Microdiffusion Methods. Blood Glucose

By E. O'MALLEY, E. J. CONWAY AND O. FITZGERALD, *From the Department of Biochemistry, University College, Dublin*

A manometric method for determining the true sugar in blood by measuring the CO_2 formed by yeast action was described by Holden [1937], and compared with the Somogyi [1927] modification of the method of Hiller, Linder & Van Slyke [1925] for determining the true sugar by a subtraction of the reducing material present after, from that present before, fermentation. A method for the estimation of blood glucose is here described in which the CO_2 evolved by yeast action on blood is measured by a microdiffusion technique. The whole procedure of fermentation, diffusion and titration is carried out in the one unit, the dimensions of which are smaller than those of the standard unit (it will be referred to as no. 2 size). The results are compared with those obtained by the Fujita & Iwatake [1931] modification of the Hagedorn-Jensen method on the same specimens of blood. In this modification the blood is deproteinized with Cd instead of Zn, and the method is claimed to give a reliable measure of the true blood glucose.

The microdiffusion conditions are different from the manometric, and an account is given of the influence of various factors before the method itself is described.

METHODS

1. *Microdiffusion apparatus*

Factors influencing CO_2 production and absorption. (*a*) *Dimensions of the microdiffusion unit.* A smaller unit than the usual standard type was considered suitable. The air volume of the standard unit— about 32 ml.—contains appreciable CO_2, and variations are likely to affect the results. Also minute leaks through defective lid contacts, which do not appreciably affect NH_3 determinations, are of importance when dealing with CO_2, and such are under better control in the smaller unit with its wider surface of contact. A smaller standard unit (no. 2) was therefore constructed with the dimensions given in Fig. 1.

The absorption rate of volatile substances such as CO_2, which are only slightly soluble (compared for example with NH_3), depends largely on the depth of the liquid diffusion layer, and is but little influenced by wide variations in the area of the absorbing or emitting surfaces. A comparison in Fig. 2 is made between the CO_2 absorption rate for

the larger and smaller units (curves C and D). The carbonate was acidified in the outer chamber, the fluid depth being 0·147 cm. in both small and large units (0·8 and 2·3 ml. respectively). The rates of absorption are similar particularly for the longer times, though the surfaces differ considerably.
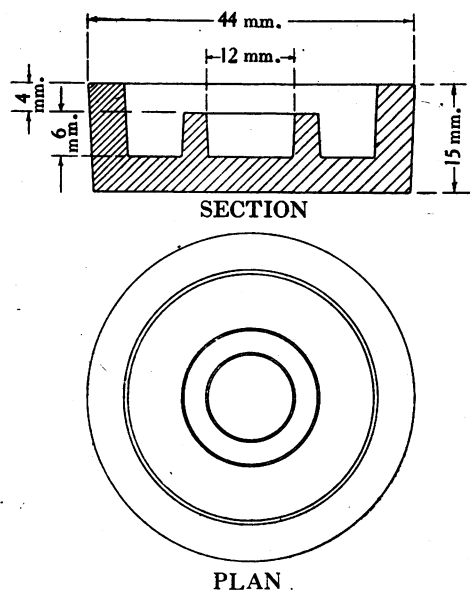

Fig. 1. Dimensions of the smaller (no. 2) microdiffusion unit (supplied by Messrs A. Gallenkamp, Finsbury Square, London, as Conway unit no. 2).

Curves B and E (Fig. 2) represent absorption curves with 0·4 and 1·6 ml. in the unit no. 2. Curve A is for 0·4 ml. with continual rotating of the unit (no. 2) and shows an absorption of 96 % in 5 min.

(*b*) *Autofermentation.* Besides the CO_2 coming from the fermentation of blood sugar, yeast steadily produces an appreciable quantity by autofermentation. Such autofermentation decreases with the age of the yeast suspension, the fall being most rapid in the first 24 hr. It is our custom when making a suspension to allow it to stand for 24 hr. at room temperature and then to place it in the refrigerator. Table 1 shows the influence of the age of the yeast suspension on the amount of

CO_2 produced by autofermentation from 0·5 ml. of a suspension (1 part in 3) of brewer's yeast (Guinness). Baker's yeast gave similar results, namely a marked decline in the autofermentation after 1 day at room temperature, and a steady fall
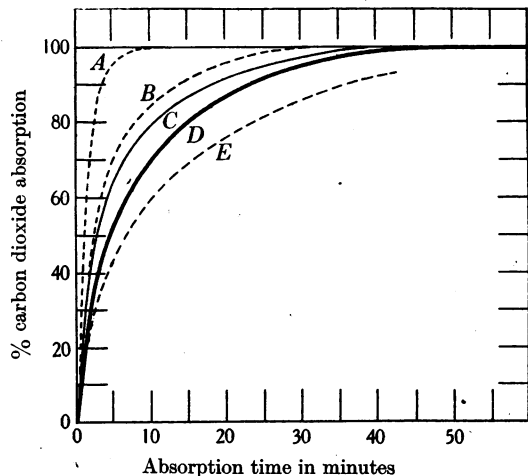

Fig. 2. Percentage absorption rate of CO_2 in the micro-diffusion unit described in Fig. 1. The lines A, B, D and E are for absorptions with 0·4, 0·4, 0·8 and 1·6 ml. fluid volume in the outer chambers, respectively. For curve A, the unit was continually rotated. For all these curves, 0·2 ml. Ba(OH)$_2$ was contained in the inner chamber. Curve C shows the absorption rate for the standard unit (no. 1) with 2·3 ml. in the outer chamber (giving same depth as 0·8 ml. in the no. 2 unit) and 1·3 ml. Ba(OH)$_2$ in the central chamber.

subsequently. The autofermentation in the method described below is equivalent to 0·03–0·06 ml. of $N/50$ acid in 90 min., but it may be somewhat higher, depending on the yeast strain and the conditions in which the yeast was grown.

Table 1. *Change of autofermentation with age in a yeast suspension*

Conditions of CO_2 absorption	Age of suspension days	CO_2 formed (ml. $N/50$ acid)
0·5 ml. of 1 in 3 suspension of	0	0·75
brewer's yeast in 2·5 ml. saline	1	0·27
in outer chamber of standard	2	0·18
unit (no. 1); absorption into	3	0·20
Ba(OH)$_2$ in inner chamber for	4	0·09
90 min. at room temperature	8	0·12

The rate of autofermentation is unaffected by wide changes in the external pH. Additions of HCl to yeast suspensions to give a concentration of 0·06N had no appreciable effect.

(c) *Yield of CO_2 from fermented glucose.* From the equation

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH,$$

1 mg. of glucose should yield 0·489 mg. CO_2 corresponding to 1·11 ml. $N/50$ acid. As is well known, less than this theoretical yield is obtained. We have found an average of 78 % with baker's yeast and of 92 % with brewer's top yeast (Guinness). Van Slyke & Hawkins [1929] found 71–79 % with baker's yeast, and Holden [1937] an average of 86 % with a certain type of brewer's yeast (L-Anheuser-Busch).

(d) *Effects of pH changes.* Over the range pH 3–5, produced by citric acid addition, there was no appreciable difference.

(e) *Effect of age and nature of fluid in which the stock yeast is suspended.* A stock baker's yeast suspension made up as 1 part in 10 of 0·5 % KH_2PO_4, left 1 day on the bench and subsequently stored in the refrigerator, showed little change in CO_2 production from glucose between 2 and 19 days. Thus, the recovery of CO_2 was 64 % of theoretical on the 2nd day and 73 % on the 19th day showing a slight rise with age; the fluctuation was around 70 % in the intervening days. After 27 days there was a marked reduction to 53 %. Other observations confirm the conclusion that yeast suspensions are serviceable for at least 2 weeks. No appreciable difference for about 2 weeks occurred if tap water or 0·9 % NaCl was substituted for 0·5 % KH_2PO_4 in suspending the yeast.

(f) *Variation in CO_2 yield from added glucose with different blood specimens.* The standard deviation from the mean value of a single formation of CO_2 from added glucose was 2·4 %, and 1·7 % for a duplicate. When blood samples from different human subjects were used instead of samples from the same subject, the variation of the CO_2 absorptions was not significantly different (2·6 %). This shows that varying the specimen of blood does not appreciably vary the CO_2 produced from a given amount of glucose. Wide variations in oxalate content of blood were also without effect. Cow's blood was found to give a result 5 % lower than that obtained with human blood.

(g) *Comparison of CO_2 formation in saline and in blood media.* We found that CO_2 formation from added glucose was a little higher when saline was substituted for the blood, and the variability greater. This could not be referred to pH effects. We have considered it advisable therefore to use the form of internal control as described below.

(h) *CO_2 formation in relation to quantity of glucose.* This was expected to show a linear relation up to a certain level. However, the CO_2 yield for small quantities of added glucose (1 mg./ml. blood) was proportionally less than for larger additions (2–4 mg./ml.). This effect was found to be suppressed by adding a little ethanol to the acidifying fluid introduced into the outer chamber. The total average CO_2 obtained in three sets of experiments in which

glucose (1–4 mg./ml.) was added to blood was 71 % of the theoretical. After 1 mg. addition it was 70 %, after 2 mg. 72 % and after 4 mg. 70 %, which values differ no more from the average than might be expected for the sampling error.

(i) *Acidifying mixture for use in the outer chamber.* With added ethanol the citric acid solution [Holden, 1937] produced a viscid mixture difficult to mix. Accordingly, we used dilute HCl, which produced a pH of 4–5 in the resulting satisfactory mixture.

(j) *Preformed CO_2 in blood and yeast.* The preformed CO_2 in the blood sample is rapidly removed on acidification and exposure to the air for 10 min. before yeast addition. The yeast suspension also contains preformed CO_2, and if many determinations are carried out there may be some difference in the CO_2 content of the yeast suspension added to the first and last samples. This possibility is overcome by adding 1·5 ml. of the yeast suspension to the inner chamber of one or more standard units (each of these 1·5 ml. additions is sufficient for seven samples) which are left exposed to the air for 15 min. before use. With the large surface exposed the preformed CO_2 quickly escapes leaving a small constant quantity dependent on the autofermentation rate.

(k) *Effect of glycolysis.* The present method, like Holden's, is independent of glycolysis up to 4 or 5 hr. exposure of blood samples to room temperature. If the samples are stored in the refrigerator much longer times may be allowed.

2. Technique for determining fermentable sugar in blood

(a) *Solutions required: N/20 Ba(OH)₂ with indicator.* Approximately $N/20$ $Ba(OH)_2$ containing 5 % of Universal indicator (B.D.H.). Before use some is run into a test-tube, and the 0·2 ml. pipette is dipped to the end of the tube. When half the solution has been used the tube is emptied and refilled with fresh solution.

N/50 HCl. For use in the horizontal burette.

Acidifying mixtures for outer chamber. (1) $N/20$ HCl containing 4 % ethanol. (2) $N/20$ HCl containing 4 % ethanol and 0·04 % glucose, which may be prepared by using a stock 2 % glucose in 2 % NaCl solution of which 2 ml./100 ml. are included.

Yeast suspension. Approximately 10 g. of yeast are made into a suspension with 100 ml. *tap* water, left for 1 day on the bench, and then placed in the refrigerator. It may be used after the 1st day for about 2 weeks. If the suspension is made in 0·5 % KH_2PO_4 it will be usable for a somewhat longer period.

Fixative for the lid of the diffusion apparatus. Good quality vaseline may be used, but a tragacanth fixative [Conway & O'Malley, 1942] has also been found very serviceable since it is water-soluble. 6 g. tragacanth are ground in a large mortar with the slow addition of 80 ml. of water; 30 ml. glycerol and 4 ml. N HCl are then added and mixed.

(b) *Procedure.* Into the inner chambers of some standard units (no. 1 size) 1·5 ml. of the yeast suspension are introduced. They are left on the bench while the others are being prepared. Duplicate samples of 0·1 ml. blood are then introduced into no. 2 units, 4 units being prepared for one specimen of blood. Two units are also set up with 0·1 ml. water. To all of these, except 2 of the 4 units with blood from the one specimen, 0·5 ml. of the acidifying mixture is added; this is mixed with the blood; 0·5 ml. of the acidifying mixture containing glucose is then introduced into the 2 remaining units.

Approximately 10 min. from the introduction of the acid, 0·2 ml. of the solution of $Ba(OH)_2$ plus indicator is introduced by a simple tube-pipette, and each unit is closed immediately by a lid smeared with tragacanth fixative. Into all the small units 0·2 ml. of the yeast suspension is now introduced, which is taken from the large units after stirring. This introduction is also made quickly from a 0·2 ml. tube-pipette, and after the unit has been closed the contents of the outer chamber are mixed by rotation. After 90 min. at room temperature the contents of the central chamber are titrated with $N/50$ HCl from the horizontal burette to pH 7·5–8·0.

(c) *Calculation.* The number of divisions on the burette corresponding to 1 mg. glucose/100 ml. blood is obtained by subtracting the mean titration for the 2 (of the 4) blood units which contain added glucose from the mean of the 2 without added glucose, this value being then divided by 200. For the remaining units the titration values are subtracted from the mean of those containing water, and divided by the result obtained above, giving the concentration as mg. glucose/100 ml. blood.

(d) *Example* (1) Mean titration of units with added glucose   11·0 (large divisions on burette)

(2) Mean titration of corresponding units without added glucose...   28·0

(3) Blank with water instead of blood   ...   36·8

Concentration of glucose in the blood

$$= \frac{(36·8 - 28·0)}{(28·0 - 11·0)} \times 200 = 104 \text{ mg./100 ml.}$$

Other blood samples require the setting up only of duplicate units as in (2).

3. Range of method

The range of the method for the blood sample which includes the internal control is 300 mg./100 ml., but for other blood samples done at the same time it is 500 mg./100 ml.

RESULTS

Results obtained with the present method and with the Fujita & Iwatake [1931] modification of the Hagedorn-Jensen procedure on the same blood specimens are in good agreement. Those for the microdiffusion procedure are a little lower on the average, as might be expected, and the difference is more marked in the fasting subject. Table 2 gives

Table 2. *Comparison of blood sugar methods*

		Hagedorn-Jensen method (modification of Fujita & Iwatake) mg./100 ml.	Microdiffusion method mg./100 ml.
		Non-fasting subjects	
1		103	108
2		137	128
3		110	112
4		154	157
5		99	95
6		107	94
	Mean	118	118
		Fasting subjects	
7		106	111
8		113	101
9		89	75
10		96	75
	Mean	101	91
Weighted mean for the two groups		111	106

a summary of the results. All determinations were made on oxalated blood, immediately after the specimen had been taken.

DISCUSSION

The present microdiffusion method involves less manipulation and fewer solutions than procedures such as the Hagedorn-Jensen, and is very suitable for a large number of determinations. Application of the method to a more complete investigation of the true glucose concentration of the blood, and to normal and pathological urines, etc., is still in an experimental stage, but the method for blood glucose appears sufficiently well developed for clinical use.

It should be noted that anti-glycolytic measures must not be used in collecting the blood, and that the method is independent of glycolysis up to 5 hr. at room temperature, or for one whole working day if the specimens are stored in the refrigerator.

SUMMARY

1. A microdiffusion method is described for the determination of blood glucose using 0·1 ml. samples. A smaller microdiffusion unit than the standard is used (Conway unit, no. 2).

2. The results obtained are very similar to those obtained with the Hagedorn-Jensen method modified by substitution of the Fujita & Iwatake [1931] procedure of deproteinization with cadmium instead of zinc, and which is claimed to give a measure of the true blood glucose.

REFERENCES

Conway, E. J. & O'Malley, E. [1942]. *Biochem. J.* **36**, 655.
Fujita, A. & Iwatake, D. [1931]. *Biochem. Z.* **242**, 43.
Hiller, A., Linder, G. C. & Van Slyke, D. D. [1925]. *J. biol. Chem.* **64**, 625.
Holden, F. R. [1937]. *J. biol. Chem.* **119**, 347.
Somogyi, M. [1927]. *J. biol. Chem.* **75**, 33.
Van Slyke, D. D. & Hawkins, J. A. [1929]. *J. biol. Chem.* **83**, 51.

Effect of Caffeine and other Iminazole Compounds on Haematins and their Derivatives

By JOAN KEILIN, *From the Molteno Institute, University of Cambridge*

It is well known that haem combines reversibly with a great variety of nitrogenous substances [Anson & Mirsky, 1925], giving the corresponding haemochromogens. This reaction is recognized by the replacement of the reddish brown colour and diffuse absorption bands of haem by the red colour and very sharp bands of haemochromogen.

By the analysis of crystalline haemochromogens [Zeynek, 1910] and by spectroscopic titration [Hill, 1926] it was shown that haemochromogens are compounds of haem with two molecules of a nitrogenous substance. Haemochromogen is easily oxidized in air, forming, within a certain range of pH, the reddish brown compound of parahaematin.