where films had been made, viable counts would not necessarily be valid.

Discussion

The failure, up to the present, to obtain good films, which was the primary object of the investigation, appears to rule out the method for this purpose unless the third method mentioned above can be made to bear fruit. However, the rapidity of preparation, together with the suggestion of better results on culture, merits further consideration. The method commends itself for the examination of cases under treatment owing to the almost complete removal of inhibitory drugs in the C.S.F. There appears to be a "planting" factor concerned in the growth of *M. tuberculosis*, and this suggests that culture results in specimens other than C.S.F. might be bettered by soaking a dry filter-paper disc in the specimen and applying this to the medium; this method, for different reasons, has been applied by Hoyt, Smith, and Gribkoff (1954) to tubercle culture.

Addendum

Since the work reported was carried out the Hemmings filter* has been introduced. It is possible with a few minor modifications to employ this filter for use with collodion membranes and thus for C.S.F. filtration.

(1) Pressures in the upper and lower chambers must remain the same. An $\frac{1}{8}$ in. hole is bored in the bottom of the upper bijou bottle. The rubber washer sealing the lower container is replaced by one of porous material or with a split aluminium washer.

(2) A thin metal plate with multiple small perforations (1/64 in.) is superimposed over the existing filter plate, in which the holes are too large.

(3) Owing to the requirement of a plane surface thrust on the membrane, which is not given by the curved lips of a bijou, the upper chamber washer must be replaced by two thin rubber washers (0.5–1.0 mm. thick) between which lies a thin metal washer.

Summary

A comparison is made of the results of direct films and culture, by the routine method and by a filtration technique, in the laboratory diagnosis of tuberculous meningitis. In a small series, better results were obtained using the routine method to prepare films, but filtration proved superior in the case of cultures.

I am indebted to Dr. K. Anderson, of the Department of Bacteriology, Guy's Hospital, London, for valuable suggestions, and to Dr. C. A. Green and Dr. J. Kennedy, of this department, for their continued interest and co-operation.

REFERENCES

- Elek, S. D., and Hilson, G. R. F. (1951). *Journal of Clinical Pathology*, **4**, 240.
 Hoyt, A., Smith, C. R., and Gribkoff, G. P. (1954). *Amer. Rev. Tuberc.*, **70**, 916.
 Silverstolpe, L. (1949). *Acta paediat. (Uppsala)*, Suppl. 77, p. 34..

* The Hemmings filter is obtainable from H. A. Jones, 26, Castle Street, Beaumaris, Anglesey.

A Micromethod for the Estimation of Serum Bilirubin

K. M. LAURENCE AND A. L. ABBOTT

From the Department of Biochemistry, Portsmouth and Isle of Wight Pathological Service, Portsmouth

(RECEIVED FOR PUBLICATION JULY 25, 1955)

As a guide in the prognosis and treatment of infants suffering from erythroblastosis foetalis, serial estimations of the serum bilirubin level have assumed greater importance than repeated haemoglobin determinations. This, however, presents a major difficulty. The small amount of blood required for the haemoglobin estimation can readily be obtained by heel puncture, but the quantity needed for the standard bilirubin determinations greatly exceeds that amount, and cannot always be obtained successfully with complete freedom from haemolysis.

The numerous methods for the measurement of serum bilirubin can be broadly classified into two main groups. The first group comprises those methods in which the proteins are precipitated after diazotization of the bilirubin and includes that of King and Coxon (1950). The second consists of those in which protein precipitation is avoided and the bilirubin is diazotized in the presence of catalysts such as urea (Powell, 1944), phenol (Patterson, Swale, and Maggs, 1952), caffeine sodium benzoate (Dangerfield and Finlayson, 1953), sodium benzoate (Jendrassik and Gróf, 1938), and ethanol (Malloy and Evelyn, 1937).

Micro-modifications of the methods of Malloy and Evelyn by Hsia, Hsia, and Gellis (1952) and of Jendrassik and Gróf by With (1943) have been suggested. For both these modifications only 0.1 ml. of serum is used, but a special micro-cell photoelectric instrument is required.

The primary object of this work was to produce a method which would require the minimum quantity of blood and would be simple to perform using standard apparatus available in most laboratories. At the same time, accuracy was required at least equivalent to the more popular methods utilizing quantities of serum up to and exceeding 1 ml. The method of Malloy and Evelyn was selected for modification, as it was the simplest available and most suited for this purpose.

Method

Reagents.—The following were used:

Diazo Reagent

Solution A	Sulphanilic acid	...	...	1 g.
	Concentrated hydrochloric acid	...	...	15 ml.
	Distilled water to	...	...	1,000 ml.
Solution B	Sodium nitrite	...	...	0.5 g.
	Distilled water to	...	...	100 ml.

For use 10 ml. of solution A is mixed with 0.3 ml. of solution B.

Absolute Methyl Alcohol

Artificial Bilirubin Standard.—This is prepared by dilution of a stock solution of phenolphthalein (50 mg./100 ml. in ethanol). Stock standard, 1 ml., is diluted to 200 ml. with M/10 sodium carbonate-sodium bicarbonate buffer, pH 10.0. At the dilution used in this test, this is equivalent to a bilirubin concentration of 8.5 mg./100 ml. serum.

Procedure

Into a test tube containing 2.9 ml. of distilled water 0.1 ml. of serum or plasma is added, followed by 0.5 ml. of freshly prepared "diazo" reagent, and finally by 2.5 ml. of absolute methyl alcohol. The contents of the tube are then mixed and allowed to stand at room temperature for 30 minutes before determining the optical density in a standard photoelectric instrument, at a wavelength of 520 μ (Ilford green filter 624 is satisfactory), distilled water being used for zero setting. The bilirubin concentration is then determined by reference to a calibration curve or by direct comparison with the artificial standard.

Calculation Using Artificial Standard

$$\frac{\text{Reading of Unknown}}{\text{Reading of Standard}} \times 8.5 = \text{bilirubin in mg./100 ml.}$$

When the bilirubin content is more than about 5 mg.% quite satisfactory results are obtained using smaller quantities of serum, such as 0.05 ml. or even less. On the other hand, with bilirubin levels below 3 mg.%, the resulting optical density is too low to give absolutely reliable readings, and more accurate results will be obtained by taking 0.2 ml. of serum and adding only 2.8 ml. of distilled water. When such modifications are made, appropriate alterations must, of course, be made in calculating the results.

Results and Comments

After choosing this method as the most practicable it was thought necessary to determine the time of maximum colour development, the linearity of the colour produced, and the reproducibility of the method. It was also decided to investigate the effects of haemolysis at varying concentrations of haemoglobin and the correlation between this method and that of a standard macro method.

Rate of Colour Development.—Several samples of sera with bilirubin concentrations ranging from 1.5 to 23.0 mg./100 ml. serum were treated as described and the optical densities of the resulting azobilirubin were plotted against the time of colour development (Fig. 1). These results showed a rapid initial production of azobilirubin within 10 minutes with practically complete colour development at 30 minutes. It was noticed in the course of this investigation that the "pure" commercial bilirubin behaved in a similar manner to foetal bilirubin, in that it required much

Fig. 1.—Graph showing the rate of colour development of "adult," "foetal," and "pure" bilirubin on treatment with "diazo" reagent. —, adult bilirubin. —x—x— foetal bilirubin. - - - - commercial "pure" bilirubin.

longer to be completely converted to azobilirubin and that the process was a more gradual one.

Linearity of Colour Production.—A stock solution containing 25 mg. "pure" bilirubin* in ethanol-chloroform mixture was prepared. In order to obtain complete solution of bilirubin 25 mg. bilirubin was refluxed gently with 75 ml. chloroform for approximately 30 minutes. The chloroform was then evaporated until only one-third of the original volume remained. The solution was then made up to 100 ml. with absolute alcohol. Serial estimations on this solution, suitably diluted, giving concentrations ranging from 2 to 25 mg./100 ml., were performed and the optical densities recorded were plotted against bilirubin concentration (Fig. 2). As the standard

Fig. 2.—Graph showing the relationship between optical density and varying concentrations of "pure" bilirubin.

* Supplied by Messrs. British Drug Houses.

solution was made up in alcohol-chloroform mixture, no distilled water was used in the azobilirubin production, this being substituted by methyl alcohol. This graph showed complete linearity up to and including a concentration of 25 mg./100 ml.

Reproducibility of Method and Bilirubin Recoveries.

—To ensure that the results obtained by this method were reproducible, 57 estimations were carried out in duplicate and plotted against each other (Fig. 3). These proved to be completely reproducible, with a standard deviation of 0.21 and a correlation coefficient of 0.976 when compared with the macro method of King and Coxon (Fig. 4).

FIG. 3.—Graph showing the reproducibility of the micro method.

FIG. 4.—Graph showing the correlation between the micro method and a standard macro method.

In any method for the estimation of bilirubin it is necessary that the method should be standardized against pure bilirubin. Several recovery experiments were performed in which varying quantities of "pure" bilirubin, ranging from 1 mg. to 10 mg./100 ml., were added to sera of known bilirubin concentrations. This gave an average recovery of 99% (Table I).

TABLE I
BILIRUBIN RECOVERIES

Test	Bilirubin Concentration	Bilirubin Added (mg.)	Theoretical Result	Actual Result	% Recovery
1	3.6	1	4.6	4.5	97.8
2	7.2	2	9.2	9.0	97.8
3	10.8	3	13.8	13.9	100.7
4	15.4	4	19.4	19.3	99.5
5	9.8	5	14.8	15.2	102.7
6	14.6	6	20.6	20.0	97.1
7	18.5	7	25.5	24.9	97.6
8	5.6	8	13.6	13.7	100.7
9	9.4	9	18.4	18.2	98.7
10	6.6	10	16.6	16.6	100

Influence of Haemolysis.—As, in many cases, serum obtained from infants shows varying degrees of haemolysis the effect of haemolysis on this method was investigated. Serial dilutions on the same serum containing respectively 0, 1, 2, 3, 4, 5, 6, and 7% haemoglobin were carried out (Fig. 5). This shows

FIG. 5.—Graph showing the effect of haemoglobin on the micro bilirubin method, and the macro method of King and Coxon (1950). — — — Micro method. —x—x—x Macro method.

that haemolysis affects the micro method to a lesser extent than it affects the macro method. Up to a concentration of 3% haemoglobin, in which the serum is bright red, the acid haematin influenced the bilirubin concentration up to a maximum of -0.2 mg./100 ml. serum in a total of 3.6 mg./100 ml. Haemolysis interferes rather less with the results with increasing bilirubin concentration, but with very low bilirubin levels, such as 0.5–1.5 mg., even 1% haemoglobin will invalidate the result. During the course of this investigation no serum was received which contained more than 2.5% haemoglobin.

In the micro method described here, the buffering effect of the protein is negligible and the acidity of the resulting solution is approximately pH 2. In the presence of mineral acids the azobilirubin formed is more violet than that of azobilirubin at a pH of 4 to 5 where the mixture is buffered to a certain extent by

the quantity of protein present in the original macro method of Malloy and Evelyn. This violet colour led us to try an alkaline solution of phenolphthalein buffered to pH 10 as an artificial standard. This solution has a maximum absorption between 560 m μ and 500 m μ with a peak at 540 m μ and was found to be stable at room temperature for a period of at least three months.

Summary

A rapid, simple and accurate method for the estimation of bilirubin in small quantities (0.1 ml. or less) of serum or plasma is described. The method is suitable for use in laboratories equipped with standard photoelectric instruments and has proved valuable, particularly in following the progress of erythroblastotic infants.

Thanks are due to Dr. E. M. Darmady for his help and encouragement.

REFERENCES

- Dangerfield, W. G., and Finlayson, R. (1953). *Journal of Clinical Pathology*, **6**, 173.
 Hsia, D. Y., Hsia, H., and Gellis, S. S. (1952). *J. Lab. clin. Med.*, **40**, 610.
 Jendrassik, L., and Gróf, P. (1938). *Biochem. Z.*, **297**, 81.
 King, E. J., and Coxon, R. V. (1950). *Journal of Clinical Pathology*, **3**, 248.
 Malloy, H. T., and Evelyn, K. A. (1937). *J. biol. Chem.*, **119**, 481.
 Patterson, J., Swale, J., and Maggs, C. (1952). *Biochem. J.*, **52**, 100.
 Powell, W. N. (1944). *Amer. J. clin. Path.*, **14**, Tech. Sect., **8**, 55.
 With, T. K. (1943). *Hoppe-Seyl. Z. physiol. Chem.*, **278**, 120.

Transistor-Amplifier Units for Absorptiometry

P. W. PERRYMAN AND D. H. RICHARDS

From the Group Biochemistry Department, Southend-on-Sea Hospital, Rochford, Essex

(RECEIVED FOR PUBLICATION AUGUST 11, 1955)

On account of the property of the junction-transistor to amplify small currents in low impedance networks this device is particularly well suited to amplify the output of selenium photo-cells. The production and use of two simple transistor units designed to increase the sensitivity of selenium-cell absorptiometers are described below.

In the commoner types of absorptiometer such as are widely used in hospital laboratories, the output of a selenium cell is coupled directly to a microammeter of some 10 micro-amps full-scale deflection. These instruments, though admirable for many routine measurements, are of insufficient sensitivity for comparison of very small differences of colour density, and for such purposes it is necessary to employ more elaborate and expensive spectrophotometric equipment.

Efforts to improve such elementary absorptiometers may be made by using good narrow-band filters to give the best possible match between the wavelength of the light used and the absorption maximum of each particular test. Such efforts are, however, frequently defeated by the impossibility of then obtaining sufficient photo-cell current to give full scale deflection for "100% transmission" owing to the low transmission of many narrow-band filters.

Even higher sensitivity may be required to permit the use of "interference filters" of still narrower band width. Since these filters are now available having any specified pass-band in the visible range to suit the absorption characteristics of any particular system of analytical interest, they offer striking possibilities of greatly improved sensitivity and selectivity of absorptiometric analyses providing adequate photo-cell sensitivity is available.

Type I Amplifier

The type I amplifier is shown in Fig. 1. The output leads from the selenium cell of a conventional photoelectric colorimeter are connected as shown, the negative lead to the "base" of a Mullard O.C.71 junction-transistor and the positive lead to the "emitter."

The negative terminal of the colorimeter galvanometer is also connected to the emitter and the positive terminal of the galvanometer to the positive terminal of a single 1.5-volt miniature dry-cell. This latter is in series with 300-ohm resistance, and finally the circuit is completed by connecting this to the "collector" of the transistor.