Determination of Blood Serum Potassium by an Improved Sodium Cobaltinitrite Method

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The object of this paper is to describe some sources of error that have been found in methods for determining serum potassium based on the sodium cobaltinitrite procedure, and to present a convenient modified method of greater accuracy and reproducibility.

We initially selected the method of Eden (1943) for determinations of potassium in cow's blood serum required for studies in milk secretion; this method combines the precipitation of potassium direct from serum as described by Peters & Van Slyke (1932) (and adapted by them from the earlier method of Kramer & Tisdall (1921*a, b*)) with the colorimetric determination of the cobalt in the precipitate devised by Jacobs & Hoffman (1931). The method did not give sufficiently reliable results and, even after a thorough trial, duplicate analyses often differed by as much as 15 %. The lengthy literature on the cobaltinitrite procedure, recently reviewed by Tinsley (1948), shows the difficulties that have repeatedly arisen in its use with biological and other materials. We had found the procedure entirely satisfactory for gravimetric determination of the larger concentrations of potassium in milk, provided it was accepted that a precipitate of constant composition is not obtained from solutions of varying potassium concentration, and that calibration with standard potassium solutions is essential (Piper, 1934). We therefore looked for sources of error peculiar to the micro-estimation of the low potassium concentrations in serum, and after a detailed study found three main sources. These errors have been overcome, and other modifications introduced, and the method thereby much improved.

EXPERIMENTAL

Many experiments were made on standard solutions of similar potassium concentration to serum, using various conditions of formation and washing of the potassium sodium cobaltinitrite precipitate, followed by its colorimetric or gravimetric estimation. The more important observations, which revealed sources of error in methods of determining serum potassium, are discussed below.

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Incomplete precipitation of potassium

In previous methods the tendency of the precipitate to dissolve in aqueous washing fluids has been realized, but the possibility has apparently not been considered of incomplete precipitation of potassium owing to the solubility of the potassium sodium cobaltinitrite in the solution from which precipitation occurs. In macro-determinations of potassium this loss by solubility can be neglected, but in serum determinations, where the ratio of potassium to water is much lower, it becomes significant, and should not be increased by diluting the serum with water. In the procedures of Peters & Van Slyke (1932) and of Eden (1943) about 0.15–0.2 mg. of potassium is precipitated from 1 ml. of serum plus 3 ml. of water by adding 2 ml. of reagent; Kramer & Tisdall (1921*a*), although mixing 2 ml. of reagent with 1 ml. of undiluted serum, subsequently add 2 ml. of water; King (1951), in a method based on that of Abul-Fadl (1949), adds 0.5 ml. of reagent to 0.2 ml. of serum but subsequently adds 1 ml. of water.

Table 1. *Loss of potassium sodium cobaltinitrite by its solubility in the reacting solutions*

(The cobaltinitrite reagent was prepared according to Kramer & Tisdall (1921*a*).)

Conditions of precipitation	Potassium sodium cobaltinitrite ppt. separating	
	Weight (g.)	Ratio of weights
(a) 6.00 mg. K in 160 ml. water mixed rapidly with 80 ml. of reagent $\equiv$ (0.150 mg. K in 4 ml. water + 2 ml. reagent) $\times$ 40	0.0349	82
(b) 6.00 mg. K in 100 ml. water mixed rapidly with 80 ml. reagent $\equiv$ (0.150 mg. K in 2.5 ml. water + 2 ml. reagent) $\times$ 40	0.0388	91
(c) 6.00 mg. K in 40 ml. water mixed rapidly with 80 ml. reagent $\equiv$ (0.150 mg. K in 1 ml. water + 2 ml. reagent) $\times$ 40	0.0426	100

The loss of precipitate entailed by such procedures is shown by the three examples of precipitation given in Table 1. In each, the same weight of potassium was treated with the same volume of reagent but in solutions of different total volume. In example (a) the relative weight of potassium, volume of solution, and volume of added reagent are the same as in the procedures of Peters & Van Slyke (1932) and of Eden (1943), but the scale has been increased 40

times so that, after 2 hr., the precipitate could readily be collected on a Gooch filter, washed with 70% (v/v) ethanol, dried and weighed. Similarly, example (c) corresponds to the addition of the reagent to 1 ml. of undiluted serum, and example (b) is intermediate. In (a) approximately 18%, and in (b) 9%, less precipitate was obtained than in (c). These results were confirmed by using one-fortieth of these amounts and estimating Co in the precipitate colorimetrically: the precipitate corresponding to (a) contained 14%, and that corresponding to (b) 8%, less cobalt than that corresponding to (c). Since the temperature and the ratio of Na to K in each solution were the same, it would be expected from the work of Piper (1934) that the composition of each precipitate would be the same; the smaller weights of precipitate in (a) and (b) must be due, not to variations in the Na/K ratio in the precipitate, but to approximately one part of the potassium sodium cobaltinitrite remaining in solution for every 16 000 parts of extra water added. The solubility of potassium sodium cobaltinitrite is one part in 1400 of water at 25° (Hodgman, 1950); the solubility here is presumably depressed by other salts. It might be expected that in serum potassium determinations the solubility of potassium sodium cobaltinitrite would be affected by the presence of serum protein, and that the loss of precipitate due to solubility would not be so great as in the example in Table 1. However, when the cobaltinitrite reagent was added directly to 1 ml. samples of sera of normal potassium concentration, the precipitates contained 15–20% more cobalt than when the serum samples were first diluted with 3 ml. of water. To get maximum precipitation in serum analyses it is therefore necessary to avoid dilution with water, and to use a reagent previously saturated with the precipitate.

It is evident that in the methods of Peters & Van Slyke and of Eden some 15–20% of the potassium in sera of normal potassium concentration remains unprecipitated, and that smaller losses of precipitate by subsequent re-solution can be expected in the procedures of Kramer & Tisdall (1921*a*) and of King (1951). This criticism may at first seem immaterial since the analytical results are always computed by comparison with the results obtained from standard potassium solutions treated similarly. To get entirely consistent results, however, it would be necessary always to ensure that the amount unprecipitated was the same by using strictly controlled conditions in which the serum was accurately diluted, the temperature kept exactly constant, and the concentrations of other salts kept the same. In practice, it has been found that the use of undiluted serum gives far more reliable results, and in our proposed procedure, although a very small amount of the potassium sodium cobaltinitrite remains dissolved in the water of the 1 ml. of undiluted serum, the variations in this amount are too small to affect significantly the accuracy of the final results.

Duration and temperature of precipitation

The precipitation of potassium sodium cobaltinitrite is very slow, and in trials with K solutions and with serum we found that, to obtain closely reproducible results, the time previously allowed for precipitation (30 min., Peters & Van Slyke, 1932; 45 min., King, 1951; 1 hr., Eden, 1943) should be increased to 2 hr. for room temperatures of 15° and above. However, when this was done the reagent, which was prepared according to Kramer & Tisdall (1921*a*), caused a flocculent precipitate, presumably of protein, to separate from undiluted serum; this difficulty was overcome by

using a reagent of approximately one-half the usual concentration. At temperatures below 15° the precipitation of K becomes progressively slower and more time should be allowed.

Temperature influences the ratio of Co to K in the precipitate (Piper, 1934), and we have found that serum analyses should preferably be made within 3° of the temperature at which the calibration was conducted.

Loss of precipitate during washing

The techniques used for washing the precipitate have been a further source of error. Kramer & Tisdall (1921*a*), after siphoning off the reagent, washed the precipitate four times by mixing the residual reagent with 5 ml. portions of water and attempting not to disturb, and so dissolve, the precipitate. This method was obviously unsatisfactory since the cobaltinitrite precipitate from 1 ml. of serum will dissolve completely in 2–3 ml. of water at 25°. It was improved by Jacobs & Hoffman (1931) who, after washing with 1 ml. of water, dispersed the precipitate twice in 70% (v/v) ethanol in which it is insoluble. King (1951) first washed the precipitate from 0.2 ml. of serum with 2 ml. of water without disturbing the precipitate, and then dispersed it in 70% (v/v) ethanol. The 70% ethanol cannot be used for the first washing since it precipitates the small amount of serum protein present. In an attempt to use for the first washing a fluid in which the precipitate is insoluble, Eden (1943) used 2 ml. of a 'saturated solution' of sodium potassium cobaltinitrite prepared by shaking the solid with water and separating the next day by centrifuging. However, we found, using standard K solutions of similar concentration to serum, that when the precipitate was dispersed in this solution for the first washing, or in the similar one recommended by Piper (1934), varying results were obtained which were up to 10% lower than when the precipitate was washed in 70% (v/v) ethanol alone: the solution was still able to dissolve the precipitate, probably owing to decomposition of the potassium sodium cobaltinitrite with which it was previously saturated.

For the first wash we have substituted 35% (v/v) ethanol which we have found does not precipitate the serum protein at this stage. Since it does dissolve a trace of precipitate, 70% (v/v) ethanol was used for subsequent washings.

Further modifications

The addition of NaNO₂ solution before precipitation, which was claimed by Kramer & Tisdall (1921*b*) to prevent the precipitate floating on the surface of the reagent and adhering to the sides of the tube, was found unnecessary.

The addition of the reagent drop by drop, which was claimed to prevent occlusion of the reagent by the precipitate (Kramer & Tisdall, 1921*a*), was also abandoned. Rapid addition of the cobaltinitrite reagent has been previously used to ensure that at each K level the same Na/K ratio is always established in the solution before precipitation begins, resulting in a precipitate of uniform composition at that level (Piper, 1934). Dropwise addition, apart from being slower, has the disadvantage that, because of the high concentration of Na in the reagent, the ratio of Na/K in the solution, and hence in the precipitate, is continually changing as precipitation occurs, and the weight of precipitate obtained depends on the rate of addition. Therefore, the rate of dropwise addition should always be kept constant,

which is not easy to do. Kramer & Tisdall (1921*a*) obtained a larger weight of precipitate when the reagent was added rapidly than when it was added slowly, and concluded that this was due to occlusion of the reagent by the precipitate. It is more probable that after rapid addition the higher Na/K ratio at the time of precipitation caused a larger weight of precipitate of lower potassium content per mole to separate.

Table 2. *Typical replicate determinations of serum potassium*

K content (mg./100 ml.)	
Serum	
Original method, Eden (1943):	
1	14.9, 16.2, 17.1, 17.5
2	15.0, 15.5, 15.7, 16.3
3	19.6, 20.4, 21.3
4	22.5, 23.4, 24.3, 25.4
Recommended method:	
5	16.7, 16.7, 16.8, 16.9
6	18.3, 18.7, 18.8, 18.8
7	18.9, 18.9, 19.2, 19.5
8	22.5, 22.5, 22.9, 22.9
9	34.3, 34.4, 34.6, 34.6

Table 3. *Further example of replicate determinations by the recommended method and recovery of added potassium*

	K content of a sample of cow's serum (mg./100 ml.)																		
	Found																		
	16.1, 16.4, 16.3, 16.1, 16.3, 16.3, 16.1																		
Mean	16.23																		
S.D.	0.13																		
	K content of a mixture of 1 vol. of the same serum with 1 vol. of a solution containing 30 mg. K/100 ml. (mg./100 ml.)																		
	<table><tr><td></td><td>Calculated</td><td>Found</td></tr><tr><td>From serum</td><td>8.12</td><td>23.1, 23.1</td></tr><tr><td>From added K</td><td>15.00</td><td>23.1, 23.0</td></tr><tr><td>Total</td><td>23.12</td><td>23.0, 23.4 23.5</td></tr><tr><td>Mean</td><td>23.17</td><td></td></tr><tr><td>S.D.</td><td>0.20</td><td></td></tr></table>		Calculated	Found	From serum	8.12	23.1, 23.1	From added K	15.00	23.1, 23.0	Total	23.12	23.0, 23.4 23.5	Mean	23.17		S.D.	0.20	
	Calculated	Found																	
From serum	8.12	23.1, 23.1																	
From added K	15.00	23.1, 23.0																	
Total	23.12	23.0, 23.4 23.5																	
Mean	23.17																		
S.D.	0.20																		

Accuracy of the improved procedure

The various modifications which are described above have been incorporated into the following procedure. Whereas previously duplicate determinations often differed by as much as 2.5 mg. K/100 ml. of serum at levels of about 20 mg. K/100 ml., they now usually agree within 0.2 or 0.3, and seldom differ by more than 0.5 mg./100 ml. In Table 2 are shown some typical replicate determinations on a number of serum samples by the original and by the improved procedure.

Table 3 shows a further set of replicate determinations on a sample of serum that was also used for an experiment on the recovery of added K. Both series of seven replicates give a coefficient of variation of just under 1%, and the recovery of K from the mixture of serum and K was satisfactory. It is evident that the presence of serum protein has not prevented the accurate estimation of added K. The method has, in fact, now reached as great a precision as can be expected from routine methods of photoelectric absorptiometry. Since the method was developed, it has been extensively used with equal success by other workers to whom it was communicated privately.

RECOMMENDED METHOD

Principle

The potassium is precipitated as potassium sodium cobaltinitrite by adding a sodium cobaltinitrite reagent to the serum in a centrifuge tube. After washing with aqueous ethanol, the precipitate is dissolved in water and the cobalt content is measured by the method of Jacobs & Hoffman (1931) from the intensity of the green colour produced on adding choline chloride and potassium ferrocyanide solutions.

Reagents

All reagents should be of A.R. quality.

Sodium cobaltinitrite reagent. Two solutions are first prepared: (A) $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (25 g.) is dissolved in 280 ml. water and then 12.5 ml. glacial acetic acid and about 10 mg. potassium chloride are added. (B) Sodium nitrite (120 g.) is dissolved in 180 ml. water. Then, 210 ml. of (B) are added to the whole of (A) and air is drawn through the solution until all nitrous fumes have been removed. The addition of potassium chloride ensures saturation of the reagent with potassium sodium cobaltinitrite. The reagent, if stored in the refrigerator at 0°, will be satisfactory for at least 1 month. Before use it is allowed to attain room temperature and, after shaking to ensure saturation at the higher temperature, it is filtered.

Ethanol. 35 and 70% (v/v).

Choline chloride. A 10% (w/v) solution stored in the cold. It is diluted to 1% just before use.

Potassium ferrocyanide. A freshly prepared 2% (w/v) solution.

Procedure

Serum. To 1 ml. of serum in a dry 15 ml. centrifuge tube graduated at 6.00 ± 0.03 ml. are added 3 ml. of the cobaltinitrite reagent from a pipette, which is blown out rapidly to aid mixing, and the mixing is immediately completed by rotating the tube between the palms of the hands. The mixture is allowed to stand for 2 hr. at a room temperature of 15° or above, centrifuged for 10 min. at about 2500 rev./min., and the supernatant liquid decanted. After wiping the mouth of the tube 5 ml. of

35% ethanol are added, using it to wash the sides of the tube, and the precipitate is dispersed in the liquid with a glass rod; after recentrifuging for 5 min. the ethanol is decanted. The tube and precipitate are washed twice more with 5 ml. portions of 70% ethanol in the same way. The precipitate is then dispersed in about 3 ml. of water, and heated in a boiling-water bath until dissolved. The solution is cooled and 1 ml. of 1% choline chloride is added and, after mixing, 1 ml. of 2% potassium ferrocyanide is added. Unless the choline chloride is well

of precipitation are adhered to, and the temperature of precipitation of serum is within $\pm 3^\circ$ of the temperature at which the standard curve was prepared the curve may be used repeatedly. With a Miller absorptiometer and Ilford Spectrum Orange Filter no. 607 the calibration curve relating $E_{1\text{ cm.}}$ and potassium concentration deviated only slightly from a straight line; it is shown in Fig. 1.

Notes on procedure

(1) If desired, 0.5 ml. of serum can be treated as described and will give a final colour which can be measured with reasonable accuracy. The procedure could no doubt be adapted for smaller volumes of serum by reducing proportionately the various volumes of reagents.

(2) The tubes must be cleaned before use in such a way as to remove all traces of potassium ferrocyanide added in a previous analysis. A satisfactory method is to leave in a solution of potassium dichromate in concentrated sulphuric acid and after thorough rinsing to heat for half an hour in boiling distilled water, and then rinse again.

(3) Although rapid mixing of the serum and reagent is essential for constant conditions of precipitation, a stirring rod should not be used. Traces of precipitate which are difficult to remove separate on the rod, and if put in subsequent solutions tend, by seeding, to increase the rate of precipitation.

(4) A useful check that the absorptiometer is giving consistent readings from day to day is provided by a solution of standard colour which can readily be prepared directly from a solution of a Co salt: e.g. 1 ml. of a solution containing 0.165 mg. Co/ml. ($0.815 \text{ g. Co(NO}_3)_2 \cdot 6\text{H}_2\text{O/l.}$) mixed with 1 ml. of 1% choline chloride and 1 ml. of 2% $\text{K}_4\text{Fe(CN)}_6 \cdot 3\text{H}_2\text{O}$ and diluted to 6 ml., gives a colour corresponding to that from an average serum.

Fig. 1. Calibration curve showing the relation between the potassium concentration of serum and the extinction of the coloured solution obtained in its estimation.

mixed with the solution before adding the ferrocyanide the full colour does not develop. The solution is made to 6 ml. and mixed again. It is then centrifuged for a few minutes to remove the slight cloudiness caused by the small amount of protein which always separates from the serum. The extinction of the resulting green solution is measured in a 1 cm. cell in a photoelectric absorptiometer at about $600 \text{ m}\mu$, or in some other suitable instrument. The colour fades slowly and should be measured within half an hour.

Standard potassium solutions. A calibration curve is prepared relating the final colour intensity to the amount of potassium initially present, by taking 1 ml. portions of standard potassium solutions and treating them in the same way as the 1 ml. of serum. Solutions containing 15.0, 20.0, 25.0 and 30.0 mg. potassium/100 ml. will give a standard curve covering the normal variations in serum potassium. Provided that the prescribed conditions

SUMMARY

1. It has been found that the low accuracy of many methods of estimating serum potassium based on the sodium cobaltinitrite procedure of Kramer & Tisdall is due mainly to three factors:

(a) The dilution of the serum with water, which can lead to as much as 20% of the potassium remaining unprecipitated owing to the solubility of potassium sodium cobaltinitrite.

(b) Allowing insufficient time for the precipitation of potassium.

(c) Dissolving some of the precipitate during washing.

2. Modification of these and other factors has given a convenient method of much improved accuracy.

3. The results of replicate determinations now agree closely, their standard deviation not exceeding 1% of their mean value, and the recovery of added potassium is satisfactory.

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Cholinesterases of the Tissues and Sera of Rabbits

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Ten years ago Mendel and associates (Mendel, Mundell & Rudney, 1943) clearly distinguished between two types of cholinesterase (ChE) in mammalian tissues, and utilized the selective substrates acetyl- β -methylcholine (MeCh) and benzoylcholine (BzCh) to measure the activities of 'true' and 'pseudo'-ChE, respectively. Other investigators have employed different nomenclature and criteria for this purpose, including over-all substrate patterns (Nachmansohn & Rothenberg, 1945; Augustinsson, 1948, 1949; Goldstein, 1951), differences in kinetics of hydrolysis (Alles & Hawes, 1940) and selective inhibition (Hawkins & Gunter, 1946; Adams & Thompson, 1948; Hawkins & Mendel, 1949). By means of these methods, the distributions of the two enzymes have been studied extensively in rat tissues (Sawyer & Everett, 1947; Ord & Thompson, 1951); their occurrence in cat tissues has been investigated histochemically (Koelle, 1950, 1951).

In recent years, evidence has accumulated for the existence of several choline ester-hydrolysing enzymes which do not conform with Mendel's classical descriptions of true and pseudo-ChE's. Sawyer (1945) reported an enzyme of guinea pig and rabbit liver which splits BzCh but not acetylcholine (ACh), and assigned to it the designation 'benzoylcholinesterase' (BzChE). Evidence of a similar enzyme has been noted in guinea pig kidney (Nachmansohn & Rothenberg, 1944) and beef kidney (Gunter, 1946; Blaschko, Chou & Wajda, 1947). The occurrence and characteristics of other choline ester-hydrolysing enzymes have been reviewed by Augustinsson (1948) and Whittaker (1951).

Ellis and associates (Ellis, Sanders & Bodansky, 1947; Ellis, 1947) found that the plasma of certain rabbits contains BzChE in addition to true or specific ChE, whereas in the plasma of others only the latter ChE is present in significant amounts. This observation was confirmed by Levine and associates (Levine, Hoyt & Suran, 1950). Levy (1951) reported on the presence of several other esterases in rabbit plasma, including a possible trace of pseudo-ChE.

With the exception of the ruminants, the plasma of most species contains predominantly non-specific or pseudo-ChE and varying amounts of specific or true ChE (Gunter, 1946; Augustinsson, 1949). In the cat, Goldstein (1951) has shown that the ChE's of the plasma are identical with those of the liver and gastric mucosa. Studies of the distribution (Koelle, 1950, 1951) and effect of inhibition (Koelle, Koelle & Friedenwald, 1950) of the non-specific ChE of the cat's ileum have indicated that this enzyme has an important role in the regulation of intestinal motility. The same conclusion was reached by Burn and associates (Burn, Kordik & Mole, 1952) in the course of an investigation of the effects of irradiation on rats. Therefore, it seemed of interest to determine whether the absence of appreciable amounts of an ACh-hydrolysing non-specific ChE in rabbit plasma was reflected by a similar situation in the gastro-intestinal tract and other tissues. Information on this point might be expected to have a bearing on the function of non-specific ChE in other species as well. To this end, the objects of the present study have been: (1) the determination of the rates of hydrolysis of ACh and other choline esters by the plasma and tissues of