THE DETERMINATION OF SODIUM IN HUMAN SERUM

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SECTION I

THE PROBLEM

Within the past few years physicians and surgeons have come to appreciate more keenly the importance of maintaining or re-establishing a normal electrolyte balance in the blood of their patients. This has led, in turn, to a greater demand for determinations of the concentration of sodium which is the chief cation in the blood plasma. To meet this demand there is need for a method which is not only accurate but reasonably rapid since it would clearly be useless to calculate the amount of intravenous fluid needed on the basis of the sodium content of the plasma many hours earlier.

The problem of devising a satisfactory method for the determination of sodium in human serum is beset with at least two serious obstacles. The relative solubility of sodium and potassium salts in aqueous solution and their insolubility in many organic solvents is well known. Methods of separating the two cations from a complex mixture by ordinary analytical techniques are therefore involved and consequently require considerable time.

Another difficulty arises which is peculiar to the determination of sodium in serum. Next to the pH, the sodium concentration of normal human plasma is the most constant

feature in blood, ranging from 300 to 330 or from 325 to 350 mg. per 100 ml., depending upon the method used for the determination. The difference between extreme normal limits is therefore only about 10 per cent, and the variation from a mean value, approximately 5 per cent. Any method to be of value must therefore be accurate to within a few per cent at least.

Many of the chemical methods available are indeed sufficiently accurate, but in general, they are so time-consuming as to be of limited value when a series of determinations is essential, as in the precise diagnosis and controlled treatment of body-fluid disturbances. By means of the flame photometer the requisite rapidity and accuracy can be achieved in the hands of skilled workers when proper precautions are exercised, but the instrument is expensive and not likely to be found in most routine hospital laboratories. The need therefore still exists for a more rapid yet accurate chemical method for the determination of sodium in serum, a method moreover, in which only small quantities of serum are required for analysis: the purpose of this investigation therefore was to develop such a procedure, which would be suitable for use in the average routine hospital laboratory.

SECTION II

REVIEW OF EXISTING METHODS

Since 1850, when Schmidt estimated sodium in the serum of choleric patients, many methods have been proposed for the determination of serum sodium. They fall into two general classes, chemical methods, and physico-chemical methods, and are therefore reviewed under these headings.

CHEMICAL METHODS

Introduction

The early methods which were chiefly gravimetric are fully reviewed by Peters and Van Slyke (1932). No attempt is made in these early methods to separate sodium from potassium in a complex solution such as blood serum. In general, the procedures follow the scheme in classical analytical chemistry, according to which heavy elements and the alkaline earths are removed prior to the ignition; the sodium and potassium are then weighed as the combined sulphates or chlorides. Potassium is then estimated in the redissolved salts by precipitation as the chloroplatinate, K_2PtCl_6, or the perchlorate, $KClO_4$, and the sodium calculated by difference. The disadvantage of such methods is

that when sodium is present in much smaller amounts than potassium, all errors are heaped on the small amount of sodium. Since sodium is always present in large excess over potassium in human serum, this error can be neglected in serum analyses. These methods are, in fact, the most accurate available to the present day, and are used in certain research problems. Being long and tedious, however, they seriously limit a study of sodium metabolism. Furthermore, 15 ml. or more of blood are required, an amount frequently unavailable, particularly in the case of infants.

Later methods depend upon the relative insolubility of certain sodium salts and the solubility of the corresponding potassium salts in various solutions.

The first of such methods, proposed by Fenton in 1898, was quite unsatisfactory. It was based on the precipitation of sodium as the salt of dihydroxy-acetone. The latter was not sufficiently insoluble for exact work, and the reagent was difficult to prepare. The method was useful, nevertheless, in that it pointed the way to a new approach in sodium determinations.

Subsequent methods in which sodium is precipitated as a complex nitrite or acetate, or as the pyroantimonate, have been more successful.

In the following review, only the more important methods are dealt with. They are grouped according to the

reagent which is used to precipitate the sodium, namely caesium bismuth nitrite, potassium pyroantimonate, and uranyl zinc acetate. Since methods using the latter have been so widely used they are considered in more detail.

Methods Using Caesium Bismuth Nitrite

By the method of Doisy and Bell (1920), sodium is precipitated as the complex sodium caesium bismuth nitrite, which has the formula, 9 $CsNO_2 \cdot 6NaNO_2 \cdot 5Bi(NO_2)_3$. This compound can be estimated by gravimetric, volumetric or colorimetric means. Weighing was not recommended by the authors due to contamination of the precipitate by a scum of bismuth subnitrite and also by potassium nitrate which crystallizes out under the conditions of the procedure. Although they also described a colorimetric estimation, based on a coupling reaction using α-naphthylamine and sulphanilic acid, the authors preferred the volumetric procedure in which the nitrite is oxidized to the nitrate on titration with standard permanganate. Despite the fact that only one ml. of serum, urine or whole blood is required for an analysis, the method has not achieved much popularity. A precipitation period of at least 24 hours at $0^{\circ}C$. is required. The caesium bismuth nitrite reagent, furthermore, was found to be quite unstable even if stored in an inert atmosphere at $1^{\circ}C$. Finally, some workers reported considerable technical difficulties,

particularly in the rather complicated titration.

Methods Using Potassium Pyroantimonate

Kramer and Tisdall in 1921 proposed a gravimetric method for the estimation of sodium in serum and other biological materials based on the precipitation of sodium as the sparingly soluble sodium pyroantimonate. According to Peters and Van Slyke (1932), however, difficulties were encountered in applying the method. These were apparently caused by the tendency for the finely divided precipitate to pass through filters, the inconstant amount of water of crystallization, and the inclusion with the precipitate of other substances which are also insoluble in alkaline media.

Independent attempts were made by Balint (1924) and Kramer and Gittleman (1924) to overcome these objections by iodometric titration of the antimony in the precipitate.

Balint also studied the optimum conditions for quantitative precipitation of sodium pyroantimonate in serum analysis. He concluded that preliminary ashing of serum was necessary; this finding was confirmed by Eisenman (Wakeman, Eisenman and Peters, 1927). Rourke (1928), notwithstanding, found ashing to be unnecessary if precipitation of proteins is avoided by adding alcohol to the serum at a low temperaure. Potassium, magnesium, calcium, sulphate, phosphate and chloride were shown not to interfere.

Most of the methods described in the above paragraphs have yielded more or less useful clinical results. In a critical study of various methods, Liegeois (1937) found that the antimoniate methods were less accurate than the triple acetate methods to be described next.

Methods Using Uranyl Zinc Acetate

Most recent procedures for the determination of sodium are based on the precipitation of sodium as one of the triple acetates first reported by Streng in 1886. The reagent most frequently employed is uranyl zinc acetate, in some modification of Barber and Kolthoff's (1928) gravimetric method.

The precipitate formed when an aqueous solution containing sodium is treated with Barber and Kolthoff's reagent is uranyl zinc sodium acetate which has the formula, $(UO_2)_3ZnNa(CH_3COO)_9 \cdot 6H_2O$. This triple salt is particularly well suited for sodium estimations. Because of its great weight, 67 times that of the sodium present, it readily lends itself to gravimetric micro-analysis. Volumetric and colorimetric adaptations have also appeared due to the ease with which some component (and hence sodium) in the precipitate can be measured.

For convenience, the uranyl zinc acetate methods are reviewed according to the technique employed in the final

stage of the analysis, namely, gravimetric, volumetric or colorimetric estimation.

Gravimetric methods. Next to the early classical methods, the gravimetric uranyl zinc acetate procedures are the most accurate available. Among the chief modifications are those of Butler and Tuthill (1931), Consolazio and Dill (1941), and Hald (1939). The first two methods differ only in the way the serum is ashed, the former employing concentrated sulphuric and nitric acids in a wet-ashing technique, whereas the latter ash serum to dryness in crucibles heated in an electric furnace. Hald, however, prefers to remove phosphate prior to ashing, a precaution of doubtful value in serum analyses because of the small amount of phosphate present.

These gravimetric procedures are much used in research and for comparison purposes when testing new methods, but their application to routine clinical work is limited by the time required for the ashing and gravimetric steps.

Volumetric methods. One of the best-known titrimetric procedures for serum sodium is that of Weinbach (1935), in which sodium is precipitated by uranyl zinc acetate from a trichloracetic acid filtrate of serum. After washing with a solution of acetone (saturated with triple salt), the precipitate is dissolved and titrated with standard alkali.

A somewhat similar procedure has been reported by Dreguss (1939), the special feature of which is the simultaneous removal of phosphate and protein by means of alcoholic zinc acetate.

An entirely different technique was introduced by Ball and Sadusk (1936) for the volumetric estimation of the triple salt precipitated from solutions of serum. It involves the reduction of hexavalent uranium in the triple salt to the quadrivalent state, and subsequent quantitative oxidation with dichromate to the hexavalent condition.

Some objections to these methods have been noted. Velarde (1947), for example, found errors ranging from -3.30 to +4.59 per cent in recovery experiments by Weinbach's method. Furthermore, in the method of Ball and Sadusk very careful control of the reduction and oxidation process is needed to obtain accurate results.

In general, the use of volumetric methods in clinical work is limited by the fact that they are inconvenient and time-consuming when numerous samples are estimated.

Colorimetric methods. Most colorimetric procedures which have been described for the estimation of serum sodium consist of the following four stages:

(1) Deproteinization

(2) Precipitation of uranyl zinc sodium acetate

(3) Washing of the precipitated triple salt

(4) Colorimetric determination of one of the constituents of the dissolved precipitate

Certain other methods include a fifth step, the removal of phosphate, but this is of no importance in serum estimations, as will be seen later. Furthermore, two methods which do not follow this general scheme have also been noted.

(1) Deproteinization - With few exceptions, all methods have employed either trichloracetic acid as a protein precipitant or an ashing procedure for the destruction of serum protein. Some methods, for example those of Hoffman and Osgood (1938) and Darnell and Walker (1940), give directions for both techniques.

It has been pointed out that trichloracetic acid filtrates yield values up to 3 per cent higher than those obtained from ashed serum. This has been attributed by Ball and Sadusk (1936) to a volume displacement of the precipitated protein, the result being an increased concentration of sodium in the filtrate. The remedy suggested by the latter investigators is either to precipitate the proteins in such a way as to cause the least bulk of precipitate to be formed, or else to add a large proportion of trichloracetic acid so as to minimize the effect due to volume displacement. The latter alternative was utilized

by Hoffman and Osgood (1938) and by Stone and Goldzieher (1949) with good results.

(2) Precipitation of uranyl zinc sodium acetate - Among the reagents used for the precipitation of sodium are uranyl zinc acetate solutions containing acetic acid and, in some cases, ethanol in varying amounts. McCance and Shipp (1931), for example, use a 50 per cent alcoholic solution, whereas Darnell and Walker (1940) prefer an aqueous reagent (Weinbach). Other investigators such as Stone and Goldzieher (1949) compromised by adding a small proportion of alcohol to their reagent. The purpose of the alcohol in all cases was to effect a more complete precipitation of the triple salt.

The triple salt is precipitated in various ways. McCance and Shipp treated 1 part of aqueous test solution with 10 parts of alcoholic uranyl zinc acetate at 0°C for 1 hour, whereas Albanese and Lein (1948) used 1 to 4 parts of aqueous solution and 10 parts of alcoholic reagent at 4°C for 1 hour. Darnell and Walker treated 1 part of aqueous solution with 5 parts of an aqueous reagent (Weinbach), followed by the addition of 2.1 parts of ethanol over a period of 30 minutes. Bradbury (1946), however, simplified the technique of Darnell and Walker by using 1 part of aqueous solution, 4 parts of aqueous reagent (Weinbach) and 2 parts

of 95 per cent alcohol, allowing the mixture to stand at room temperature for 20 to 30 minutes. Fowweather and Anderson (1948) found it necessary to alter the method of Bradbury by treating 1 part of the sodium-containing solution with 2 parts of aqueous reagent, 3 parts of absolute alcohol, and 2 parts of water at 3°C. for 2 hours. In the method of King et al (1942), 1 part of aqueous solution is treated with 2 parts of aqueous reagent and 5 parts of ethanol at 0°C. for an overnight period.

(3) Washing of the precipitated triple salt - Among the wash reagents which have been used in colorimetric methods to remove excess reagent from the precipitated triple salt are the following: (i) 95 per cent alcohol (Albanese and Lein, 1948), (ii) absolute alcohol, saturated with triple salt to prevent re-solution of the precipitate (King et al, 1942), (iii) glacial acetic acid, saturated with triple salt (Salit, 1932), (iv) ethyl acetate - acetic acid solution (Darnell and Walker, 1940), (v) 95 per cent alcohol - glacial acetic acid solution, saturated with triple salt (Hoffman and Osgood, 1938; Stone and Goldzieher, 1949).

It was shown by Hoffman and Osgood in 1938 that saturated solutions of the triple salt in alcohol, glacial acetic acid or acetone produced precipitates of uranium

salts when added to uranyl zinc acetate. Since the intensity of the colour of the dissolved triple salt is dependent upon the amount of uranium present, a slight positive error is produced because of extra precipitation of the triple salt. Hoffman and Osgood (1938) claim that in their method this positive error is balanced by an equal negative error due to incomplete precipitation of the triple salt. This type of compensation no doubt also operates to a certain extent when using the other wash reagents mentioned above.

The chief disadvantage of the Hoffman -Osgood and Darnell-Walker methods is the fact that two additional washings with ether are required to remove the acetic acid left after the first washing, thus greatly lengthening the procedures. This difficulty is overcome in the Stone and Goldzieher procedure where the washed triple salt is dissolved in strongly alkaline solution (thereby neutralizing any acid from the wash reagent) prior to further treatment with hydrogen peroxide.

(4) Colorimetric determination of one of the constituents of the dissolved precipitate - Most colorimetric uranyl zinc acetate methods depend upon the measurement of the colour produced when the dissolved triple salt is treated with one of the following: potassium ferrocyanide, sulphosalicylic acid or hydrogen peroxide. In a number of

other methods no further reagent is used to intensify the weak yellow colour of the uranyl ion.

The use of potassium ferrocyanide in sodium methods originated with Barrenscheen and Messiner (1927), who applied the well-known colour reaction between uranium salts and the ferrocyanide ion (in weakly acid solution) to the colorimetric estimation of the uranyl ion (and hence sodium) in the triple salt. The deep, plum-red colour produced was more easily and accurately estimated when visual colorimetric instruments were used than was the untreated solution of triple salt.

Numerous modifications using ferrocyanide have appeared, notably those of McCance and Shipp (1931), Salit (1932) and King et al (1942). All these investigators were aware of the fact that the colour produced due to uranyl ferrocyanide was not stable, but that after some minutes it became darker owing to the formation of colloidal zinc ferrocyanide. No serious errors due to this cause were observed so long as the colours of the test and standard solutions were compared simultaneously in a visual colorimeter within 10 or 15 minutes after the addition of ferrocyanide. When photoelectric colorimeters were introduced, however, this colour instability assumed greater significance (Fowweather and Anderson, 1948) since in these instruments no direct, simultaneous comparison between test

and standard solutions is made. Other objections to the use of ferrocyanide have been reported, such as sensitivity to changes in pH, temperature and reagent concentrations.

Darnell and Walker (1940) treated the dissolved triple salt with sulphosalicylic acid and sodium acetate. The deep yellow colour obtained is stable, but does not obey Beer's law exactly, and a calibration curve must be used.

A number of investigators, including Krakusin (1948) and Albanese and Lein (1948) simply dissolved the triple salt in water and measured the intensity of the yellow solution. These methods, in general, lack sensitivity and are subject to large experimental errors. The Krakusin method is examined in greater detail in Section III.

Stone and Goldzieher (1949) treated the dissolved precipitate with hydrogen peroxide in alkaline solution to give an orange-red colour. This method also is discussed more fully in Section III.

The two methods which differ fundamentally from the others are due to Bradbury (1946) and Fowweather and Anderson (1948). It occured to Bradbury that since the uranyl ion made the reagent a bright yellow, the supernatant should lose colour in proportion to the amount of triple salt precipitated. He found this to be the case; his method, therefore, and a modification by Fowweather and

Anderson depend upon the colour of the reagent after precipitation of the triple salt. Washing, re-centrifuging, weighing, transfer and colour development or titration are eliminated, thus effecting a useful saving of labour compared with other methods and, moreover, avoiding possible losses, either mechanical or by solution in washing, which are associated with the washing process. In Section III a more critical analysis of the Fowweather and Anderson method is presented.

PHYSICO-CHEMICAL METHODS

Introduction

Various attempts have been made to apply physico-chemical concepts to the essentially analytical problem of the determination of sodium in serum. They have resulted, for the most part, in methods which are restricted to particular situations and require expensive, specialized equipment which is not found in the ordinary hospital laboratory. One of these methods, namely polarography, will be dealt with briefly. Greater consideration, however, will be given to the use of the flame photometer which has made a strong bid for popularity in situations where large numbers of sodium and potassium determinations are made.

Polarography

Polarographical determinations of sodium in serum have been made by Prinsen-Geerlings (1937). His procedure is simply to ash 0.1 ml. of serum in the presence of sulphuric acid at 500°C., extract the ash and use the solution thus obtained in a polarograph. In this instrument current voltage lines are recorded automatically and can be related to the concentration of the ion in question. Prinsen-Geerlings gives no data regarding the accuracy of his method, but according to Zlotowski and Kolthoff (1942), this technique is subject to an error of at least 3 per cent.

Flame Photometry

The flame photometer was developed by Barnes et al (1945) for the rapid quantitative determination of small concentrations of the alkali metals, primarily sodium and potassium, in aqueous solution. The instrument was soon applied in medical research and clinical medicine, where exceptionally rapid analyses are often essential in the proper assessment of sodium and potassium balance in various metabolic disturbances.

The physical basis of flame photometry is complex and need not be considered here, but the method itself is simple and can be summarized as follows. A solution of the test

material, appropriately diluted, is sprayed as a fine mist into the air intake of a low temperature gas flame under controlled conditions. After vaporization, light of a characteristic wavelength is emitted by excited atoms of the metal. After passing through a suitable optical system the light is directed onto a photo-sensitive element. The resulting electrical energy is measured by a galvanometer and compared with that found for known standard solutions.

In direct flame photometry a single optical system and one photocell are employed. The deflection of the galvanometer is thus a direct measure of the concentration of metal in the solution. Although detailed procedures for serum and other biological materials are available (Hald, 1947), the method is no longer recommended because of serious errors due to fluctuations in gas and air pressure, variations in viscosity and the effect of foreign molecules.

In internal standard flame photometry (Berry et al, 1946) a constant proportion of lithium is added to all standard and test solutions. A dual optical system and two photocells are so arranged that the electrical energy produced by lithium light falling on one cell is opposed by the electrical energy produced by sodium or potassium light falling on the other cell. The two energies are balanced by a potentiometer, and a calibration curve is

prepared by plotting potentiometer readings against concentrations of known standard solutions. The principle of the method is simply that any factor or variation in experimental conditions which affects the intensity of the internal standard similarly affects the intensity of the unknown, so that the ratio of intensities remains constant. In this way most of the serious errors associated with direct flame photometry are greatly decreased.

Papers by Domingo and Klyne (1949), Spencer (1950) and Fox (1951) should be consulted for further information regarding this new technique.

FURTHER COMMENT ON METHODS

From consideration of the methods already mentioned certain critcisms may be made.

Gravimetric methods remain the methods of choice where accuracy is the prime requisite of the result. When 10 or more ml. of serum are available the classical chloroplatinate method is unexcelled, but if only 1 ml. of serum is available the methods of Butler and Tuthill or Consolazio and Dill are indicated. The chief disadvantage of such methods is that they are involved and become very tedious and time-consuming when multiple analyses are demanded.

Volumetric methods have been used with success by

some people, and are useful where high sensitivity is essential. Accuracy approaching that of gravimetric methods has been achieved with as little as 0.1 ml. of serum by the reduction method of Ball and Sadusk, but the reduction and oxidation steps need very careful control. In general, however, methods involving titration are no more accurate than colorimetric methods, and lend themselves less readily to routine analysis.

Colorimetric methods have become increasingly popular in recent years. This is due in part to the great sensitivity attainable, but also to the ease with which numerous samples can be manipulated in the final stage of the estimation.

Limitations of colorimetric methods have nevertheless been noted. Fowweather and Anderson, for example, have drawn attention to the instability of the colour produced by the addition of potassium ferrocyanide to a solution of uranyl zinc sodium acetate. The effect of this instability, while not serious when visual colorimeters are used, is significant when photoelectric colorimetry is employed, as will be shown in the next section. The use of sulphosalicylic acid in the involved method of Darnell and Walker results in a coloured solution which does not obey Beer's law. The recent method of Stone and Goldzieher , it will be remembered, employs hydrogen peroxide in the final stage,

but this leads to difficulties referred to more fully later. Those methods in which no colour intensifier is used suffer from a lack of sufficient sensitivity to detect small changes in concentration.

Applications of the flame photometer to serum sodium analyses have yielded variable but encouraging results. The extreme simplicity and rapidity of this technique make possible mass electrolyte balance studies, which by the best chemical procedures would be impossible. Occasionally, however, the flame photometer yields erratic results. The cause of this temperamental behaviour may be difficult to discover, and may require that the instrument be returned to the factory for repairs. A delay of several months is not uncommon before the instrument can be put back in service. In the meantime, chemical methods (usually colorimetric) must be resorted to if data are urgently required. Furthermore, at the present time, flame photometry is out of the reach of average or small laboratories due to the expense of the instrument and the necessity for an operator specially trained in the new technique.

It may be concluded, therefore, that until flame photometry is more widely available, colorimetric methods for serum sodium based on the uranyl zinc acetate procedure best meet the demands of the average routine biochemical laboratory.

SECTION III

INVESTIGATION OF SOME EXISTING METHODS

INTRODUCTION

A review of the methods available for the determination of serum sodium has established that colorimetric methods based on the triple acetate of Kolthoff are the most suitable for routine clinical work. Limitations of these procedures have been discussed and the need for a more satisfactory procedure has been pointed out.

As already mentioned, the present section deals with an experimental investigation of four recent colorimetric methods. These were selected for study because the methods seemed to be suitable for routine use but had not yet received adequate evaluation, criticism or comment in the literature.

INVESTIGATION OF THE METHOD OF KING ET AL (1942)

This method is typical of those methods which employ potassium ferrocyanide to impart an intense plum red colour to the weak yellow colour of the uranyl ion. Methods of this type have been criticized by Fowweather and Anderson (1948) on the grounds that the intensity of the uranyl ferrocyanide solution increases with time and hence is not a

suitable reagent for use in methods employing photoelectric colorimeters. It seemed desirable, therefore, to investigate this charge, and to determine what effect such colour instability, if present, would have on the accuracy of the results.

Preparation of Reagents

1. Standard sodium chloride solution (containing 0.75 mg. per ml.) - Dry analytical grade sodium chloride (191 mg.) was dissolved in water and made up to 100 ml.

2. Trichloracetic acid - Seven grams of trichloracetic acid were dissolved in water and made up to 100 ml.

3. Uranyl zinc acetate reagent - Twenty grams of uranyl acetate, $UO_2(CH_3COO)_2 \cdot 2H_2O$, 60 grams of zinc acetate, $Zn(CH_3COO)_2 \cdot 2H_2O$, and 60 ml. of glacial acetic acid were added to 320 ml. of distilled water and warmed gently until dissolved. After standing 24 hours the solution was filtered into a dark bottle, and stored in the refrigerator. This solution was filtered immediately before use.

4. Saturated alcoholic uranyl zinc sodium acetate - Eight ml. of uranyl zinc acetate reagent were mixed with 10 ml. of 50 per cent alcohol saturated with sodium chloride; 20 ml. of absolute alcohol were added, and after standing in the refrigerator overnight, the supernatant solution was decanted. The precipitate was washed several

times with alcohol, drained, dried and then shaken with 100 ml. of absolute alcohol. This solution was also stored in the refrigerator and filtered immediately before use.

5. Potassium ferrocyanide - Twenty grams of potassium ferrocyanide were dissolved in water and made up to 100 ml.

6. Dilute acetic acid - One-half ml. of glacial acetic acid was diluted to 100 ml. with water.

Method

To 0.5 ml. of serum was added 1.5 ml. of 7 per cent trichloracetic acid. The mixture was shaken well and filtered after 5 minutes; 0.2 ml. of the filtrate (equivalent to 0.05 ml. of serum) was transferred to a centrifuge tube containing 1.0 ml. of absolute alcohol and 0.4 ml. of uranyl zinc acetate reagent. The contents were mixed and kept in the refrigerator overnight; they were then centrifuged for 15 minutes. The supernatant solution was decanted, the tube allowed to drain on a filter paper for 10 minutes, and the lip dried. Five ml. of absolute alcohol saturated with sodium zinc uranyl acetate were added, the contents mixed by rotating the tube, centrifuged for 15 minutes and drained as before. The precipitate was then dissolved in 10.0 ml. of dilute acetic acid. To this solution 0.25 ml. of 20 per cent potassium ferrocyanide was added and mixed. This solution was allowed to stand in the dark for 5 minutes to

develop the colour. One ml. of the coloured solution was then diluted to 10.0 ml. with 0.5 per cent acetic acid and mixed. The resulting coloured solution was compared with that produced from a standard sodium chloride solution, 0.2 ml. (equivalent to 0.15 mg. of sodium) of which had been treated simultaneously in the same way as the deproteinized serum. The Coleman Junior Spectrophotometer was used with the wavelength set at 520 millimicrons (equivalent to a green filter). Thus far the method outlined is the procedure of King et al.

In order to test the effect of time on the coloured uranyl ferrocyanide solution the following technique was followed.

The serum and standard solutions, in duplicate, were read in four closely matched cuvettes at 3 minute intervals for 30 minutes, starting 3 minutes after the dilution of 1.0 ml. of coloured solution to 10.0 ml. Reading were also taken after 45, 60 and 180 minutes. Individual closely matched cuvettes were used for each solution in order to make possible the repetition of four readings every 3 minutes.

Calculations

The following relation was used to calculate the sodium concentration:

$$Na = \frac{D_T}{D_S} \times 0.15 \times \frac{100}{0.05} = \frac{D_T}{D_S} \times 300 \text{ mg. per 100 ml.}$$

where D_T and D_S refer to the density of test and standard solutions, respectively.

In order to determine what effect colour instability would have on the accuracy of the results, two series of calculations based on the above relation were carried out using the optical density values obtained after various intervals of time, t, had elapsed, where t represents the interval of time following the preparation of the final coloured solution.

In the <u>first series</u> of calculations, the values for the concentration of sodium for given values of t are based on corresponding values of D_S and D_T; that is,

$$Na = \frac{D_T}{D_S} \times 300 \text{ mg. per 100 ml.}$$

In the <u>second series</u>, the values for the concentration of sodium for given values of t are based on corresponding values of D_T, and one constant value for D_S, namely $D_S = 0.582$ when $t = 3$ minutes (see Table I); that is,

$$Na = \frac{D_T \times 300}{0.582} \text{ mg. per 100 ml.}$$

The significance of these two series of calculations

is that they indicate to what extent colour instability would affect results if the optical densities of the standard and test solutions were measured (1) simultaneously, as in visual colorimetry, or (2) at different times, as in photoelectric colorimetry, where the common procedure (unless the effect of colour instability is fully appreciated) would be to read the standard once, and then to read in succession a series of test solutions .

Results

The results of the experiment are recorded in Table I. A steady, gradual increase in optical density with time is noted for both standard and test solutions. The effect of the colour instability of the final solution on the accuracy of the results is indicated in the fourth and fifth columns (which list the results of the first and second series of calculations).

It can be seen from these calculations that results based on values of D_S and D_T which are obtained simultaneously show good agreement over a reasonable interval of time (fourth column), but that results do not agree when D_T is measured some time later than D_S (fifth column).

Discussion

The data presented above verify the observations of

TABLE I

EFFECT OF COLOUR INSTABILITY IN METHOD OF KING ET AL

Interval of time following preparation of final coloured solution	Optical density of final coloured solutions		Concentration of sodium in serum. Mg. per 100 ml. (a) when standard is read simultaneously with test, (b) when standard is read at 3 minutes	
	Standard solution	Test solution	(a)	(b)
t (minutes)	D_S	D_T	$Na = \frac{D_T \times 300}{D_S}$	$Na = \frac{D_T \times 300}{0.582}$
3	0.582	0.602	310	310
6	0.588	0.609	310	314
9	0.595	0.615	310	317
12	0.603	0.624	310	322
15	0.609	0.633	312	326
18	0.614	0.639	312	329
21	0.621	0.645	312	
24	0.628	0.651	310	
27	0.633	0.657	311	
30	0.639	0.655	312	
45	0.622	0.684	315	
60	0.672	0.706	315	
180	0.682	0.715	315	

Fowweather and Anderson, namely, that the intensity of the colour due to uranyl ferrocyanide increases quite rapidly with time. This colour instability, however, was found to have a negligible effect on the final result so long as standard and test solutions were read simultaneously, but when an interval of 3 or more minutes elapsed between the reading of the standard and the test, the calculated results were in error by at least one per cent. For a 15-minute interval this error amounted to about six per cent. The latter situation could easily arise when numerous solutions are analyzed by means of a photoelectric colorimeter, in the manner referred to previously (see Calculations).

It is clear, therefore, that the use of potassium ferrocyanide in sodium analyses may lead to appreciable errors when photoelectric colorimetry is employed.

A further disadvantage which is immediately appreciated in clinical applications of the method of King et al is the fact that an overnight precipitation is necessary.

Conclusions

1. The method of King et al (1942), like other methods which employ potassium ferrocyanide to intensify the colour of the dissolved uranyl zinc sodium acetate, suffers from the defect that colour instability of the final solution may lead to significant errors when photoelectric

colorimetry is employed.

2. The necessity for a long precipitation period detracts from the clinical usefulness of the method.

METHOD OF FOWWEATHER AND ANDERSON (1948)

The Fowweather and Anderson method, in which the colour of the excess reagent is measured, was tried next since it was most attractive from the standpoint of simplicity.

Preparation of Reagents

1. Trichloracetic acid solution. Twenty grams of trichloracetic acid were dissolved in water and made up to 100 ml.

2. Weinbach's uranyl zinc acetate reagent. Solution A: Uranyl acetate (7.7 grams) and 1.4 ml. of glacial acetic acid were dissolved with gentle heating and stirring in 40 ml. of water, and the volume made up to 50 ml. in a volumetric flask. Solution B: Zinc acetate (23.1 grams) and 0.7 ml. of glacial acetic acid were dissolved by gentle heating and stirring in 40 ml. of water and the volume made up to 50 ml. in a volumetric flask.

Solutions A and B were mixed while hot, allowed to stand 24 hours, and filtered.

3. Standard sodium chloride solution - (containing 500 mg. of sodium per 100 ml.) - Dry, analytical grade sodium chloride (12.7090 grams) was dissolved in water and made up to 1 litre.

4. Standard sodium chloride solutions - (containing 400, 350, 300, 200, and 100 mg. of sodium per 100 ml., respectively) - These solutions were obtained by suitable dilution of the 500 mg. standard.

Method

To 1.0 ml. of serum were added 1.0 ml. of water and 1.0 ml. of trichloracetic acid solution. After mixing and allowing to stand for 10 minutes, the solution was filtered. To 1.0 ml. of protein-free filtrate in a small conical flask were added 2.0 ml. of the Weinbach uranyl zinc acetate reagent, 3.0 ml. of absolute alcohol and 2.0 ml. of water. After mixing thoroughly, the flask was stoppered and allowed to stand in the refrigerator for two hours. The contents of the flask were then centrifuged and the colour of the supernatant fluid was determined in a Coleman Junior Spectrophotometer with the wavelength adjusted to 430 millimicrons. The optical density of this fluid was subtracted from the reading obtained with a similar mixture (the reagent blank) containing 1.0 ml. of water instead of 1.0 ml. of filtrate, and the difference referred

to a graph obtained by plotting the corresponding values from a series of standard solutions. This gave the sodium content of the serum.

Results

Table II gives the optical density readings and the calculated differences in optical density for a given series of solutions. In Fig. 1 the differences between the readings of the reagent blank and the standard solutions, $(D_B - D_S)$, have been plotted against the respective concentrations giving a calibration curve for the method.

When $D_B - D_S$ for the serum is referred to the curve, a value of 327 mg. of sodium per 100 ml. is obtained by interpolation.

Discussion

From the data and calibration curve it can be seen that the difference in optical densities between two samples of serum containing 300 and 350 mg. of sodium per 100 ml. is very small, approximately 0.020. Put another way, a difference of 2.5 mg. of sodium per 100 ml. of serum is required to produce a unit change in the third decimal place of the density reading. Since this decimal must be estimated from the galvanometer scale with an accuracy not better than 0.001, it follows that a given reading may

TABLE II

DATA OBTAINED BY THE METHOD OF

FOWWEATHER AND ANDERSON

Solution treated with uranyl zinc acetate reagent	Optical density of excess reagent after precipitation of sodium	Difference in optical densities $D_B - D_S$ *
Blank (0 mg. Na per 100 ml.)	0.500	
Standard(500 mg. Na per 100 ml.)	0.272	0.228
Standard(400 mg. Na per 100 ml.)	0.328	0.172
Standard(350 mg. Na per 100 ml.)	0.348	0.152
Standard(300 mg. Na per 100 ml.)	0.368	0.132
Standard(200 mg. Na per 100 ml.)	0.420	0.080
Standard(100 mg. Na per 100 ml.)	0.468	0.032
Serum	0.360	0.140

* D_B and D_S represent the optical densities of excess uranyl zinc acetate reagent after precipitation of sodium in the blank and standard (or serum) solutions, respectively.

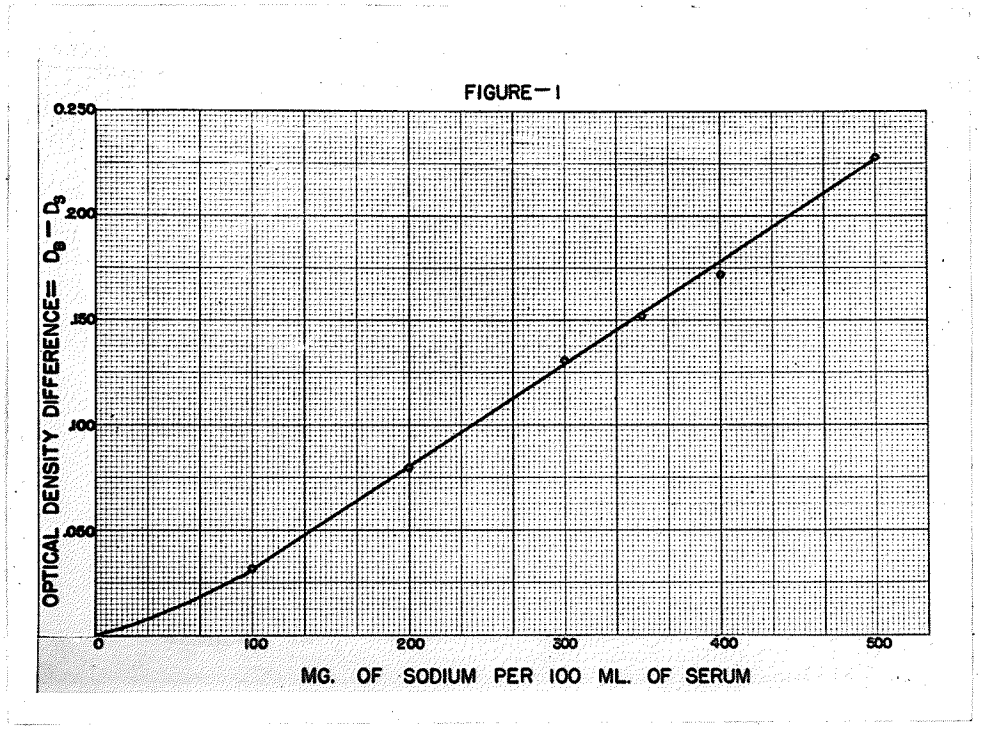

Figure 1

Calibration curve for sodium content of serum.

Method of Fowweather and Anderson.

be in error by 5 mg. per 100 ml. or more. Furthermore, since two readings are involved in each estimation, the possible error is doubled, that is, 10 mg. per 100 ml., and this due solely to inaccuracies in reading the scale.

It is thus evident that the method lacks sufficient sensitivity to detect small deviations from the normal serum sodium concentrations.

Other disadvantages of the method are that it requires one ml. of serum, and a two-hour precipitation period.

Conclusion

The method of Fowweather and Anderson, despite its extreme simplicity, is not satisfactory as a routine method for clinical use because it lacks sensitivity and rapidity, and because at least one ml. of serum is required.

THE METHOD OF KRAKUSIN (1948)

The next method to be tried was that of Krakusin. Certain features, which detract from the simplicity and convenience sought for in a rapid method, can be noted from a casual examination. Among these are (1) it is necessary to ash the serum, (2) the washed precipitate of sodium uranyl zinc acetate has to be dried, (3) hot water (60°C.) is used to dissolve the precipitate, and (4) the

final coloured solution is warmed to a definite temperature before colorimetric estimation.

The precipitation period, on the other hand, is relatively short, 30 to 45 minutes, and the colour of the dissolved triple salt which is measured directly, is not subject to the instability found when ferrocyanide is added. Highly reproducible results are also claimed for this method.

These features, plus the fact that the method can also be used for the determination of sodium in urine, were considered sufficient reasons for an experimental test.

Preparation of Reagents

1. Uranyl zinc acetate reagent (According to Butler and Tuthill, 1931) -

Solution A: To 40 grams of uranyl acetate in a one-litre beaker were added 24 grams (23 ml.) of 30 per cent acetic acid and water to make 260 grams.

Solution B: To 110 grams of zinc acetate were added 12 grams (11.5 ml.) of 30 per cent acetic acid and water to make 260 grams.

Both Solutions were covered and warmed on a steam bath until, with stirring, complete solution of the salts was effected. Solution B was then added to Solution A while hot, and allowed to stand for 24 hours before using.

A small amount of triple salt, prepared as described below, was added to the mixture. The latter was shaken several times before using, and filtered to ensure saturation at the temperature of the analysis.

2. Alcohol (95 per cent) saturated with precipitated triple salt - The uranyl zinc sodium acetate was prepared according to the method of King et al (q.v.). This was then shaken up with 500 ml. of 95 per cent alcohol and stored in the refrigerator. The solution was filtered before use.

Method

One ml. of serum was pipetted into a large Pyrex test tube (200 by 25 mm.); a small glass bead, 1 ml. of 4 N sulphuric acid and 0.5 ml. of concentrated nitric acid were added. The mixture was digested over a Bunsen flame; when charring appeared the flame was removed. After cooling for about one minute a few drops of concentrated nitric acid were added and digestion resumed. The addition of nitric acid and heating were continued until the solution cleared. Heating was continued for a few minutes to drive off excess acid. The tube was allowed to cool and 4 to 5 drops of water were added. The solution was then poured into a 10 ml. volumetric flask and the tube rinsed three times with 2 ml. of distilled water, the rinsings being added to the flask. The volume was made up to 10 ml. with water.

One ml. of the ashed serum solution was added dropwise to 10 ml. of uranyl zinc acetate in a 15 ml. centrifuge tube with constant stirring. After allowing the mixture to stand for 30 to 45 minutes with occasional stirring, the stirring rod was withdrawn and washed with 3 ml. of reagent, the latter being added to the solution already in the centrifuge tube. The tube was then stoppered and centrifuged at 2000 r.p.m. for 5 to 10 minutes. Aspiration of the supernatant, washing of the precipitate with 5 ml. of 95 per cent alcohol saturated with triple salt, stoppering and centrifuging followed. The washing process was repeated, after which the tubes were dried at 60 to 70°C. in an oven for 5 to 10 minutes. Eight ml. of hot water (60°C.) were added to dissolve the precipitate. After cooling to room temperature, the tube was made up to the mark with water and mixed. The temperature was brought to 25°C. in a water bath and the colour of the dissolved precipitate read at 430 millimicrons on the Coleman Junior Spectrophotometer. The concentration of the sodium in the original serum was then obtained by reference to a calibration curve.

The calibration curve was prepared by plotting optical densities obtained when 1 ml. of each of five standard sodium chloride solutions was treated in the same way as the ashed serum solution. The standard solutions contained 0.05, 0.04, 0.03, 0.02, and 0.01 meq. of sodium per

ml. of solution. These concentrations are equivalent to 500, 400, 300, 200, and 100 meq. per litre, or 1150, 920, 690, 460, and 230 mg. per 100 ml. of the original serum or urine.

The following day a second series of standard solutions containing 0.045, 0.035, 0.025, 0.015, and 0.006 meq. of sodium per ml. was similarly carried through the above procedure, the purpose being to add intermediate points to the calibration curve originally prepared.

Results and Discussion

It was found that the points from the second series of standard solutions did not fall on the original curve, but considerably above it. When the experiment was repeated with the same series of standard solutions a similar day to day variation was observed.

Fig. 2 illustrates the magnitude of the error involved in a serum estimation due to this imperfect reproducibility by showing the results of two series of standard solutions plotted as separate calibration curves (I and II, respectively). The test sample of serum yielded an optical density of 0.199, which when referred to Curve I gave a result of 160 meq. of sodium per litre, whereas the corresponding value when referred to Curve II was 130 meq. It is evident from this that the method does not yield the

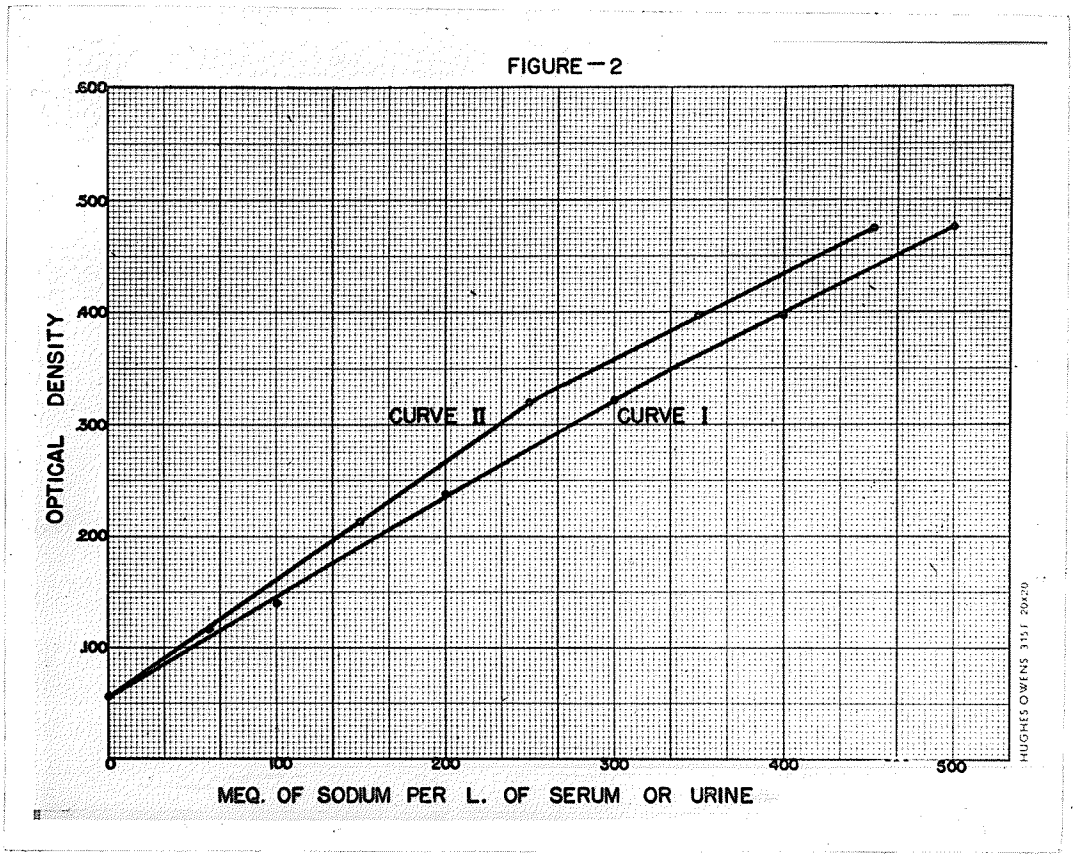

Figure 2

Calibration curves for sodium content of serum and urine (method of Krakusin). Curves I and II were prepared from data obtained on two different days. They illustrate a day to day variation in results by this method. Furthermore, since the extreme range of concentrations in health and disease is between 250 and 350 mg. per 100 ml. (equivalent to 108 and 152 meq. per litre), it is evident that only a small portion of the curves is useful for serum estimations.

necessary degree of reproducibility, despite the cumbersome, tedious procedure with its controlled temperatures for drying and dissolving the precipitate, and for reading the final colour.

It may be noted further that these calibration curves are more suitable for urine analyses where wide variations in sodium concentrations are commonly found, but that for serum analyses, where most values fall between the narrow limits, 137 to 147 meq. per litre (315 to 340 mg. per 100 ml.), the corresponding optical density values are confined to a very small portion in the lower part of each curve. Like the Fowweather and Anderson method, therefore, this method is insensitive to small changes in serum sodium concentrations.

Conclusions

The method of Krakusin has been found to lack the simplicity, reproducibility and sensitivity required of a routine method for the estimation of sodium in serum.

THE METHOD OF STONE AND GOLDZIEHER (1949)

Stone and Goldzieher proposed a method in which simplicity, they claimed, could be achieved without sacrifice in accuracy. Their method was outlined for serum and urine in a preliminary report (Goldzieher and Stone, 1949),

but was later modified to increase the accuracy of the method for serum.

It is a conventional procedure, consisting of the usual four steps, deproteinization, washing, and colour development. Because it will be convenient to refer to later the method is given in detail here.

Preparation of Reagents

1. Uranyl zinc acetate. A solution of 14 ml. of glacial acetic acid in 750 ml. of distilled water was brought almost to a boil. 77 grams of uranyl acetate were added. To this mixture 231 grams of zinc acetate, divided into five or six portions, were added with frequent stirring. Finally, 7 ml. of glacial acetic acid were added, and the solution was cooled to room temperature and diluted to 1 litre. Thereafter, 200 ml. of 95 per cent ethanol were added, and the solution was refrigerated overnight and filtered.

2. Triple salt. To 10 or 15 ml. of the above solution there was added enough of a concentrated sodium chloride solution to remove all but a trace of yellow colour from the supernatant liquid. The precipitate was collected by filtration and washed five times with glacial acetic acid and five times with ether, then dried.

3. Wash reagent. A solution of 425 ml. of 95 per cent ethanol and 75 ml. of glacial acetic acid was saturated

at room temperature with pure triple salt (see above). The reagent was stored in a brown bottle.

4. Ammonium carbonate. A saturated aqueous solution.

5. Hydrogen peroxide. 30 per cent (Superoxol).

6. Trichloracetic acid. A 10 per cent aqueous solution.

Procedure

To 9.0 ml. of 10 per cent trichloracetic acid was added 1.0 ml. of serum dropwise. The mixture was shaken or stirred thoroughly and centrifuged.

One ml. of the protein-free filtrate was pipetted into a 15 ml. graduated centrifuge tube and 6.0 ml. of the uranyl zinc acetate reagent were added. The solutions were mixed well by holding the tip of the centrifuge tube between the thumb and forefinger and rotating it rapidly back and forth. The tube was then allowed to stand for 20 minutes, during which it was twirled twice by the same manoeuvre in order to stir up the precipitate. The tube was then centrifuged at high speed for 7 minutes following which the supernatant was decanted and the tube drained for 1 minute.

Five ml. of the wash reagent were added, care being taken to wash down the sides of the tube well. The contents of the tube were mixed by twirling and then centrifuged for

7 minutes. The supernatant was decanted and the tube drained as before.

The precipitate was dissolved in a few drops of distilled water and 6 ml. of ammonium carbonate were added. Then 1 ml. of 30 per cent hydrogen peroxide was added and the volume adjusted to 15.0 ml. with distilled water. After mixing by inversion, the solution was read in a Coleman Junior Spectrophotometer with the wavelength set at 460 millimicrons. The optical density of the solution was finally related to the concentration of sodium by means of a calibration curve . The latter was prepared by running known sodium chloride solutions through the entire procedure.

Discussion

Two features of the method are of particular interest. It will be remembered that whereas King *et al* use an overnight precipitation period, Stone and Goldzieher require only 20 minutes by using a reagent containing alcohol and through proper choice of reactant concentrations. The colour development, moreover, depends upon the reaction between the uranyl ion, in strongly alkaline solution, and hydrogen peroxide producing a complex of reddish-yellow colour. Under the conditions of the determination, they claimed that Beer's law is obeyed when the coloured solution

is examined at a wavelength of 415 millimicrons, but that the absorption is so great with solutions corresponding to normal sodium concentrations that optical density readings fall outside the useful region of the galvanometer scale. They therefore selected a wavelength of 460 millimicrons, since although adherence to Beer's law is sacrificed and a calibration curve must be used, greater accuracy is achieved due to the fact that normal readings fall approximately in the most accurate (middle) portion of the scale.

Stone and Goldzieher observed that after a variable period of time there was a tendency for the formation of bubbles along the walls of the cuvettes used in the colorimetric estimation. They suggested that where a large number of specimens is to be handled, the bubble nuisance can be eliminated by adding the peroxide to a few tubes at a time. In the writer's experience this precaution frequently proved to be inadequate, as bubbles often formed immediately. The determination was thereby rendered useless.

Their uranyl zinc acetate reagent, however, was good and was subsequently used.

Conclusions

The method of Stone and Goldzieher, although simple to perform and capable of high accuracy, is not thoroughly dependable at all times due to the unpredictable formation

of bubbles in the final coloured solution. It is necessary, furthermore, to use a calibration curve, the disadvantages of which are discussed in the next section.

Apart from these criticisms the method seems to be the most suitable yet described for routine estimations of serum sodium.

SUMMARY

Four recent colorimetric methods for the estimation of serum sodium have been investigated experimentally. These were, respectively, the methods of King et al (1942), Fowweather and Anderson (1948), Krakusin (1948) and Stone and Goldzieher (1949).

It was concluded that all four methods possessed certain features which made them unsuitable for routine estimations in hospital biochemical laboratories.

SECTION IV

THE DEVELOPMENT OF A NEW METHOD

Introduction

In view of the inadequacies of recent colorimetric methods, the development of a new method employing uranyl zinc acetate was undertaken.

It was necessary at the outset to establish criteria which the new method must satisfy and to decide in what way the problem of devising such a method might best be approached.

The preliminary work which followed these considerations fell into two parts: the search for a suitable reagent to intensify the colour of the dissolved triple acetate, and the successful application of this reagent in a new method.

In order to facilitate presentation, the method finally chosen is given at the end of this section and further experimental work is dealt with in Section V.

Requirements of the New Method

Since the method to be developed was intended for routine estimations in hospital biochemical laboratories, the following requirements were considered essential.

(1) Rapidity. The method should permit a determination on a given sample of serum to be performed in one hour or less.

(2) Accuracy. An average accuracy of one per cent should be obtained over the range of concentrations encountered clinically, namely, 250 to 350 mg. of sodium per 100 ml. of serum.

(3) Dependability. Reliable results should be given under ordinary laboratory conditions.

(4) Use of small amounts of sample. One-half ml. of serum should be adequate for the usual analysis, but it should be possible to use even smaller amounts, particularly when a series of determinations is to be done on an infant.

(5) Use of ordinary laboratory equipment. In the majority of situations special or complicated equipment might not be available, and for this reason should not be required in a method for general use.

It has been seen that colorimetric methods satisfy most of the above requirements, but that, in general, rapidity is gained at the expense of either accuracy or dependability.

Approach to the Problem

It was felt that an attempt should be made to satisfy all the requirements referred to above in a new

colorimetric procedure for the determination of sodium in serum, since colorimetric methods , where applicable, are usually the methods of choice for routine analyses.

In the development of a new colorimetric method the four stages already mentioned were considered separately.

(1) Precipitation of serum proteins. Trichloracetic acid is without question the best reagent for the purpose. It has been used almost exclusively in recent methods in lieu of the more tedious ashing procedure.

A number of investigators have nevertheless reported discrepancies between sodium values obtained from ashed serum and those from trichloracetic acid filtrates. The latter were found to give consistently positive errors of one to three per cent, or even somewhat higher. It was shown that the error could not have arisen from sodium impurities in the trichloracetic acid. The explanation advanced in some quarters, therefore, is that the precipitated protein occupies a definite volume and consequently raises the concentration in the supernatant. Ball and Sadusk (1936) note that this discrepancy becomes negligible when a large proportion of trichloracetic acid is added to serum in the preparation of the protein-free filtrate.

(2) Precipitation of sodium. The uranyl zinc acetate method of Stone and Goldzieher is obviously the most

satisfactory which has been proposed so far. Their reagent and precipitation procedure were therefore adopted tentatively in preliminary work.

(3) Washing of precipitate. The wash reagent of Stone and Goldzieher was likewise chosen. It is effective in removing excess uranyl zinc acetate reagent without dissolving the precipitate, and is more pleasant to use than certain other reagents used for this purpose.

(4) Development of final colour. A new method of developing the colour of the dissolved precipitate was imperative for a satisfactory colorimetric method. This was therefore the first and most important aspect of the problem to be attacked.

The removal of phosphate prior to or following the precipitation of sodium was not considered necessary. In the first place, assuming that the uranyl phosphate formed has the formula, $UO_2 \cdot HPO_4$, the maximum error introduced would be 0.7 per cent for serum containing the abnormally high concentration of 10 mg. of phosphate per 100 ml. The error caused by normal amounts of phosphate in serum can therefore be neglected. In the second place, removal of phosphate would necessitate an additional step, thereby lengthening the procedure.

Search For a Suitable Colour Intensifier

A few preliminary attempts were made to find a suitable reagent to intensify the colour of the triple salt, uranyl zinc sodium acetate, when this is dissolved in water.

Among the various reagents tried were aliphatic α-hydroxy- and keto acids, and aromatic phenolic acids well known to give characteristic colours with uranyl salts (Muller, 1919). Of the forty five compounds tested, only eight gave coloured solutions which could be utilized for colorimetric estimation. These were (1) 2,4 dinitro-1-naphthol-7-sulphonic acid, (2) ascorbic acid, (3) pyrogallol, (4) sulphosalicylic acid, (5) hydroquinone, (6) pyrocatechol, (7) salicylic acid, and (8) sodium salicylate.

Sodium salicylate was selected for further study because it yields an intense orange colour with the uranyl ion. A preliminary test, moreover, showed that the coloured solution produced was remarkably stable with time, and also that the intensity varied but slightly with temperature. An absorption curve prepared from a spectrophotometric examination (Fig. 3) revealed that the absorption increases continuously with decreasing wavelength over the range measured, that is, between 410 and 700 millimicrons.

Having obtained this information attempts were made to apply salicylate to Stone and Goldzieher's method.

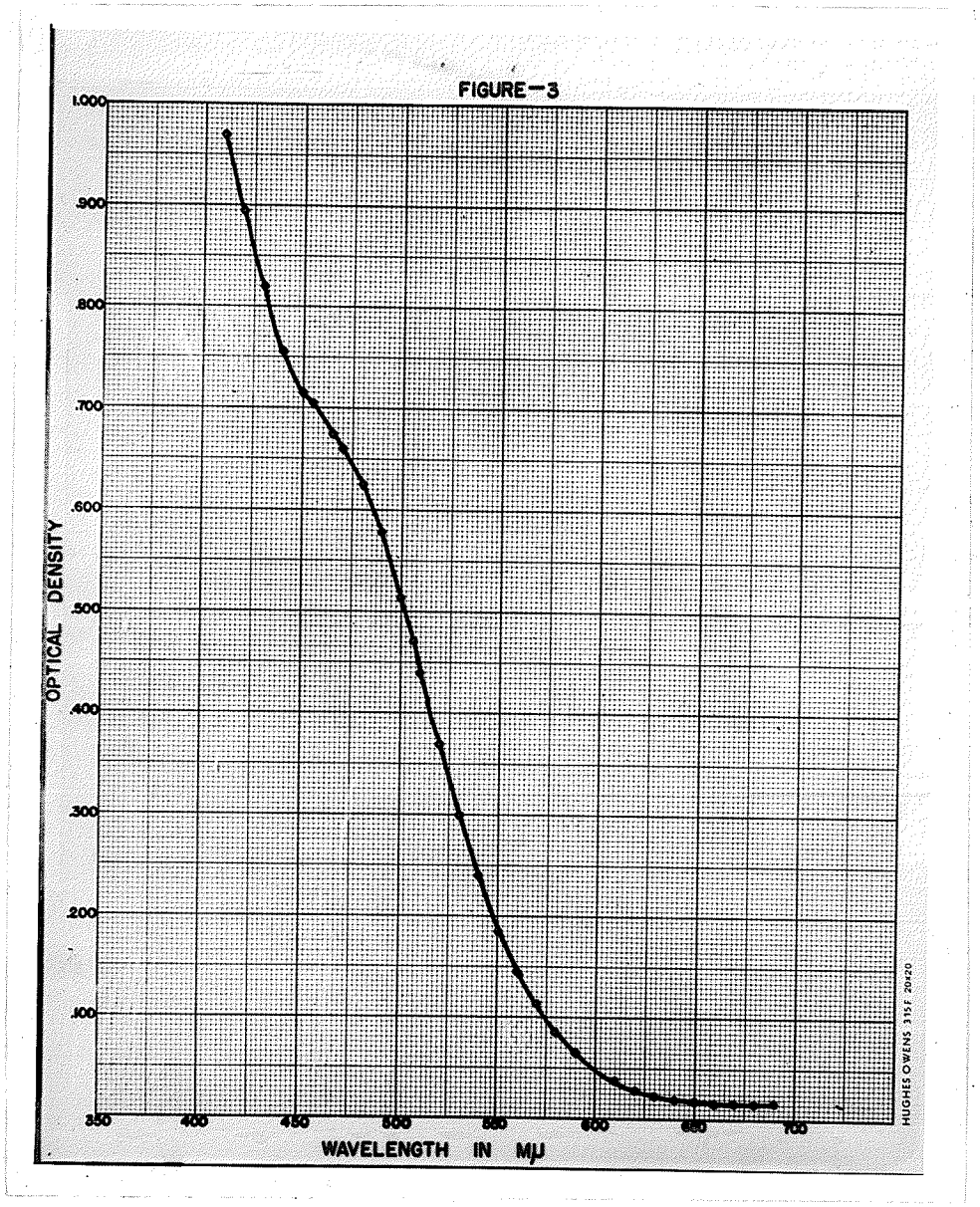

Figure 3

Absorption curve of a uranyl zinc sodium acetate solution treated with sodium salicylate

Attempted Application of Salicylate

Preliminary attempts. At first the method of Stone and Goldzieher was followed without change insofar as deproteinization of serum, precipitation of sodium and washing of the precipitate were concerned. The final colour, however, was produced in the following way.

The washed precipitate was dissolved in 10 ml. of distilled water, and to the resulting pale yellow solution 1 ml. of 10 per cent sodium salicylate was added, yielding a deep orange solution. This was further diluted to 15 ml. and the resulting intensity compared in a Coleman Junior Spectrophotometer with a solution which had been treated with hydrogen peroxide (according to Stone and Goldzieher) instead of with salicylate.

This experiment showed that the colour produced by salicylate was more intense than that produced by peroxide, the optical densities being 1.06 and 0.592, respectively, at a wavelength of 460 millimicrons.

Since a reading of 1.06 falls on that portion of the scale at which instrumental errors are relatively great, further readings of the solution were taken at longer wavelengths, in order to determine the most suitable wavelength. Table III shows that at wavelengths of 520 and 550 millimicrons the galvanometer readings were brought within the

TABLE III

VARIATION IN OPTICAL DENSITY WITH WAVELENGTH FOR A SOLUTION OF URANYL ZINC SODIUM ACETATE TREATED WITH SODIUM SALICYLATE IN A PRELIMINARY MODIFICATION OF THE METHOD OF STONE AND GOLDZIEHER

Wavelength	Optical density
460	1.06
490	0.900
520	0.700
550	0.314

most accurate and useful portion of the scale.

The next step was to determine the relation between the concentration of sodium in the original solution and the optical density of the final solution.

This was accomplished by treating standard sodium chloride solutions in the manner indicated above for serum and plotting the results. The curves obtained for various wavelengths (Fig. 4) are similar to those found by Stone and Goldzieher. It will be seen that a linear relationship between concentration and optical density does not exist.

Although such curves could be used for calibrating the instrument (as Stone and Goldzieher did), it was felt that a comparison between the serum and a standard solution prepared in parallel would provide a sounder basis for a method. The disadvantage of calibration curves is that given concentrations of the substance analyzed may not always give the same readings at different times. Two reasons for the slight discrepancies occasionally observed have been discussed by Delory (1949). The first is slight changes in photometric factors such as the behaviour of the photocell or the properties of the light filters, and the second is the effects of time, temperature, external lighting or deterioration of reagents. When, however, a standard solution is prepared and read in parallel with each batch of tests, such changes are allowed for since both standard

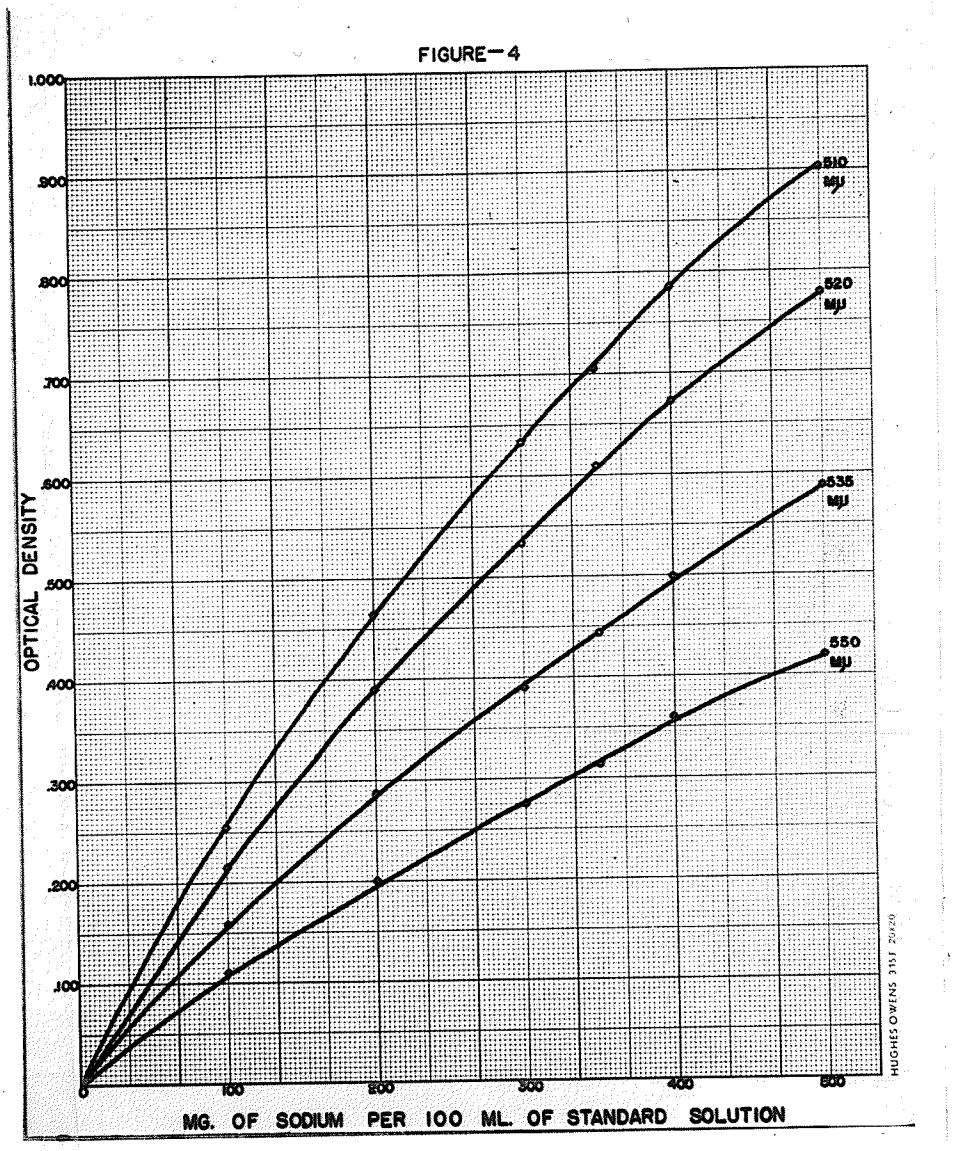

Figure 4

The relation between optical density (measured at 510, 520, 535 and 550 millimicrons) and sodium content of standard solutions in a preliminary application of sodium salicylate

and test solutions are affected equally.

It was therefore decided to find out whether proportionality between optical density and concentration might be attained by changing conditions in one or more stages of the method.

Further modifications to establish proportionality between optical density and concentration. One way which has sometimes been used to obtain better proportionality is to dilute the solution to be tested.

In the present situation it was possible to dilute either the original serum (or standard), or the final coloured solution, or both. Experiments were accordingly performed with standard sodium chloride solutions (containing 100 to 500 mg. of sodium per 100 ml.) in the manner indicated on page 53, except that in one case initial dilutions were doubled, and in another case final dilutions were doubled. Since in both cases the optical densities of the final solutions were much lower than previously found, it was possible to read the solutions at shorter wavelengths, that is, closer toward the peak absorption characteristic for a solution of uranyl zinc sodium acetate treated with salicylate (cf. Fig. 3). The results for wavelengths between 415 and 520 millimicrons when plotted gave curves similar to those shown in Fig. 4. It is apparent , therefore, that mere dilution of initial or final solutions was

not effective in producing linearity, even though shorter (and hence theoretically more suitable) wavelengths were used in the measurement.

This problem of establishing conditions whereby linearity between optical density and concentration could be achieved was then investigated from another point of view, namely, by change in the conditions for precipitation.

Among the various changes introduced were three which ultimately gave the desired results. The first of these was the use of only 3 ml. of uranyl zinc acetate reagent instead of the 6 ml. previously used to precipitate the sodium contained in 1 ml. of trichloracetic acid filtrate. The second change concerned the preparation of a more dilute filtrate by using only 0.5 ml. of serum (or standard) to 10 ml. of trichloracetic acid instead of 1.0 ml. of serum to 9 ml. of trichloracetic acid. The third change involved a slight modification in the preparation of the uranyl zinc acetate reagent. Whereas Stone and Goldzieher allowed the freshly prepared reagent to stand overnight in the refrigerator and then filtered , the reagent was now allowed to stand overnight at room temperature. To this reagent a small amount of the triple salt was purposely added to ensure saturation at all times. A portion of this reagent was then filtered just before using.

A small change was also introduced into the washing

procedure of Stone and Goldzieher's method. Instead of using 5 ml. of wash reagent, only 3 ml. were used. Although this did not affect the method in any way, it did result in a saving of some reagent.

Tests on standard solutions. When the method with the various modifications described above was applied to a series of standard solutions containing 100, 200, 300, 400 and 500 mg. of sodium per 100 ml., proportionality was good although there was a falling off at higher concentrations.

Further tests were then made with standard solutions containing 250 to 350 mg. of sodium per 100 ml., since this is the range of clinical significance in serum analyses. Blank solutions in which distilled water was substituted for standard solutions were carried simultaneously through the whole procedure to allow for traces of sodium in the reagents and filter paper, and also for trace residues of uranyl salts deposited in the centrifuge tube during the washing procedure. Using the Coleman Junior Spectrophotometer, best results were obtained when the optical densities were measured at 460 millimicrons. A typical result shown in Fig. 5 illustrates the close approximation to linearity now achieved.

Application of Beer's law. In view of linearity it

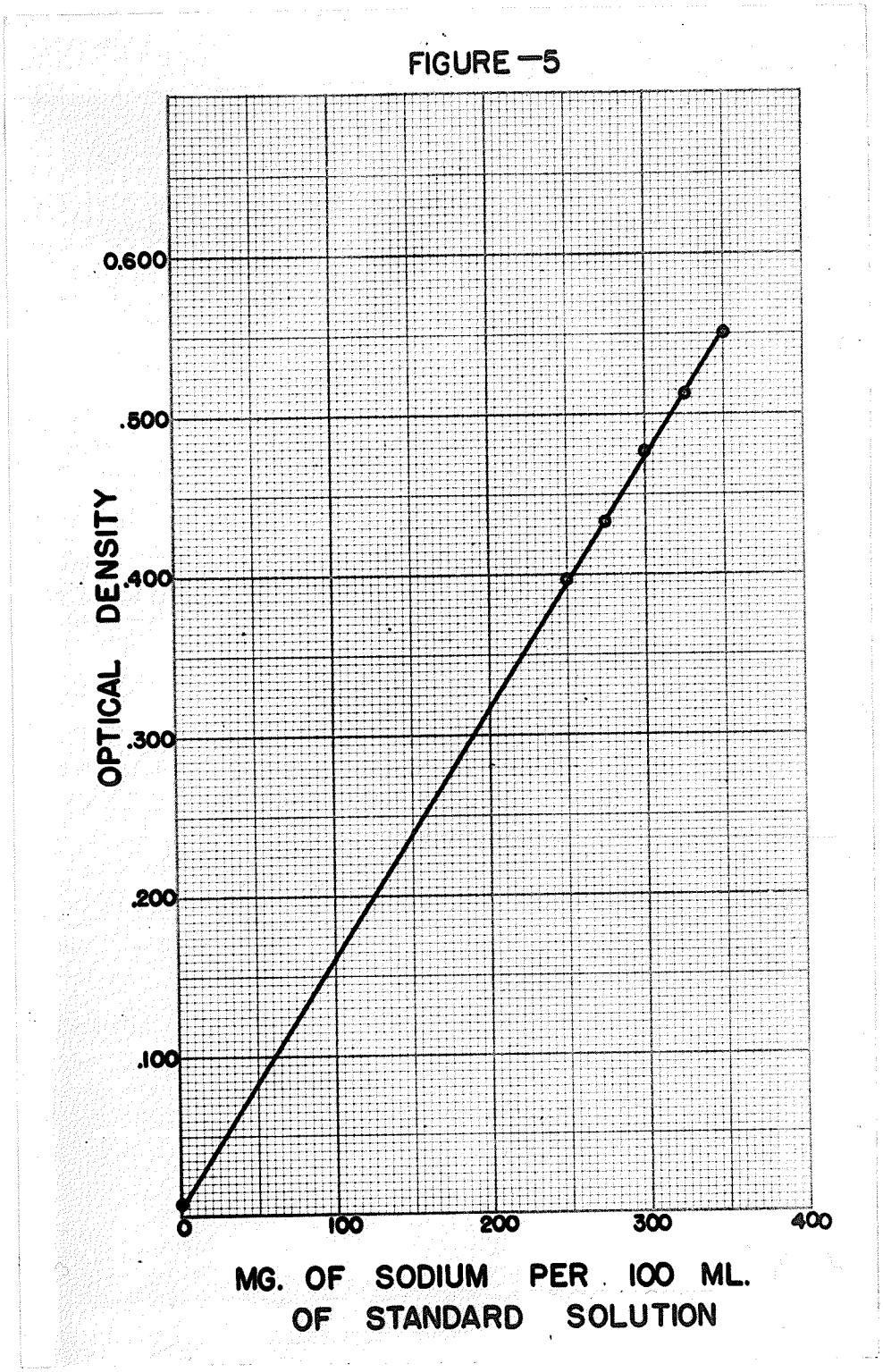

Figure 5

The relation between optical density (measured at 460 millimicrons) and sodium content of standard solutions in a modified method employing sodium salicylate

was justifiable to dispense with a calibration curve and to take advantage of the more desirable procedure referred to earlier, namely, the comparison of a test solution with a standard solution which is prepared and read in parallel with the test. In consequence, calculations based on Beer's law were made using the general expression for photoelectric colorimetry,

$$\frac{D_T - D_B}{D_S - D_B} = \frac{C_T}{C_S}$$

where D_T, D_S and D_B represent the optical densities of the test, standard and blank solutions, respectively, and C_T and C_S the concentrations of test and standard. The validity of these calculations is indicated in Table IV, which gives the results for four known solutions treated as "tests", when a solution containing 350 mg. of sodium per 100 ml. was used as a "standard". Good agreement was observed between true and calculated values and also between duplicates.

Stability of the coloured compound. The coloured compound produced by the interaction of sodium uranyl zinc acetate with sodium salicylate was found to be stable over periods of time up to 24 hours. It will be remembered that the addition of potassium ferrocyanide to a solution of the triple salt produces a plum-red colour which steadily darkens with time at a rate which may introduce appreciable

TABLE IV

TEST OF CALCULATIONS BASED ON BEER'S LAW

IN MODIFIED METHOD USING SODIUM SALICYLATE

Optical density of final coloured solution	Mg. of sodium per 100 ml. of original solution	
	True concentration	Calculated concentration *
0.550	350	
0.007	0	
0.397	250	251
0.397	250	251
0.430	275	273
0.435	275	276
0.475	300	302
0.477	300	303
0.511	325	325
0.513	325	326

* Calculations were based on the following form of Beer's law:

$$C_T = \frac{D_T - D_B}{D_S - D_B} \times C_S$$

where D_T, D_S, and D_B refer to the optical densities of test, standard and blank, respectively, and C_T and C_S refer to the concentrations of the test and standard, respectively. The solution containing 350 mg. of sodium per 100 ml. was taken as the "standard"; hence the above expression transforms to:

$$C_T = \frac{D_T - 0.007}{0.543} \times 350$$

errors in photoelectric colorimetry. By way of comparison the effect of time on the two coloured compounds is illustrated in Fig. 6. The uranyl ferrocyanide solution was produced during the course of a sodium determination by the method of King et al (1942).

As a result of these considerations the following method was proposed.

THE PROPOSED METHOD

Preparation of Reagents

1. Uranyl zinc acetate reagent. A solution of 14 ml. of glacial acetic acid in 750 ml. of distilled water was brought almost to a boil. To this mixture were added in succession, with stirring, 77 grams of uranyl acetate, 231 grams of zinc acetate (in five or six portions), and 7 ml. of glacial acetic acid. The solution was cooled to room temperature and diluted to one litre. Thereafter 200 ml. of 95 per cent ethanol were added and the solution allowed to stand overnight. A small amount of precipitate was formed (due to sodium impurities in the reagents), but a further amount of triple salt, prepared as described below, was added to ensure saturation of the reagent at all times. This solution was shaken and filtered immediately before use.

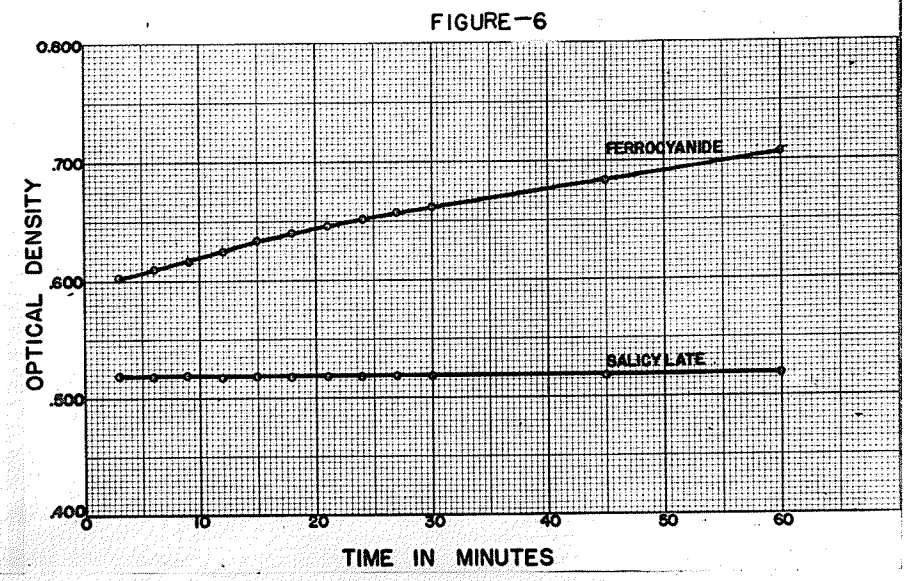

Figure 6

The effect of time on the intensity of uranyl zinc sodium acetate solutions after treating either with sodium salicylate or with potassium ferrocyanide

2. Triple salt. To 10 or 15 ml. of the above solution there was added enough of a concentrated sodium chloride solution to remove all but a trace of yellow colour from the supernatant liquid. After 15 minutes, the precipitate was filtered off in a sintered glass crucible, washed five times with glacial acetic acid and five times with ether, then dried for about an hour in a desiccator.

3. Wash reagent. A solution of 425 ml. of 95 per cent ethanol and 75 ml. of glacial acetic acid was saturated at room temperature with pure triple salt (prepared as above). This reagent was stored in a brown bottle, and was shaken and filtered before use.

4. Trichloracetic acid. Ten grams of sodium-free trichloracetic acid were made up to 100 ml. with distilled water.

5. Standard sodium chloride solution (contains 350 mg. of sodium per 100 ml.). Sodium chloride, 0.8896 gram, was dissolved in water and made up to 100 ml.

6. Sodium salicylate. Ten grams of sodium salicylate were made up to 100 ml. with distilled water.

Procedure

By means of an Ostwald pipette exactly 0.5 ml. of serum was carefully pipetted into a test tube. Ten ml. of the trichloracetic acid reagent were added and mixed well

with the serum; the precipitated protein was then filtered off. One ml. of the protein-free filtrate was transferred to a 15 ml. centrifuge tube and 3 ml. of the uranyl zinc acetate reagent added. After mixing thoroughly by twirling the tube, the mixture was allowed to stand for 20 minutes. During this time the precipitate which formed was agitated twice in such a way as to distribute it thoroughly throughout the solution to induce a more rapid and complete precipitation. After centrifuging for 7 minutes at 2000 r.p.m., the supernatant was decanted and the tube drained briefly in an inverted position on a pad of filter paper. Three ml. ofalcoholic wash reagent were added, a small portion being blown gently into the tube so as to cause complete suspension of the precipitate, and the balance utilized for washing down the inside wall of the centrifuge tube. Centrifuging, decanting and thorough draining were repeated as above. The precipitate was dissolved in 14.0 ml. of distilled water. After adding 1.0 ml. of the sodium salicylate solution, the colour was read in a Coleman Junior Spectrophotometer with the wavelength adjusted to 460 millimicrons, or in an Evelyn Photoelectric Colorimeter using a blue filter.

Parallel determinations on 0.5 ml. of the standard sodium chloride solution and 0.5 ml. of distilled water (the reagent blank) were performed simultaneously.

Calculation

The concentration of sodium, Na, in mg. per 100 ml. of serum was calculated by means of the following equation

$$Na = \frac{D_T - D_B}{D_S - D_B} \times 350$$

where D_T, D_B and D_S refer to the optical densities of the test, blank and standard solutions, respectively.[1]

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[1] Subsequent to the development of the method outlined in this section, a method for serum sodium in which sodium salicylate is used in the colour development stage has been published by Butterworth (1951). However, it makes use of an artificial blank solution to achieve linearity, a device which leaves out of consideration the true purpose of a reagent blank, namely, to allow for impurities introduced by reagents or by contamination.

SECTION V

AN EVALUATION OF THE PROPOSED METHOD

The proposed method may be assessed from the following aspects: rapidity, accuracy and general suitability for routine estimations.

Rapidity

It is readily appreciated that the rapidity with which a sodium determination can be performed plays an extremely important part in the clinical diagnosis and treatment of electrolyte disorders. Because results obtained by existing chemical methods were frequently not available until after their usefulness had been lost, sodium determinations were not called for as often as their importance warranted. By the present method one hour is ample time for a single determination on serum whereas two hours is quite sufficient for six or eight simultaneous estimations. This new method should therefore contribute toward a wider clinical application of serum sodium estimations in a variety of electrolyte investigations.

Accuracy

The accuracy of the method has been tested by comparison with the standard gravimetric procedure of Butler

and Tuthill, by recovery experiments and by comparison with a flame photometer.

Comparison with the gravimetric method of Butler and Tuthill. Samples of serum obtained from the Routine Biochemical Laboratory of the Winnipeg General Hospital were analyzed for sodium by the proposed method and by the standard uranyl zinc acetate method of Butler and Tuthill (1931).

One of the samples was dialyzed prior to analysis in order to obtain a low concentration of sodium, such as might be encountered in a severe case of Addison's disease. The dialysis was performed by placing about 25 ml. of the serum in a small cellulose bag and suspending this in running water for about two hours.

Table V gives the results for ten samples of serum, the dialyzed sample being listed first. With one exception all colorimetric results are within two per cent of the gravimetric values, the average difference by the two methods being one per cent. Differences greater than this can be attributed -- in part, at least -- to an additive effect since the gravimetric values themselves are subject to small errors.

Recovery experiments. Recovery experiments were performed on three samples of pooled serum which were again

TABLE V

COMPARATIVE SODIUM ANALYSES ON HUMAN SERUM
BY THE GRAVIMETRIC METHOD OF BUTLER AND TUTHILL (1931)
AND BY THE PROPOSED COLORIMETRIC METHOD

Concentration of sodium in mg. per 100 ml. of serum		Difference
Gravimetric analysis	Colorimetric analysis	%
254	250	- 1.3
322	320	- 0.6
325	329	+ 1.2
327	322	- 1.5
329	331	+ 0.6
332	342	+ 3.0
334	334	+ 0.0
335	334	- 0.3
345	346	+ 0.3
349	354	+ 1.4
		Mean 1.0

supplied by the Routine Biochemical Laboratory of the Winnipeg General Hospital. Two of these samples (about 220 ml. of each) were dialyzed to remove much or most of the sodium originally present, and varying amounts of sodium (as sodium chloride) were then added to two 100-ml. portions of each. Sodium chloride was similarly added to four 50-ml. portions of undialyzed serum.

The results (Table VI) show that the mean recovery of added sodium was 98.9 per cent, while the recovery range varied between 96 and 103 per cent.

Comparison with the flame photometer. In view of the fact that the flame photometer has recently been introduced for routine serial determinations of sodium in serum, particularly in certain fields of research, it was thought that a comparison of analyses between the proposed method and the flame photometer would be of value. For this purpose fourteen samples of serum obtained from normal adults were analyzed by both methods.

The flame photometer values were provided through the kindness of Dr. M. Ferguson and Miss Joy Macy, of the Department of Physiology and Medical Research, University of Manitoba. A Barclay internal standard flame photometer was used in the estimations, the method being briefly as follows.

TABLE VI

RECOVERY OF SODIUM ADDED TO SERUM

BY PROPOSED METHOD

	Mg. of sodium added per 100 ml. of serum	Mg. of sodium found per 100 ml. of serum	Recovery %
Dialyzed serum	0	102 *	
	200	297	97.5
	250	348	98.4
Dialyzed serum	0	25.7*	
	250	279	101.1
	300	320	98.1
Serum	0.00	345.5*	
	39.35	386	102.9
	78.70	421	96.0
	118.1	459	96.1
	157.4	505	101.3
		Mean	98.9

* These values were confirmed by gravimetric determinations according to the method of Butler and Tuthill (1931).

To 0.1 ml. of serum in a 100 ml. volumetric flask were added 20 ml. of a stock lithium sulphate standard solution containing 1000 p.p.m. of lithium , and distilled water to the mark. After setting the instrument to zero with a solution containing 200 p.p.m. of lithium, the potentiometer reading of the test solution was compared with a calibration curve for standard sodium chloride solutions containing 1, 5, $7\frac{1}{2}$, 10, $12\frac{1}{2}$ and 15 p.p.m. of sodium and 200 p.p.m. of lithium.

The results given in Table VII show satisfactory agreement between the two methods. The largest single difference noted is 5.5 per cent. Other investigators have found differences of the same order when comparing flame photometer values with those obtained by chemical means. Fox (1951), for example, also found differences up to 5.5 per cent when comparing values obtained by his flame photometer with those obtained by the gravimetric method of Consolazio and Dill (1941). Indirectly, therefore, the results in Table VII lend support to the close agreement between colorimetric and gravimetric methods reported in Table V.

General suitability for routine estimations. The suitability of the proposed method for routine analyses may be judged from the way in which the criteria established in

TABLE VII

COMPARATIVE SODIUM ANALYSES OF NORMAL HUMAN SERA

BY A FLAME PHOTOMETRIC METHOD

AND BY THE PROPOSED COLORIMETRIC METHOD

Concentration of sodium in mg. per 100 ml. of serum		Difference
Flame photometric analysis	Colorimetric analysis	%
303	304	+ 0.3
298	308	+ 3.4
314	309	- 1.6
306	313	+ 2.3
340	325	- 4.4
323	325	+ 0.6
344	325	- 5.5
343	329	- 4.1
340	330	- 2.9
328	331	+ 0.9
320	331	+ 3.4
352	337	- 4.3
346	338	- 2.3
337	339	+ 0.6
	Mean difference	- 1.0

Section IV have been satisfied. Rapidity and accuracy, the most important requirements, have already been dealt with. The other criteria are considered next.

Months of service in the Routine Biochemical Laboratory of the Winnipeg General Hospital have proved the dependability of the method. No special training on the part of the analyst is required to carry out the simple procedure. It is only necessary, as in other methods for sodium, to pay particular attention to such matters as exact measurements of all substances, and cleanliness of glassware. Since photoelectric colorimeters are standard equipment in any biochemical laboratory, their operation presents no problem.

The use of small quantities of serum is, of course, a definite advantage in routine estimations. Although 0.5 ml. of serum is usually available, it may occasionally be necessary to use less, as in the case of infants. The proposed method therefore has the advantage that it can be applied in such cases merely by decreasing the amounts of serum and trichloracetic acid used (to 0.2 and 4.0 ml., respectively) in the preparation of the protein-free filtrate.

Summary

The proposed method has been evaluated and found

satisfactory as a routine method for hospital biochemical laboratories for the following reasons:

1. A given determination can be performed within one hour, while as many as eight samples can be handled simultaneously and completed in about two hours. The result of a test or series of tests can therefore be made available to the physician before it has lost its significance.

2. It is capable of the required degree of accuracy, namely one per cent, as shown by recovery experiments and comparisons with a well-known gravimetric method. Supporting evidence is furnished by comparisons with a flame photometric method.

3. It is simple to perform, dependable, and requires no special apparatus.

4. Small amounts of serum are adequate, the amount normally required being 0.5 ml., although as little as 0.2 ml. may also be used.

SECTION VI

CONCLUSIONS

Although the history of sodium determinations in human serum goes bach a century, the last fifty years have failed to produce any method more accurate than the early macro gravimetric methods of classical analytical chemistry. A high order of accuracy, nevertheless, has been attained in more recent micro gravimetric methods based on the precipitation of sodium as the triple salt, uranyl zinc sodium acetate. Because gravimetric analyses are time-consuming and laborious, however, their effective use in routine clinical chemistry has been much restricted.

In attempts to overcome the disadvantages of gravimetric methods, numerous volumetric and colorimetric adaptations have been evolved. These have, in general, sacrificed accuracy for rapidity. In certain cases where both requisites appear to have been reconciled, other features have tended to mar the reliability and elegance of the methods.

The flame photometer, while capable of exceptional rapidity and a fair degree of accuracy, as yet is not available in many routine hospital laboratories due to its expense, limited usefulness and necessity for a specially trained operator.

In view of the increasing interest and importance attached to body-fluid electrolyte studies, it became evident that a more satisfactory chemical method for the determination of serum sodium was needed. The present investigation was therefore concerned with the development of such a method.

The proposed procedure depends upon the precipitation of sodium as uranyl zinc sodium acetate, and colorimetric estimation of the uranyl ion in the redissolved precipitate after reaction with sodium salicylate. The coloured solution is very stable indefinitely, and little affected by ordinary temperature changes.

Since the method is intended for use in routine hospital laboratories, the fulfillment of certain criteria was essential. By enabling an analysis accurate to within one per cent to be performed within an hour the method renders a useful clinical result. The application of simple laboratory techniques and apparatus, furthermore, makes the method available to any hospital laboratory. An additional advantage is the fact that the method can be adapted to very small amounts of serum. This is an important factor since the amount of blood available from an infant is often very limited.

Finally, it might be added that the method has been successfully used for the past nine months in the biochemical laboratory of the Winnipeg General Hospital.

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