XLVII. THE DETERMINATION OF CALCIUM IN BLOOD-SERUM AND CEREBROSPINAL FLUID.

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THE method for the estimation of calcium in general use is that of Kramer and Tisdall [1921] as modified by Clark and Collip [1925], depending on the direct precipitation of the serum-calcium as oxalate and titration of the latter with potassium permanganate.

Although this method has the advantage of simplicity the estimation cannot be made on fatty or opaque sera. This, coupled with the fact that in all specimens the calcium oxalate precipitates char on ignition, suggests that variations in the KMnO_4 titration may be partially due to the amount of organic matter adsorbed by the precipitate. Hirth and Klotz [1923] compared three sera by KMnO_4 and acidimetric methods, obtaining an average difference of 0.6 mg./100 cc., which they attributed to the titration by the KMnO_4 of organic matter. Later, Stanford and Wheatley [1925], by a similar comparison, obtained values differing by 4 %, which they attributed to personal error, their absolute recoveries by the acidimetric method being 3–7 % lower than by the KMnO_4 method.

There is, moreover, some doubt as to whether all the non-diffusible calcium, found by von Meysenbug *et al.* [1921] to form 30–40 % of the total serum-calcium, is precipitated; Van Slyke and Sendroy [1929] obtained 5–15 % more calcium when precipitated from deproteinised serum than when precipitated directly.

It is also questionable whether, in view of these difficulties, the clinical significance frequently attached to minor variations in serum-calcium, of the order of 0.3 mg./100 cc. as estimated by the Clark and Collip method, is justified.

In the endeavour to devise a procedure that will avoid these difficulties and give figures more approaching absolute values, the following methods have been investigated and compared.

(1) The Clark and Collip procedure and the incineration method of Trevan and Bainbridge [1926], *i.e.* the direct KMnO_4 titration as compared with the acidimetric titration of the ignited calcium oxalate precipitate.

(2) The Clark and Collip procedure and the method of Halverson and Bergeim [1917] as modified by Van Slyke, in the latter case deproteinising with acid of such strength that all the calcium is brought into solution and subsequently precipitating the calcium as oxalate and titrating with KMnO_4 .

(3) The techniques for purification by washing of the calcium oxalate precipitate of Clark and Collip [1925], Halverson and Bergeim [1917] (as modified by Van Slyke and Sendroy [1929]), and Stanford and Wheatley [1925], since it is to the washing technique that the maximum error (± 3 %) of the Clark and Collip method is usually attributed. Clark and Collip require one washing with 2 % NH_4OH with draining by inversion of the tube; Halverson and Bergeim

require two washings with 2 % NH_4OH , withdrawing the supernatant fluid by suction; Stanford and Wheatley require two washings with saturated calcium oxalate solution.

(1) *Comparison of the Clark and Collip and Trevan and Bainbridge methods.*

For the Trevan and Bainbridge estimations, the precipitates were dissolved in $N/100 \text{ H}_3\text{PO}_4$ (leaving overnight) and the titration made against $N/100 \text{ NaOH}$, using bromophenol blue as the indicator and taking as the end-point a colour between buffer solutions p_{H} 4.0 and 4.2. It was found that, after gradual heating of the oxalate precipitates in the oven at 160° for an hour, subsequent heating over the direct Bunsen flame for 20–30 seconds gave the highest recovery from CaCl_2 solution and the least difference between the two methods when applied to serum.

Estimations were made mostly on pooled sera and C.S.F. and at least two estimations were made on each sample. For each method, most of the duplicates agreed to ± 1 % for the C.S.F. specimens; and, for sera, about one-half agreed to ± 1 % and the remainder to ± 2 %.

Forty-eight samples of serum were compared, and in every case the Clark and Collip method gave the higher figure; the average values being 10.4 mg./100 cc. (C. and C.) and 9.7 mg./100 cc. (T. and B.), with an average difference of 0.7 mg./100 cc. (highest 1.7, lowest 0.2).

Fourteen samples of C.S.F. gave average values of 5.7 mg./100 cc. (C. and C.) and 5.4 mg./100 cc. (T. and B.), with an average difference of 0.3 mg./100 cc. (highest 1.0, lowest 0.0).

Of the calcium added to 5 samples of serum and C.S.F. in the form of a 10 mg./100 cc. CaCl_2 solution; 106 and 101 % were the respective average C. and C. recoveries; and 98 % the average T. and B. recovery from both serum and C.S.F.

The absolute recoveries from CaCl_2 solutions were 100 % by C. and C. (average 9 estimations) and 98 % by T. and B. (average 11 estimations).

Table I.

Serum		mg. Ca/100 cc.			
		10.0	10.2	10.3	10.4
C.S.F.	C. and C.	9.1	9.6	9.2	10.0
	T. and B.	5.0	5.8	5.6	5.7
	C. and C.	4.9	5.6	5.4	5.4
	T. and B.				

Table II.

Serum	0.1 mg. Ca added to 2 cc. sample		Recovery %
	mg. Ca/100 cc.		
C. and C.	10.1	15.4	106
"	10.4	15.7	106
"	9.5	14.7	104
"	10.7	16.1	108
T. and B.	10.0	14.6	92
"	9.9	14.9	100
"	9.7	14.9	104
"	10.1	15.1	100

Table III.

	C.S.F.	0.1 mg. Ca added to 2 cc. sample mg. Ca/100 cc.	Recovery %
C. and C.	5.8	11.0	104
"	5.6	10.6	100
"	5.7	10.7	100
"	5.5	10.6	102
T. and B.	5.6	10.4	96
"	5.4	10.4	100
"	5.5	10.5	100
"	5.0	10.0	100

Hendriks [1929], in comparing the C. and C. and T. and B. methods on sera, obtained (as an average of 8 estimations) values of 10.7 mg./100 cc. and 10.5 mg./100 cc. respectively; but, in this investigation, the differences were higher throughout.

The results are further exemplified in Tables I, II and III, which represent absolute comparisons on four specimens of serum and C.S.F. respectively.

It will be observed that the KMnO_4 titration gives a higher figure than the acidimetric titration following ignition of the oxalate precipitate.

(2) *Comparison of the Clark and Collip and Halverson and Bergeim methods.*

The method of Halverson and Bergeim was carried out in the manner described by Peters and Van Slyke [1932]. The washing technique adopted was that of Clark and Collip in each case.

Twelve samples of serum were compared, and in most cases the H. and B. method gave the higher figure; the average values being 11.4 mg./100 cc. (H. and B.) and 11.1 mg./100 cc. (C. and C.), with an average difference between the two methods of 0.3 mg./100 cc. (highest 1.3, lowest, -1.1). Three extremely fatty sera estimated by the H. and B. method gave normal values.

Six samples of C.S.F. gave the same average value of 5.7 mg./100 cc. by each process, with an average difference of 0.0 (highest 0.3, lowest -0.3).

Of the calcium added to 4 samples of serum and C.S.F. in the form of a 10 mg./100 cc. CaCl_2 solution, the average H. and B. recoveries were 99 % from serum and 100 % from C.S.F.

The H. and B. method gave an average absolute recovery from CaCl_2 solution of 102 % (average 4 estimations).

KMnO_4 and incineration methods, carried out in duplicate after deproteinisation on 5 cc. serum and C.S.F., gave similar values (serum, 11.1 H. and B., 11.2 T. and B.; C.S.F., 6.6 H. and B., 6.7 T. and B.). This is in agreement with the work of Heiduschka and Schmidt-Hebbel [1932].

These results are further exemplified in Tables IV, V and VI, which represent absolute comparisons on four specimens of serum and C.S.F. respectively.

Table IV.

		mg. Ca/100 cc.			
Serum	C. and C.	11.3	11.9	11.0	11.2
	H. and B.	11.6	11.8	11.6	11.8
C.S.F.	C. and C.	5.4	6.0	5.5	5.6
	H. and B.	5.7	5.7	5.6	5.6

Table V.

Serum	0.1 mg. Ca added to 2 cc. sample		Recovery %
	mg. Ca/100 cc.		
H. and B.	10.9	15.7	96
"	11.1	16.1	100
"	9.1	14.7	96
"	12.1	17.4	106

Table VI.

C.S.F.	0.1 mg. Ca added to 2 cc. sample		Recovery %
	mg. Ca/100 cc.		
H. and B.	5.6	10.7	102
"	5.3	10.3	100
"	5.5	10.5	100
"	5.6	10.6	100

It will be observed that, in most instances, the H. and B. method gives the higher value. The relative significance in respect to the suggested organic matter attached to the C. and C. oxalate precipitate, and the increase of calcium concentration due to separation of combined calcium by acid deproteinisation, will be discussed later.

(3) *Washing methods.*

The results of the three methods carried out on calcium oxalate precipitated without deproteinisation and after deproteinisation are set out in Tables VII and VIII.

Table VII.

		Serum-Ca in mg./100 cc.														
Serum		No.	1	2	3	4	5	6	7	8	9	10	11	12	13	Av.
Without de- proteinising	C. & C.	11.0	10.9	—	—	—	—	—	—	11.2	10.7	—	—	—	—	11.0
	H. & B.	10.8	10.6	—	—	—	—	—	—	11.0	10.8	—	—	—	—	10.8
After de- proteinising	C. & C.	—	—	11.0	10.7	10.4	11.0	—	—	—	—	—	—	—	—	10.5
	H. & B.	—	—	11.6	10.8	10.4	11.0	—	—	—	—	—	—	—	—	11.0
Without de- proteinising	H. & B.	—	—	—	—	—	—	—	10.9	11.0	10.8	—	10.8	—	—	10.9
	S. & W.	—	—	—	—	—	—	—	11.2	12.0	11.9	—	12.0	—	—	11.8
After de- proteinising	H. & B.	—	—	—	11.0	—	11.0	—	—	—	—	—	—	10.5	10.5	10.7
	S. & W.	—	—	—	11.8	—	11.8	—	—	—	—	—	—	10.7	10.5	11.0

Table VIII.

		C.S.F.-Ca in mg./100 cc.				
	C.S.F. No.	1	2	3	4	Average
Without depro- teinising	C. & C.	—	—	5.5	5.6	5.5
	H. & B.	—	—	5.5	5.6	5.5
After depro- teinising	C. & C.	5.7	—	5.6	—	5.6
	H. & B.	5.8	—	5.7	—	5.7
Without depro- teinising	H. & B.	—	5.7	5.5	—	5.6
	S. & W.	—	6.0	6.0	—	6.0
After depro- teinising	H. & B.	5.8	—	5.7	—	5.7
	S. & W.	6.8	—	5.4	—	6.1

As will be seen from the Tables, in the case of serum, after deproteinisation the H. and B. technique gave higher figures than that of C. and C., and without deproteinisation, slightly lower results. In the case of C.S.F. there was no difference between the C. and C. and H. and B. techniques.

The S. and W. technique gave higher values than that of H. and B.; the difference being less after deproteinisation than without deproteinisation.

DISCUSSION.

(1) The Trevan and Bainbridge method was found to be more liable to personal error than the Clark and Collip method because it was not always easy to adjust the time of incineration so that the minimum quantity of CaO was formed or so that no fusion with the glass of the test-tube occurred. The absolute recoveries, and the agreement of KMnO_4 and incineration values after deproteinisation, however, suggest that personal error did not account significantly for the lower T. and B. results. Further, although a micro- and not a micrometer burette was employed, the agreement of duplicates would indicate that this factor also did not account significantly for the lower results.

It seems probable that the discrepancy between the two methods was caused firstly by the titration by the KMnO_4 of organic matter, which might augment the true value by about 0.3 mg./100 cc. (corresponding to one drop of $N/100$ KMnO_4), and secondly by the combination of protein-sulphur and -phosphorus with the calcium during the incineration, which might diminish the true value also by about 0.3 mg./100 cc. (corresponding to one drop of $N/100$ NaOH).

A comparison of the results supports this explanation. The difference between the results obtained by each method was less for C.S.F. than for serum, which contains more protein than C.S.F.; and the accuracy obtained in the agreement of duplicates was greater for C.S.F. Further, the recoveries of added calcium also appear to relate to the protein content. The average recovery from serum of 106 % by the C. and C. method may be explained by the greater adsorptive surface provided by the added calcium; and for C.S.F., the recoveries of 101 % (C. and C.) and 98 % (T. and B.) were respectively greater and less only by the small amounts corresponding to the small protein content.

(2) The average of 3 % more calcium obtained by the Halverson and Bergeim method as a result of deproteinising, although not within the 5–15 % range of Van Slyke and Sendroy, may suffice to justify the method in preference to that of Clark and Collip. A further advantage is that fatty and opaque sera can be successfully analysed. The removal of the protein also gives greater relative accuracy to the titration.

The C.S.F. protein-calcium was insufficient to augment the average H. and B. value; although, in one instance, an extra 3 % was obtained.

99 % of added calcium was recovered by the H. and B. method from serum, and 100 % from C.S.F., there being, owing to the deproteinisation, no organic matter to be adsorbed.

(3) It is evident from the results on the washing technique that the method of Halverson and Bergeim is to be preferred, because this tends when compared with the C. and C. technique to lower the results obtained from non-deproteinised serum (probably owing to the solubility of titratable protein in the extra washing fluid) and to raise those obtained from deproteinised serum (there being in this case a finer precipitate, which tends to drain away upon inversion of the test-tube during the C. and C. washing).

Although the saturated calcium oxalate of Stanford and Wheatley fulfilled the author's requirements as to KMnO_4 titration, the results were so high as

to suggest a withdrawal of oxalate from solution; this effect being more apparent where there was an adsorptive surface, *i.e.* in the case of non-deproteinised serum.

CONCLUSIONS.

(1) Owing to errors associated with adsorbed organic matter and incomplete precipitation, calcium estimations (by KMnO_4 or acidimetric methods), effected on non-deproteinised serum and C.S.F., cannot be relied upon to give absolute values, or values such that significance can be attached to minor variations.

(2) The above errors may be eliminated by adopting the method of Halverson and Bergeim [1917] as modified by Peters and Van Slyke [1932]; including the Halverson and Bergeim washing with removal of the mother-liquors by suction, as modified by Van Slyke and Sendroy [1929].

(3) Fatty and opaque sera can be successfully analysed by the above method.

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