TECHNICAL METHODS

A SIMPLE APPARATUS FOR THE ESTIMATION OF THE DIFFUSIBLE FRACTION OF CALCIUM IN SERUM

BY

GWENDOLINE I. M. CARLIER

From the Skin Hospital, Birmingham

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This is a modification of the method of Updegraff, Greenberg, and Clark (1926) which depends upon the diffusion under reduced pressure of calcium ions in serum through a collodion sac into water. The calcium in the diffusate is estimated by the method of Clark and Collip (1925) and a correction applied for the diffusible ions which remain in the sac on the establishment of equilibrium.

Apparatus

The following apparatus is required: a centrifuge tube, graduated in 1/10 ml.; a shortened Folin-Wu blood-sugar tube with the bulb filed off; two corks, accurately bored, soaked in paraffin wax; a tube of narrow bore, fitted with a rubber collar; a bent tube inserted in the lateral hole of the top cork; a collodion sac stored at 4°C. in normal saline; sewing-cotton, No. 24, 36 or 50, but no finer, or it cuts the sac; a filter-pump, preferably fitted with a non-return valve.

Technique

A sac is removed from storage, dried outside with a soft cloth and inside with a throat-swab. Then 2 ml. of serum is pipetted into the dry sac, which is wiped with the swab at the open end to remove any trace of serum. This is important.

The sac is then firmly tied over the rubber collar with a reef-knot.

The tube D (Fig. 1) with its attachments is inserted into the centrifuge tube, containing 5 ml. of distilled water, and the serum and water levels provisionally adjusted.

The tap is then cautiously turned on and the pressure raised to 120 mm. Hg or more, according to the strength of the sac.

Diffusion at this pressure is allowed to proceed for five hours. As the water level rises, it becomes necessary to raise tube B to readjust the meniscus of the serum to that of the water from time to time. If the corks are efficiently waxed this does not break the vacuum.

The tube C is connected to the vacuum-pump via an empty wash-bottle (to form an "air-cushion" in case the water pressure varies) and a manometer.

It is advisable to saturate the air entering at A by passing it through a water bottle to avoid evaporation.

After the five hours tube C is loosened and slowly withdrawn before the tap is turned off.

Tube D is disconnected *at once* (to prevent any osmosis) and the end of the sac drained against the mouth of the centrifuge tube. The water level is read on the scale and recorded. It is much easier to estimate the volume retained in the sac in this way than by direct measurement of the contents, which might involve rupture of the sac.

The sacs can be used over and over again for months if they are carefully untied, washed out under running water, and stored as described.

The calcium in the diffusate is estimated by the Clark and Collip method, using the measured volume of distilled water in a similar centrifuge tube as a blank.

Collodion Sacs

Dry nitroxyline cannot be sent by post, so the collodion supplied by the makers already contains an unknown amount of solvent.

About 15 g. of B.D.H. collodion is dissolved in 15 ml. of ethanol. About 80 ml. of ether is added and mixed in a wide-mouthed conical flask, tightly corked over tinfoil, and incubated at blood-heat for several days, with occasional stirring, until a homogeneous sol is obtained, free from bubbles. More ether can be added if the sol is too viscous to pour.

The sol is poured into two 3 in. $\times$ $\frac{7}{8}$ in. (internal diameter) tubes, which must be filled to the top, centrifuged at 1,500 r.p.m. for two minutes, *slowly* inverted with brisk rotation, and drained upside down for 10 minutes. A second coating is applied by refilling, centrifuging again for two minutes, and draining for not less than 20. Exposure to the air during draining governs the fineness of the pores. The tubes are then completely immersed in cold water to toughen the membrane. Beware of little bubbles at this stage. The tubes must be left under water long enough to impart the necessary toughness—at least half an hour. All-night soaking does them no harm. The sacs are withdrawn from the tubes under water on the tip of the little finger, inspected for mechanical leaks, and tested for correct permeability after cutting with scissors to a suitable length.

FIG. 1.—The apparatus described. A = air-inlet, B = piston-tube, C = to manometer and vacuum-pump, D = shortened Folin-Wu blood sugar tube, E = graduated centrifuge tube, F = collar made from 0.5 cm. rubber tubing, G = cotton, H = collodion sac, I = serum, J = water.

Test for Leakage.—The outside is dried with a soft cloth, the sac nearly filled with water, gently blown into, and the outside inspected for droplets.

Permeability Tests and Controls.—Since the permeability of the sac is the vital point in these estimations, every new sac should be tested against normal serum to ascertain that it is (a) impermeable to protein, and (b) porous enough not to hinder the transmission of the diffusible calcium ions.

Serum, 2 ml., is pipetted into the dried sac, which is then "run" under analytical conditions for not less than four hours. The Clark and Collip analysis is then begun and a biuret test carried out on the first centrifugate. Unless this is negative the sac is too porous and must be discarded. By the above process (Hatschek, 1920; Ostwald, 1920; Ostwald, 1917) this seldom happens, and the sacs are usually retentive enough to withhold the protein-calcium complex and to transmit only the diffusible ions at negative pressures up to 180 mm. Hg.

But a sac can be too retentive. That low results are suspect was indicated by Updegraff *et al.* (1926). As a matter of experience it is a simple matter to recognize a sac that is faulty by the slow rising of the water level in the centrifuge tube. Any sac that transmits less than 0.5 ml. in five hours should be regarded with suspicion. If the sac at this stage appears neither too porous nor too retentive the analysis should be completed. If the result lies within the normal range of 4.1 to 5.8 mg. per 100 ml. the sac can be taken as satisfactory. A further control can be applied by estimating the total calcium on a second sample of the same serum and calculating the diffusible calcium as a percentage of that. Moritz

(1925) established that the diffusible fraction varied between 45% and 55% of the total calcium in normal sera.

Correction for the Permeability of Individual Sacs.—Within the desirable limits of permeability some sacs allow of more diffusion than others in the five-hour run. The most serviceable sacs are those which transmit between 0.6 and 1.0 ml. in five hours. This makes no difference to the end-result so long as a correction for the amount of diffusible calcium still in the sac at the end of the experiment is applied. To make the correction it is essential to know the volume of liquid inside the sac. This can be arrived at by measuring the volume that has diffused, subtracting this from the original 2 ml., and then subtracting the volume occupied by the serum-protein content of 2 ml. For normal sera the protein volume can be assumed to be 0.1 ml. per 2 ml. serum, but where there is any reason to suspect an abnormal serum protein content this must, of course, be estimated and the correction for the volume it occupies calculated from the percentage so determined.

Specimen Calculations

Data as Ascertained	Patient A	Patient B
Ca in diffusate, by titration ..	5.7 mg. %	5.3 mg. %
.. Ca per 2 ml. of diffusate ..	0.114 mg.	0.106 mg.
Water at outset ..	5.0 ml.	5.0 ml.
Serum at outset ..	2.0 "	2.0 "
Water volume after diffusion ..	6.1 "	5.6 "
.. Volume diffused ..	1.1 "	0.6 "
Volume retained ..	0.9 "	1.4 "
Calculation of Correct Percentage		
Ca in each external ml. ..	$\frac{0.114}{6.1}$	$\frac{0.106}{5.6}$
	=0.0187 mg.	=0.0189 mg.
Volume retained ..	0.9 ml.	1.4 ml.
Minus volume of protein in 2 ml. serum ..	0.1 "	0.1 "
.. True volume ..	0.8 "	1.3 "
.. Diffusible Ca in sac ..	0.0187×0.8	0.0189×1.3
	=0.015 mg.	=0.0246 mg.
.. Diffusible Ca in 2 ml. ..	$0.114 + 0.015$	$0.106 + 0.0246$
	=0.129 mg.	=0.1306 mg.
.. Diffusible Ca in 100 ml. ..	$\frac{12.9}{2}$	$\frac{13.06}{2}$
	=6.45 mg.	=6.53 mg.

If high accuracy is not required, the result, corrected to ± 0.1 mg., may be read off from Fig. 2.

Effects of Variations

Temperature.—Moritz (1925) found that sera left at room temperature for 48 hours yielded higher results than fresh sera or sera stored for the same time in a refrigerator. His experimental error for the diffusible calcium estimated on rabbit serum is of the order of ± 0.3 mg./100 ml. for sera stored under refrigeration up to 48 hours as against the fresh sample and is considerably lower for sera similarly stored for half the time. For this reason all sera for diffusible calcium estimation should be kept under conditions that are carefully standardized.

Laboratory temperature is not without influence on the texture of the collodion sacs. Working

FIG. 2.—Correction chart. The corrected percentage is found by following the diagonal line from the point of intersection of the ordinate and abscissa (as experimentally determined) to the right-hand column, where the result is read off.

a continental climate, Updegraff discovered that sacs made on a hot day tended to be unduly retentive. Here, efficient central-heating is more to be feared. Sacs made in chilly weather and stored as described will generally be found to conform to the requisite permeability range when they are tested before being used.

Pressure.—No attempt was made to control the CO_2 pressure by Updegraff and her team or by

me, in view of the findings of von Meysenbug, Pappenheimer, Zucker, and Murray (1921).

Small variations in the Hg pressure, as controlled by the devices specified above, produce no noticeable effect. A sharp increase in water pressure ruptures the sac.

Time.—Variations in the time factor were measured by Moritz (1925), who showed that the calcium in the diffusate reached a maximum in about four hours.

Experimental Error

Duplicate estimations using (a) two different sacs of satisfactory, but not identical, permeability, and (b) the same sac on two successive days, have been found to agree within ± 0.25 mg./100 ml. in this laboratory.

Application

As the main application of the method here is in the biochemical control of vitamin D therapy, and as biochemical toxæmia, according to Dawson and Dolby (1947; personal communication), supervenes at a concentration of 7.5 mg. diffusible calcium per 100 ml. serum (an increase over normal which is well beyond the experimental error), the method has proved useful in forestalling possible abnormal calcification. Our experience of the estimation in this hospital confirms the findings of these two authors, and our results are in close agreement with theirs.

Summary

A convenient routine method for the estimation of the diffusible fraction of calcium is described. A time-saving correction chart is included for use in routine clinical determinations.

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