The Precision of a Direct-reading Flame Photometer for the determination of Sodium and Potassium in Biological Fluids

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Flame photometry is now well established as a simple method for the determination of sodium and potassium. It has proved particularly valuable for the rapid estimation of these ions in serum, urine and other biological fluids. An early flame photometer was constructed by Barnes, Richardson, Berry & Hood (1945), and since then many instruments have been described (e.g. Hald, 1947; Domingo & Klyne, 1949; Spencer, 1950; Ryssing, 1951). Several commercial models are now available, such as the Beckman DU flame spectrophotometer, the Perkin-Elmer 52A, and in this country the model constructed by Evans Electroselenium Ltd. (Harlow, Essex).

More recently, attention has been directed to the many sources of interference involved in this method. Errors have, for instance, been traced to viscosity (Wynn, Simon, Morris, McDonald & Denton, 1950; Spencer, 1950), and to the presence of other substances, such as urea (Spencer, 1950), glucose (Hald, 1947), acid (Wallace, Holliday, Cushman & Elkington, 1951), and phosphate (Crismon, 1948). Some workers have reported mutual interference between sodium and potassium (Kiers & Speck, 1950; Wallace *et al.* 1951), and others have found difficulty in excluding the calcium emission while measuring sodium (Domingo & Klyne, 1949; Wallace *et al.* 1951).

In view of the increasingly wide use of simple direct-reading flame photometers, especially in clinical laboratories, it was considered appropriate to describe, for comparison, the results obtained with our instrument, which has been in continuous use for 6 years.

Special reference has been made to the nature and limits of interference in simple solutions, in urine, and in serum. In addition, the flame spectrum has been analysed, and the efficiency of the filter systems assessed.

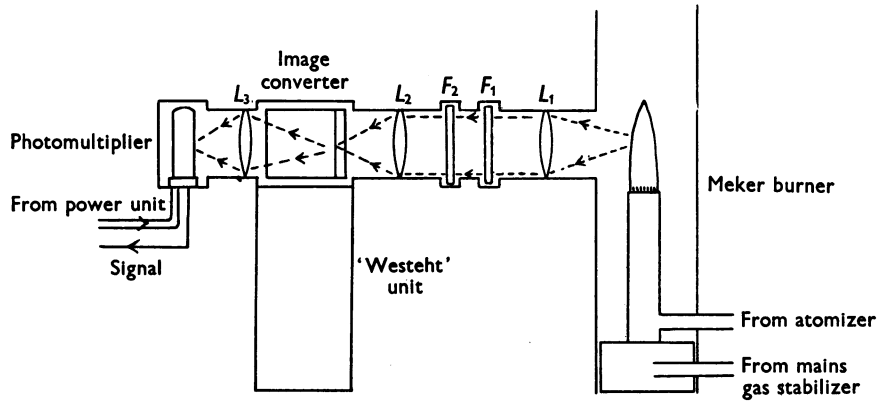

Fig. 1. General layout of flame photometer. L_1, L_2, L_3 are convex lenses. F_1, ON20 filter; F_2, either Na or K filter combinations. Arrows indicate path of light. Distance from flame to photomultiplier, 57 cm.

APPARATUS

The photometer is of the direct-reading type, without internal standard. It consists of the following components: (1) atomizer, (2) burner, (3) optical system of lenses and filters, (4) image converter, (5) photomultiplier and power pack, and (6) galvanometer, with shunt and backing-off circuit. The general layout is shown in Fig. 1.

Atomizer. Oxycillin model, designed for penicillin sprays by Oxygenaire Ltd. (8 Duke Street, London, W. 1), fed by compressed air from a cylinder fitted with pressure and flow gauges. Air flow to the atomizer is maintained at 7 l./min. Trapped spray may be led to waste, or returned to the reservoir, depending on the volume of sample available.

Burner. Meker type Bunsen burner, with flame nozzle of 12 mm. diam., supported in a vertical metal cylinder of bore 5 cm., having opposite the flame a window, 6 × 12 mm., closed by a sheet of mica.

Mains gas is used, the pressure being maintained at 1·5 cm. water by a commercial gas stabilizer Type GLC60D (Evered and Co., 23 Albemarle Street, London, W. 1), which is inserted in the lead to the burner.

Optical system (Fig. 1). This consists of a double condenser with room for insertion of filters (F_1, F_2) in the collimated beam between the two convex lenses (L_1, L_2). A further convex lens (L_3) condenses the light from the image converter on to the photomultiplier. The lenses (3·8 cm. diam. 5 cm. focal length) and filters (5 cm. square) are mounted in a horizontal metal cylinder (4 cm. diam., 55 cm. long) which is supported opposite the window in the burner housing.

Filters. For Na estimations, an interference filter with peak transmission at 590 mμ. (Barr and Stroud Ltd., Anniesland, Glasgow) is combined with an auxiliary filter, Wratten no. 16 (Kodak Ltd., Kingsway, London, W.C. 1). For K estimations, an interference filter with peak transmission at 767 mμ. is used, with a Wratten 88A as auxiliary filter. A heat-resisting filter ON 20 (Chance Bros., St James Square, London, S.W. 1) is used during both estimations.

Image converter (Fig. 1). The photomultiplier (see below) is relatively insensitive to the K 766·0 mμ. radiation. To extend the sensitive range to this region, an image converter is used (no. CV147 or 148, obtainable from surplus radio stores). This is energized with 2–5 kv (variable) from a Westeht unit (Westinghouse Brake and Signal Co., York Way, London, W. 1). The converter stays permanently in position, but is not energized during Na estimations.

Photomultiplier and power pack. Photomultiplier Type 931A (Radio Corporation of America), energized by 500–1000 v (variable) from a stabilized power unit, Cintel Type 1000/5 (Cinema-Television Ltd., Worsley Bridge Road, London, S.E. 26).

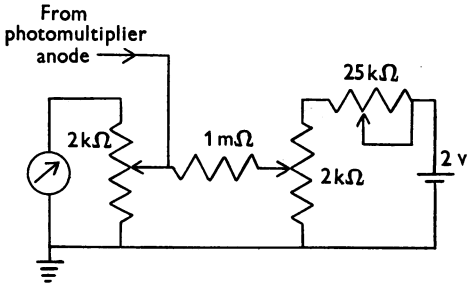

Fig. 2. Shunt and backing-off circuit.

Galvanometer, shunt, and backing-off circuit. Galvanometer, Tinsley Type SS645, 20 cm. scale, sensitivity 1240 mm./μA, slightly overdamped to give a period of about 3 sec. Shunt and backing-off circuits are shown in Fig. 2. The shunt alters the sensitivity of the detection unit, without altering the degree of galvanometer damping. The backing-off circuit is used to balance the small signal from photomultiplier dark current and flame background radiation.

OPERATION

Sodium estimations. The flame is lit, Na filters inserted and the power unit switched on. The instrument is then left for 15 min. Air is turned on, and the flow adjusted to 7 l./min. The gas flow is adjusted so that the cone of the flame is flat. A standard NaCl solution (5 μg. Na/ml.) is atomized, and the galvanometer reading adjusted to nearly full-scale deflexion with the shunt. Distilled water is then atomized, and the reading adjusted to zero with the backing-off circuit. The exact reading of the NaCl standard is taken, and the instrument is then calibrated and ready for use. Unknown solutions diluted to contain 2–5 μg. Na/ml. are atomized, and the readings compared directly with that for the standard (see pp. 216–217).

The usual dilution for urine is 1:500. Dilutions of 1:1000 and 1:200 or less are sometimes necessary. The dilution for serum is 1:1000.

Potassium estimations. K filters are inserted and the image converter switched on. The same routine is followed as with Na estimations, but using a KCl standard (5 μg. K/ml.). For serum, it is usually more convenient to use a more dilute standard (2 μg. K/ml.).

The dilution for urine is usually 1:500, rarely 1:1000. The dilution for serum is 1:100.

RESULTS

Spectroscopic analysis of flame emission

In order to determine whether emissions other than those of Na or K were passing the filter systems, a Hilger D246 spectrometer with glass prism was inserted in the light beam between flame and image converter. The image converter was energized, and the flame spectrum was scanned between 400 and 1500 mμ. The result of a spectral analysis when a solution of KCl and NaCl (containing 5 μg./ml. of each cation) was being atomized is shown in Fig. 3. Similar qualitative results are obtained when serum or urine dilutions are atomized, no additional emission bands being observed. It will be seen from the figure that apart from the Na and K emissions, there are no deflexions at wavelengths shorter than 800 mμ. Between 800 and 1250 mμ., however, there is a broad emission from the flame itself. This remains constant in intensity and distribution whether distilled water, serum or urine dilutions or simple solutions are atomized, or when air alone is blown through.

These results were confirmed over wavelengths shorter than 800 mμ. by photographing the flame emission with a Hilger medium quartz spectrograph, allowing a 20 min. exposure, and using a Kodak IIL plate.

Efficiency of filter combinations

With the monochromator in position, the filters were placed in turn between the flame and the entry slits of the monochromator.

Sodium filters. With the monochromator set to transmit the Na emission at 589 mμ., the Na interference filter was placed in position. The image converter was not energized. The standard NaCl solution (5 μg. Na/ml.) was atomized, and the deflexion adjusted to read 15 cm. A solution of $CaCl_2$ (500 μg. Ca/ml.) was atomized, and the flame

spectrum scanned between 530 and 660 mμ. Apart from a small deflexion centred on 589 mμ. (due to Na impurity in the sample), deflexions were observed at 554 and 624 mμ. only. These amounted to 0·2 cm. each, indicating a relative intensity of Ca lines of less than 0·02 % of Na. Further scanning of the flame emission while a solution of KCl (500 μg. analysed in Table 1. These form a convenient method for determining the linearity of response for standard dilutions, and for estimating the error of dilution and reading under normal working conditions. It was found convenient to express readings for the 2, 3 and 4 μg. K or Na/ml. standards as a percentage of that for the 5 μg./ml. standard.

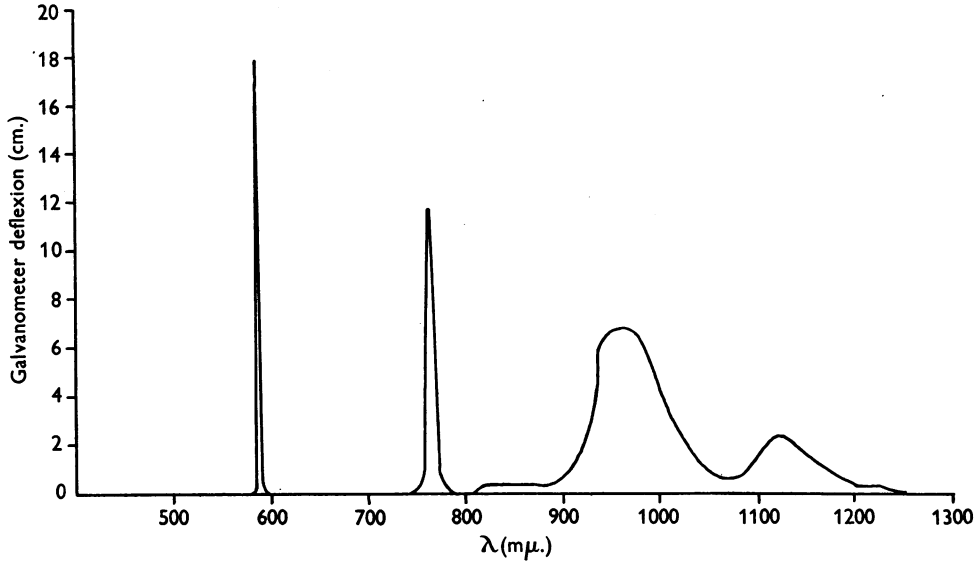

Fig. 3. Tracing of a flame spectral analysis, during the atomizing of a solution containing NaCl or KCl. Na and K emissions at 590 and 766 mμ. respectively. Flame background emission between 800 and 1250 mμ.

K/ml.) was being atomized showed that the Na interference filter transmitted the weak K emission band at 394 mμ. This was effectively excluded by the Wratten no. 16 auxiliary filter. It was unnecessary to exclude the filter band-passes above 589 mμ., due to the steep fall in sensitivity of the photomultiplier at longer wavelengths.

Potassium filters. The image converter was energized, and the K interference filter with the Wratten 88A was placed in position. The standard KCl solution (5 μg. K/ml.) was set to read 15 cm. deflexion at 766 mμ., and the flame spectrum scanned as before between 400 and 1300 mμ. No deflexion was observed when NaCl or $CaCl_2$ solutions, containing 500 μg./ml. of the cation respectively, were atomized. In addition, deflexion from the flame background radiation was eliminated.

Interposition of the Chance ON 20 filter alone was found to reduce the flame background radiation by approximately 80 %.

Reproducibility

A number of readings for standard NaCl and KCl solutions, collected over a period of months, are

It will be noted that the mean readings for each standard lie on a curve, a finding which is in agreement with those of other workers (Overman & Davis, 1947; Gilbert, Hawes & Beckman, 1950).

Table 1. *Relation between standard solution readings*

Standard solution (μg./ml.)	No. of readings	Mean reading (as % of that for 5 μg./ml.)	Standard deviation
		Sodium	
2	17	41·15	0·55
3	24	61·82	0·54
4	24	81·33	0·72
5	24	100·00	0·58
			(Mean, **0·60**)
		Potassium	
2	23	40·90	0·71
3	23	62·18	0·65
4	23	81·44	0·57
5	23	100·00	0·78
			(Mean, **0·68**)

However, in the range 1–5 μg./ml. K or Na, the curve is slight, and if the concentration of an unknown solution is calculated by reference to the nearest

standard, the error is negligible. In practice (see p. 215), the reading from the 5 μg./ml. standard only is obtained, the readings for the remaining standards being calculated from Table 1.

Interference in simple solutions

Mutual interference of sodium and potassium. KCl was added to a standard solution of NaCl (4 μg. Na/ml.) in increasing amounts, so that the Na/K ratio (w/w) varied between 4:1 and 1:20. The apparent Na content of these solutions was compared with that of the standard NaCl solution alone. The results are shown in Fig. 4, where the Na/K

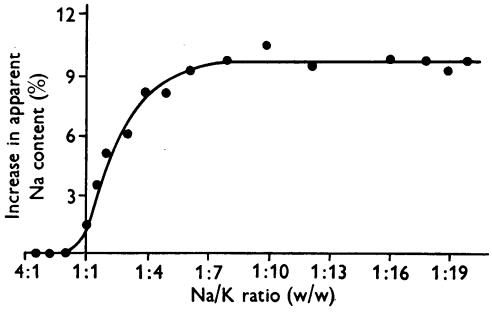

Fig. 4. Effect of increasing KCl additions on the apparent Na content of a standard NaCl solution (4 μg. Na/ml.). The percentage increase in apparent Na content is plotted against the Na/K ratio (w/w).

ratio is plotted against the apparent increase in Na content, expressed as a percentage. There is no increase in the apparent content until the Na/K ratio falls below 2:1. At lower ratios, the apparent content rises to a maximum of 10 % at Na/K ratio 1:7, after which there is no further rise with increasing KCl additions.

Similar increases in apparent K content were observed when increasing quantities of NaCl were added to a standard KCl solution under similar conditions.

Interference by other salts found in urine. Solutions of $CaCl_2$, $(NH_4)_2HPO_4$, K_2SO_4 and KCl respectively, were made up as shown in Table 2, the concentrations being the highest likely to occur in urine. The salts were dissolved in 0·1 N-HCl in order to simulate routine urine analysis. (Sufficient acid to obtain the above normality is added to urine samples as a preservative.) These solutions were further diluted 1:500 as for urine, 4 μg. Na/ml. (as NaCl) were added to each, and the apparent Na content compared with that for the 4 μg. Na/ml. standard alone. The results in Table 2 show that each foreign salt produces a significant increase in the apparent Na content. (Allowance was made in each case for a small blank reading due to Na impurity.) The addition of acid in the concentration used here was shown to have no effect on the readings.

Interference by glucose and urea. Glucose and urea were found to have no effect on Na or K readings when present in w/v concentrations up to 0·05 and 0·075 %, respectively. These concentrations are much greater than those likely to be found in urine or serum samples after appropriate dilution.

Interference in urine

Addition experiments. Specimens of urine (51) were chosen with an apparent Na concentration of about 2 μg./ml. after dilution ranging from 1:25 to 1:1000. Sufficient NaCl was added to each to raise the Na concentration by exactly 2·5 μg./ml. The readings before and after NaCl addition were compared. The results in Table 2 show the Na 'found' was equal to Na 'added', within the expected error

Table 2. *Addition of sodium and potassium to various constituents of urine, to urine and to serum*

(1) Substance	(2) Conc. (g./100 ml. 0·1 N-HCl)	(3) No. of observations	(4) Na or K added (μg./ml.)	(5) Na or K found (mean) (μg./ml.)	(6) Mean increase in Na or K (%)	(7) Standard deviation	(8)* Expected standard deviation	(9) Range (%)
				Addition of Na				
$CaCl_2$	0·10	5	2·50	2·70	+8·0	—	—	±0·5
$(NH_4)_2HPO_4$	0·63	5	2·50	2·66	+6·5	—	—	±0·25
K_2SO_4	0·55	5	2·50	2·65	+6·0	—	—	±0·5
KCl	0·40	5	2·50	2·65	+6·0	—	—	±0·5
Mixture of above salts in same concentrations	—	5	2·50	2·75	+10·0	—	—	±1·0
Urine	—	51	2·50	2·50	−0·04	0·05	0·04	—
Serum	—	29	2·50	2·50	±0	0·04	0·04	—
				Addition of K				
Urine	—	28	2·50	2·52	+0·71	0·05	0·05	—
Serum	—	76	2·50	2·61	+4·25	0·05	0·05	—

* Each experiment involves two readings on the galvanometer. The expected S.D. of the result will be $\sqrt{2}.a^2$, where a is the mean standard deviation of the Na or K estimation (Table 1, column 4). The calculated figures appear in column 8.

(column 8). Analysis of the results showed that there was no significant variation referable to the dilution.

These findings are in contrast to Na addition to the composite solution of urinary salts shown in Table 2, column 1, where Na found was 10 % higher than Na added.

A further twenty-eight urine samples were chosen with a K concentration of about 2 μg./ml. after dilution between 1:300 and 1:1000. KCl was added instead of NaCl, as in the previous experiment. Similar recoveries were obtained, as shown in Table 2.

Comparison with chemical methods. Twenty-four estimations of the K content of ten normal urine samples were carried out by the method of Rieben & Van Slyke (1944). Six of these samples were also estimated by a modification of the platinichloride

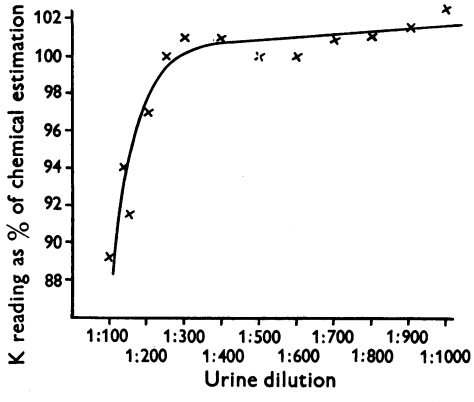

Fig. 5. Effect of dilution on the apparent K content of a urine sample. The increase in apparent K content (expressed as a percentage of the chemical estimations) is plotted against the dilution.

method of Tenery & Anderson (1940), close agreement between the two methods being obtained. These results were then compared with the same number of estimations on the same urine samples by flame photometry, the dilution being 1:500 in each case. Close agreement with the chemical methods was found, the mean variation between chemical and flame analyses being 0·5 % with a range of ± 2·4 %.

Chemical Na analyses were carried out in duplicate on ten futher specimens by the method of Dreguss (1939), as modified by Aitken & Preedy (1953). Urines for these analyses were chosen in which the Na/K ratio (w/w) was 2:1 or above, in order to minimize K precipitation. The absence of significant K precipitation in these particular specimens was established spectrographically (Aitken & Preedy, 1953). The mean variation between chemical and photometric findings was 1 %, with a range of + 1 to − 2·3 %.

Dilution experiments. Eight normal specimens of urine were serially diluted between 1:100 and 1:1000, and the apparent Na and K content of the dilutions was estimated photometrically, by direct comparison with NaCl or KCl standards of concentration 2–20 μg. Na or K/ml. The results were similar in each case. A typical example is shown in Fig. 5 where the apparent K content, expressed as a percentage of the chemical estimation, is plotted against the dilution. Dilution has no significant effect on the expected Na or K concentration over the range 1:250 to 1:1000, the values obtained agreeing with the chemical findings. At lower dilutions, the observed values became progressively lower than expected. Ashing of urine samples at 460–480° for 12 hr. in platinum crucibles before dilution did not alter these findings.

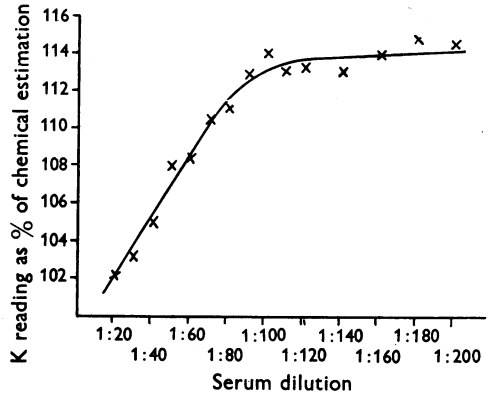

Fig. 6. The effect of dilution on the apparent K content of a typical serum sample. The increase in K reading (expressed as a percentage of the chemical estimation) is plotted against the dilution.

Interference in serum

Addition experiments. Addition of Na (as NaCl) to twenty-nine specimens of serum was carried out as shown in Table 2. Na found was equal to that added within the expected error. The dilution of serum was 1:1000.

However, when similar additions of K (as KCl) were made to seventy-six samples of serum, diluted 1:100, K found was significantly greater than K added, the mean increase being 4·25 %.

Comparison with chemical methods. Using Aitken & Preedy's modification of Dreguss's method, twenty Na results on ten specimens of serum were compared with those obtained photometrically. The mean variation between results by the two methods was 0·7 %, with a range of ± 2·0.

In the case of K, however, when fifty analyses on twenty-five specimens of serum by the method of Rieben and Van Slyke were compared with the photometric results on the same sera, it was found

that the photometric results were higher than the chemical results by a mean of 13 %, range ± 1·5. Dilutions of 1:100 or 1:150 were used for photometry, the same results being obtained.

Dilution experiments. Serial dilution between 1:700 and 1:1300 of five serum samples caused no significant alteration in expected Na reading.

Dilution was, however, found to have a considerable effect on expected K values. Ten specimens of serum were serially diluted between 1:20 and 1:200. The results were similar in each case, and typical findings with one serum are given in Fig. 6. The apparent K content, expressed as a percentage of the chemical estimation, is plotted against the dilution. The photometric result is progressively depressed at dilutions lower than 1:100, but at this and higher dilutions the reading remains steady at approximately + 13 % of the chemical figure. Ashing of serum samples (as for urine) did not significantly affect results.

DISCUSSION

With the instrument described, flame photometric analysis of Na and K in simple solutions of NaCl and KCl is reproducible with a high degree of precision which compares favourably with that of most chemical methods (Table 1).

In urine and serum, the validity of flame photometric Na and K analyses has been assessed by addition experiments, by comparison with chemical methods, and by observing the effect of serial dilution.

The great majority of urine samples require dilution between 1:300 and 1:1000. Within this dilution range, it has been shown that recovery of added K or Na is accurate within the expected error limits (Table 2), that photometric results for both cations are in close agreement with those obtained chemically, and that serial dilution has no significant effect on the photometric estimations. It would therefore appear that, within the above range, flame-photometric estimation of Na and K in urine is satisfactory without further correction, and without using the various composite reference standards recommended by several authors (Wynn *et al.* 1950; Mosher *et al.* 1949; Kapuscinski, Moss, Zak & Boyle, 1952).

Some concentrated urines of very low Na content may, however, require dilution below 1:300, and in these, depression of the Na reading might be expected to occur as in Fig. 5. However, eleven such urines, requiring dilution between 1:25 and 1:250, were included in the Na-addition experiments (Table 2). It was found that Na recoveries from these urines were as accurate as for those requiring higher dilution, suggesting that depression of the reading did not, in fact, occur. Unfortunately, this finding could not be confirmed by chemical Na estimations on the same urines, owing to the limitation of the method at low Na/K ratios (Aitken & Preedy, 1953).

Concentrated urines of very low K content, on the other hand, are seldom if ever met with and present no practical difficulty.

With serum Na estimations, Na additions were recovered within the expected limits (Table 2), comparison of flame photometric and chemical results showed good agreement, and there was no apparent interference referable to dilution between 1:700 and 1:1300. Flame photometric estimation of serum Na therefore appears satisfactory.

However, in the case of serum K estimations, the mean recovery of K was 4·25 % higher than the K added (Table 2), the mean photometric results were 13 % higher than those obtained chemically, and furthermore serial dilutions between 1:20 and 1:200 showed that the photometric analysis is considerably affected by the dilution (Fig. 6).

Serum K estimations are therefore subject to a large systematic error, but although the causes contributing to this finding were not identified, the error is constant to ± 1·5 %, and determinations to this accuracy can therefore be made by applying an arithmetical correction. It will be noted that the K recovery experiments did not indicate the full extent of the error, possibly due to alteration of the original Na/K ratio by the KCl addition.

For serum K estimations, it would appear advisable to use dilutions between 1:100 and 1:150 where the dilution curve (Fig. 5) is flatter, and the estimations therefore less likely to be affected by alteration in other serum constituents.

The effect of dilution upon photometric determinations of both urine Na and K and serum K is presumably due to altered concentration of inorganic material, since it is not significantly affected by ashing.

Reliable chemical methods for estimating Na and K in urine and serum are laborious and time-consuming, requiring 12–24 hr. for completion. In addition, they are not always free from interference effects (Aitken & Preedy, 1953). Flame photometry, therefore, remains the method of choice where many samples have to be analysed, and where rapid results are required, such as in hospital laboratories. But it appears essential to recognize that the method may be subject to interference, at least with direct-reading instruments. The nature and extent of any interference should, therefore, be established by checking results over a wide range for each type of sample by recovery experiments, by comparison with chemical methods, and by serial dilution.

SUMMARY

1. A direct-reading flame photometer for the estimation of sodium and potassium in urine and serum is described, which has been in continuous use for six years.

2. The flame spectrum has been analysed and the efficiency of the filter combinations assessed.

3. The operation of diluting and reading of samples containing 2–5 μg. sodium or potassium/ml. has a mean standard deviation of 0·03 μg./ml.

4. The validity of sodium and potassium estimations in urine and serum, by direct comparisons with standard solutions of sodium or potassium chloride, has been assessed by addition experiments, comparison with chemical methods and serial dilutions.

5. Urinary sodium and potassium estimations are free from interference at sample dilutions between 1:300 and 1:1000. At lower dilutions readings of both sodium and potassium are depressed.

6. Serum sodium estimations are free from interference, but serum potassium estimations (dilution 1:100) are subject to a systematic error of + 13 % of the chemical estimation. Estimations accurate to ± 1·5 % can, however, be made by applying an arithmetical correction. At lower dilutions, progressive depression of the potassium reading occurs.

We would like to record our indebtedness to Dr G. H. Beaven for much valuable assistance, to Miss E. H. Aitken for technical help, and to Prof. C. Wilson for laboratory facilities.

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The Chemical Nature of the Second Hydrogen Peroxide Compound formed by Cytochrome *c* Peroxidase and Horseradish Peroxidase

2. FORMATION AND DECOMPOSITION

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Spectrophotometric titration of the second intermediate compound formed by horseradish peroxidase or cytochrome *c* peroxidase and hydrogen peroxide, using the reducing agents ferrocyanide ion, ferrous ion and ferrocytochrome *c* showed that this intermediate compound is one oxidation equivalent above the original ferric state of the enzyme (George, 1952*a*, *b*, 1953), e.g.

$$\text{compound II} + e^- \rightarrow \text{ferric enzyme.} \qquad (a)$$

This compound can therefore be regarded as containing iron with an effective oxidation number of + 4, and it differs in two important respects from the classical type of 'enzyme-substrate complex' with which it has long been identified (Chance, 1943, 1949*a–c*, 1951*a*, *b*). First, the substrate, hydrogen peroxide, is not a component part of the structure, because the intermediate is only one oxidation equivalent, and not two, above the ferric state of the enzyme. Secondly, the intermediate cannot dissociate to give the free enzyme and a valency saturated product: it can only be reduced to the free enzyme.

In this paper experiments on the formation and decomposition of the second intermediate compound are described which reveal a further feature