in working out a method, which for the first time offers an opportunity of measuring the activity of the calcium ions or at least a value analogous to this.

For this purpose we make so to say a print, a snap-shot of the ionic condition of the liquid. A very small quantity of a fatty acid (stearic acid) solved in a volatile, indifferent solvent (gasoline of low boiling point) is placed on the surface of the liquid to be examined which is kept in a flat basin (spreading-basin). The solution spreads itself over the surface and

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[8]) L. SEEKLES, E. HAVINGA, J. DE WAEL, Tijdschr. v. Diergeneesk. 69, 179 (1942).

[9]) A more ample description of the method will be published elsewhere in due time. For a provisional communication we refer to E. HAVINGA, Chem. Weekblad 39, 266 (1942).

after the solvent is evaporated, under circumstances aptly chosen, the stearic acid is left on the surface as a monomolecular film. The hydro-polar character of the fatty acid is manifested in the immersion of the carboxyl-group, the paraffine rest of the fatty acid "protruding above the water".

The carboxyl groups immersed ionize partly, the liberated hydrogen-ions diffusing into the underlying solution (hypophasis). On the other hand: part of the positive ions from the hypophasis occupy a number of places near to the ionized carboxyl groups which originally were occupied by hydrogen ions. It is particularly the calcium ions which show a strong tendency to salt formation with the carboxyl groups and an equilibrium is formed between film and hypophasis, dependent on the ionic condition of the hypophasis. So the composition of the film reflects the ionic condition in the liquid underneath. *The quantity of ions absorbed is so small that practically no change in the equilibrium between the different fractions of calcium and other elements in the hypophasis occurs.*

To check matters, solutions of known ionic strenghth are prepared, similar to the possible ionic concentration of the unknown liquid, the activity of calcium ions of which we wish to determine.

An example of such a test liquid comparable with the ultrafiltrate of blood serum is the following one.

Ca^{++} 2 mg %
Mg^{++} 0,25 mg %
Na^{+} 330 mg %
} In addition to the negative Cl^{-} and HCO_3^{-}-ions, not reacting with the ionised carboxyl groups of the film.

pH (20° C) = 7,50 (measure for the activity of hydrogen ions).

After the equilibrium between film and hypophasis has been established we collect the film quantitatively by compressing it and skimming it off from the surface and next we determine its content of the different ions as well as the "free" carboxyl groups — that is to say not bound to the ions.

The results with the "hypophasis" mentioned above as an example were as follows:

Bound by Ca: 65 %
" " Mg: 2,3 %
" " Na: 8 %
} of the carboxyl groups originally present in the film.

Free carboxyl groups: 24,7 %.

Figure 2 represents, in the form of a verification curve, some of our results, obtained with simplified "synthetic serum ultrafiltrates".

Verification curves having been determined with hypophases of known composition, the spreading of stearic acid can be performed on the surface of a liquid the calcium ions-activity of which we wish to state.

The calcium ions content, *i.e.* the calcium ions activity of the liquid, then follows immediately from the calcium content of the film, by reading the graphic. Critical examination shows, that the possibility of applying this method is based upon the simultaneous determination of the activity in the hypophasis and the "concentration" in the surface layer for at least one ionic species. Evidently this can be done for the hydrogen ions (determination of pH by means of the glass electrode and of the COOH groupings of the monomolecular layer).

Finally just a few words as to the difficulties, experienced when performing the spreading-method. They are principally of an analytical chemical nature.

The extremely small quantities of the different ions contained by the removed film, cause the application of special methods of research and of special apparatuses. Therefore, before we were able to analyse these monolayers we were obliged to work out special ultra-microchemical

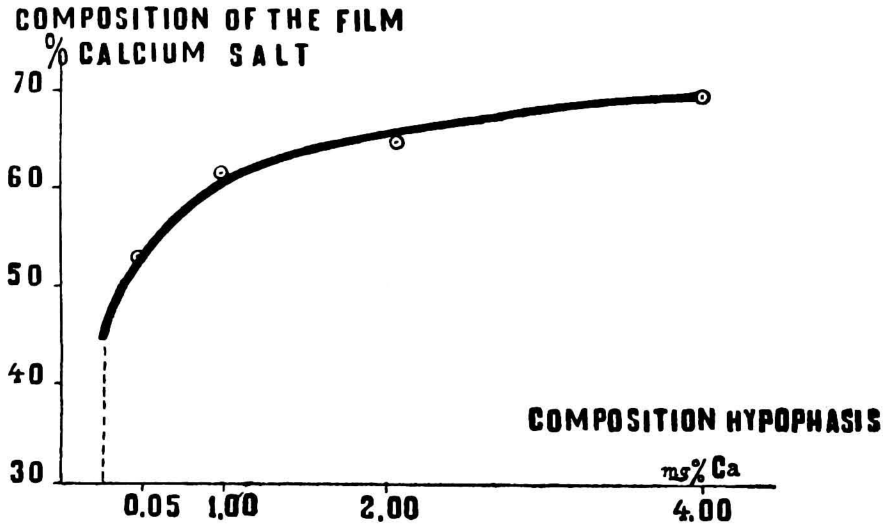

Fig. 2. Curve, demonstrating the Calcium content of the surface layer as a function of the Ca++ concentration of the hypophasis ($pH_{20} = 7{,}50$).

methods. With the aid of these we now succeed in determining quantities of calcium from 10 to 1 γ and quantities of magnesium from 1 to 0.1 γ [10]) with an error of some per cent only. Two more important consequences follow from the description of the method-of-spreading given.

In the first place the nature of the method raises the expectation that in principle it may also be applied for the determination of the activity of other ions.

The property of stearic acid, of reacting in the first place with calcium ions, renders this substance pre-eminently suitable for the determination of the activity of calcium ions. We hope that in future it may appear possible to use other substances for spreading which combine preferably with other ions and so enable us to determine the activity of these ions. In principal the method of spreading is a universal method of research for the determination of the activity of ions in (biological) liquids and of the

[10]) E. HAVINGA, A. F. K. BUYS BALLOT; Rec. Trav. Chim. **61**, 849 (1942).

antagonism of ions in interfaces. So we may cherish the hope that in years to come numbers of biological problems which up till now could not or could not efficiently be studied, may further be solved. It is a great difficulty, however, that according to our experience gathered up till now, the presence of protein has a disturbing influence. Therefore in any case we shall have to work with ultrafiltrates. For the rest it may have become clear that the practical performing of the method of spreading research requires great technical skill and relatively a great deal of biological material. This is an objection to its being generally applied in laboratory and clinic. We therefore now carry out experiments in order to find a method feasible by simple means and by small quantities of material — therefore practical — which is to be verified by the universal, exact — but very complicated-method of working described before.

Summary.

The authors give a brief survey of the diseases in man and animals which are characterized by abnormal values of calcium in the body fluids. The different forms in which calcium occurs in blood and the importance of calcium ions activity for physiological phenomena, diagnostics and therapeutics are mentioned. In simple solutions of calcium salts the ionic activity has been estimated by means of conductivity measurements. It was shown that solutions of calcium lactate and particularly of calcium gluconate contain less calcium ions than equimolar solutions of calcium chloride. In consequence a subcutaneous injection of calcium chloride solutions causes considerable lesions in the subcutaneous tissue, as compared with solutions of calcium gluconate. The activity of (calcium) ions in liquids of complicated composition is estimated by means of a new method in which a monomolecular layer (of stearic acid) is brought on the surface of the liquid. Then the film is skimmed off from the surface and the contents of calcium (and other cations) and of free carboxyl groups are estimated by means of ultra-microchemical methods. From the calcium value of the film the calcium ion activity of the liquid can be calculated by comparison with films spreaded on solutions of known ionic concentrations.