FIG. 2. *Foetal hepatocytes after four days in culture.* ×900.

sions centrifuged at 500 r.p.m. for five minutes at 4°C. The packed cells were washed once in the growth medium and centrifuged again before resuspending them in a minimal amount of the growth medium. The liver cells were subsequently cultured after implantation by the method of Zuckerman, Tsiquaye, and Fulton (1967).

The cultures were examined by phase-contrast illumination and by fluorescence microscopy after staining with acridine orange. A practically confluent viable monolayer of cells was obtained after 24 hours in culture, and after 48 hours a number of mitoses were observed in the preparations (Fig. 1). Cell debris was present in small quantities, particularly at the periphery of the culture and after four days the cells were healthy and viable (Fig. 2).

By this technique it is regularly possible to obtain at least 32 separation cultures from the cell suspension contained in a single ampoule.

It is a pleasure to thank members of the Tissue Bank of the Royal Marsden Hospital. Miss Susan Baker, B.Sc., and Miss Lorna Dunkley provided excellent technical assistance.

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Rapid method for the estimation of plasma haemoglobin levels

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The increasing use of extracorporeal circulation in cardiac surgery has led to a renewed interest in the levels of plasma haemoglobin in view of the possible dangers to patients. During the course of recent studies with different pumps and oxygenators a need was felt for a reasonably accurate and simple method for this estimation. A spectrophotometric method of analysis seemed to offer the best solution to the requirements.

The simple measurement of the optical density of a specimen at a characteristic absorption band for oxyhaemoglobin does not give satisfactory results because of the presence of bilirubin and small quantities of other pigments. However, when optical density measurements need to be made in the presence of a background level of other absorbing materials it is possible to correct for the non-specific light absorption in a manner which has been described by Allen (1950) for ketosteroids and by Rimington and Sveinsson (1950) for porphyrins.

An inspection of the absorption curve of oxyhaemoglobin suggested that the same principle could be used for its estimation in plasma and this has been applied in the method described.

MATERIALS

INSTRUMENT   The Unicam S.P. 600 spectrophotometer with 1 cm. cells was used throughout and special care was taken to check the wavelength calibration by means of a Didymium glass filter.

STANDARDS   A stock solution of oxyhaemoglobin was made by haemolysing the red cells of pooled heparinized blood with distilled water, the solution then being clarified by centrifugation. Suitable dilutions of this solution were made in phosphate buffer (pH 7·5) to obtain final concentrations of oxyhaemoglobin to cover the range 5-75 mg. per 100 ml. The haemoglobin content was found by making parallel dilutions in a modified Drabkin's solution (Dacie and Lewis, 1963a), and the cyanmethaemoglobin concentration of each dilution was found by reference to a known cyanmethaemoglobin standard solution (B.D.H.).

BLOOD SAMPLES   Samples were obtained either by clean venepuncture with a wide-bore needle, or, in the theatre, directly into the syringe from the oxygenator. The specimens were transferred to heparinized plastic centrifuge tubes and centrifuged at 1,500 r.p.m. for five minutes. The plasma was removed and centrifuged a second time to remove all cells.

METHOD

PRINCIPLE   The absorption peak at 576 mμ is selected, as over the range 560-592 mμ the background spectrum of bilirubin and other minor pigments approximates closely to the straight line required in the mathematical correction of Allen (1950) and Rimington and Sveinsson (1950) (Fig. 1). When this is applied to oxyhaemoglobin it can be shown that: (2 × optical density 576 mμ) − (optical density 560 mμ + optical density 592 mμ) = constant × haemoglobin in mg. per 100 ml.

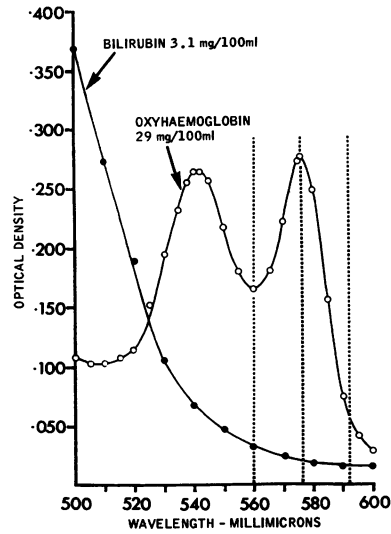STANDARDIZATION OF INSTRUMENT   The optical densities of the standard oxyhaemoglobin solutions are determined at 576 mμ (y), the absorption peak, and at two points equidistant from it; 560 mμ (x) and 592 mμ (z). The function $2y - (x + z)$ is then calculated for each standard and plotted against the oxyhaemoglobin concentration in mg. per 100 ml. A straight line relationship is obtained (Fig. 2).

DETERMINATION OF HAEMOGLOBIN CONCENTRATION OF UNKNOWN SAMPLES   The samples are examined undiluted and, their optical densities at 560 mμ, 576 mμ, and 592 mμ having been determined, the value of $2y - (x + z)$ is calculated for each. The level of haemoglobin in mg. per 100 ml. is found by referring this function to the standard graph. Results are read to the nearest 0·5 mg.

DISCUSSION AND RESULTS

The method was tested in recovery experiments, an example of which is given in Table I. A good degree of correlation (Table II) was obtained in a comparison with the method described by Dacie and Lewis (1963b). The technique described offers many advantages in speed and simplicity over the chemical procedure and, since the latter involves the use of the carcinogen benzidine, it is better avoided.

FIG. 1. *Spectrophotometric absorption curves of oxyhaemoglobin and bilirubin.*

TABLE I

Sample	Haemoglobin Added (mg.%)	Amount Found (mg.%)	Amount Expected (mg.%)	Difference (mg.%)
Plasma	—	0·5	—	—
Plasma	7·5	8·5	8·0	+0·5
Plasma	29·5	30·0	30·0	0
Plasma	59·0	60·0	59·5	+0·5
Plasma	117·5	121·0	118·0	+3·0

TABLE II

	Benzidine Method (mg.%)	Present Method (mg.%)
Pre-operative level	8·0	8·0
Specimen 1	21·0	23·0
2	30·5	31·5
3	34·5	38·0
4	41·0	43·0

The interference of bilirubin is allowed for by a mathematical correction which assumes that the spectrum of bilirubin is a straight line between the wavelengths employed. This assumption is valid for normal levels of bilirubin but at higher values the line becomes more curved introducing a negative error. Thus, in the presence of 10 mg.% bilirubin, the method will underestimate by approximately 2 mg.% haemoglobin. However, this defect is unlikely to be of importance when the method is used for estimations in cardiac surgery and, if necessary in other situations, a correction can be made.

SUMMARY

A spectrophotometric method for the estimation of plasma haemoglobin is described with many advantages in speed and simplicity. It has particular relevance to the field of cardiac surgery.

FIG. 2.   *Standardization graph.*

Specific determination of plasma and urinary lactose

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The recognition of three cases of lactose intolerance in the Sheffield Children's Hospital led to an investigation into the specific determination of plasma and urinary lactose levels following lactose loading tests.

Using the method of Reithel (1962) (see below) it was found that aqueous solutions of lactose (0.1 ml.) were readily and consistently determined even at levels down to 5 mg. per 100 ml.

As plasma glucose interferes with the determination it was destroyed by glucose oxidase and the oxidase itself was removed during the subsequent deproteinization.

DETERMINATION

The method depends upon the measurement of glucose liberated from lactose by hydrolysis with β-galactosidase, by the following steps:—

1 Lactose→glucose + galactose. With a comparatively high concentration of hexokinase and adenosine triphosphate, the glucose is immediately converted to glucose-6-phosphate, as in step 2.

2 Glucose + A.T.P.→glucose-6-phosphate + A.D.P. The glucose-6-phosphate produced is oxidized by glucose-6-phosphate dehydrogenase in the presence of nicotinamide-adenine dinucleotide phosphate (N.A.D.P.).

3 Glucose-6-phosphate
  →6 -phosphogluconate + N.A.D.P.H. + H^+.

The increase in the amount of N.A.D.P.H. measured by the increase in optical density at 340 mμ is directly proportional to the amount of glucose liberated from the original lactose.

In Reithel's method, reaction 1 is made rate-limiting in order to resist the transglucosidase activity of the enzyme,

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Rapid method for the estimation of plasma haemoglobin levels—concluded.

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β-galactosidase. Plasma that has been either frozen or freshly obtained is mixed with an equal quantity of glucose oxidase reagent containing catalase. The latter reagent is included in order to give rapid decomposition of the hydrogen peroxide produced by the oxidase, because a previous series of unsatisfactory results was attributed to hydrogen peroxide or other peroxides interfering with the dehydrogenase enzyme.

The buffering capacity of the plasma is sufficient to ensure complete removal of plasma glucose by the oxidase reagent.

METHOD

REAGENTS 1 Salt solution. Potassium chloride and magnesium chloride: 20 g. $MgCl_2.6H_2O$ and 7.5 g. KCl per 100 ml. of deionized water.

2 Adenosine triphosphate[1] (A.T.P.): 20 mg. per ml. in deionized water.

3 Nicotine-adenine-dinucleotide phosphate[1] (N.A.D.P.): 20 mg. per ml. in deionized water.

4 Hexokinase[1]: 10 mg. per ml.

5 Glucose-6-phosphate dehydrogenase[1]: 5 mg. per ml.

6 β-Galactosidase[2]: 20 mg. per. ml. in deionized water.

7 'Tris' 2-amino -2- (hydroxymethyl) 3-propane-1:3-diol: 0.5% solution in deionized water.

8 Glucose oxidase reagent: 9 parts of Fermcozyme[3] 1 part catalase[4] solution.

Fresh heparinized plasma should be used immediately or stored below 0°C. The plasma (0.05 ml.) is mixed with an equal quantity of glucose oxidase reagent in a micro-tube (0·4 ml. polypropylene) and incubated at 56°C for 20 minutes, thereafter 0·05 N acetic acid (0·1 ml.) is added and the contents are mixed on a vibrator.

Deproteinization is completed by heating the tubes in a water bath at 70°C for three minutes and then cooling rapidly. After centrifugation 0·12 ml. of the supernatant is transferred to a 0·5 ml. microcell in a spectrophotometer, reading at 340 mμ (we have used the Optica instrument). An equal volume of 0·5% Tris solution is added and the contents shaken, giving a final pH of 7·4. The following solutions are then added to the cell in the stated order.

(i) Salt solution	..	..	..	..	5 microlitres
(ii) N.A.D.P.	..	..	..	..	10 microlitres
(iii) A.T.P.	..	..	..	..	10 microlitres
(iv) Hexokinase	..	..	..	..	5 microlitres
(v) G-6P.D.	..	..	..	..	10 microlitres

the cell volume is made up with deionised water.

The cell contents are thoroughly mixed and the absorbance is read at 340 mμ. If the reading is not steady it should be allowed to become so before the optical density is noted. At this point the absorbance at 400 mμ. is also taken.

β-Galactosidase solution, 20 microlitres, is added and the absorbance at 340 mμ is allowed to become steady before reading; once it has done so a reading at 400 mμ