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UNIVERSITY OF LOUISVILLE

A STUDY OF THE DIFFUSIBILITY AND NON-DIFFUSIBILITY
OF MAGNESIUM AND CALCIUM IN NORMAL BLOOD SERA.

A Dissertation

Submitted to the Faculty

Of the Graduate School of the University of Louisville

In Partial Fulfillment of the

Requirements for the Degree

Of Master of Science

Department of Chemistry

By

LOUIS J. HALLE

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1931

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HISTORICAL

Investigation of the literature, concerning the diffusibility of various inorganic constituents of blood serum, shows definitely that potassium, sodium, phosphate and chlorine are wholly diffusible (1,2) through a collodion sac. However, it has been found that magnesium and calcium are not entirely diffusible. Very little experimental work has been done to determine the condition of magnesium in blood serum. Just how much is diffusible and just how the non-diffusible magnesium is combined in the blood serum has been left almost untouched experimentally. In 1920, Cushny (2) working on 2 samples of ox-serum, filtered through a membrane 47% and 54% of magnesium. Bernhard and Beaver (3) in 1926, employed electro-dialysis and noted that magnesium was 42% ionizable. Recently, Stary and Winternitz (4) found that in human and horse serum, magnesium was about 81% diffusible. The ultrafiltration procedure was done according to Zsigmondy using filter paper saturated with a 10% glacial acetic acid-collodion solution.

As to the state of calcium in blood serum many results have been recorded. It has been found (5,6) that disorders of calcium metabolism

are not always indicated by changes in the calcium content of blood or other body fluids. Studies, hence, have been made by numerous investigators of the condition of calcium in blood serum in order to throw some light on the means by which the assimilation and utilization of calcium is accomplished. Calcium is found in the blood serum, both in a diffusible form and also a non-diffusible form, which is said to be bound to the serum proteins. The pioneer experimenters in this field were Rona and Takahashi (7) who in 1911, using compensation dialysis found that 60-75% of the calcium in horse, pig, and cow serum was capable of diffusing through a membrane. Von Meysenbug (8) and his co-workers using parchment membranes and dialyzing against calcium-free Ringer's solution, noted that a progressive diffusion of calcium resulted in 5 to 7 days, and that 90% of the calcium had diffused out. Richter and Quittner (9) later employed a patented Zsigmondy-Bachman filter. They obtained 5 to 7%, unusually low values for filterable calcium. In 1920, Cushny (2) using relatively large quantities filtered ox-serum through collodion membranes under 150 mm. of mercury pressure. He obtained results which corroborated those of Rona and Takahashi. (7) Neuhausen and Pincus (1) also employed ultrafiltration

and stated that 50-70% of calcium in pig serum was diffusible. In their work 30 c.c. of serum was put in a sac and filtered at pressures varying from 120-180 mm. of mercury. As Moritz (10) pointed out in his experimentation, the methods employed by Cushny, (2) and Neuhausen and Pincus, (1) could not be used on small quantities of blood serum. Moritz, therefore, developed a micro method for determining diffusible calcium, employing a combination of ultrafiltration and diffusion. In this method, a sac containing 2 c.c. of serum was immersed in distilled water and then negative pressure was applied. Updegraff, Greenberg, and Clark (11) introduced slight changes in the latter's procedure. Their determinations showed that calcium in human blood serum was from 45 to 55% in the diffusible form. Both the ultrafiltration results with no water outside of the sac, and the combination, dialysis-ultrafiltration results with water or a isotonic salt solution outside of the sac, checked closely. Recently, Greenberg and Gunther (12) simplified their ultrafiltration procedure by using a regulating cylinder that automatically maintained a constant filtering pressure. Greenberg and Gunther abandoned the combination of ultrafiltration and diffusion to eliminate the possible changes in equilibrium between

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the diffusible and the non-diffusible calcium of the serum. Their procedure produced similar results as obtained by Updegraff, Greenberg and Clark. (11)

The purpose of this investigation was to determine the distribution of the diffusible and non-diffusible magnesium in human, beef, sheep and hog blood serum. Since it is necessary as a matter of procedure to remove the calcium before determining the magnesium, it was thought that a check on the previous calcium determinations reported in the literature might well be recorded; so as to show that the ultrafiltration apparatus was operating efficiently. The effect, of allowing blood to stand in the refrigerator for a period of time, on the total and the diffusible magnesium and calcium in blood serum was also recorded.

DESCRIPTION OF ULTRAFILTRATION APPARATUS

To determine the diffusible magnesium and also calcium, the ultrafiltration procedure as described by Greenberg and Gunther (12) is used. The diagram (Figure A) of the ultrafiltration apparatus with an automatic pressure-regulating cylinder is essentially the same as reported by them. The regulating valve is made as follows: A glass cylinder (a) of 200 mm. in height is fitted with a 3 hole rubber stopper; 2 bent glass (b,c) tubes, as shown are used to

Figure A. ULTRAFILTRATION APPARATUS

connect between the suction pump and the rest of the apparatus with rubber tubing; a third straight piece of capillary tubing (d) of about one 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 mercury below atmospheric pressure, when suction is employed, if the regulating tube is accurately set. It is also desirable to have a wash bottle (e) between the regulating cylinder and the water pump to trap any mercury which splashes over, or any water that is sucked back from the tube (f) leading from the water pump. In order to measure the amount of ultrafiltration the apparatus is fitted with a Folin-sugar tube (h) having a constricted portion of 5 c.c. capacity at the bottom. Also the glass tubing (i), connecting the parlodion sac with the outside air, is connected to a flask (j) containing distilled water to keep the air saturated with water vapor, and thus prevent evaporation. No attempt was made to control the pressure of carbon dioxide, as it has been demonstrated by Von Meysenbug (8) and Neuhausen and Pincus (1) that the carbon dioxide

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tension is not an important factor in this connection. Greenberg and Gunther (12) also investigated the influence of change of pH by changing the carbon dioxide tension on the distribution between diffusible and non-diffusible calcium. They found that between the range of pH 7 to 8 there was no influence on diffusibility in the short time allowed for ultrafiltration.

PREPARATION OF PARLODION SAC

The preparation of the parlodion sac requires utmost care in order to obtain correct ultrafiltration results. The parlodion solution used, is made up of 7 grams Du Pont's parlodion in 60 c.c. of ether and 40 c.c. of absolute alcohol, to which is added 5 c.c. of ethylene glycol. This solution is completely permeable to magnesium and calcium and holds back the serum proteins completely. The sacs are cast as described by Updegraff, Greenberg and Clark. (11) In making a membrane a pyrex glass tube, 7 cm. long and having an inside diameter of 14 mm. at the open end and about 9 mm. at the closed end, is used. It is important that the closed end be well rounded off and the glass well annealed and free from frosting, otherwise the membranes may be granulated and frosted

at this point. The tube is filled to the top by letting a stream of the parlodion solution flow in, care being taken that no air bubbles collect along the sides. The tube is then inverted on the neck of an Erlenmeyer flask and rotated rapidly in a slanting position until drops begin to fall quite slowly. The operation is repeated and then the sac allowed to dry for about 10 minutes with the open end of the tube down. The tube is then immersed in cool distilled water. It has been pointed out, by Nelson and Morgan (13) and Pierce (14), that a very important condition favoring high permeability is the immersion of the membrane in water as soon as the point of jellification is reached. If placed in the water before jellification the phenomenon known as "blushing" or whitening of the film is produced (13). The sac is then detached by means of a pair of forceps from one side of the upper end of the tube and gently a stream of water from a wash bottle is forced down between the wall of the tube and the sac. This gradually separates the sac from the tube and ultimately the sac comes out whole. A convenient way to test for leaks, also described by Updegraff, Greenberg and Clark (11), is

to fill the sac with water and push it on a No. 0 one hole rubber stopper in which a glass tube is inserted. After drying the outside of the sac with filter paper, any minute leaks can be detected by blowing into the tube while holding the sac securely on the stopper. The sacs are usually stored in a physiological saline solution, and placed in the refrigerator until required for use.

ULTRAFILTRATION PROCEDURE

To carry out a diffusibility determination, a parlodion sac is taken from the storage jar and carefully dried inside and out with filter paper. Then 3 to 4 c.c. of serum are introduced into the sac with the aid of a pipette. The sac is then slowly worked on to the stopper (k) and is tied with a stout cotton thread. To make it leak proof, the sac is further sealed to the stopper by painting it with a little collodion solution. The Folin tube is then fitted tightly on to the apparatus by a rubber stopper (l). To start the ultrafiltration the rubber tube leading from the water pump is connected to the apparatus by the bent glass tube (b). The water is then turned on until a slight bubbling of the mercury is seen in the regulating tube. After

the first few minutes, the ultrafiltration is stopped in order to wipe the few drops appearing on the outside of the sac. This represents the displaced capillary water of the parlodion sac which otherwise would dilute the ultrafiltrate and bring on lower results for diffusible magnesium and calcium. The ultrafiltration is allowed to go on for 3 hours. In that time approximately half the serum introduced into the sac is ultrafiltered into the Folin sugar tube. Then using a micro-burette, graduated in 0.02 c.c., an amount of distilled water is added up to the 5 c.c. capacity mark. This accurately gives the amount of ultrafiltrate that has come through. Then, 4 c.c. of the fluid containing the ultrafiltrate are measured into a 15 c.c. centrifuge tube and the regular procedure is followed as for the total magnesium and calcium in blood serum, except that no further water is added when precipitating the calcium.

ANALYTICAL METHODS

In order to determine the magnesium in blood serum, the usual manner of first removing the calcium as an oxalate is employed. The calcium is determined according to Clark and Collip's (15) modification of the Kramer-Tisdall method. The magnesium is determined from the supernatant fluid of the calcium

oxalate precipitate by using a combination of Cohen's (16) and Denis' (17) method. Using Denis' method of precipitating magnesium as magnesium ammonium phosphate, low results were obtained at first. It might be mentioned that Weaver and Reed (18) also obtained low magnesium results and also had to modify the method of Denis. However, Cohen's method of precipitation gave better results. The following is a detailed description of the procedure used.

Determination of calcium:

About 12 to 15 c.c. of whole blood is obtained and immediately centrifuged at 2,000 revolutions per minute for 20 minutes in order to separate the serum from the plasma. Then 2 c.c. of the serum is used to determine the total magnesium and calcium, and the remainder is used to determine their diffusibility. Into a 15 c.c. centrifuge tube, 2 c.c. of the clear serum is carefully introduced with the aid of a pipette, then 2 c.c. of distilled water and 1 c.c. of 4 percent ammonium oxalate solution are added and the tube thoroughly mixed. The centrifuge tube should have an outside diameter of 6-7 mm. at the 0.1 c.c. mark. Mixing is aided by holding the tube at the mouth and giving it a circular motion by tapping the lower end. The tube is then allowed to stand for about 1 hour to insure complete precipitation. The

contents are again mixed and then centrifuged for about 5 minutes at 1500 revolutions per minute. The supernatant fluid is carefully poured off and while the tube is still inverted it is allowed to drain in a rack for 5 minutes, resting the mouth of the tube on a piece of filter paper. The supernatant fluid containing the magnesium is set aside for a time and the calcium determination continued. The mouth of the tube containing the calcium precipitate is then wiped dry with a soft cloth. The precipitate is stirred up and the sides of the tube are washed with 3 c.c. of dilute ammonia directed in a very fine stream from a pipette. The suspension is centrifuged and drained again as before. Now, 2 c.c. of approximately normal sulphuric acid is added, this being blown from a pipette directly upon the precipitate so as to break up the mat and facilitate solution. The tube is placed in a boiling water bath for about 1 minute. Then it is titrated immediately with 0.01 normal potassium permanganate to a definite pink color which persists for at least 1 minute. A micro-burette graduated in 0.02 c.c. is used for the titration.

Calculations:

Since 1 c.c. of 0.01 N potassium permanganate is equivalent to 0.2 mg. calcium, the number of mg. of

calcium per 100 c.c. of serum therefore equals

$$\frac{100 \times 0.2 \times X}{2}$$

From X, which is the number of cubic centimeters of permanganate required, subtract the number of cubic centimeters of permanganate required to titrate 2 c.c. of the sulphuric acid to the usual end point, before making the calculation.

The total calcium and diffusible calcium obtained from specimens of (a) human, (b) beef, (c) sheep, (d) hog blood serum are shown in table 1.

Determination of Magnesium:

To determine magnesium, 4 c.c. of the supernatant fluid, free from calcium, is measured out by pipette. To this is added 2 c.c. of a mixture containing 1 volume of a 2 percent aqueous solution of acid ammonium phosphate and 2 volumes of concentrated ammonium hydroxide. After thoroughly mixing the contents the tube is allowed to stand over night. The next day the tube is centrifuged at 3,000 revolutions per minute for 10 minutes. By careful decantation the supernatant fluid is removed without disturbing the precipitate of magnesium ammonium phosphate. The precipitate is then disturbed by gently tapping the lower end of the tube and washed by carefully pouring down the sides of the tube, 5 c.c. of a mixture containing 2 volumes of absolute alcohol and 1 volume of concentrated ammonium hydroxide. The tube is then

again centrifuged at 3,000 revolutions per minute for 7 minutes. This operation is repeated twice. After the third washing the supernatant fluid is removed by careful decantation and the tube containing the precipitate is allowed to stand in a warm place until the ammonia has evaporated. From this point the procedure of Denis is followed. The precipitate of magnesium ammonium phosphate is dissolved in 10 c.c. of 0.1 N hydrochloric acid and transferred to a 25 c.c. volumetric flask, in order to continue the colorimetric determination. As a standard, 10 c.c. of a solution of magnesium ammonium phosphate (containing .02 mg. magnesium in 10 c.c.) in 0.1 N hydrochloric acid, is introduced into another 25 c.c. volumetric flask. To each flask there is added 2 c.c. of a 2.5 percent solution of ammonium molybdate in water and then 1 c.c. of 0.25 percent aminonaphtholsulphonic acid solution. The contents are mixed and allowed to stand for 5 minutes, and then compared in a colorimeter.

Calculations:

Since 4 c.c. of the 5 c.c. of the supernatant fluid obtained from 2 c.c. of serum is used this will correspond to 1.6 c.c. of serum. As the standard contains .02 mg. of magnesium thus, mg. of magnesium per 100 c.c. of serum equals $\frac{\text{Reading of Standard} \times .02 \times 100}{\text{Reading of Unknown}}$

The total magnesium and diffusible magnesium obtained

from specimens of (a) human, (b) beef, (c) sheep, (d) hog blood serum are shown in Table 2.

In Table 3 and 4 there is recorded, the effect of contact of blood serum and plasma while standing in an air-tight jar in the refrigerator. Successive determinations show that there is no alteration in the total or diffusible calcium in blood serum. Greenberg and Gunther (12) in their work, also found no change in the calcium content. However, magnesium shows a definite alteration in both the total and diffusible content in blood serum. This change in concentration might be attributed to the fact that magnesium is almost equally distributed between the serum and cells with, if anything, a slight excess in the corpuscles. The concentration of magnesium in the blood cells varies from 2.3-4 mg. per 100 c.c. (20). Thus on standing, there is a change in the equilibrium which favors the magnesium in the blood serum. Kramer and Tisdall (21) account for the stability of calcium in that it is found almost, if not quite exclusively in the serum. All this tends to show that the separation of serum from blood plasma, for magnesium determinations, must be made as soon as possible.

TABLE I
(a) HUMAN BLOOD SERUM

Total Calcium mg.	Diffusible Calcium mg.	% Diffusible Calcium
10.1	4.94	49.6
10.26	5.25	51.2
9.8	4.74	48.4
10.06	4.76	47.4
10.3	5.56	54.06
9.7	4.82	49.7
10.16	5.34	52.6
9.5	4.63	48.8
9.9	4.87	49.2
10.6	5.41	53.6
10.2	5.32	52.2
10.04	5.1	50.8
10.4	5.86	56.4
9.4	4.35	46.3

TABLE I
(b) BEEF BLOOD SERUM

Total Calcium	Diffusible Calcium	% Diffusible Calcium
10.26	5.99	58.4
10.3	5.90	57.3
10.7	7.10	66.4
10.46	6.32	59.7
10.36	6.44	62.2
9.9	6.0	60.7
10.2	6.57	64.5
10.3	6.35	61.7
11.1	6.68	60.2
10.4	5.86	56.4
9.72	5.42	55.8
10.2	5.98	58.7
10.5	6.59	62.8

TABLE I

(c) SHEEP BLOOD SERUM

Total Calcium	Diffusible Calcium	% Diffusible Calcium
11.64	6.2	53.3
11.7	6.11	52.3
11.68	6.33	54.2
11.5	5.72	49.8
11.76	6.18	52.6

(d) HOG BLOOD SERUM

Total Calcium	Diffusible Calcium	% Diffusible Calcium
10.46	6.57	62.9
9.8	5.66	57.8
10.1	5.87	58.2
10.2	6.23	61.1

TABLE 2

(a) HUMAN BLOOD SERUM

Total Magnesium	Diffusible Magnesium	% Diffusible Magnesium
2.4	1.99	83.0
2.37	1.88	79.6
2.2	1.76	80.2
2.1	1.68	80.3
1.9	1.51	79.7
2.25	1.82	81.0
2.51	2.06	82.6
2.62	1.03	77.8

TABLE 2
(b) BEEF BLOOD SERUM

Total Magnesium	Diffusible Magnesium	% Diffusible Magnesium
2.1	1.73	82.6
1.9	1.52	80.4
2.06	1.7	82.7
1.97	1.56	79.6
2.2	1.62	80.7
2.16	1.64	76.3

TABLE 2

(c) SHEEP BLOOD SERUM

Total Magnesium	Diffusible Magnesium	% Diffusible Magnesium
2.14	1.7	79.8
2.4	1.86	77.5
2.1	1.64	78.2
1.86	1.38	74.4
1.94	1.41	72.7

(d) HOG BLOOD SERUM

Total Magnesium	Diffusible Magnesium	% Diffusible Magnesium
2.4	1.91	79.6
2.34	1.75	74.8
2.38	1.86	78.4
2.51	2.0	80.2

TABLE 3

THE EFFECT OF CONTACT OF SERUM AND

PLASMA ON CALCIUM

(a) HOG BLOOD SERUM

	Total Ca.	Diffusible Ca.	% Diffusible Ca.
First			
Day	10.1	5.87	58.2
Second			
Day	10.1	5.85	57.9
Third			
Day	10.1	5.87	58.2
Fourth			
Day	10.1	5.88	58.7

(b) BEEF BLOOD SERUM

	Total Ca.	Diffusible Ca.	% Diffusible Ca.
First			
Day	9.9	5.94	60.2
Second			
Day	9.9	6.06	61.3
Third			
Day	10.08	6.08	60.5
Fourth			
Day	9.9	6.0	60.7

TABLE 4
THE EFFECT OF CONTACT OF SERUM AND
PLASMA ON MAGNESIUM

(a) HOG BLOOD SERUM

	Total Mg.	Diffusible Mg.	% Diffusible Mg.
First			
Day	2.38	1.86	78.4
Second			
Day	2.64	2.11	80.2
Third			
Day	2.9	2.47	85.3

(b) BEEF BLOOD SERUM

	Total Mg.	Diffusible Mg.	% Diffusible Mg.
First			
Day	2.2	1.62	80.7
Second			
Day	2.76	2.29	83.2
Third			
Day	2.93	2.53	86.4

DISCUSSION

Ultrafiltration as employed in this investigation offers a simple means of determining the condition of magnesium in blood serum. However, ultrafiltration, and also dialysis, has its disadvantages in that neither method gives the actual ionized and non-ionized magnesium or calcium present in blood plasma. As Stewart and Percival (19) point out, the method of dialysis used by Moritz (10), and Updegraff and her co-workers (11), can give results which may approximate the true results, if the time required for ultrafiltration is short and the rate of adjustment of the equilibrium within the membrane is small. They cannot, however, give the true results.

Similar objections may be raised against the method of compensatory dialysis as proposed by Ven Meysenbug and his co-workers (8), for compensation, to be complete, must take account not only of magnesium and calcium, but of all other diffusible substances as well. However, the various types of diffusions employed give readily comparative values of the amount of substance that is diffusible through a membrane. Stary and Winternitz (4) obtained almost the same results for diffusible magnesium using ultrafiltration, as they did by using compensation

dialysis. Furthermore, consideration has to be given, not only to the varying permeability of the membranes, but also to the pressure applied when the filtration is carried out. Richter and Quittner (9) using very low pressure found that only 5-7% of the calcium filtered. Very high pressures are objectionable, for apart from the technical difficulty of obtaining a protein free filtrate, they tend to destroy any labile compounds between the protein and the inorganic ions.

It is of paramount importance to know that the ultrafiltration method used is correct, in order to show that the results of this experiment on magnesium are valid. It is gratifying to note that the diffusible calcium of pig serum obtained in these determinations agree with that of Neuhausen and Pincus (1) who state that on the average, 60% is in a diffusible form. Likewise the diffusible calcium results of beef serum check with the results of Greenberg and Gunther (12) who record that 57% is diffusible. The diffusible calcium determinations of human blood agree with those of Snell and Wales (6) who point out that normally 45-55% of the serum calcium is diffusible. The results also check with the work of Greenberg and Gunther (12), Updegraff, Greenberg and Clark (11), and also with

that of Neuhausen and Pincus (1). It is also to be noted that the data in Table 1a on the diffusible calcium of human serum agree closely with the results of analysis of calcium in cerebrospinal fluid. Halverson and Bergeim (22), Pincus and Kramer (23), and Meritt and Bauer (24) report that spinal fluid calcium averages 5 mg. per 100 c.c., which is approximately one-half the normal calcium concentration of blood serum. The calcium content of the cerebrospinal fluid is considered by Cameron and Moorhouse (25) as an accurate physiological representation of the diffusible calcium in blood serum, assuming that the spinal fluid is protein free. However, it cannot be said definitely that the remaining non-diffusible calcium in blood serum is bound to serum proteins. The data of the spinal fluid calcium only substantiates it to some extent, as it is not definitely known whether the spinal fluid is absolutely protein free (11). Salvesen and Linder (5) show, in cases of nephritis, that the decrease in the concentration of calcium in serum parallels the decrease in the concentration of protein. Greenwald (26) maintains that some of the calcium is bound to an organic substance in a citrate like combination.

Though the evidence points more towards the idea that part of the calcium is protein bound, further investigation is needed to yield a more complete theoretical interpretation.

Examination of Table 2 shows that diffusible magnesium of human serum is in agreement with the results of Stary and Winternitz (4) who record that actually 81% is in a diffusible form. This result does not take into consideration the volume correction which they bring in. It is interesting to point out that the magnesium content of the cerebrospinal fluid is 20-30% higher than the total magnesium content in blood serum (16). The diffusible magnesium, thus does not seem to bear the similar relationship to the spinal fluid content as is shown by the diffusible calcium. More direct investigation is needed to explain in what manner the non-diffusible magnesium is tied up in blood serum.

SUMMARY

- 1- It has been shown that magnesium is diffusible from 72 to 84 percent in human, beef, sheep and hog blood serum.
- 2- Corroboration, of the diffusibility of calcium in human, beef and hog blood serum recorded by various experimenters, is offered.
- 3- Calcium in sheep blood serum has been found to be about 52 percent diffusible.
- 4- It has been shown that blood on standing in the refrigerator has a decided effect on the total and diffusible content of magnesium in beef and hog blood serum. There is no effect, however, on the serum calcium or its distribution.

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