LXXXV. A MICRO-METHOD FOR TITRATING THE BICARBONATE IN PLASMA.

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IN the early method of Zuntz [1867] and its various modifications by Lassar [1874], Jaquet [1892], Loewy [1894], Wright [1897] and Snapper [1913] a dilute solution of acid was added to a known volume of serum until a drop of the mixture changed the tint of an indicator paper, litmus, lakmoid or neutral red as the case might be. The particular end-point depended upon the predilection of the observer. In terms of modern physical chemistry, the serum was titrated to an arbitrarily chosen p_H .

Hans Meyer [1883] pointed out 42 years ago that the alkalinity of serum, determined in this way, was a function of the indicator employed, and further that the amphoteric proteins took part in the reaction. He came to the conclusion that the only definite and important measurement of blood alkalinity was its CO_2 combining power. The general use of the gasometric method of Van Slyke and Cullen [1917] indicates the recognition of the soundness of Meyer's reasoning.

It is difficult to say what is the p_H of a drop of serum which just changes the colour of red litmus paper because of the considerable "salt" error of azolitmin. Kolthoff [1922] found the error to be as much as $+1.0$ p_H in the presence of 0.5 normal NaCl ; that is the hydrogen ion concentration was ten times what the colour indicated. The more the NaCl is concentrated in the drop by evaporation the greater will be the salt error. We find that a sensitive glazed red litmus paper is slightly blued when a drop of $M/15$ phosphate solution of p_H 6.50 remains upon it for a few minutes, but is not allowed to dry. A similar solution of p_H 6.45 does not produce an appreciable alteration of colour. It would appear, therefore, that one is titrating to an end-point of p_H 6.47 but as the phosphates are concentrating all the time in the drop, the real p_H will be a little lower from the increasing "salt error" of the azolitmin.

Titration even to 6.5 indicates an alkalinity of the blood considerably greater than that determined gasometrically. In addition to the base balanced by HCO_3' a variable amount associated with the proteins is titrated, depending upon the litmus paper used, the time allowed for the CO_2 to escape from the

drop and the degree of change in tint which the observer decides upon as his end-point.

Although these indicator-paper methods afford useful comparative information, a method which only measures the base present as bicarbonate is preferable. Granted that the proteins, especially haemoglobin, are mostly responsible for the buffer action, it is only the alkaline bicarbonate and the respiration which are available for *adjusting* the reaction of the blood. Consequently, during recent years, the gasometric method of Van Slyke and Cullen [1917] has been generally adopted.

The method about to be described was devised by one of us, whilst abroad during the war, to measure the bicarbonate in blood-serum under circumstances in which the means to carry out Van Slyke and Cullen's method were not available.

It seemed likely that by titrating a small quantity of serum diluted with NaCl solution, and shaking out the CO₂ meanwhile, an end-point could be found at which the value obtained corresponded with the amount of alkali, as bicarbonate, determined gasometrically after the serum had been brought into equilibrium with alveolar air. After trial of titration to different end-points, it was found that when the indicator used was neutral red, the end-point sought was when the colour matched that of a *M*/15 solution of phosphates of p_H 7.07 to which a similar amount of indicator had been added. That the neutral point is the most suitable end-point is fortuitous. The reaction is a little acid of this, as neutral red has a small protein error. The real p_H is about 7.0.

The method was demonstrated at a meeting of the Biochemical Society in November, 1918, but was not published at the time because neutral red was not an entirely satisfactory indicator owing to gradual precipitation when the neutral point was approached. It was hoped to overcome this difficulty or to substitute another indicator. We have not been able to find another indicator to replace neutral red but have succeeded in obviating the separation

Table I.

The volumes per cent. of CO₂ in plasmas calculated from the titration values compared with those found by Van Slyke's method. 0.25 cc. was used for the titration and 1.0 c.c. for the gasometric estimation.

Specimen	CO ₂ calculated	Van Slyke CO ₂ observed
Smith plasma	60.0	61.2
Horse serum	41.0	43.3
1229 plasma	58.0	57.6
1372 „	59.5	58.5
1890 „	66.0	64.8
1890 B „	66.0	63.0
Spencer „,†	70.0	65.0
Roper „	65.0	66.9
Hawes „	68.0	66.0
Simkins „	68.0	68.5
Bass „	59.0	61.9
Pearce „	66.0	63.9
Platt „	62.0	63.0

† This plasma was haemolysed.

of the indicator by titrating in the presence of 16 % alcohol. This amount of alcohol does not appreciably alter the sensitivity of neutral red to hydrogen ions and facilitates the shaking out of CO_2 by lessening frothing.

During the last few years we have used the method in clinical and experimental work and found it handy and reliable. Its merits are the simplicity of the apparatus and the small quantity of serum (0.25 cc.) required. It affords results which agree with the gasometric with an error not exceeding $\pm 5\%$ as is seen from the observations in Table I. In these the gasometric estimations were made by one observer and the titrations by another.

Meanwhile, Van Slyke, Stillman and Cullen [1919] have described a titration method, with neutral red as indicator, as an alternative to the gasometric method. Their method differs somewhat from ours. To 2 cc. of plasma, 5 cc., *i.e.* excess, of 0.02 *N* HCl is added and the CO_2 shaken out. The acidified serum is diluted with 20 cc. of water and titrated back with 0.02 *N* NaOH to an end-point corresponding with the colour of *M*/8.5 phosphate solution of p_{H} 7.4. The results recorded agree with the gasometric titration within 7 %.

Van Slyke [1922] has recently elaborated this method and increased its accuracy by using the actual plasma, collected without loss of CO_2 and diluted to a similar extent, as the standard to titrate to. This procedure obviates a number of small errors and renders the method unexceptionable, but the determination cannot be carried out upon less than 10 cc. of blood.

EXPERIMENTAL PROCEDURE.

Collection of the samples of blood. The blood is drawn from the ear into a collecting tube made from a piece of glass tube of 7 mm. internal bore, sealed at one end and drawn out at the other until the internal bore is about 1 mm. The length from the sealed end to the shoulder should be about 4 cm. It will then conveniently contain up to 1 cc. of blood. The collecting tube must have a small hole blown near the drawn-out end to permit the air to escape as the blood enters (see Fig. 2). Before use a few crystals of oxalate and a tenth of a cc. of liquid paraffin are introduced into it. A free puncture of the skin is made and the capillary end applied to the drop of blood, which then flows into the tube and under the paraffin. The plasma is separated from the corpuscles by centrifuging.

The amount of CO_2 lost and consequent emigration of H and Cl ions out of the corpuscles by collecting blood in this way is not sufficient to introduce a serious error. The discrepancy would appear to be about 2 %. It was determined by the following experiments in which the amount of exposure to air closely simulated that which occurs when blood is drawn from the ear.

A piece of rubber pressure tubing bearing a screw clip was placed on the upper end of a 1 cc. pipette; the tip was put into some blood which had been collected under paraffin and the pipette filled by suction. The screw was tightened and the pipette clamped in a holder. By relaxing the screw clip the blood was allowed to escape from the pipette in drops. As each drop formed

it was run into one of the blood-collecting tubes under paraffin. When sufficient blood had been collected in the tube the plasma was separated by centrifuging. At the same time some of the original blood was centrifuged. Observations were made upon four samples of blood. In Table II below, the titration values, expressed as volumes CO_2 % in the plasmas, are set forth.

Table II. *Volumes CO_2 %.*

Sample	Blood taken direct	Same blood allowed to drop from pipette
1	51.0	49.6
2	55.4	54.5
3	64.5	63.0
4 a	40.0	39.6
4 b	40.7	39.6

Fig. 1. Colorimeter stand.

Fig. 2.

The amount of bicarbonate in blood drawn from the ear by a free puncture is lower than in venous blood. In this respect capillary blood approximates more closely to arterial blood, as Lündsgaard and Moller [1922] found for oxygen content.

Blood drawn in this way gives sufficiently consistent results, as the following observations show. The blood was collected from the finger at intervals of about 15 minutes and the plasma titrated.

Sample	Length of time taken in collection	Vols. % CO_2
1	1.0 cc. in 10 minutes	59
2	1.0 " 5 "	62
3	0.8 " 5 "	62
4	0.8 " 5 "	61

However the blood be obtained, if the titration is not proceeded with at once, the plasma is best stored in tubes coated with paraffin wax.

Apparatus required for the titration.

1. A few test tubes of equal bore made from the same piece of clear white resistance-glass. Convenient dimensions are 8 cm. long and an internal diameter of 8 mm.
2. A 100 cc. flask of hard glass (*e.g.* Jena) with rubber stopper¹ bored to take one of the test tubes.
3. A micro-burette: a 1 cc. pipette graduated in 100ths with rubber tube, nozzle and pinch-cock answers the purpose.
4. A pipette graduated to contain 0.25 cc.
5. A simple colorimeter (Fig. 1) shaped like a short test tube rack with three holes to take the test tubes. Suitable dimensions are 9 cm. long, 6 cm. high and 5 cm. wide. The inner surface is blackened and one of the open sides is covered with a tissue paper screen. The holes are best placed as close as possible to the paper screen.

Chemicals required.

1. Standard $N/10$ H_2SO_4 from which $N/100$ can be made up as required.
2. Standard phosphate of p_H 7.07 made up as follows:

Potassium dihydrogen phosphate $M/5$	33.33 cc.
Caustic soda $N/10$	43.66 cc.
CO_2 -free distilled water to	100 cc.
3. Neutral red 0.05 % dissolved in water.
4. Sodium chloride 3.5 %.
5. Alcohol 40 %.
6. A solution of burnt sugar which has been carefully neutralised and to which toluene has been added to prevent moulds growing in it.

Details of the titration.

0.25 cc. of plasma is drawn up into the graduated pipette and delivered into the flask. The pipette is washed out to the mark three times with the saline. 1 cc. of the 40 % alcohol is then added, so that the flask contains 2 cc. The contents are mixed and the rubber stopper carrying one of the test tubes inserted. The flask is inverted, so that the contents run down into the test tube, and placed in this position in the colorimeter.

2 cc. of the phosphate solution are placed in another of the tubes and this is put in the colorimeter beside the plasma. A small quantity of the solution of burnt sugar is then added to it with a fine dropping pipette until it matches the colour of the plasma. Another cc. of phosphate is added and five to eight drops of the indicator, the depth of colour chosen depending on personal preference.

¹ The stoppers must be clean. They should be moistened with petrol and rubbed with a rag until all sulphur is removed. Subsequently they should be boiled first in soda, then in weak acid and thoroughly washed with distilled water.

Acid is then run into the flask from the micro-burette until the contents become bright pink. The fluid is well shaken by swirling it round the flask, the air being frequently changed by inserting a piece of wide bore glass tubing and inhaling through it. Exhalation in the immediate neighbourhood of the flask must be avoided. As the CO_2 is removed the colour of the fluid changes to an orange tint again. The shaking out process is repeated after each addition of acid. As the end-point approaches, single drops of acid are added and the stopper is placed in the flask, the flask inverted and the colour of the contents compared with that of the phosphate tube.

The amount of acid added when the plasma is just alkaline to the phosphate, matches it, and is just acid are noted, and the mean between the first and last readings is taken as the end-point. With a little practice the alkaline and acid points lie 0.01 on either side of the match. Much more shaking is required to get rid of the last traces of CO_2 than might be anticipated. It is necessary to continue to add acid until the fluid remains definitely pink and it is impossible to alter the colour by further shaking and aeration.

If the end-point is accidentally overshoot it is possible to titrate back with $M/50$ sodium bicarbonate, shaking out meanwhile. Sodium hydroxide *must not* be used, as it is difficult to shake in CO_2 from atmospheric air in a reasonable length of time.

DISCUSSION.

Our method is frankly empirical but it is appropriate to discuss why it is necessary to titrate to an apparent end-point of p_{H} 7.07 (really p_{H} 7.0) to arrive at the alkali present as bicarbonate instead of to the p_{H} of blood which is about 7.3 after cooling to 18° .

The reason appears to be that the whole of the bicarbonate cannot be estimated unless the air with which it is brought into equilibrium is entirely free from CO_2 . In the dilute solutions of bicarbonate with which we are working the small amount left undetermined is very significant. If, on the other hand, the solution is entirely freed from CO_2 and bicarbonate, by acidifying and shaking out the CO_2 , practically the whole of the bicarbonate can be estimated by titrating back to the p_{H} of blood with NaOH . This is the principle of the method of Van Slyke, Stillman and Cullen [1919].

The amount of bicarbonate untitrated, when 0.25 cc. of 0.02M NaHCO_3 made up to 2.5 cc. with saline is estimated by our method can be derived from Hasselbalch's [1917] formula

$$p_{\text{H}} = p_{\text{K}_1} + \log \frac{[\text{Bic.}]}{[\text{CO}_2]}.$$

Let us assume that the air of the laboratory contains 0.05 % CO_2 and that the titration is carried to an end-point of p_{H} 7.4 and the final volume is 3 cc.

Taking the value of p_{K_1} at 18° as 6.22, which is what we have found for

0.02M NaHCO₃ in the presence of 0.85 % NaCl (Warburg [1922] found 6.24 under similar circumstances) we have

$$\log \frac{[\text{Bic.}]}{[\text{CO}_2]} = 1.2,$$

or $[\text{Bicarbonate}] = 13.1 \times [\text{CO}_2]$.

The molecular concentration of CO₂ in water at 18° which has been brought into equilibrium with air containing 0.05 % CO₂ is

$$\frac{0.05 \times \alpha^{18^\circ} \text{CO}_2^*}{100 \times 22.3} = 0.000021.$$

Multiplying by 13.1 gives 0.000275 for the molecular concentration of the bicarbonate in the 3 cc. of liquid at the end of the titration.

At the beginning, the solution contained $0.02 \times 0.25 = 0.005$ millimols NaHCO₃; at the end, $0.000275 \times 3 = 0.000825$ millimols, *i.e.* 16.5 % of the bicarbonate is untitrated.

Pursuing the same reasoning; supposing the titration were carried to a p_{H} 7.07, the millimols of bicarbonate left at the end would be 0.00045, or 9 % is untitrated.

As, when serum is substituted for pure NaHCO₃ solution, the value obtained agrees with the gasometric when the titration is carried to p_{H} 7.07, some compensation must occur. In serum, leaving aside the NaCl and the small amount of phosphate, the basic ions are balanced by the ions of two weak acids, H₂CO₃ and protein. The amount of base balanced by the latter depends on the hydrogen ion concentration. If titration is taken to p_{H} 4.7, the isoelectric point of serum albumin, the whole of this base can be titrated. If the titration is taken to a c_{H} short of this only a proportion of the base will be titrated. It would appear that when acid is added until the $p_{\text{H}} = 7.07$, the amount of base balanced by protein which is titrated about equals the amount of bicarbonate untitratable.

To ascertain whether varying the p_{H} by about 0.3, *i.e.* doubling the c_{H} , would increase the base balanced by HCO₃' at the expense of that balanced by protein anions sufficiently to effect the above compensation, we determined the "alkali reserve" of a serum gasometrically, according to the method of Van Slyke, firstly when the serum was brought into equilibrium with 41.7 mm. pressure of CO₂ and secondly with 86.6 mm. pressure of CO₂. In the first case the volume CO₂ % (as bicarbonate) obtained was 50.2, in the second 55.1.

The bicarbonate in the serum was therefore 0.0223M and 0.0245M respectively; an increase of 10 %. The pressure of CO₂ was 2.08 times as great in the second as in the first of our experiments. This would diminish the p_{H} by 0.32, approximately the difference between 7.4 and 7.07. It appears, therefore, that, under the conditions obtaining in our method, the bicarbonate untitratable in the presence of air containing 0.05 % CO₂ and the amount of protein base titrated by changing the end-point 0.3 p_{H} are of the same order of mag-

* α^{18° CO₂ is coefficient of absorption of CO₂ in water at 18°.

nitude. We conclude, therefore, that the compensation is brought about in this way.

About 9 % of the bicarbonate is untitrated when the air contains 0.05 % CO_2 . If the amount of CO_2 in the air of the room increases, the untitratable bicarbonate increases but the amount of protein base titrated does not vary. Consequently, a lower value is found. This is quite obvious when working in an ill-ventilated laboratory; in fact, variation in the CO_2 of the air is the chief source of error inherent in the method.

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