ON THE DETERMINATION OF DIFFUSIBLE AND NON-DIFFUSIBLE SERUM CALCIUM.

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INTRODUCTION.

Ultrafiltration offers an empirical means of determining the calcium distribution in the blood. The same objections that apply to ultrafiltration also apply to compensation dialysis in that most probably neither method gives the actual ionized and non-ionized calcium present in blood plasma. It is interesting to note in this connection that the dialysis experiments, in vivo, of Stewart and Percival (1), in which the living peritoneal cavity is employed as a dialysis sac, yield about the same values for diffusible calcium as are obtained by the ultrafiltration method. The analysis of cerebrospinal fluid also yields similar results (2). Because of its comparative simplicity and rapidity, ultrafiltration offers the readiest means of determining the distribution of calcium in blood serum. The results obtained by this method have been consistent and offer results of readily comparative value, even if perhaps they are at present not completely theoretically interpretable.

Ultrafiltration as a means of determining the diffusible calcium of blood serum, with large samples of blood, has been employed by many investigators among whom may be mentioned Cushny (3), Neuhausen and Pincus (4), Richter-Quittner (5), and Pincus, Peterson, and Kramer (6). Moritz (7) developed a micro method for determining diffusible calcium, employing a combination of ultrafiltration and diffusion. The method of Moritz was improved in some respects by Updegraff, Greenberg, and Clark (8). Since
then a number of studies in which use is made of this micro method have appeared by Lui (9) and Reed (10).

In the course of the recent studies on blood calcium carried out by us, a number of improvements have been made in the technique of the ultrafiltration procedure and certain experiments carried out having both a practical and theoretical bearing on the determination of the true distribution of the diffusible and non-diffusible calcium of the serum. In connection with the improvements in technique, a system of analysis has been developed for total calcium, diffusible calcium, inorganic phosphate, and serum proteins on the serum from about 12 cc. of blood, with the use of the colorimetric method for serum proteins and the supernatant fluid from calcium analysis for phosphate determination already published by us (11, 12).

**EXPERIMENTAL.**

*Ultrafiltration Procedure.* For determining diffusible calcium, ultrafiltration alone is now used by us to obtain a protein-free filtrate. No water or aqueous solution is placed in the outside container in contact with the collodion membrane. We have abandoned the combination of ultrafiltration and diffusion to eliminate any doubt that diffusion equilibrium may not be established between the serum in the collodion sac and the external solution in the course of time of the Moritz procedure (7), and also to avoid possible changes in equilibrium between diffusible and non-diffusible calcium. This subject will be considered again in connection with the subject of the nature of the equilibrium between diffusible and non-diffusible serum calcium.

The apparatus used is essentially the same as that described by Updegraff, Greenberg, and Clark (8). However, instead of using the mercury syphon illustrated in Fig. 2 of that publication, to maintain the external reduced pressure required for filtration, use is now made of a water- or motor-driven vacuum pump. The mercury syphon required almost constant attention to keep the pressure difference at the required level. By the use of a vacuum pump and a regulating valve, the proper pressure is maintained automatically for any desired length of time. A regulating valve that is very satisfactory for the purpose is quite easily made. A glass cylinder of 200 mm. or more in height is fitted with a 3-hole
rubber stopper; two bent glass tubes as shown are used to connect between the vacuum pump and the rest of the apparatus with rubber tubing; a third straight piece of capillary tubing of about 1 mm. bore is inserted through the third hole into the mercury contained in the cylinder, to a depth of 150 mm. below the surface of the mercury. This automatically keeps the vacuum at 150 mm. of Hg below atmospheric pressure when a water pump is employed.

![Diagram of ultrafiltration apparatus with an automatic pressure-regulating cylinder.](http://www.jbc.org/)

By accurately setting the regulating tube, a manometer to show the pressure difference can be dispensed with. It is also desirable to have a wash bottle between the regulating cylinder and the pump to trap any mercury splashed over, or any water sucked back from the pump. When it is desirable to measure the amount of ultrafiltrate that comes through, the filtration apparatus is fitted into a Folin sugar tube with dimensions of 2.5 cm. inside diameter and 19 cm. long, having a constricted portion of 5 cc.
at the bottom. In Fig. 1 there is shown a sketch of such an ultrafiltration apparatus with a manometer omitted.

**Collodion Solution.**—The selection of a satisfactory collodion solution is of paramount importance for obtaining correct ultrafiltration results. The parlodion solution containing olive oil, introduced by Moritz, has been abandoned by us because it does not give uniformly permeable membranes. We have tried out various solutions made from parlodion, Merck's and Schering's collodion. Parlodion was found to give a slower rate of filtration than the other two mentioned, which were about equal in this respect. No one of the three collodions in simply alcohol-ether solution gave sacs, however, that were uniformly completely permeable to calcium chloride. To obtain completely satisfactory membranes, we found it necessary to add either glycerol or ethylene glycol to the alcohol-ether collodion solution (13). About 5 per cent of glycerol or ethylene glycol added to parlodion or Schering's collodion was found to give suitable membranes, although those with glycerol had a tendency to be weak. Collodion membranes with ethylene glycol were considerably stronger. Our final choice was a solution of 7 per cent Schering's collodion in 60 parts of ether and 40 parts of absolute alcohol, to which there are added 5 cc. of ethylene glycol per 100 cc. of collodion solution. This solution proved to be completely permeable to calcium and to hold back the serum proteins completely. Each stock of collodion solution prepared is tested by us for permeability by filtering a calcium-containing solution, with a calcium concentration of about 10 mg. per 100 cc. through a number of sacs. The sacs are cast as described by Updegraff, Greenberg, and Clark (8), with two dippings of collodion solution. After tying on the collodion sac to the rubber stopper of the filtering apparatus, it has been found useful to seal the sac to the stopper by painting it with a little collodion solution. This makes a leak-proof seal when the collodion dries.

A necessary precaution in carrying out the ultrafiltration is to wipe off the first few drops of fluid that appear on the outside of the sac the first few minutes after filtration is started. This represents the displaced capillary water of the collodion sac. If this is not done, the values obtained for diffusible calcium will be a little low, as has been shown by Neuhausen and Pineus (4). To determine
the magnitude of the dilution possible due to capillary water, in the size of the membrane employed by us, a test run was carried out. It was found that the same sample of serum gave a 6.5 per cent lower value for diffusible calcium in a sac left unwiped than in one that was wiped when 4 cc. of serum were introduced into the sac and about 2 cc. ultrafiltered. As a still further test, four cast sacs were wiped dry with filter paper, weighed, dried overnight in 105° in an electric oven, and the loss in weight determined. The loss of weight was found to be 0.745 gm. for the four sacs or an average of 0.186 gm. per sac. This indicates that there are less than 0.2 cc. of water contained in the pores of a membrane sac. With the 3 or 4 cc. of serum employed for ultrafiltration by us, this amount of water cannot cause more than a 10 per cent dilution, even if the first extruded drops are not wiped off.

**Analytical Methods.**

For calcium, the Tisdall modification of the Kramer-Tisdall method is employed except that precipitation is allowed to continue for at least 2 hours before centrifuging (14). In washing the calcium oxalate precipitate, a 2 per cent ammonia solution saturated with calcium oxalate is used, as recommended by Stanford and Wheatley (15). Samples containing as much calcium as is usually found in 2 cc. of serum are titrated with 0.005 N potassium permanganate that is freshly diluted as needed from a 0.05 or 0.1 N stock solution. With less amounts of calcium, as in the samples of the ultrafiltrate for diffusible calcium, the Van Slyke and Sendroy gasometric method (16) is used to measure the amount of calcium oxalate precipitate. We found about 5 minutes shaking was required for complete evolution of the CO₂ rather than the 3 minutes stated by Van Slyke and Sendroy. In the communication of Updegraff, Greenberg, and Clark ((8) p. 95) it was pointed out that in the ultrafiltrate samples films of calcium oxalate very often remained floating on the surface after the tubes had been centrifuged. To avoid such films, we have adopted the procedure of adding 1 cc. of supernatant fluid from the determination of total serum or previously decalcified serum in place of an equivalent amount of distilled water in setting up the tubes for analysis.
The determination of inorganic phosphate is made by Fiske and Subbarow's method on serum (17) or on the supernatant fluid from calcium analysis (12), and colorimetric analysis for serum proteins was carried out as previously mentioned (11).

**System of Analysis.**—About 12 cc. of blood are drawn for the purpose of this analysis. After separating the serum, 2 cc. of the serum are pipetted out for total calcium analysis, 0.5 cc. for protein analysis, and the remainder is used for ultrafiltration. The inorganic phosphate is determined in the supernatant fluid from the total calcium determination. In the procedure there is no need to measure carefully the amount of serum used for ultrafiltration. The ultrafiltration is allowed to go on for from 3 to 4 hours. In that time approximately half the serum introduced in the collodion sac is ultrafiltered. The calculations involved are straightforward. Each sample taken represents the conditions of the original serum except for a correction for the volume of the serum proteins which are, of course, absent from the ultrafiltrate liquid.1

1 If less serum is available, the complete analysis can be carried out on as little as 3 cc. of serum by following this procedure, although the results obtained are subject to greater errors. In the alternate method, 3 or 4 cc. of serum are accurately pipetted into a collodion sac that has been wiped dry. After the collodion sac has been fitted onto the rubber stopper, the filtration apparatus is fitted into a Folin sugar tube with dimensions of 2.5 cm. inside diameter and 19 cm. long, having a constricted portion of 5 cc. at the bottom (see Fig. 1). This conveniently allows the determination of the amount of ultrafiltrate that has come through, by measuring the amount of water required to fill the 5 cc. portion. A micro burette graduated in 0.02 cc. is very convenient for this purpose.

At the end of the filtration, 4 cc. of the fluid containing the ultrafiltrate are measured into a 15 cc. centrifuge tube and 1 cc. of 4 per cent ammonium oxalate added for diffusible calcium analysis. The supernatant liquid after centrifuging is poured off to be used for inorganic phosphate determination. On the concentrate left in the colloidion sac there are determined proteins and calcium. The contents of the sac are transferred by puncturing the end to the constricted portion of one of the above Folin sugar tubes, the top of which has been cut off a few cm. above the constricted portion. The concentrate is made up to 5 cc. by filling to the 5 cc. mark with water. A 3 cc. or other convenient aliquot is taken for calcium analysis. The serum proteins are determined on a 0.5 cc. portion in the usual manner.

**Calculation.**—For the protein results it is only necessary to remember that all the protein is retained in the colloidion sac. If a 3 cc. sample has been used, the 0.5 cc. aliquot from the Folin tube represents 0.3 cc. of serum. The rest of the calculation is as given in the formula ((11) p. 549).
Experiments on Interpretation of Calcium Partition.

Effect of Time of Contact of Serum with Clot on Serum Calcium.—It is important to know whether after the clot is once formed there is any change in the amount or the distribution of the calcium in the serum if the clot is allowed to remain in contact with the serum. Aside from the theoretical implications of this problem, we were interested from the practical point of deciding when best a separation of serum from blood could be made. Blood that is centrifuged immediately after clotting does not give a separation of as much serum as blood that has stood sufficiently long for the clot to retract. The subject also concerned us because many of our samples, coming from San Francisco to Berkeley, required some 6 hours to be brought for analysis. A number of experiments, therefore, were carried out to determine the effect of time of contact of serum with the clot. Freshly obtained beef blood was separated into a number of fractions which were centrifuged and the serum separated from the clot at varying intervals of time. The total calcium and, in one series, the diffusible calcium were determined on these serum fractions. All analyses were performed in duplicate. It took $\frac{3}{4}$ of an hour after the blood was obtained to reach the laboratory and to start centrifuging the first sample. The blood, on reaching the laboratory, was still warm. It was found, as is shown in Table I, that the calcium in the serum is independent of the time of contact with the blood clot.

Effect of Carbon Dioxide Tension on Calcium Distribution.—Theoretically, it is to be expected that diffusible calcium will increase with lowered pH and decrease with increased pH of blood.
### TABLE I.
**Effect on Serum Calcium of Time of Contact of Serum with Clot.**

<table>
<thead>
<tr>
<th>Serum sample No.</th>
<th>Time of centrifuging</th>
<th>Total Ca. (mg. per 100 cc.)</th>
<th>Diffusible Ca. (mg. per 100 cc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.30 a.m.</td>
<td>9.25</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>5.30 p.m.</td>
<td>8.90</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>10.00 a.m.</td>
<td>9.30</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>following day.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9.30 a.m.</td>
<td>9.90</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>1.00 p.m.</td>
<td>9.85</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>4.30 &quot;</td>
<td>9.85</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11.00 a.m.</td>
<td>7.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.00 p.m.</td>
<td>7.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.00 a.m.</td>
<td>7.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>following day.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.00 p.m.</td>
<td>7.65</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE II.
**Effect of pH As Produced by Changes in Carbon Dioxide Tension on Calcium Distribution of Serum.**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment of serum.</th>
<th>pH when put in ultrafiltration sec.</th>
<th>Total Ca. (mg. per 100 cc.)</th>
<th>Diffusible Ca. (mg. per 100 cc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Original serum.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some CO₂ removed by evacuation.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ bubbled into serum.</td>
<td>About 8.0</td>
<td>9.9</td>
<td>3.3</td>
</tr>
<tr>
<td>2</td>
<td>Original serum.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some CO₂ removed by evacuation.</td>
<td></td>
<td>7.2</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>CO₂ bubbled into serum.</td>
<td></td>
<td><em>7.0</em></td>
<td>9.9</td>
</tr>
<tr>
<td>3 A</td>
<td>Original serum.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some CO₂ removed by evacuation.</td>
<td></td>
<td>About 8.0</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>CO₂ bubbled into serum.</td>
<td></td>
<td><em>7.0</em></td>
<td>8.1</td>
</tr>
<tr>
<td>3 B</td>
<td>Original serum.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some CO₂ removed by evacuation.</td>
<td></td>
<td>About 8.0</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>CO₂ bubbled into serum.</td>
<td></td>
<td><em>7.0</em></td>
<td>8.0</td>
</tr>
<tr>
<td>4</td>
<td>CO₂ tension not kept constant.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; kept constant.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>&quot; &quot; not kept constant.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; kept constant.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
serum. However, experiments by von Meysenbug, Pappenheimer, Zucker, and Murray (18) and Neuhausen and Pincus (4) on changing the pH by changing the carbon dioxide tension, showed no appreciable changes in the values of diffusible and non-diffusible calcium. Owing to the theoretical expectations, there is a strong tendency to doubt the results of these authors.

To check for ourselves the effect of pH on the calcium distribution and to make sure that we were justified in neglecting this factor, a number of experiments were carried out on the effect of changes in carbon dioxide tension. The results are summarized in Table II. In Experiments 1 to 3 beef serum was divided into three fractions; one of the fractions was left untreated, CO₂ was bubbled through one portion until the pH was about 7.0, and the third was placed in a vacuum and CO₂ extracted until the pH became about 8.0. The three fractions were then ultrafiltered and the calcium distribution determined. In all the experiments except No. 2, the resulting calcium distribution is independent of the treatment. In Experiment 2 there is a decrease in the diffusible calcium of the evacuated fraction and an increase in the saturated fraction that is outside of the experimental error. This is perhaps due to the evacuation and saturation with carbon dioxide having been carried out to a greater degree in this particular experiment. In Experiment 3 the serum was divided into two portions and then each portion was independently separated into three fractions and treated as described above. The analytical results of the two portions are in very good agreement.

Still another experimental procedure was devised to test the effect of carbon dioxide tension. By the simple procedure of putting some sodium bicarbonate into a phosphate buffer solution of pH 7.4 (0.1 gm. of sodium bicarbonate to 100 cc. of 0.2 M buffer solution), a solution is obtained with a carbon dioxide tension of the same value as that of normal blood. By putting such a solution in the saturating bottle shown in Fig. 1 and connecting to the ultrafiltration apparatus, the filtering serum is maintained at the carbon dioxide tension of the buffer solution throughout the filtration.² Experiments 4 and 5 of Table II give the results of comparing the calcium distribution of pooled human serum

² Because of the simplicity, we are now using such a solution as a regular part of the ultrafiltration procedure.
filtered in connection with the carbon dioxide tension-regulating solution and without. The results show that the same values of diffusible calcium are obtained by either procedure.

The results of Table II substantiate the claim that changes in carbon dioxide tension corresponding to the pH range of about 7 to 8, do not influence the values of diffusible and non-diffusible calcium.

The question arises as to the reason for this seeming independence of calcium distribution on the reaction of the serum. This question for the present cannot be completely or definitely answered. The work of Marrack and Thacker (19) and of Loeb and Nichols (20) indicates, as will be more completely discussed in a following section, that the diffusible calcium, non-diffusible calcium, and the protein content of the serum are functionally related. From the results obtained, it must be concluded that either over the pH range of 7 to 8 there is practically no change in the amount of calcium bound by the serum proteins, or that changes in calcium accompanying changes in reaction are very slow.

Theory of Ultrafiltration.—An understanding of what takes place in the ultrafiltration process is a necessary preliminary to evaluating properly the significance of the values of diffusible calcium obtained by this procedure. Ultrafiltration is an irreversible process and in general there should be a continuous change in the composition of the ultrafiltrate liquid as the colloidal constituents become more and more concentrated. With blood serum, as water and electrolytes are removed by ultrafiltration, the proteins and non-colloidal constituents in combination with the proteins become more concentrated. The ionic strength (21) of the residual solution becomes increased in proportion to the effect of increasing protein. With changes in ionic strength, there are to be expected changes in the equilibrium between diffusible and non-diffusible colloidal constituents.

A number of authors have claimed, and the statement is again brought up by Stewart and Percival (1), that the amount of pressure applied in ultrafiltration influences the value of diffusible calcium obtained by tending to break up labile compounds. This point of view is not well grounded. Since from the thermodynamic standpoint the only influence that pressure can have on equilibrium in aqueous solution is by changes produced in the partial molal volumes of the constituents (21 p. 204), in the light of the
This is a factor that modifies the obtained diffusible calcium from the true diffusible calcium of the serum. From the many studies carried out on the subject, we know that, with the exception of calcium and magnesium, all other ionic constituents are completely or practically completely diffusible. Then it is only because the completely diffusible constituents, chiefly sodium chloride, are present in relatively such large amounts as compared to the non-diffusible constituents and because of the small equivalent concentration of the proteins of the serum, that values of diffusible calcium obtained by ultrafiltration represent practically the true original diffusible calcium.

### TABLE III.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beef.</td>
<td>103.5</td>
<td>109.0</td>
<td>1.055</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>99.5</td>
<td>107.6</td>
<td>1.08</td>
</tr>
<tr>
<td>3</td>
<td>Rabbit.</td>
<td>107.4</td>
<td>117.2</td>
<td>1.09</td>
</tr>
<tr>
<td>4</td>
<td>Sheep.</td>
<td>103.0</td>
<td>111.3</td>
<td>1.08</td>
</tr>
<tr>
<td>5</td>
<td>Beef.</td>
<td>101.9</td>
<td>114.2</td>
<td>1.12</td>
</tr>
<tr>
<td>6*</td>
<td>Pig.</td>
<td>106.0</td>
<td>117.0</td>
<td>1.10</td>
</tr>
<tr>
<td>7*</td>
<td>&quot;</td>
<td>105.5</td>
<td>117.3</td>
<td>1.115</td>
</tr>
<tr>
<td>8*</td>
<td>&quot;</td>
<td>94.8</td>
<td>102.2</td>
<td>1.08</td>
</tr>
</tbody>
</table>

* Data recalculated from Neuhausen and Pincus (4), Table II, p. 102.

Another consideration of importance is the influence of the Donnan equilibrium theory as extended to heterogeneous equilibria by Wilson and Wilson and others (22, 23). According to this development, it is postulated that there is the same spatial arrangement of ionic constituents in the vicinity of a colloidal micelle as there is obtained with a membrane interposed. On this view, ultrafiltration would give in essence the same experimental results small compressibility of such solutions it is obvious that pressures of even several atmospheres can have very little effect on the values of diffusible calcium. Where differences have been claimed as in the low results obtained by Richter-Quittner (5) with a low filtration pressure, the results are probably due to imperfect filtering membranes.
as a membrane distribution experiment, if the amount of ultrafiltrate fluid removed were too minute to produce appreciable changes due to concentration of the colloidal constituents. No experimental evidence is as yet available to test this idea generally. For blood serum from a comparison of the ultrafiltration results of a completely diffusible constituent such as chloride ion, we can make a test as to whether the ultrafiltration process gives results analogous to a membrane equilibrium distribution. Loeb and Nichols (20) have determined the distribution ratios of chloride and calcium in membrane equilibrium experiments with blood serum. Using a saline solution of about the same concentration as is present in blood for the external solution, they obtained an average ratio of chloride in the external fluid to chloride in the serum of 1.10:1. The chloride in ultrafiltrate and serum has been determined in a few instances by Neuhausen and Pincus (4). To obtain more extensive data, we also have made a number of measurements. The Van Slyke method as modified by Wilson and Ball (24), was used by us in making our chloride determinations. The results are given in Table III. All the analytical results for the chlorides in Table III are given in millimols per liter of serum or aqueous solution. Neuhausen and Pincus' values have been recalculated to this unit. No correction is made for the volume occupied by the proteins. It is to be observed that the chloride ratios of ultrafiltrate to serum approximate the membrane equilibrium ratios obtained by Loeb and Nichols. The presumption is then that ultrafiltration results on blood serum are equivalent to membrane distribution figures.

The alternate interpretation would be that the difference in chloride between ultrafiltrate and serum is due to the specific volume and the water of hydration of the serum proteins. But such an interpretation would apply equally to the membrane distribution experiments of Loeb and Nichols (20). Since this interpretation implies that the Donnan effect between serum and aqueous solution is zero, it is highly improbable.

Equilibrium between Diffusible and Non-Diffusible Serum Calcium.—Updegraff, Greenberg, and Clark obtained essentially identical figures for diffusible calcium when there was employed ultrafiltration alone or combined diffusion and ultrafiltration with the filtering sac immersed in water, isotonic sodium chloride, or
calcium-free Ringer's solution. On the basis of these results they concluded that diffusible and non-diffusible calcium varied independently of each other. The published results since then of Marrack and Thacker (19) and Loeb and Nichols (20) show that such a conception is erroneous. Instead, at least qualitatively, the results show that at constant pH, the amount of non-diffusible calcium is determined by the protein content and the height of the diffusible calcium concentration of the blood stream. The reasons for the contrary results of Updegraff, Greenberg, and Clark at present are difficult to place. Imperfect diffusion between inner and outer liquids or slowness of readjustment of equilibrium with changes of the calcium concentrations are possible explanations. The apparent non-effect of changes in carbon-dioxide tension of the value of diffusible calcium also may perhaps be due to a slowness in readjustment of equilibrium. As has already been pointed out, because of these doubtful points, the combined diffusion and ultrafiltration method has been abandoned by us in favor of pure ultrafiltration.

Since the non-diffusible calcium is a function of the protein and diffusible calcium concentration when the pH is constant, it becomes a problem of considerable interest to determine whether the relation between these factors can be fitted to a mathematical expression. Some of the data of Loeb and Nichols (20) we found suitable for the investigation of such a relationship, and we also found that what provisionally we shall call the "ionized" calcium and "protein-bound" calcium are related to each other according to the Langmuir adsorption isotherm (25). To make the matter clear, it is to be noted, as stated by Loeb and Nichols ((20) p. 688), that the factors governing the diffusibility of the blood serum calcium in a dialysis system are the Donnan membrane equilibrium and the formation of calcium protein complex ions. The mathematical relations governing the Donnan membrane equilibrium are only of concern in the present instance for calculating the ionized and protein-bound calcium. It is the relationship between the calcium of the protein complex and the calculated ionic calcium that we are primarily interested in. From the ratio of chloride in the serum to chloride in the aqueous solution, the amount of calcium in the serum to be expected from the Don-
nan distribution can be calculated if the calcium content of the aqueous solution is known. This follows from the equation

\[ [\text{Ca}_s] = r^2[\text{Ca}_aq] \]

in which \([\text{Ca}_s]\) and \([\text{Ca}_aq]\) are the calcium concentrations of serum and aqueous solutions respectively, and \(r\) is the ratio of chloride in aqueous solution to serum. The value of calcium calculated for the serum in this way, we have designated ionic calcium. The figures obtained in this way subtracted from the total calcium of the serum give the protein-bound calcium. Such calculations have been carried out by Loeb and Nichols. However it must be noted that Loeb and Nichols, in their analysis on serum, use the volume of serum as the standard for their calculations without taking into account the volume occupied by the protein of the serum. Using the value of 0.75 for the specific volume of the serum proteins (26), we have recalculated the results of Loeb and Nichols on the basis of millimols per liter of protein-free solution. This correction tends to increase the value of chloride and calcium in the serum. The ratios of chloride in aqueous solution to chloride in serum become less than that obtained without the correction and the calculated ionic calcium from the Donnan distribution is also less. This necessarily increases the values for protein-bound calcium.

It is found if the reciprocal of the ionic calcium is plotted against the protein concentration divided by the protein-bound calcium a straight line is obtained. When the values uncorrected for serum proteins, as calculated by Loeb and Nichols, are so plotted, not nearly so good a fit is obtained. The reciprocal of the concentration of calcium in the aqueous solution can equally well be used in place of the reciprocal of the ionic calcium of the serum. In both cases straight lines are obtained, but of course with different slopes. A plot of this type yields a straight line when Langmuir's adsorption isotherm is obeyed. We are not implying by this that the protein-bound calcium is adsorbed by the serum protein. It is significant, as Hitchcock (27) has shown,

\footnote{We only wish to imply by the term ionic calcium calculated by Equation 1, that it is an approximation to the calcium activity of the serum and therefore mostly ionized in contrast to the protein-bound calcium which presumably is non-ionized.}
that for homogeneous systems the Langmuir equation follows from the law of mass action if one constituent in the system is constant.

The recalculated results of Loeb and Nichols that were found suitable for plotting (the first three sets of experiments of Table I, (20) p. 690) are given in Table IV and the plot obtained is shown in Fig. 2. The points of the data fit two lines, Serums 1 and 3 determining the Curve I, and Serum 2, Curve II. This is perhaps to be expected as with altered ratios of albumin to globulin differ-

Fig. 2. Illustration of Langmuir’s isotherm governing the amount of protein-bound calcium with changes in ionic calcium in serum. Ordinate = $\frac{P}{[Ca]_p}$; abscissa = $\frac{1}{[Ca^{++}]}$. ○, × represent points determining Curve I from Serums 1 and 3, Table IV, respectively. □, △, data of Curve I plotted against reciprocal of calcium of aqueous solution.

ences in the values of protein-bound calcium are to be expected. Curve III is the data of Serums 1 and 3 plotted against the reciprocal of the calcium of the aqueous solution. For Curve I, which is fitted by two experimental series and is therefore the most reliable, we have evaluated the constants of the equation.

The equation is

$$y = 52.6x + 20.4$$
in which \( y = \frac{P}{[Ca]_p} \) and \( x = \frac{1}{[Ca^{++}]} \) where \( P \) represents the protein and \([Ca]_p\) the protein-bound calcium. The units used are gm. per liter for protein and millimols per liter for calcium. In the form of the Langmuir equation, there is obtained

\[
\frac{[Ca]_p}{P} = \frac{[Ca^{++}]}{52.6 + 29.4 [Ca^{++}]} \tag{3}
\]

Either equation shows that as the ionic calcium is increased indefinitely, the limiting value of the protein-bound calcium approaches 0.34 millimol per gm. of protein per liter. This amounts, in a solution containing 7.6 per cent protein to 2.55
millimols per liter, or 10.2 mg. of protein-bound calcium per 100 cc. The limiting value of protein-bound calcium given by Curve II is even less. This shows that in blood serum the limiting amount of calcium that can be bound by the protein, i.e. the non-diffusible calcium, even if the calcium concentration is infinitely increased, is not of much greater magnitude than the non-diffusible calcium already present.

**SUMMARY.**

1. Improvements in the technique of ultrafiltration including a regulating cylinder that automatically maintains a constant filtering pressure and suitable collodion solutions are described.

2. A system of analysis for total calcium, diffusible calcium, inorganic phosphorus, and serum proteins on the serum from 12 cc. of blood or even less is outlined.

3. It is shown that once the blood clot is formed the time of contact of the serum with the clot has no effect on the serum calcium or its distribution for periods of 24 hours or even longer.

4. The influence of change of pH by changing the CO₂ tension on the distribution between diffusible and non-diffusible calcium has been reinvestigated and, between the range of pH 7 to 8, found to have no influence on the distribution in the time allowed for ultrafiltration.

5. The hypothesis that the same spatial arrangement of ions exists in the neighborhood of a charged colloidal particle as would be obtained by a membrane distribution, in which event ultrafiltration should give results equivalent to a membrane distribution, has been experimentally tested. From analysis of chloride it has been found that practically the same ratios of chloride in filtrate to chloride in serum are obtained by ultrafiltration as by membrane distribution, which favors the spatial arrangement hypothesis.

6. From the data of Loeb and Nichols (20) it has been shown that at constant pH and protein content, the distribution of protein-bound and ionic calcium, with increasing calcium concentration, conforms to the Langmuir adsorption isotherm. From this equation it is seen that the limiting value of protein-bound calcium when the ionic calcium approaches infinity is of the same order as is present in normal blood serum.
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David M. Greenberg and Lewis Gunther


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