LXXVII. THE STATE OF CALCIUM IN BODY FLUIDS.

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IN the absence of a direct method of estimating calcium ion, we depend for our conception of the state of calcium in body fluids on indirect evidence. This evidence, which is mainly based on dialysis experiments and on the low solubility of calcium carbonate and calcium phosphate, we propose to consider.

(1) DIALYSIS EXPERIMENTS.

It has been shown frequently that serum¹ in a dialysing sac is in equilibrium with an external fluid containing a lower concentration of calcium. This unequal distribution is generally ascribed to the proteins in the serum, but as far as we know no quantitative experiments have been made to decide whether the protein will account for the effect. We therefore have dialysed proteins against calcium solutions. In case some other undialysable calcium compound might be present in serum we have first made sure that our protein solutions were free from calcium.

In our first experiments the dialysis was performed at room temperature, which varied during the day from 18° to 28°.

The proteins were prepared from horse plasma or serum. The serum used in Exps. 6, 7, 8, 14, 15 and 16 was dialysed against 0.9 % NaCl; the serum protein in Exps. 9 and 17 was precipitated by alcohol and ether in the cold [Hardy and Gardiner, 1910], and dialysed against 0.9 % NaCl; both contained less than 0.3 mg. calcium per 100 cc. The albumin was crystallised from plasma; the globulin was precipitated three times with half-saturated ammonium sulphate; both albumin and globulin were dialysed against 0.9 % NaCl until they contained only faint traces of sulphate and were proved free from calcium. Before dialysis the protein solutions were brought to p_H approximately 7.4 with CO₂-free sodium hydroxide.

The collodion sacs were made according to Looney's [1922] formula but with 0.9 % pyroxylin (B.D.H.); they were tested for impermeability to protein and attached to short pieces of pressure tubing with rubber bands

¹ We do not find convincing Vines' [1921] thesis that calcium is in different states in serum and plasma; for he appears to have ignored the difference of calcium concentration in serum and whole blood, and to have used blood heated to 55° for half an hour as unchanged blood.

and a smear of fresh collodion solution. After filling with protein solution (about 5 cc.) they were closed by inserting glass rods into the pressure tubing. They were then placed in 500 cc. of solution in duro-glass flasks, containing 0.9 % NaCl, varying quantities of CaCl_2 , and phenolsulphonephthalein as indicator. In both these and the preliminary dialyses CO_2 -free air was passed continuously through the external fluid to ensure absence of CO_2 and keep the fluid stirred; this air was first passed through water and toluene, so that the solutions were saturated with toluene; there was no sign of growth of bacteria. Dialysis was continued two or three days.

Calcium was estimated by Clark's [1921] method, protein by a micro-Kjeldahl method, and the reaction by comparison with standard phosphate solutions. The results are shown in Table I, exps. 1 to 8.

Table I. *Partition of calcium between protein solutions and protein-free solutions.*

No.	Temp.	Protein solution		p_H	External solution				Protein used
		Protein g. per 100 cc. = Pr	Calcium mg. per 100 cc. = Ca_p		HCO_3^- Mol. $\times 10^3$	P Mol. $\times 10^3$	Calcium mg. per 100 cc. = Ca_e	$\frac{\text{Ca}_p - \text{Ca}_e}{\text{Ca}_e \times \text{Pr}} \times 100$	
1	Room	2.23	11.0	7.5	0	0	9.8	5.5	Globulin
2	"	1.81	11.0	7.4	0	0	9.8	6.8	Albumin
3	"	3.02	11.8	7.3	0	0	9.6	7.7	"
4	"	5.55	8.8	7.6	0	0	6.0	8.5	"
5	"	4.3	9.3	7.48	0	0	7.3	6.35	"
6	"	5.4	3.5	7.45	0	0	2.7	5.5	Dialysed serum
7	"	6.87	8.6	7.70	0	0	6.0	6.3	"
8	"	6.67	13.3	7.67	0	0	9.8	5.3	"
9	37°	4.5	6.1	7.00	0	0	3.9	12.5	Serum protein
10	"	5.4	10.9	7.30	0	0	6.1	14.5	Albumin
11	"	3.4	7.65	7.20	5.5	0	5.65	10.4	"
12	"	5.4	5.0	7.30	11	0	3.1	11.4	"
13	"	5.25	10.4	7.85	5.5	0	5.25	16.0	"
14	"	1.8	5.45	7.55	11	0	4.56	10.9	Dialysed serum
15	"	3.2	7.15	7.40	11	0	5.3	11.1	"
16	"	3.3	3.7	7.30	11	0	2.8	9.7	"
17	"	3.2	6.65	7.50	27	1	4.9	10.9	"
18	"	3.3	7.6	7.45	27	1	5.5	11.5	"
19	"	4.5	9.3	7.3	27	1	6.2	11.1	Serum protein
20	"	5.35	8.7	7.55	27	1	5.45	11.2	Albumin

Later we repeated dialyses in a water-bath at 37°. These dialyses we made as before except that only 50 cc. of the external fluid was used, contained in 8×1 inch Pyrex test-tubes; NaHCO_3 was used to buffer the solution in Exps. 11 to 20, and in Exps. 17 to 20 phosphate added to reproduce body conditions; in all NaCl was added in appropriate amounts to keep the concentration approximately 0.15 *M*. In Exps. 11, 13, 16, 18 and 20 toluene was not used, and 3.5 cc. of distilled water saturated with thymol were used in making up the external solution. For fear of bacterial action we only kept up the dialysis for 24 hours, and therefore reduced the volume of protein solution to 3 cc., and added to it before dialysis enough calcium chloride solution to bring the concentration of calcium in it to about 1 mg. per 100 cc. less than the final concentration expected. The tubes were gently rocked throughout the time of dialysis. In these and subsequent experiments the calcium

oxalate precipitate in the calcium estimation was washed with saturated calcium oxalate solution as recommended by Stanford and Wheatley [1925].

In some of the external solutions used in these experiments the solubility products of calcium carbonate and phosphate were exceeded though no precipitation occurred. We therefore needed to know whether in these solutions the calcium was present as ion or as colloidal calcium carbonate or phosphate. It is possible to settle this question in artificial solutions in the following way. When calcium carbonate is formed in a solution containing calcium chloride and a bicarbonate the following reaction may be considered to take place:

So that with the formation of CaCO_3 in such a solution the concentration of H_2CO_3 rises and that of HCO_3^- falls, the hydrogen ion concentration rises, and the rise of hydrogen ion concentration serves as a measure of the degree to which CaCO_3 is formed.

For this method we have two similar apparatus of equal volumes as shown in Fig. 1. Into the bulb *A* of each was introduced 10 cc. of a solution of sodium bicarbonate and sodium chloride (total concentration 0.30 *M*), into the bulb *B* of one (with the apparatus horizontal) 10 cc. of a solution of calcium chloride, into *B* of the other 10 cc. of distilled water (neutral). All the solutions contained phenolsulphonephthalein as indicator. A CO_2 -air mixture was then passed through both the apparatus, while these were rotated, with care to avoid mixing the fluids, for half an hour. The stopcocks were closed, the whole warmed to 37° , and finally the fluids were mixed by standing the apparatus vertically. The volume of each apparatus was 130 cc., so that the gas space was 110 cc. It is then possible to calculate the change in p_{H} resulting from the formation of a given amount of CaCO_3 .

Suppose that by the formation of CaCO_3 the CO_2 content of the gas phase is increased by *Y* millimols, this will increase the CO_2 pressure at 37° by $\frac{760 \times 310}{273} \times \frac{Y \times 22}{110}$ mm. Hg. With this increase

of pressure there will be an increase of dissolved CO_2

$$= \frac{0.53}{22} \times \frac{310}{273} \times \frac{Y}{5} \times 20 \text{ millimols in the 20 cc. of fluid.}$$

If the HCO_3^- concentration is reduced by *X* mille-equivalents per litre (that is if $X/2$ millimols of CaCO_3 are formed), $X/2$ millimols of CO_2 are set free per litre of solution, or $\frac{X}{2} \times \frac{20}{1000} = \frac{X}{100}$ per 20 cc. of solution. This CO_2 equals the increase of CO_2 in liquid and gas phases, so that

$$\frac{X}{100} = Y + 0.024 \times \frac{310}{273} \times \frac{Y}{5} \times 20 = 1.11 Y, \text{ or } Y = \frac{X}{111}.$$

If $X = 1$, $Y = \frac{1}{111}$, so that increase of CO_2 pressure

$$= \frac{760 \times 310}{273} \times \frac{Y}{5} \text{ mm. Hg} = 1.56 \text{ mm. Hg.}$$

If the initial CO_2 pressure is 30 mm. Hg, and the initial HCO_3^- concentration is 0.033 M , the change in p_{H} will be

$$-\log \frac{30}{33} + \log \frac{31.56}{32} = 0.035.$$

Now a difference of p_{H} of 0.025 between the fluids in the two apparatus can be seen easily, so that there can be no difficulty in detecting the change due to the formation of less than 0.5 millimol of CaCO_3 per litre, that is, the conversion of 2 mg. of calcium ion per 100 cc. into calcium carbonate.

In repeated experiments with such pressures of CO_2 and concentrations of bicarbonate, and 10 mg. of Ca per 100 cc. in the final mixture, that is a solution highly supersaturated with CaCO_3 , in one of the tubes we have not found any appreciable difference of p_{H} until precipitation began, which might not be for several days in spite of repeated vigorous shaking.

In the same way, when CaHPO_4 or $\text{Ca}_3(\text{PO}_4)_2$ is formed in a phosphate mixture containing about 1 millimolar phosphate at p_{H} about 7.4 a still greater change of p_{H} will occur. We have made solutions of millimolar phosphate and calcium chloride (10 mg. calcium per 100 cc.) in 0.9 % sodium chloride at about p_{H} 7.4, and sealed them in duro-glass tubes. These solutions, which were made up sterile, remained without change in p_{H} for weeks.

We therefore conclude that the calcium remains as ion in such supersaturated solutions until precipitation occurs.

The excess of calcium in the protein solution is independent of the bicarbonate and phosphate concentration. At 37° and p_{H} 7.3–7.55

$$\frac{\text{Ca}_p - \text{Ca}_s}{\text{Ca}_s \cdot \text{Pr}} = 0.11$$

at higher p_{H} the values found for this expression are higher. The values found when no buffer was used, Exps. 9 and 10, were higher; this is due to the fact that, in order to get the p_{H} at the end of dialysis as high as 7.0, alkali had to be added at times¹, with the result that the actual p_{H} during the dialysis in these experiments was higher than that found at the end; the calcium seems to dialyse out from the protein solutions more slowly than it goes in so that the figures found for the calcium distribution in these experiments are those for a higher p_{H} than that found at the end of the dialysis.

The values for $\frac{\text{Ca}_p - \text{Ca}_s}{\text{Ca}_s \cdot \text{Pr}}$ at room temperature are irregular for the same reason

and owing to the variation of temperature; they are definitely lower than those found at 37° .

¹ The appearance of a small amount of acid was probably due to the action of the toluene on the rubber; it was greater at 37° than at room temperature.

The excess of calcium in the protein solutions is far greater than the Donnan equilibrium will account for.

Suppose that the protein-free solution contains

sodium ion; 155 mg. equivalents per 100 g. water,
calcium ion; 3 mg. equivalents per 100 g. water,
chlorine ion; 158 mg. equivalents per 100 g. water,

and the protein solution

sodium ion; $[\text{Na}]$ mg. equivalents per 100 g. water,
calcium ion; $[\text{Ca}]$ mg. equivalents per 100 g. water,
chlorine ion; $[\text{Cl}]$ mg. equivalents per 100 g. water.

With protein concentration 5 % the base combined with protein at p_{H} 7.4 is, according to Van Slyke, Wu and MacLean [1923], approximately 9 mg. equivalents per 100 g. water.

$$\text{Then} \quad [\text{Ca}] + [\text{Na}] = [\text{Cl}] + 9 \quad \text{and} \quad \frac{[\text{Na}]^2}{155^2} = \frac{158^2}{[\text{Cl}]^2} = \frac{[\text{Ca}]}{3}.$$

$$\text{Therefore} \quad [\text{Ca}] + \frac{[\text{Ca}]^{\frac{1}{2}}}{3^{\frac{1}{2}}} \times 155 - \frac{3\frac{1}{2} \times 158}{[\text{Ca}]^{\frac{1}{2}}} - 9 = 0,$$

whence $[\text{Ca}] = 3.22$.

Since 100 cc. of the protein-free solution contain practically 100 g. water, and 100 cc. of the protein solution only 95 g. water [Van Slyke, Wu and MacLean, 1923], we find that with a calcium concentration in the external fluid of 3 mg. equivalents per litre, or 6 mg. per 100 cc., there will be according to the Donnan effect alone $3.22 \times \frac{95}{100} = 3.06$ mg. equivalents of calcium per litre in the protein solution, or 6.12 mg. per 100 cc. That is, the difference of concentration produced by the Donnan effect is almost negligible.

Nor does it seem possible that the difference is due to the effect of the greater ionic strength of the protein solution on the activity of the calcium ion. In Exp. 7, Table I, the chlorine concentrations in the external fluid and protein solutions were 157 and 144 millimolecular. The water in 100 cc. of the external fluid was 99 g., of the internal fluid 94.2 g.; so that there were 158.4 millimols of Cl to 100 g. of water in the external fluid and 152.9 to 100 g. of water in the protein solution. This last is under 1 % higher than would be expected from the Donnan effect (151.7), so that the ionic strength of the protein does not reduce the activity of the chlorine ion by more than 1 %, and therefore could not have sufficient effect on the activity of the calcium ion to produce the unequal distribution we have found. This distribution is therefore probably due to the formation of undissociated calcium protein compounds, of the formation of which there is other evidence [see Greenberg and Schneider, 1926].

We therefore consider such solutions containing protein and calcium to contain calcium ion, in approximately the same concentration as the protein-free fluid against which they were dialysed (slightly higher owing to the Donnan effect), and an undissociated calcium protein compound, the amount

of which is proportional to the concentration of protein in the protein solution and to the concentration of calcium ion in the external solution. If two fluids containing calcium in concentrations Ca_{p_1} and Ca_{p_2} , and protein in concentration Pr_1 and Pr_2 are in equilibrium they will be in equilibrium with the same protein-free fluid, so that

$$Ca_{p_1} - Ca_s = 0.11 Pr_1.Ca_s,$$

and

$$Ca_{p_2} - Ca_s = 0.11 Pr_2.Ca_s,$$

and for a series of such fluids in equilibrium, if the protein concentration is represented by x and the calcium concentration by y ,

$$y - Ca_s = 0.11x.Ca_s \dots\dots\dots(1)$$

a straight line which cuts the axis of y in $y = Ca_s$, giving the calcium ion concentration of a protein-free solution in equilibrium with these fluids, and, approximately, the calcium ion concentration in the protein solutions.

Table II. *Protein and calcium concentrations in body fluids in five cases of heart failure [Salvesen and Linder, 1923] and one normal subject.*

Subject	Fluid	Protein g. per 100 cc.	Calcium mg. per 100 cc.
16 a (29. iii. 23)	Plasma	6.12	10.4
" "	Ascitic	2.66	7.5
" "	Oedema	0.29	6.3
16 b (6. iv. 23)	Plasma	5.72	9.0
" "	Pleural	1.72	6.9
17	Plasma	5.85	8.5
" "	Oedema	0.24	5.8
18	Plasma	6.24	9.3
" "	Ascitic	2.24	6.9
" "	Oedema	0.35	5.1
19	Plasma	6.57	9.8
" "	Pleural	1.76	6.8
20	Plasma	5.95	8.4
" "	Pleural	1.88	5.9
J. M.	Plasma	7.3	10.6
"	Blister	4.9	9.1

In Table II are given the results of what are practically *in vivo* dialysis experiments, the calcium and protein in body fluids in 5 cases of heart failure from the paper of Salvesen and Linder [1923] and in a normal person. In Fig. 2 the protein concentration of the fluids is plotted as abscissa and calcium content as ordinate. The points obtained lie, within experimental errors, on straight lines given by the equation (1) with values of Ca_s ranging from 5.0–6.0. The distribution of calcium in these fluids is therefore what would be found in a series of protein solutions which would be in equilibrium with protein-free fluids containing from 5.0 to 6.0 mg. of calcium ion per 100 cc.—the concentration of calcium found in the practically protein-free body fluid, cerebrospinal fluid. We find therefore that the distribution of calcium in body fluids, including cerebrospinal fluid, is such as would occur if they contained on an average about 5.5 mg. calcium per 100 cc. *as ion*, and in addition undissociated calcium proportional to the protein concentration.

This is the simplest conception of the distribution found, but evidence (which we shall discuss later) derived from the solubility of calcium carbonate

suggests that calcium ion cannot exist permanently in these fluids in concentrations above 2.5 to 3 mg. per 100 cc. It is therefore necessary to consider the possibility of obtaining the same distribution with a lower calcium ion concentration. This would involve a hypothetical diffusible undissociated calcium compound Ca X, and an indiffusible undissociated compound Ca Y, proportional to the protein concentration—possibly colloidal CaCO_3 or $\text{Ca}_3(\text{PO}_4)_2$ carried by the protein. The distribution in serum might then be

Ca ion	...	...	2.5 mg. per 100 cc.		
Ca X	...	...	3.0	"	"
Ca protein compound	2		"	"	"
Ca Y	...	...	3.5	"	"
Total	...	...	11.0	"	"

Supposing such a serum were dialysed against a large volume of a fluid containing 5.4 mg. of calcium ion per 100 cc., and sufficient bicarbonate and

Fig. 2. Relation between protein and calcium concentration in body fluids.

phosphate to keep any colloidal CaCO_3 or $\text{Ca}_3(\text{PO}_4)_2$ from dissolving, we should get:

External fluid					
Ca ion	...	...	5.4 mg. per 100 cc.		
Serum					
Ca ion	...	...	5.5 mg. per 100 cc.		
Ca protein compound	4.0		"	"	"
Ca Y	...	...	3.5	"	"
Total	...	...	13.0	"	"

that is, the total calcium in the serum would definitely increase. Even if the original distribution in the serum were

Ca ion	...	...	2.5 mg. per 100 cc.
Ca X	...	...	5.0 " "
Ca protein compound	...	...	2.0 " "
Ca Y	...	...	1.5 " "
Total	...	...	11.0 " "

which would give a higher value for the calcium content of protein-free fluids than is ever found, the serum would not lose calcium on dialysis against a fluid with 5.4 mg. of calcium ion per 100 cc.

Table III. *Result of dialysing serum and plasma against large volumes of solutions of calcium ion.*

Subject	Internal fluid				External fluid			
	Protein before dialysis g. per 100 cc.	Protein after dialysis g. per 100 cc.	Calcium before dialysis mg. per 100 cc.	Calcium after dialysis mg. per 100 cc.	p _H	Calcium mg. per 100 cc.	HCO ₃ ⁻ Mol. × 10 ³	P Mol. × 10 ³
1 G. D. T. serum	7.7	7.7	11.2	11.0	7.45	5.4	30	0.4
2 G. D. T. plasma	7.6	7.6	11.4	11.3	7.30	5.4	30	0.4
3 J. M. serum	7.3	7.3	10.65	9.2	7.45	5.0	30	0.4
4 J. M. plasma	7.3	7.25	10.55	9.2	7.45	4.9	30	1.0
5 J. M. serum	7.4	7.4	10.8	11.8	7.70	6.56	32	0.4

We therefore tried this experiment with normal serum and heparin plasma (Table III). Since calcium carbonate appears to be more soluble at room temperature than at 37° [Kugelmass and Shohl, 1924] it was necessary not to let the blood cool, for if it did colloidal CaCO₃, if present, might dissolve and remain in supersaturated solution on reheating. We therefore maintained the blood, and plasma or serum separated from it, at 37° ± 2° throughout. The dialysis which lasted 24 hours was performed in an incubator, using about 4 cc. of serum or plasma and 500 cc. of external solution in a duro-glass flask. Toluene was added to check bacteria and precautions were taken to avoid contamination; there was no suggestion of bacterial growth. The collodion membranes used in these experiments, especially 1 and 2, were less permeable than usual, ensuring that no loss of protein took place; they were permeable to glucose but not to phenolsulphonephthalein. The external flask was not shaken or stirred. In Exps. 1, 2, 3 and 5 the phosphate concentration was that found in cerebrospinal fluid; this is lower than that in serum but since the solubility product of Ca₃(PO₄)₂ was exceeded, any colloidal Ca₃(PO₄)₂ present in the serum or plasma would not have dissolved; from Exps. 3 and 4 we see that the phosphate concentration made no difference.

In Exps. 1 and 2 we find no increase of calcium in the serum or plasma, but a slight loss; although it is probable that final equilibrium was not obtained in these experiments, the change that took place is in the opposite direction to that which would have happened if a compound such as Ca Y were present. In Exps. 3, 4 and 5 we find a distribution of calcium such as would be accounted for by the protein alone according to equation (1); if any such compound as Ca Y were present the final concentration of calcium in these sera should have

been higher; these three experiments give as the value for the calcium ion concentration of a protein-free fluid which would be in equilibrium with the serum, 5.8 mg. per 100 cc. in agreement with that inferred from the experiments on J. M. (Table II and Fig. 2). These experiments therefore show that there is no such compound as Ca Y, and if such a compound is not present we have to fall back on the simpler explanation that the body fluids contain from 5 to 6 mg. of calcium ion per 100 cc. together with un-ionised calcium proportional to the protein concentration.

The calcium of cerebrospinal fluid is then all in the form of ion, and gives an approximate figure for the calcium ion concentration in other body fluids, slightly low owing to the Donnan effect and possibly to reduction of calcium ion activity by protein.

(2) SOLUBILITY OF CALCIUM CARBONATE AND CALCIUM PHOSPHATE.

If the calcium ion concentration in body fluids, for example cerebrospinal fluid, is as high as we have concluded, the solubility products of CaCO_3 , as generally accepted, and of $\text{Ca}_3(\text{PO}_4)_2$ [Holt, La Mer and Chown, 1925] are exceeded. This would mean that the fluids are in unstable equilibrium and calcium carbonate and phosphate should in time precipitate from them, more especially in the presence of the solid calcium carbonate and phosphate of bone. With respect to $\text{Ca}_3(\text{PO}_4)_2$, Holt, La Mer and Chown [1925] and Holt [1925] have shown that serum and cerebrospinal fluid are supersaturated¹. However CaCO_3 separates out from supersaturated solutions more readily than does $\text{Ca}_3(\text{PO}_4)_2$; it is therefore necessary to consider the question in relation to CaCO_3 also.

From the equation $\text{Ca}^{++} + \text{CO}_3^{--} = \text{CaCO}_3$ we get

$$f_{\text{Ca}^{++}} [\text{Ca}^{++}] \times f_{\text{CO}_3^{--}} [\text{CO}_3^{--}] = K_1 f_{\text{CaCO}_3} [\text{CaCO}_3] = K_1 c \dots\dots(2),$$

when the fluid is saturated with CaCO_3 .

And from $\text{H}^+ + \text{CO}_3^{--} = \text{HCO}_3^-$ we get

$$f_{\text{H}^+} [\text{H}^+] \times f_{\text{CO}_3^{--}} [\text{CO}_3^{--}] = K_2 f_{\text{HCO}_3^-} [\text{HCO}_3^-] \dots\dots\dots(3)$$

where $f_{\text{Ca}^{++}}$, f_{H^+} , $f_{\text{CO}_3^{--}}$, $f_{\text{HCO}_3^-}$ and f_{CaCO_3} are the activity coefficients of Ca^{++} , H^+ , CO_3^{--} , HCO_3^- , and CaCO_3 .

From (2) and (3)

$$[\text{Ca}^{++}] = \frac{K_1 c}{K_2} \times \frac{f_{\text{H}^+} [\text{H}^+]}{[\text{HCO}_3^-]} \times \frac{1}{f_{\text{Ca}^{++}} \times f_{\text{HCO}_3^-}} \dots\dots\dots(4)$$

or

$$F = \frac{[\text{Ca}^{++}] [\text{HCO}_3^-]}{f_{\text{H}^+} + [\text{H}^+]} = \frac{K_1 c}{K_2} \frac{1}{f_{\text{Ca}^{++}} \times f_{\text{HCO}_3^-}} \dots\dots\dots(5).$$

Equation (4) gives the concentration of calcium ion that can exist in solution in stable equilibrium in presence of solid CaCO_3 , in terms of quantities

¹ These results would probably be equally well explained by the supposition that calcium phosphate was present in the fluids in colloidal solution and separated out on prolonged shaking with $\text{Ca}_3(\text{PO}_4)_2$.

that can be measured—hydrogen ion activity (usually called hydrogen ion concentration), and concentration of HCO_3^- (equal to the total bicarbonate concentration), and bringing in a constant $\frac{K_1 c}{K_2}$ and two terms $f_{\text{Ca}^{++}}$ and $f_{\text{HCO}_3^-}$ which vary with the ionic strength of the solution. Equation (5) also gives the expression F , which is equivalent to a solubility product, in terms of the constant and $f_{\text{Ca}^{++}}$ and $f_{\text{HCO}_3^-}$.

This is the form of equation required by the theories of complete dissociation of strong electrolytes and the mutual effects of ions, which are found to agree better with the observed solubility products of salts than the older theory of incomplete dissociation, neglecting the mutual effect of ions. Now the estimations of F at 38° of Kugelmass and Shohl [1924] were made without the addition of salt to make the total ion concentration up to that found in body fluids. If the relations between activity coefficient and ionic strength¹ found by Bronsted and La Mer [1924], $\log f = -0.49v^2\sqrt{\mu}$, continue to hold at the higher concentrations with which we have to deal, the value of F should be four times as great at the ionic strength of body fluids as at the ionic strengths in the experiments of Kugelmass and Shohl; this would allow a calcium ion concentration such as we suppose to be present in cerebrospinal fluid. We have therefore estimated the value of F with varying ionic strengths (Table IV).

Table IV. *Solubility relations of calcium carbonate in presence of other salts.*

Expt. No.	μ	$\sqrt{\mu}$	Cl Mol. $\times 10^3$	K Mol. $\times 10^3$	Mg Mol. $\times 10^3$	P Mol. $\times 10^3$	HCO_3^- Mol. $\times 10^3$	p_{H}	Ca Mol. $\times 10^4$	$\log F$	F	No. of days shaken
1	0.077	0.28	48.7	0	0	0	27.3	7.31	5.30	2.46	288	1
2	0.0875	0.30	60.5	0	0	0	26.0	7.38	4.65	2.46	288	2
3	0.093	0.31	52.0	0	0	0	40.2	7.50	2.54	2.51	324	1
4	0.114	0.34	85.5	0	0	0	27.2	7.34	4.80	2.46	289	3
5	0.143	0.38	115.0	0	0	0	27.5	7.36	5.33	2.54	345	2
6	0.143	0.38	116.0	0	0	0	26.8	7.36	5.48	2.515	328	2
7	0.145	0.38	117.0	0	0	0	26.5	7.35	6.80	2.60	399	4
8	0.145	0.38	117.0	0	0	0	27.0	7.35	6.95	2.62	417	2
9	0.1615	0.40	131.6	0	0	0	28.5	7.32	7.40	2.64	437	1
10	0.164	0.405	133.6	0	0	0	28.7	7.32	7.2	2.635	432	1
11	0.180	0.425	154.5	0	0	0	23.5	7.24	9.9	2.61	408	1
12	0.159	0.40	134.0	5	2	0	22.7	7.32	11.1	2.72	525	1
13	0.159	0.40	133.0	5	2	0	23.3	7.41	10.6	2.77	589	1
14	0.162	0.40	135.0	5	2	0	24.0	7.27	13.45	2.78	603	3
15*	0.169	0.41	135.0	5	2	0	30.0	7.37	22.3	—	—	1
16*	0.161	0.40	129.0	0	0	0	30.8	7.35	13.3	—	—	2
17	0.160	0.40	131.0	5	2	5	25.0	7.49	30.0	—	—	3
18	0.157	0.40	128.0	5	2	5	25.0	7.37	15.2	—	—	3
19	0.165	0.405	134.0	5	2	5	27.0	7.445	30.0	—	—	2
20	0.165	0.405	135.0	5	2	10	24.5	7.42	25.6	—	—	1
21	0.165	0.405	135.0	5	2	16.7	26.0	7.37	27.9	—	—	3

* In Exps. 15 and 16, 0.1 cc. of serum was added to the bicarbonate solution used.

¹ Ionic strength of solution

$$= \frac{1}{2} (m_1 v_1^2 + m_2 v_2^2 + \dots)$$

where m_1, m_2 are the molar concentrations, and v_1, v_2 the valencies of the ions in the solution.

Methods.

10 cc. of a solution containing sodium bicarbonate and the other salts used to make up the ionic strength with phenolsulphonephthalein as indicator were placed in a duro-glass test-tube, and a CO_2 -air mixture passed through it, then 10 cc. of a solution of CaCl_2 through which the same gas mixture had been passed were added, and finally solid calcite dropped in; the gas mixture was passed through again after each addition. Finally the entrance and exit tubes were closed and the stopper sealed in with paraffin wax. The tubes were then placed in a water-bath at 37° and shaken continuously. After shaking p_{H} was estimated by comparison with phosphate standards, warmed to 37° ; 0.03 was subtracted from the observed value to allow for the effect

Fig. 3.

Fig. 4. Relation between $F = \frac{[\text{Ca}^{++}][\text{HCO}_3^-]}{f_{\text{H}^+}[\text{H}^+]}$ and ionic strength.

of heating on the p_{H} of the standards [Hastings and Sendroy, 1924]; when the ionic strength was about 0.80, 0.04 was added to the p_{H} to correct for the effect of ionic strength on the indicator, and at ionic strengths between this and 0.155 corresponding corrections were made [Martin and Lepper, 1926]. The tubes were connected with a centrifuge tube and mercury reservoir as shown in Fig. 3, and the gas and then the fluid forced into the centrifuge tube; the fluid was thus collected in the centrifuge tube under its own gas mixture and loss of CO_2 was avoided. To avoid excessive cooling the mercury was used warm. The fluids were then rapidly centrifuged and calcium and bicarbonate estimated in them at once; calcium as before, and bicarbonate by the titration method of Van Slyke [1922]. In Exp. 19, in which we wished to detect a

change of reaction should any occur, we used the apparatus shown in Fig. 1, filled according to the technique described on p. 582; the fluid was removed and analysed as above. The chloride concentration was also estimated. Fig. 4 shows $\sqrt{\mu}$ plotted as abscissa and $\log F$ as ordinate; we have inserted the values of F found by Kugelmass and Shohl recalculated on the supposition that the salts are fully dissociated. The increase of F with increasing ionic strength is less than would be found if the formula $-\log f = 0.49v^2\sqrt{\mu}$ was correct at the concentration used. This formula would give the line A_1A_2 . The values for activity coefficient given by Lewis and Randall [1923], line B_1B_2 , fit our results fairly well.

The practical result of these experiments, from the point of view of our problem, is that although the value we find for F at the ionic strength of body fluids is much higher than that found by Kugelmass and Shohl at lower ionic strengths, still it gives lower concentration of Ca ion, at the hydrogen ion activity and bicarbonate concentration of cerebrospinal fluid, than our dialysis experiment would lead us to suppose present (2.7 mg. per 100 cc. instead of 5 to 6 mg.).

When in similar experiments magnesium and potassium also were added rather higher values for F were obtained; when small quantities of protein salts also were added higher values still were obtained, but this was probably due to delay only, as some precipitation always took place. When however phosphate was added there was no precipitation with much higher values of F . This does not appear to be due to delay merely, as without phosphate present the precipitation appeared to take place, to a great extent, directly the calcite was added, while in these experiments no precipitation occurred after 2 days' shaking. The absence of precipitation was not due to the formation of colloidal calcium phosphate, for in Exp. 19 with 0.0005 M phosphate the whole phosphate present could only hold up 0.00075 M calcium leaving in solution 0.00225 M calcium, which still would give much higher values for F than we had found in experiments without phosphate present. Nor was the calcium in the form of colloidal CaCO_3 since there was no change in p_{H} .

The increase of F in the presence of phosphate brings us to another aspect of the question. The values for F that have been applied to this question of calcium in body fluids have been obtained by experiments with calcite as the solid phase, but there is no evidence that calcite exists in the body. The calcium carbonate which, according to Bassett [1917], is probably present in bone as such, is apparently in an amorphous form which is more soluble than calcite; this will alter the value of c in equation (5), and allow correspondingly higher values of F . It is possible that the presence of phosphate interferes in some way with the formation of crystalline calcite, and leads to the failure of precipitation found in our experiments.

We have deduced from theoretical considerations and found experimentally that the nearer approach to body conditions with regard to ionic strength of the fluid leads to higher values of F and allows higher calcium ion concentra-

tion; experimentally, we have evidence that by more nearly approaching body conditions by making the composition of the salt mixture the same as found in body fluids still higher values are obtained. We conclude that the arguments based on the insolubility of CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$ which have been employed to give an upper limit to possible concentration of calcium ion in body fluids, are not applicable to body conditions, and therefore do not invalidate our deduction from other considerations that body fluids contain from 5 to 6 mg. calcium ion per 100 cc.

We do not propose to discuss the work of Brinkman and Van Dam, as it has been dealt with by Schulten [1926]; owing to supersaturation with calcium oxalate the values for calcium ion obtained by this method are too low.

DISCUSSION.

If the calcium ion concentration in body fluids were expressed by equation (5), a very satisfactory explanation of the tetany of alkalosis would follow. The bulk of evidence indicates that the tetany following parathyroidectomy is due to reduction of total calcium, and it would agree well with this if the tetany of alkalosis could be considered to be due to the reduction of calcium ion concentration consequent on the reduction of the ratio $\text{H}^+/\text{HCO}_3^-$. Our conclusions however will not admit this attractive theory, more especially in the case of hyperpnoea tetany, in which the tetany comes on in about 10 minutes; now we have shown in Exp. 19, Table IV, that reduction of calcium ion concentration to values in agreement with equation (5) does not take place in fluids resembling body fluids in 2 days, if at all; it is therefore improbable that such reduction of Ca ion concentration can take place sufficiently rapidly in the body to account for the onset of hyperpnoea tetany, even if it occur in time with prolonged alkalosis.

One of the most plausible theories of the cause of epilepsy is that advanced by Bigwood [1924], that a slight alkalosis results in reduction of calcium ion concentration which, acting presumably on a hyperexcitable cortex, causes the fits. This theory also must be rejected if our conclusions are correct.

However we have found that a reduction of hydrogen ion concentration leads to an increase of calcium combined in an un-ionised form with protein; this would account for the increase of total serum calcium found by Stewart and Haldane [1924]—an increase of calcium in the protein-rich serum at the expense of fluids of lower protein concentration. This increase of calcium protein compound would be associated, temporarily at any rate, with a reduction of calcium ion, but the reduction would be much less than that which would occur if equation (5) were followed. Thus a change of p_{H} from 7.4 to 7.8 would, if equation (5) were applicable, produce a reduction of calcium ion concentration of 60 %; while such a change of p_{H} would, as a result of the increase of the calcium protein compound, produce in serum alone a reduction of calcium ion from 5.4 to 4.5 mg. per 100 cc; with other fluids, containing less protein to draw upon, the reduction of calcium ion would be less. It is

however possible that this effect may contribute to the production of hyperpnoea tetany.

The work of Salvesen and Linder [1923] indicates that it is the concentration of calcium uncombined with protein, which we consider to be calcium ion, that is kept constant in body fluids. In chronic alkalosis therefore we might expect that there would be no reduction of calcium ion, in spite of the increased amount of calcium combined with protein, since excretion would be reduced or absorption increased to make up the deficiency of ion. We should expect therefore that the chronic slight alkalosis found by Bigwood could not lead to a reduction of calcium ion concentration; this is borne out by the normal concentrations of calcium in the cerebrospinal fluid of epileptics found by Hamilton [1925]; for, as we have shown, the calcium in the cerebrospinal fluid is wholly ionised and approximately equal to the calcium ion concentration in other body fluids.

The un-ionised calcium protein compound is the so-called indiffusible calcium in serum. The values usually found for it are lower than would be found at body temperature, since most estimations of indiffusible calcium have been made at room temperature. However, the formula used for calculating the percentage of diffusible calcium

$$= \frac{(\text{Ca in external fluid after dialysis}) - (\text{Ca in external fluid before dialysis})}{\text{Original Ca in serum}} \times 100$$

is incorrect as it assumes that the calcium protein compound remains constant with varying calcium ion concentrations.

SUMMARY.

(1) Calcium forms an un-ionised compound with proteins; the formation of this compound accounts for the so-called indiffusible calcium of serum.

(2) The calcium of body fluids is partly in the form of calcium ion, partly in the form of un-ionised protein compound. The calcium ion concentration of plasma is slightly higher than the calcium concentration of cerebrospinal fluid.

(3) The calcium ion concentration of body fluids is not regulated by the solubility of calcium carbonate and calcium phosphate.

(4) Epilepsy is not caused by a reduction of calcium ion concentration in body fluids.

(5) The value of the expression $\frac{[\text{Ca}^+][\text{HCO}_3^-]}{f_{\text{H}}[\text{H}^+]}$ in solutions saturated with calcite increases with increasing ionic strength of the solution.

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