173. ESTIMATION OF OXYHAEMOGLOBIN AND OF METHAEMOGLOBIN BY A PHOTOELECTRIC METHOD

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THE oxyhaemoglobin content of blood can be rapidly and accurately estimated with the help of a photoelectric colorimeter. In the absence of oxyhaemoglobin or of other pigments, methaemoglobin can be determined in the same way. In whole blood the presence of met- or of sulph-haemoglobin or of other pigments can be detected. The experiments also indicate that the use of 0.4 % ammonia as a diluent for blood for spectrophotometric measurements is not safe. Better results can be obtained with a concentration of 0.04 % ammonia.

Instrument

The photoelectric colorimeter used for these experiments was a Hilger "Absorptiometer" [Hilger, 1936]. The light source of this is a projector lamp from the white light of which suitable spectral bands can be selected with the help of filters. Two beams of light fall on two photocells which are connected in opposition across a galvanometer so that, when the photoelectric currents given by the cells are equal, the galvanometer shows zero deflexion. The instrument works with a compensation-null method which ensures a high accuracy. The accuracy of the absorptiometer used in the present experiments was better than 0.3 %.

The optical densities of the solutions to be measured are read off directly from the instrument scale. The optical density is the logarithm of the ratio of the intensity of the incident light to the intensity of the transmitted light. This function has the advantage that it is approximately linear with the concentration of the absorbing substance. However, since the light source of the absorptiometer is not monochromatic, the density is not strictly proportional to the concentration and therefore calibration curves are necessary.

Estimation of oxyhaemoglobin

To prepare a calibration curve for oxyhaemoglobin, whole blood of known haemoglobin concentration was diluted with 0.04 % ammonia¹ in various proportions and the optical densities of the diluted solutions were read with a green or a blue filter.² In the present experiments the resulting calibration curve with the green filter was actually a straight line over the range of haemoglobin concentrations from 0.5 to 1.1 mg./ml. (see curve 1, Fig. 1). Calibration curves prepared with normal blood samples of different origin showed a variation up to 1 %. This means that if two bloods give the same density reading for example at a dilution of 1 in 100, they may differ as much as 1 % at a dilution of for instance 1 in 300.

¹ 0.04 ml. of ammonia (sp.gr. 0.880) made up to 100 ml. with water.

² Chance Bros. Colour filters, Nos. 5 and 6 respectively.

The actual estimation of oxyhaemoglobin was carried out as follows. The blood specimen to be examined, which contained sodium citrate or potassium oxalate as anticoagulant, was diluted with 0.04 % ammonia to a concentration giving a density reading of 0.25–0.5, one part in two hundred usually being a suitable ratio, and the oxyhaemoglobin concentration was read off directly from the calibration curve. The time required for the whole estimation was only

Fig. 1. Calibration curves for oxyhaemoglobin. Curve 1: green filter; 1 cm. cell. Curve 2: "Spectrum Orange" filter; 4 cm. cell.

2–3 min., and the relative accuracy was limited only by the 1 % uncertainty of the calibration curve explained in the previous paragraph. The absolute accuracy depends naturally on the method by which the haemoglobin content of the calibration solutions has been determined. In the present experiments this has been done with a Hilger spectrophotometer.

Estimation of methaemoglobin

In the absence of oxyhaemoglobin or of other pigments, methaemoglobin can be estimated in a similar way. The solutions, both for the calibration and for the experiments described below, were prepared by diluting one part of blood, containing anticoagulant, with four parts of water, together with a few drops of a freshly prepared concentrated solution of potassium ferricyanide. The absorption of blue light by the ferricyanide prohibits the use of blue or violet filters, but at these concentrations its effect is negligible with green, yellow or red filters. The concentrations of methaemoglobin in the calibration solutions were calculated from the haemoglobin concentration of the original blood.

Detection of other absorbing substances

If, using monochromatic light, calibration curves for oxyhaemoglobin are prepared at two different wave-lengths, the optical densities at these wave-lengths for a solution which contains only oxyhaemoglobin should correspond to the same concentration; but if another pigment, e.g. met- or sulph-haemoglobin, or haematin, is also present, they will not do so, and the presence of this foreign pigment can be detected. A marked effect would be expected with these substances since they absorb at long wave-lengths much more intensely than does haemoglobin itself [cf. Heilmeyer, 1933].

Using the absorptiometer, this principle was applied by constructing calibration curves for pure oxyhaemoglobin solutions (a) with the Chance's green filter

mentioned above and a 1 cm. cell; and (b) with an Ilford "Spectrum Orange" filter,¹ which has a narrow range of transmission at about 5900 Å, with a 4 cm. cell (see Fig. 1). In this way, suitable density readings for both filters were obtainable with one solution. The blood to be tested was diluted with 0.04 % ammonia to a suitable concentration and the apparent concentration was determined with either filter. If these two apparent concentrations were not the same, a foreign pigment was present.

As an example, it was found that in a solution containing 0.8 mg. haemoglobin per ml. the presence of 3 % of methaemoglobin increased the reading with the "Spectrum Orange" filter by 10 %, while that with the green filter was unchanged.

Normal blood samples of different origin differed from each other up to about 5 % in the corresponding density readings, i.e. solutions of different normal bloods, giving the same reading with the green filter, gave up to 5 % different density readings with the "Spectrum Orange" filter. Such variations have been observed by other workers using spectrophotometric methods [cf. Heilmeyer, 1933]. This 5 % uncertainty would correspond in the case of the example cited above to 1.5 % uncertainty in the detection of methaemoglobin. This means that the presence of more than 1.5 % methaemoglobin can be detected with certainty, and also very small concentrations of other foreign pigments. The method will not, however, detect carboxyhaemoglobin, in view of the similarity of its absorption to that of oxyhaemoglobin; nor can it be used for very turbid blood, since light scattering then affects the density readings.

The use of 0.4 % ammonia as diluent

Readings of the type described above revealed that in 0.4 % ammonia the conversion of oxyhaemoglobin into methaemoglobin, and of methaemoglobin into haematin proceeds much faster than is usually supposed, and considerable changes were found to occur within 1 hr. after the blood was diluted. The changes are most marked during estimations involving methaemoglobin and could lead to serious errors in spectrophotometric measurements.

Table 1 shows the conversion of oxyhaemoglobin into methaemoglobin by ammonia at room temperature. It contains successive readings on different samples of the same blood, using the "Spectrum Orange" filter. 1 % increase in

Table 1. *Conversion of oxyhaemoglobin into methaemoglobin in presence of ammonia*

		("Spectrum Orange" filter)				
Time after dilution		20 min.	80 min.	4 hr. 20 min.	18 hr.	43 hr.
0.4 % ammonia: Sample 1	1	0.163	0.171	0.184	0.228	0.281
	2	0.162	0.171	0.185	0.228	0.282
	3	0.163	0.170	0.185	0.227	0.282
0.04 % ammonia: "	4	0.163	0.165	0.167	0.173	0.182
	5	0.162	0.164	0.167	0.169	0.180
	6	0.163	0.164	0.166	0.171	0.177

absorption corresponds to about 0.3 % conversion, so that a considerable change is evident in the solutions in 0.4 % ammonia, even after intervals of less than an hour; but those in 0.04 % ammonia are stable over a much longer period.

¹ The transmission of this "Spectrum Orange" filter was found to be very sensitive to heat radiated from the lamp. The resultant error could be avoided by allowing it to cool for about 2 min. between successive readings.

Since in experiments of this type the successive readings are being taken on the same blood, the detection of changes in the blood is not subject to the uncertainty caused by the 5 % variation of different blood specimens explained in the previous section.

Table 2 contains similar data for the production of alkaline haematin from methaemoglobin. Readings were taken in this case with the green filter. 1 % decrease in absorption now represents roughly 3.5 % conversion. Similar results were obtained with other blood samples. Once again, the solutions in 0.04 % ammonia are more stable.

Table 2. *Conversion of methaemoglobin into haematin in presence of ammonia*

		(Green filter)			
Time after dilution with ammonia ...	Ammonia concentration %	8 min.	4 hr.	40 hr.	pH measured by H ₂ electrode
Sample 1	0.4	0.410	0.350	0.337	10.84
" 2	0.4	0.412	0.352	0.338	
" 3	0.1	0.422	0.398	0.372	
" 4	0.1	0.419	0.395	0.370	
" 5	0.04	0.419	0.413	0.396	
" 6	0.04	0.419	0.414	0.398	9.86
" 7	0.02	0.422	0.418	0.411	
" 8	0.02	0.424	0.418	0.410	

In a further series of experiments, it was found that the rate of conversion of methaemoglobin is almost independent of the time interval between the preparation of the initial methaemoglobin solution and its dilution with ammonia; but it is slightly influenced by the ratio in which it is diluted.

The results indicate that the use of 0.04 % ammonia is safe for most purposes, provided that the solutions are not allowed to stand for too long. Lower concentrations of ammonia are not suitable, since the solutions may become turbid owing to precipitation of blood proteins. Some light is also shed on the recorded inconsistency of spectrophotometric measurements on methaemoglobin at high pH values, such as solutions in sodium carbonate [e.g. Denes, 1930; see also Haurowitz, 1924]. The solutions of methaemoglobin in 0.4 % ammonia used in the present experiments had pH about 10.9, so that readings at higher alkalinity could not be expected to give reproducible results.

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