XLIII. A METHOD FOR THE ESTIMATION OF SMALL QUANTITIES OF CALCIUM.

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OUR knowledge of calcium metabolism is limited, and probably hampered, by the lack of a good and accurate method of estimating small quantities of calcium. In quite a number of biological problems the estimation of amounts of calcium of the order of 0.1 mg. is frequently desired. The method about to be described is applicable to even smaller quantities than this; and is, we believe, remarkably accurate if used with reasonable care, and superior to those in use at the present time.

The modern methods are of two types (*a*) biological, (*b*) chemical. The biological methods depend on the fact that calcium is necessary for blood coagulation. Wright [1912] and later Vines [1921] have developed methods with this fundamental fact as basis. Inasmuch as the nature of the coagulation process is only imperfectly known these methods suffer from being built on an insecure foundation and are liable to break down for unknown reasons. They are, however, sensitive and within limits not clearly defined useful. If a method equally sensitive with a secure basis were available they would rarely be employed. The more recent chemical methods such as those of Howland and Kramer [1920], Kramer and Tisdall [1921] are difficult to use, and consisting as they do of microtitrations of oxalate by *N*/100 potassium permanganate are liable to error. Thus small amounts of organic matter such as carbon from imperfect incineration of a biological product, dust, or even the organic material in poor distilled water, may each and all cause significant error. The weak solution does not keep well, and the end point is not sharp. With elaborate precautions and in the hands of the select few they give good results; but we obtained very disappointing results on trying them out.

The principle of the method. The calcium is separated in the first place as oxalate and the oxalate is converted into calcium alizarinate under defined conditions. The crystalline calcium alizarinate is collected and washed on a Gooch filter and decomposed by oxalic acid. The alizarin is dissolved in alcohol made up to known volume with a relatively large volume of dilute ammonia, and the resultant solution of ammonium alizarinate is compared in a Dubosc colorimeter with a standard dilution of ammonium alizarinate. The primary

separation as oxalate is necessary since in spite of many trials no clean separation of magnesium and calcium alizarinates proved feasible. Since the calcium in the alizarinate is only one-sixth the weight of the alizarin, the initial weight of calcium to be estimated is multiplied by six; and since the colorimetric comparison is carried out against a 0.001 % solution of alizarin, the method is very sensitive. Given cleanliness, reagents free from calcium, pure alizarin, and avoiding undue hurry, 0.1 mg. calcium may be estimated, to 0.002 or 0.005 mg. without much trouble.

Precipitation of calcium oxalate. This is done along the usual lines and may be carried out on the ash of a biological product, or sometimes, as in the case of blood serum, directly. After careful incineration the ash is dissolved in 0.5 cc. of *N* HCl and transferred with about 2 cc. wash water to a centrifuge tube. One cc. of a saturated solution of ammonium oxalate is added, followed by 2 cc. of a saturated solution of sodium acetate, to which some ammonium oxalate has previously been added to exclude calcium contamination. The whole is mixed and allowed to stand for three hours, or if more convenient till next day. In our experience three hours' standing, especially in the case of direct estimations in blood serum, gives more uniform results than shorter periods, though most of the oxalate separates as a rule in a much shorter time. The oxalate precipitate is centrifuged to the bottom of the tube (two minutes at 4000 per min. is sufficient) and the supernatant fluid sucked off as completely as possible without disturbing the oxalate precipitate at the bottom of the tube. Three cc. of a 0.1 % solution of ammonium oxalate are then added, the precipitate stirred up and the centrifugation repeated at once. The supernatant fluid is once more sucked off as completely as possible. In this way all the calcium is obtained as oxalate free from all but traces of magnesium, and with only a *little* excess of oxalate.

Conversion of oxalate into alizarinate. The oxalate is dissolved in 0.5 cc. of *N* HCl, and transferred to a clean test-tube. The centrifuge tube is washed out four times with water. The resultant dilute solution in the test-tube should measure 8 to 10 cc. A small volume is disadvantageous, as oxalate may separate at a later stage if the volume is kept too small. Excess of an alcoholic solution of alizarin is then added (1 cc. of a 1 in 1000 solution is sufficient for quantities of calcium of about 0.1 mg.). The test-tube is then warmed on a water bath to about 80° C., and five drops of strong ammonia solution added. The contents of the tube turn deep purple on mixing. It is best to add at this stage a minute amount of crystalline calcium alizarinate though it is not essential. The step ensures the full and ready separation of the calcium alizarinate in a crystalline form which facilitates filtration later. The tube is kept warm for about one hour then set aside to cool and stand till the following day. The whole of the calcium should then have separated in clumps of blue black microcrystalline needles at the bottom of the tube and the supernatant fluid should be pale in colour.

The calcium alizarinate is filtered off on a Gooch crucible with a fine asbestos

layer at a water pump, and the precipitate and filter washed with dilute ammonia. At this point care must be taken not to suck much air through the filter since acids, even the carbonic acid of the air, will decompose the calcium salt. Paper filters are not so good as asbestos. The ordinary papers contain significant amounts of salts which interfere; and extracted papers have their own difficulties.

The delivery from the Gooch crucible is now arranged to return fluid to the test-tube in which crystallisation of the alizarinate took place, and 1 cc. of a strong solution of oxalic acid in 50 % alcohol allowed to run all over the asbestos and precipitate. The calcium alizarinate decomposes at once and the blue black colour becomes orange. The alizarin set free is washed through with warm 95 % alcohol along with excess of oxalic acid into the test-tube. The asbestos should now be pink or very pale purple. The nature of this colour is unknown, but it does not appear to be due to any calcium compound and is usually greater when the oxalate is precipitated directly from blood serum, and less in the case of ash estimations. The alcoholic solution of alizarin is transferred to a 50 cc. standard flask, the test-tube washed out with dilute ammonia, the contents of the flask made just alkaline with ammonia, and the volume made up to the mark with water.

The standard solution for comparison is made up as follows. Two cubic centimetres of an accurate $M/1000$ solution of alizarin in alcohol are run into a 50 cc. standard flask, followed by 1 cc. of the oxalic acid solution in 50 % alcohol, and a volume of alcohol approximately equal to that used in extracting the alizarin from the Gooch crucible when dealing with the unknown. Water is added, the contents of the flask are made just alkaline with ammonia, and the volume made up to the mark. In this way a perfect colour match is readily obtained as the reactions of the test and standard solutions are nearly the same. A large excess of ammonia in one flask yields a purple tint as compared with a red one. A small difference in ammonia content is insignificant owing to the buffer effect of the oxalate.

The unknown and the standard solutions are then compared in a Dubosc colorimeter. 20 to 25 mm. depth of the standard solution yields a suitable tint for colour match.

The alizarin in the unknown solution is calculated from the depth of the column of fluid which gives a perfect match with the standard, and this figure divided by six gives the weight of the calcium.

The method is simple and though time must be spent over the whole process owing to the waits for complete precipitation at two stages, the actual time spent on manipulation is not great, and if a series of estimations is being done the time spent on any one is still smaller. It may be stated that there are many indications that if still smaller amounts than 0.1 mg. are in question, the use of small scale apparatus would enable these to be estimated with fair accuracy.

EXPERIMENTAL.

The composition of the calcium alizarinate as prepared from the oxalate, under the defined conditions of excess alizarin, in the presence of ammonia, and in weak alcoholic solutions, was determined by incineration of three separate specimens of the crystalline solid; conversion of the calcium carbonate so obtained to sulphate; and weighing after heating and cooling:

Alizarinate taken	Sulphate	Calcium	Theory
0.2424 g.	0.1023	0.0301	0.0307
0.4855 g.	0.2053	0.0604	0.0614
0.2535 g.	0.1111	0.0327	0.0321

0.2535 g. calcium alizarinate dried over sulphuric acid heated to

110° C. lost	0.0158 g.	1H ₂ O = 0.0144 g.
120° C. „	0.0213 g.	
160° C. „	0.0276 g.	2H ₂ O = 0.0288 g.
190° C.	began to decompose.	

The weighing of the heated alizarinate was difficult since there was gain in weight during the weighings. The data show that under the conditions of experiment a salt of the composition $\text{Ca}_1\text{Aliz}_1 + 2\text{H}_2\text{O}$ separates. We also have evidence of the formation of another calcium salt of a different composition when there is not excess of alizarin present.

The method was tried out on an artificial salt mixture, made up to imitate the ash of blood serum but with the magnesium in excess, of the following composition:

NaCl	...	...	4.6340
KH ₂ PO ₄	...	...	0.0395
MgSO ₄ (dry)	...	...	0.7577
CaCO ₃	...	...	0.2410
HCl	...	...	8 cc. Water to 500 cc.

A trace of iron was present probably due to impurity in the magnesium sulphate. This was no disadvantage since this metal may be present in blood serum ash from haemoglobin.

The following table shows some of the results we obtained when estimating various amounts of the salt mixture:

Solution taken cc.	Ca found mg.	Calculated mg.	Difference mg.
0.9	0.171	0.1737	-0.0027
0.7	0.140	0.135	+0.005
0.6	0.117	0.115	+0.002
0.5	0.102	0.096	+0.006
0.4	0.0815	0.077	+0.0045
0.3	0.056	0.058	-0.002

Repeated estimations of quantities of about the amount normally present in blood serum gave the following figures:

Calcium taken mg.	Found mg.	Difference mg.
0.116	0.113	-0.003
0.116	0.118	+0.002
0.116	0.1196	+0.0036
0.096	0.098	+0.002
0.096	0.098	+0.002
0.096	0.096	0.000

Estimations on mixed samples of human blood serum are given below. It will be observed that the figures for the ash of the serum and the direct determinations are practically the same. Other figures show that the agreement is even closer if 24 hours be allowed for the separation of the oxalate in the case of the direct estimation. Other experiments on heated serum indicate that after heating some of the calcium becomes bound to the serum proteins and cannot be estimated directly:

	Ca in ash, mg.	Ca directly without ashing, mg.
Sample 1	{ 0.098 0.100	0.096 0.095
Sample 2	{ 0.097 0.094	0.091 0.093

SUMMARY.

A method for estimating small quantities of calcium is described. The method is colorimetric and depends on the insolubility of calcium alizarinate. It is accurate on 0.1 mg. to 0.002 mg.

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