VIII. A MICRO-METHOD FOR THE ESTIMATION OF THE HYDROGEN ION CONCENTRATION OF CAPILLARY BLOOD.

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THE method is, in principle, essentially that of Hawkins [1923], which in turn was a modification of that of Cullen [1922], using blood instead of plasma. Hawkins collected 0.25 cc. of blood from a vein in a graduated pipette, delivered it into neutralised saline containing indicator under paraffin, centrifuged down the red cells and matched the supernatant fluid against standard phosphates.

Hollo and Weiss [1924] adopted a procedure similar to that of Hawkins but differing in some details.

Our method differs from that of Hawkins in that only a single drop, about 20 mm.³, of blood is required. Further, the tube containing the diluted blood is sealed up, the use of liquid paraffin is avoided and loss of CO₂ cannot occur during the centrifugalisation.

The plasma is diluted 30 times, which, as Cullen [1922] has shown, is sufficient to reduce the influence of the proteins on the indicator used to nearly negligible proportions. This dilution also renders the disturbing effect of the colour of the serum inappreciable. The diluting fluid contains sufficient oxygen to complete the oxygenation of the haemoglobin. Accordingly, the p_H measured is that of the sample when fully oxygenated, without loss of CO₂ and cooled to room temperature. We employ capillary blood which, as Lundsgaard and Moller [1922] and Verzar and Keller [1923] have shown, is 90–95 % saturated with oxygen. The method may, therefore, be taken as measuring the p_H of arterial blood cooled down to 18°. The correction to be applied to this measurement to arrive at the p_H of the same blood at the temperature of the body will be discussed later.

DETAILS OF THE METHOD.

Requirements.

(1) A freshly prepared saline solution containing 0.7 % NaCl and 0.2 % potassium oxalate or 0.2 % sodium fluoride. Shortly before use, one volume of 0.02 % phenol red is added to 10 volumes of saline and the p_H adjusted to about 7.5 with *N*/50 NaOH containing the same amount of indicator. It is kept in a hard glass bottle protected from CO₂.

(2) A set of standard tubes made of resistance glass, 7 cm. long and 2.5 mm. internal bore. These are filled with $M/15$ phosphate solutions of p_H varying from 7.3 to 7.6 at intervals of 0.05, to which one-tenth volume of 0.02 % phenol red has been added, and sealed off.

The phosphate solutions can be made from appropriate mixtures of $M/15$ NaH_2PO_4 and Na_2HPO_4 obtained from Sørensen's [1912] chart, or Table IV, p. 509, in Cullen's [1922] paper which is derived from Sørensen's data. Another convenient way is to mix a $M/15$ solution of phosphates of p_H 7.5 with one of p_H 6.5 or 10.5 respectively, according to the graph supplied by Dale and Evans [1920]. Their graph is drawn from data calculated from Sørensen's values. These standards must be made up frequently as they fade.

(3) A number of pieces of resistance glass tubing about 8 cm. long and of 2.5 mm. internal bore drawn out at each end to capillary dimensions so as to leave about 7.5 cm. from shoulder to shoulder. The glass tubing must be selected and prepared as described below under "special precautions."

Procedure.

A mark is made on one of the pieces of resistance tube at 0.5 cm. from the shoulder of one end, which we will call A. The tube is then filled from end B up to this mark with the saline containing phenol red and placed ready in a horizontal position. A large drop of blood is obtained from the finger or ear by a deep puncture and the end B of the tube, already filled with saline, is placed in it. End A is slightly depressed and the blood is allowed to flow in, pushing the saline in front of it, until the saline reaches *the beginning* of the capillary at end A. When this has occurred, end B is wiped dry and end A sealed in a peep-flame. As this end cools, blood is sucked into end B which is also sealed either in the flame or with sealing-wax. The blood and saline are at once mixed by rotating the tube between the fingers to avoid clotting. The corpuscles are then separated by spinning in a hand centrifuge for a couple of minutes and the colour of the mixture of saline and plasma compared with that of the phosphate standards.

The matching is done by holding the tubes in a good light at an angle of 45° to a piece of white paper lying on the bench. The tubes should be held by the capillary ends so that they are not warmed by contact with the hand. Greater accuracy is obtained if they are placed in test-tubes of water at 18° containing a slip of white filter paper as background.

If the unknown is found to lie between say 7.35 and 7.4 another standard of p_H 7.375 may be made up and the relation of the unknown to this determined. With a little practice, and p_H around 7.4, it is possible to place the unknown between two standards 0.025 p_H apart. The range of p_H over which such fine adjustment can be made will depend on the colour vision of the individual worker. For most purposes, however, intervals of 0.05 in the p_H of the phosphate standards are close enough. This enables a determination to be made within $\pm 0.02 p_H$.

Special precautions.

1. The stock phosphate solutions must be made up with the greatest accuracy attainable. We test ours electrometrically when made up and from time to time afterwards. They must be kept in hard glass bottles and protected from evaporation and from CO_2 when withdrawing some by a trap of soda-lime on the inlet tube. It is convenient to have a micro-burette attached to each. The arrangement is that usually employed for standard $\text{Ba}(\text{OH})_2$. It is also described by Stitt [1921]. In this case the solutions do not come into contact with laboratory air after they are transferred to the bottles. Even with this precaution, however, watch must be kept upon the more alkaline solution.

2. The indicator should only be added to the saline solution shortly before use and the p_H adjusted to 7.5. The error which may arise from using saline of a p_H differing markedly from that of the blood is indicated in Table I. If the p_H of the blood is found to be abnormally high or abnormally low it is advisable to adjust the p_H of the saline to the level found and make further observations.

Table I. *The effect of the hydrogen ion concentration of the diluting fluid on the p_H found.*

p_H of saline used for dilution	p_H of the serum found
7.2	7.55
	7.56
7.35	7.575
	7.575
7.75	7.625
	7.625

The sample of serum employed was saturated with hydrogen containing about 4 % CO_2 and its p_H electrometrically was 7.59.

3. The whole reliability of the method depends on the small glass tubes not furnishing alkali. Only resistance glasses are suitable and these must be individually tested as described below, as glass from the same batch varies.

Pieces of tubing are washed and left standing in boiled distilled water containing phenol red. If after a few hours the contents of the tubing become pinker, the glass is unserviceable. If no change in colour occurs, the tubing is dried, cut into lengths and the ends drawn out.

As most resistance glass yields alkali temporarily after being melted in the flame, the tubes are rinsed in distilled water and left to soak for some hours in boiled out distilled water containing phenol red. If no pink colour develops inside the tubes they are fit for use. They are rinsed with distilled water and dried with alcohol. *They must not be dried in a hot oven.*

4. In filling the tubes the minimum of air necessary for easy sealing should be left and entrance of CO_2 from the peep-flame during sealing avoided.

If it occurs a fall in the p_H of the saline in the neighbourhood of the seal will be obvious.

5. Unless NaF is used, separation of the corpuscles and comparison with the standards should be undertaken forthwith. With oxalate, the p_H gradually falls from glycolysis, as first shown by Evans [1922]. At room temperature no change can be detected after an hour, but at 38° a fall is discernible after a quarter of an hour. The use of fluoride as anti-coagulant inhibits glycolysis (Evans). If 0.2 % NaF is employed the p_H generally remains constant for 12 hours at 18° . This is a convenience in clinical work, as the sample of blood can be taken and mixed with the saline at the bedside and the remaining stages proceeded with at leisure.

6. The colorimetric observations must be made at or about 18° . The p_H attributed to a particular colour of the indicator in Sørensen's various phosphate mixtures is only valid for 18° so that if the comparison is made at another temperature the result will be incorrect. When using phenol red the error from this cause is of no great moment provided the temperature change does not exceed $\pm 3^\circ$, as this would lead to a difference of only 0.015 in p_H . Greater accuracy is obtained if the standard tubes and the blood-containing tubes are placed in test-tubes of water at 18° as described above, p. 38.

Magnitude of the error due to loss of CO_2 from the blood.

The effect of loss of CO_2 from a small volume of blood might have been expected to invalidate our method, but it is surprising how slowly a drop of blood loses enough CO_2 appreciably to alter its p_H .

We collected some blood from the left ventricle of a rabbit under ether in a syringe which had been rinsed out with 10 % oxalate. The p_H of this blood was determined firstly without any exposure to air by placing the tip of the capillary end of one of our tubes inside the nozzle of the syringe. Subsequently the opportunity for the blood to lose CO_2 was varied in the following manner. Drops of the blood were expressed on to a greasy slide and allowed to remain for different lengths of time before being run into the tubes. The p_H was determined as described above.

After exposure of the blood for 1 minute an alteration in the p_H was not appreciable. After 3 minutes' exposure the p_H had risen 0.05. After 5 minutes the p_H was 0.11 and at 7 minutes 0.17 higher. As was to be expected the effect of loss of CO_2 from a drop of plasma could be detected much sooner. At the end of 1 minute's exposure on a slide the p_H of the plasma had risen 0.06, at the end of 2 minutes 0.09, after 7 minutes 0.19.

As the blood can generally be collected and the tubes sealed off in 30 seconds the error due to loss of CO_2 from the blood in this time is negligible, but if more than a minute is taken the results will be a little too high.

The effect of dilution.

We have found, in agreement with Cullen [1922], that it is immaterial whether the blood is diluted 1/15 or 1/20, so that the rough measurement afforded by the relation of the relative length of the column of blood to the column of saline is sufficient to obviate the protein error of phenol red, provided always that at least a 1/15 dilution is made. Plasma or serum should be diluted 1/30.

The effect of dilution on the p_H found with capillary blood and with a serum equilibrated with a hydrogen CO_2 mixture, the p_H of which was determined electrometrically to be 7.59, is shown in Table II.

Table II.

Fluid examined	Dilution	p_H found
Capillary blood	1/ 5.5	7.4
	1/ 7.5	7.4
	1/14	7.525
	1/15	7.5
Serum saturated with H_2 , CO_2 mixture	1/13	7.525
	1/15	7.525
	1/25	7.55
	1/30	7.575
	1/40	7.575

The measure of agreement obtained by repeated observations on the same sample.

A number of consecutive readings of the p_H of oxalated blood collected in a syringe from the left ventricle of an anaesthetised rabbit are shown in Table III. The low p_H is due to the anaesthetic. The first five observations were made by passing the capillary end of the tube inside the tip of the syringe in order to collect the sample of blood. In the last two observations (6 and 7) a drop of blood was pressed to the end of the syringe and exposed to the air during the very short time required for the collection of the sample. Again, it will be seen that the difference between the p_H in these two methods of sampling was within the experimental error of the method.

Table III. *The p_H of arterial blood of a rabbit under ether.*

Number of sample	p_H found	
1	7.24	
2	7.225	
3	7.24	
4	7.21	
5	7.225	Mean of 5 observations 7.227
6	7.235	
7	7.235	Mean of 2 observations 7.235

The results obtained from successive samples of capillary blood from the finger are shown in Table IV. The samples were taken at intervals of 15 minutes and the blood diluted 1/15 with saline of p_H 7.45.

Table IV.

Date of observation	p_H found
4. vi. 25	7.52 (temp. 22°)
	7.53
	7.56
5. vi. 25	7.54 (temp. 21°)
	7.52
	7.56
	7.55

*Comparison of the results obtained by the colorimetric method with
electrometric measurements.*

Evans [1921] came to the conclusion that there was a considerable and fundamental discrepancy between colorimetric and electrometric determinations of the reaction of blood or bicarbonate solutions; and that the former indicated a p_H 0.2 higher than the latter. He was of the opinion that the colorimetric was correct. In view of Evans' observations, Cullen and Hastings [1922] compared carbonate and phosphate solutions equilibrated with definite CO_2 pressures, but found agreement between the results by the two methods. Verney and Bayliss [1923] also failed to find any systematic difference, either with $NaHCO_3$ solution or blood.

Hastings and Sendroy [1924] have recently collected together thirty comparative observations of the p_H of plasma by the electrometric and colorimetric methods, made by Cullen, by Hawkins and by themselves. The average deviation in p_H was only 0.003. We have also made observations on this point and find that there is no serious discrepancy between the two methods either with bicarbonate solutions or plasma provided the concentration of Na^+ is about the same as that in blood plasma.

For the electrometric observations we employed a hydrogen electrode which permitted agitation and deoxygenation of the liquids examined. The other half-cell was a 3.5 N KCl calomel electrode. The chain was tested before use with Michaelis' "standard acetate" (0.1 N $HA + 0.1 - Na\bar{A}$) in the hydrogen electrode.

We regard the value 4.626 for the p_H of "standard acetate" at 18° derived from Walpole's [1914] observations to be more nearly correct than the value 4.604 now attributed to it by Michaelis and Kakinuma [1923]. This is, however, a matter of no moment for the present investigation, as the p_H values of the phosphate solutions used for colorimetric comparison were also arrived at on the basis that the p_H of standard acetate is 4.626 and that the E.M.F. against a 3.5 N KCl calomel electrode is 0.5218 at 18°. Any small discrepancy between the E.M.F. obtained and 0.5218 was allowed for in the subsequent determinations.

A saturated KCl bridge was employed and it was assumed that diffusion potential was eliminated. The E.M.F. observed was corrected for dry hydrogen at 760 mm. pressure.

As the electrometric determination can only be made on completely reduced blood, whereas the colorimetric is only suitable for oxygenated blood, comparison of the two methods cannot be made with whole blood. The entrance of oxygen into the haemoglobin molecule increases its acid dissociation constant, according to Hastings, Van Slyke and others [1924], about 30 times, according to Brown and Hill [1923] 69 times. This makes a considerable difference to the p_H of blood, if the CO_2 formed cannot get away.

We were, therefore, constrained to use serum or plasma for comparing the two methods. To test our micro-method horse serum was brought into equilibrium with hydrogen containing 3.5 % CO_2 and the p_H determined electrometrically by one of us and colorimetrically by the other, both observations being made at 18° . By the electrometric method the p_H was found to be 7.59, by the colorimetric it was found to be between 7.55 and 7.60 and indistinguishable from 7.575. Another experiment was made with human plasma in equilibrium with hydrogen containing 6.37 % CO_2 at 19° . The p_H arrived at electrometrically was 7.197; duplicated colorimetric observations gave 7.18 and 7.22.

The agreement is good and we conclude that we can determine the p_H of a drop of serum or plasma by our micro-method with a precision not far short of that which we attain with larger quantities electrometrically and we have no reason to suppose that the results with blood would not be equally satisfactory, were we able to make the comparison.

Correction to be applied to colorimetric determinations at 18° to obtain the p_H of the same blood at 38° .

In the case of plasma, this correction can be ascertained by determining its p_H at 38° electrometrically and, without loss of CO_2 , at 18° or 20° colorimetrically. Cullen [1922] did this and found that, on the average, the p_H of human plasma was 0.22 and of horse serum 0.16 higher at 20° than at 38° . We have repeated Cullen's experiment with a sample of horse serum, using the micro-method, and obtained 0.18 rise for 20° fall in temperature.

Cullen's results showed rather wide variations when using the sera of different species of animal but, as the effect of temperature will vary with the relative concentration of CO_2 , NaHCO_3 and proteins in the particular serum, this is not surprising.

Cullen's experiments, however, fail to decide what is the appropriate correction to apply to observations of the p_H of plasma at room temperature to obtain the p_H of the corresponding blood at 38° . Nor does the manoeuvre of Hastings and Sendroy [1924], who determine the p_H of diluted plasma at 38° and allow for the increased dissociation of the indicator, succeed in determining this. In all these experiments the corpuscles were not separated from the plasma at body temperature, so that a "true plasma" was not investigated.

Hasselbalch [1917] found the effect of temperature on the p_H of blood

and serum, at constant CO_2 pressure, to be very different. It would be unsafe, therefore, and in view of the high value for the heat of ionisation of haemoglobin found by Brown and Hill [1923], to assume, without further enquiry, that when the total CO_2 , bound and free, remains constant the effect of changing the temperature from 18° to 38° is the same with blood as it is with plasma. In a following paper we shall deal with the effect of temperature upon the p_{H} of plasma and blood respectively. So far, our results indicate that the p_{H} of both is very similarly affected by change in temperature, when the total CO_2 in the system remains constant. Accordingly, if Cullen's correction of -0.2 be applied to the p_{H} of human blood determined colorimetrically at 18° - 20° the result will closely approximate to the p_{H} of the arterial blood at body temperature.

SUMMARY.

A method by which the p_{H} at room temperature of a drop of capillary blood can be ascertained with an error of ± 0.03 is described.

The correction necessary to convert this to the p_{H} of the blood at 38° is discussed.

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