a tetracoccus, but the majority grow in single form. The incorporation of glass beads into the nutrient broth culture medium enabled the bacterial clumps to be readily dispersed on shaking during the 48-hour growth period. The culture was then killed in 10% formol saline, washed in normal saline, and the final suspension standardized to give a concentration of 5×10^6 organisms per ml. The stability of the culture was found to be satisfactory. Dilutions prepared from the original master sample were counted daily in the first instance, and then at weekly intervals or longer over a period of nine months. These results are shown in Table III. Thereafter aliquots of the *Micrococcus roseus* bacterial suspension were counted regularly and used as a standard for the Coulter counter.

TABLE III

STABILITY OF THE *MICROCOCCUS ROSEUS* SUSPENSION

Day	*Count*	*Day*	*Count*
0	667·5	39	672·0
1	670·8	46	688·0
2	669·8	53	694·0
3	665·8	83	690·0
4	675·4	113	695·0
11	670·0	171	700·0
18	690·0	201	690·0
25	665·0	232	680·4
32	675·0	260	665·9

Counts are expressed as N $\times 10^3$ per cmm

REFERENCES

Brecher, G., Schneiderman, M., and Cronkite, E. P. (1953). *Amer. J. clin. Path.*, **23**, 15

Bull, B. S., Schneiderman, M. A., and Brecher, G. (1965). *Ibid.*, **44**, 678.

Eastham, R. D. (1963). *J. clin. Path.*, **16**, 168.

—— (1965). *Ibid.*, **18**, 248.

Eggleton, M. J., and Sharp, A. A. (1963). *Ibid.*, **16**, 164.

Williamson, J. R. (1966). A practical integrated method for the electronic counting of platelets, leucocytes and erythrocytes. In *Advances in Medical Laboratory Technology*, Vol. IV, edited by F. J. Baker. Butterworth, London.

APPENDIX

The particle-free saline is prepared as follows:

A fresh 0·9 w/v sodium chloride solution is filtered initially through a glass fibre filter[1]. This is followed by pumping the roughly filtered solution through a 0·8 mμ millipore cellulose acetate filter. The resulting solution is a satisfactory diluting fluid for platelet counting, because the background count is invariably less than 100 counts per 0·5 ml aliquot.

[1]Glass fibre filter, type M. 2 in. diameter (Gelman Instrument Co. Michigan, USA)

Separation of haemoglobins on cellulose acetate

J. KOHN *From Queen Mary's Hospital, Roehampton, London*

Since cellulose acetate was first recommended for separation of abnormal haemoglobins (Kohn, 1958 and 1968) a number of papers have been published and various modifications recommended (*eg*, Friedman, 1962; Bartlett, 1963; Marengo-Rowe, 1965; Rosenbaum, 1966; Graham and Grunbaum, 1963). There seems to be little doubt that for routine purposes cellulose acetate is a very suitable medium for the separation of abnormal haemoglobins. The advantages over filter paper are speed and much neater and clearer separation patterns. With very few exceptions there is no need for the more sophisticated and complicated procedures such as agar gel, starch, or polyacrylamide. It was thought worthwhile to report a technique which is simple, reliable, and gave consistently satisfactory separations of abnormal haemoglobins as well as of Hb A_2 and Hb F (Figs. 1 and 2). A slight modification recently introduced proved to be particularly useful. By applying relatively high voltage across a narrow bridge gap and using standard equipment available in any laboratory, perfectly satisfactory separation of abnormal haemoglobins can be achieved within eight to 15 minutes (Fig. 1a). A discontinuous buffer system was found to be particularly useful.

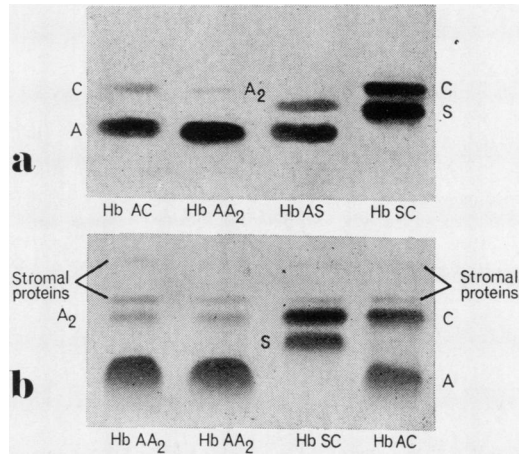

FIG. 1. *Haemoglobin separation:* a *300 V applied. 10 minutes' run. 3 cm wide bridge gap. Unstained strip. Note Hb A_2 migrating with Hb C mobility and absence of stromal protein bands.* b *200 V applied. 25 minutes' run. 5 cm bridge gap. Ponceau S. stained strip. Note the presence of two stromal bands, one of them nearly as strong as the A_2 band. This might lead to confusion.*

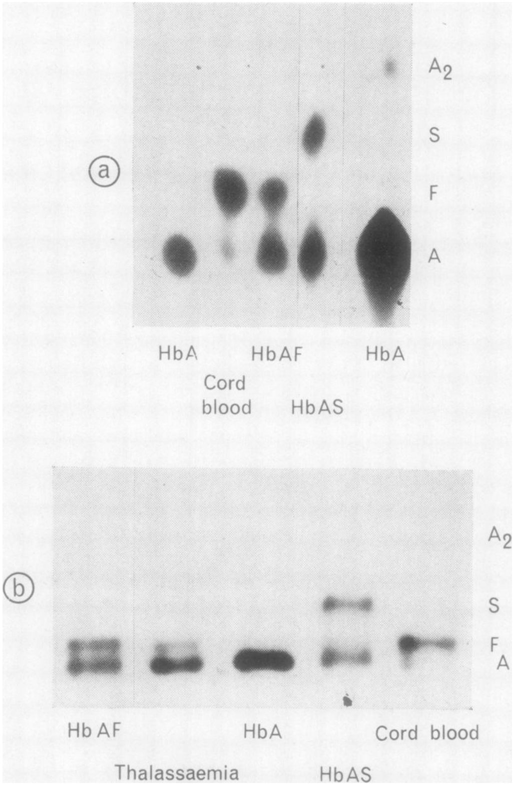

FIG. 2 (a) '*Dot' application of minute sample volumes. Note separation of Hb F. To demonstrate Hb A_2 somewhat larger sample applied. 90 minutes' run at 240 V.* (b) *Separation of Hb F. Sample of HbAF prepared by mixing cord blood with HbA haemolysate. 100 minutes' run at 230 V. Ponceau S stain.*

The composition of the buffer is as follows:

AT THE ANODE

0·26M TRIS buffer *p*H 9·1 (twice diluted Craddock Watson solution)

Tris (hydroxymethyl-aminomethane)	25·2 g
EDTA (ethylenediamine tetraacetic acid)	2·5 g
Boric acid	1·9 g
Distilled water to	1,000 ml

AT THE CATHODE

A barbitone buffer, *p*H 8·6

Sodium diethylbarbiturate	5·15 g
Diethylbarbituric acid	0·92 g
Distilled water to	1,000 ml

For the impregnation of the cellulose acetate strip itself, both buffers are mixed together in equal volumes.

Sheets of cellulose acetate, rather than strips, are recommended; all available brands can be used. Celagram[1] cellulose acetate was used in our experiments and is particularly suitable for the 'high voltage' technique. The bridge gap need not be larger than 5 to 6 cm. For the 'high voltage' technique a gap of 3 cm is recommended. An electrophoretic tank with adjustable bridge pieces (*eg*, Shandon tank U77) is particularly convenient; alternatively a flat frame with the required gap is easily constructed and can be fitted over the partitions in most tanks.

[1]Shandon Scientific Co.

The samples are best applied midway between cathode and anode. It is important that the application line should be thin and straight and the sample volume small, not more than 0·5 μl/cm (of approximately 10% haemoglobin solution). Carlsberg micropipettes, 'pulled out' capillaries, or a fine brush are very convenient for the purpose. A brush with a fine tip is a most useful device for the application of very small samples. When coming in contact with the strip it should be kept moving along a straight edge. Between applications the brush is rinsed in water. Subsequent blotting will reveal any residual contamination. Population screening can most conveniently be carried out by applying the samples in the form of tiny drops approximately 0·1 μl suitably spaced on a sheet or strip of cellulose acetate (Fig. 2a). For Hb A_2 determination, however, relatively large sample volumes have to be applied. Known control haemoglobin solutions (*eg*, AS and AC) are applied at suitable intervals for comparison.

The best results were achieved with a bridge gap of 5 to 6 cm and a potential gradient of 200 to 240 V obtained by applying constant current of 0·2 to 0·3 m amp/cm width. Time of electrophoretic run: 30 to 90 min, depending on the type of separation required; for Hb F, for instance about 90 minutes. The optimal conditions, however, are best worked out by preliminary trials using marker haemoglobins. 'High voltage electrophoresis' conditions, 100 V/cm for instance, can be easily obtained on standard laboratory equipment by applying 300 V across a bridge gap of 3 cm. Using this technique a perfectly satisfactory separation is achieved in eight to 15 minutes. The short gap and hence the ample supply of buffer, as well as the relatively very short running period, almost entirely prevent overheating. Electrophoresis is continued until the applied samples are separated satisfactorily. The electrophoretic run is best monitored by watching the migration of the heterozygous marker haemoglobins, *eg*, AS or AC. When separation is complete the cellulose acetate strips are dried or fixed as for routine protein electrophoresis. Staining is not usually necessary. Some authors recommend protein stains but this may lead to confusion as the stromal proteins, *eg*, carbonic anhydrase, will, of course, stain as well. Specific haemoglobin stains unfortunately may be carcinogenic and the colours obtained are unstable. Good results were obtained by using a 0·5 to 1% Guaiacum (not carcinogenic) solution in ethanol to which a few drops of hydrogen peroxide had been added just before use.

QUANTITATION OF HB A_2

Using the electrophoretic procedure as described above, Hb A_2 migrates with Hb C mobility and can be readily separated. The sample is applied somewhat nearer the anode than recommended before. Hb A_2 is best estimated by elution of the separated bands rather than by scanning,

mainly due to the great disproportion of the optical density of the Hb A and Hb A_2 bands. In order to obtain measurable amounts of Hb A_2 for elution without staining at least 20 to 30 μl of a 10% Hb solution should be applied, preferably as one long streak or a series of shorter streaks (depending on the size and form of cellulose acetate available). If a micro-densitometer is available, obviously smaller samples can be applied. Satisfactory elution can be achieved if the sample volume to substrate surface (length of application site) ratio is not too high. Overloading leads to incomplete elution which in turn will result in relatively too low Hb A values and in consequence too high Hb A_2 values. About 2 μl per cm of application line creates conditions compatible with adequate elution. The Hb A_2 band is cut out close to the band margins. The haemoglobin trail between the HbA and Hb A_2 bands belongs to the Hb A band. The cut-up fragments of the Hb A_2 band (or bands) are eluted (preferably with agitation) in 3 to 4 ml of barbitone or Tris buffer (as for electrophoresis) or Drabkin's solution. Similarly the corresponding A band or bands is cut out from the same strip and placed in 30 to 40 ml of eluent. The eluate is centrifuged and the supernatant read in a suitable colorimeter at 413 mμ or 540 mμ, in cuvettes with at least 1 cm light path.

Alternatively, the bands can be stained, *eg*, with Ponceau S, eluted and the eluate is read at the appropriate wavelength, *eg*, 525 mμ for Ponceau S. The advantages of staining the bands is that much smaller volumes of the sample can be used; only about 3 to 4 μl of the haemolysate needs to be applied, bearing in mind that 1 μl/cm of the application line should not be exceeded. Care should be taken that the stromal protein bands are recognized and not mistaken for haemoglobin and subsequently measured together with Hb A_2.

The choice of method is largely a matter of personal preference. Using the techniques described, the normal range of Hb A_2 varied between 1·5 and 3·3% and did not exceed 3·4%.

REFERENCES

Bartlett, R. C. (1963). *Clin. Chem.*, 9, 325.
Friedman, H. S. (1962). *Clin. chim. Acta*, 7, 100.
Graham, J. L., and Grunbaum, B. W. (1963). *Amer. J. clin. Path.*, 39, 567.
Kohn, J. (1958). *Trans. roy. Soc. trop. Med. Hyg.*, 52, 304.
(1968). In *Chromatographic and Electrophoretic Techniques*, 2nd ed., edited by Ivor Smith, vol. 2, p. 84. Heinemann, London.
Marengo-Rowe, A. J. (1965). *J. clin. Path.*, 18, 790.
Rosenbaum D. L. (1966). *Amer. J. med. Sc.*, 252, 726.

Apparatus for shell freezing liquids in the standard blood transfusion bottles

G. W. R. DIKE, F. E. BEW, AND G. G. HOPWOOD
From the Oxford Haemophilia Centre, Plasma Fractionation Laboratory, Churchill Hospital, Oxford, M.R.C. Central Workshop, Hut 22, Churchill Hospital, Oxford, and Grant Instruments (Cambridge) Ltd, Barrington, Cambridge

Lyophilization (drying from the frozen state) is widely used for the preservation of biological materials. The physics of the drying process requires that for the best results there should be a large surface for evaporation, with a minimal depth of frozen material. This has led to the so-called 'shell freezing' in which the container is rotated during freezing in such a way as to coat the inside surfaces of the bottle with the material to be dried. There have been several attempts to deduce theoretically the physical structure of the frozen mass which provides the optimum conditions for the drying (Rowe, 1964) and the quality of the product (Greaves, 1946; Rosenburg, 1964). In the preparation of proteins with biological activity, the quality of the product, *ie*, speed of solution on adding water and the preservation of biological activity, are more important than achieving the maximum speed of drying.

The best material appears to result from prefreezing in such a way as to produce small crystals (Greaves, 1946). It has been found that if the material is to be dried in the cylindrical bottles of the type commonly used in the Blood Transfusion Service good results are obtained by rotating the bottle about its vertical axis during freezing, the speed of rotation being high enough to cause the liquid to rise up the walls of the bottles and distribute itself evenly over the inner periphery. During the freezing period the centrifugal force causes the ice crystals, as they are formed, to move towards the centre of the bottle, away from the cold outer surface, thus ensuring that small ice crystals form. Greaves (1946) describes a method of spin freezing using air as coolant but a liquid coolant such as alcohol offers many advantages.

We found that apparatus available commercially for vertical spin freezing was extremely expensive and cumbersome and lacked versatility. Some designs did not make provision for maintaining sterility within the bottle being frozen. We set out to design an apparatus which would incorporate the following points:— (1) It should be relatively inexpensive and compact. (2) The cold bath should be readily adaptable to other purposes. (3) The spinner assemblies should be independent units permitting easy replacement in the case of breakdown. (4) It should be possible to use bottles with a bacteriological filter cap in place ready for drying under sterile conditions. This implies that the bath liquid must not