DISCUSSION

The new micro-method for determination of glycogen in tissues gives reasonably accurate results with 25–75 mg. of muscle or 10 mg. of liver. These results are in good agreement with those obtained by Pflüger's method of extraction; however, the new method has several advantages. In the first place, the glycogen content of a small piece of tissue can be readily determined. Fifteen to twenty determinations can be done on a single rat muscle weighing 2 g.; this would make it possible, for example, to determine the distribution of glycogen within the muscle itself. Similarly, the small amount of liver tissue necessary for the determination would allow the estimation of glycogen in biopsy specimens. Furthermore, the new method is simpler and more rapid than the classical methods; the glycogen content of twenty tissue samples can be determined in about 2 hr.

The procedures described above are concerned only with the determination of the total glycogen content of tissues. It seems possible, however, that two different forms of glycogen may exist in the tissues. As noted previously, only part of the glycogen can be extracted from the tissues with cold trichloroacetic acid, and it is necessary to heat the suspension or to destroy the tissue by alkaline hydrolysis before the remaining glycogen can be brought into solution. The results obtained by Bloom *et al.* (1951) and by Bloom & Knowlton (1953) seem to indicate that the relationship between these two glycogen fractions in muscle can be altered by subjecting the animal to various experimental conditions. The new micro-method for the determination of glycogen could easily be adapted to a study of this problem by carrying out the extrac-

tion with deproteinizing solution in two stages, that is, first in the cold and subsequently at 100°.

SUMMARY

1. A simple micro-method for the determination of glycogen in tissues has been described. The tissue is extracted with a solution of trichloroacetic acid at 100° and the glycogen in the extracts is determined, without previous hydrolysis, by the colorimetric method described by Mendel *et al.* (1954).

2. A procedure is described by which the glucose can be extracted from the tissue and determined separately.

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Spectrophotometric Studies of the Reaction of Methaemoglobin with Hydrogen Peroxide

1. THE FORMATION OF METHAEMOGLOBIN-HYDROGEN PEROXIDE

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The unstable red substance formed when hydrogen peroxide is added to an acid methaemoglobin solution (Kobert, 1900) was shown by Keilin & Hartree (1935) and Haurowitz (1935) to be a well-defined compound apparently analogous to the azide, fluoride, cyanide and hydrosulphide compounds of methaemoglobin, with absorption bands at 545 and 589 m μ , and requiring for its formation

1 molecule of hydrogen peroxide for each iron atom of haematin. The compound is unstable, and in the subsequent reaction part of the methaemoglobin is recovered whilst the remainder is lost by oxidative degradation to unidentified products (Keilin & Hartree, 1935).

We have shown that in the reaction between human haemoglobin and hydrogen peroxide in the

presence of an excess of sodium dithionite at pH 8.5, which results ultimately in partial oxidation to choleglobin, a transient intermediate compound is formed, which subsequently reverts, in the main, to haemoglobin by a rather slow reaction (Dalziel & O'Brien, 1951, 1952*a*). The spectral absorption of this transient compound resembles, but is not identical with, that of methaemoglobin-hydrogen peroxide. Lemberg, Legge & Lockwood (1939) postulated the occurrence of a haemoglobin-hydrogen peroxide compound as the precursor of choleglobin in the coupled oxidation of oxyhaemoglobin and ascorbic acid. The same workers remarked that methaemoglobin, as well as haemoglobin and oxyhaemoglobin, yielded choleglobin on reaction with hydrogen peroxide in the absence of reducing agents, and assumed that methaemoglobin was reduced to haemoglobin by the peroxide and thence formed haemoglobin-hydrogen peroxide (Lemberg, Legge & Lockwood, 1941). In seeking to clarify the relationship between the transient compound which occurs in the reaction of haemoglobin, dithionite and hydrogen peroxide, and methaemoglobin-hydrogen peroxide, we have made spectrokinetic studies of the reaction of human methaemoglobin and hydrogen peroxide.

In this paper, experiments with a rapid-reaction apparatus (Dalziel, 1953) are described which show that catalase-free human methaemoglobin is quantitatively converted into methaemoglobin-hydrogen peroxide by an excess of hydrogen peroxide at pH's 6.0 and 8.5. The spectral absorption of the compound in both the visible and Soret regions has been measured, and approximate kinetic and apparent equilibrium data have been obtained. Some observations on the reaction of the compound with sodium dithionite at pH 8.5 are also described. In view of the variability of published spectrophotometric data for methaemoglobin, extinction coefficients for the latter are recorded. Studies of the consecutive reaction of the compound in the presence of excess of peroxide will be described in a second paper. An abstract of the work has been published previously (Dalziel & O'Brien, 1952*b*).

EXPERIMENTAL

Spectrokinetic measurements

The changes of spectral absorption accompanying the reaction were recorded in a form of Hartridge-Roughton rapid-reaction apparatus, consisting essentially of a Beckman D.U. photoelectric spectrophotometer and a constant-flow apparatus. A detailed account of the apparatus has already been published (Dalziel, 1953). The reactants are stored in vessels surrounded by water in a lagged pressure tank, and are driven by gas pressure through capillary jets into a common tube, where they mix completely in 0.01 sec. The reacting mixture flows at a constant rate down the tube and can be analysed spectrophotometrically at selected points. Each of the two jets is connected through a three-way stopcock to two storage vessels, in order that calibration fluids or solvent may be alternately run with the reaction mixture; the analysis is then independent of spectrophotometric constants, and of the path length and optical qualities of the observation tube. If the temperature of the reactants in the pressure tank is within about 1° of room temperature, the temperature of the mixture does not change by more than 0.1° during flow through the leads and observation tube. Slower reactions are followed, after stopping the flow, in an extension to the observation tube surrounded by a jacket through which water at the same temperature as the reactants circulates.

The half-reaction time for methaemoglobin-hydrogen peroxide formation under the conditions of our experiments was found to be of the order of seconds, and most of the reaction could be followed by stopped-flow measurements. In some experiments it was desirable to make measurements in both the visible and the Soret regions of the spectrum with the same pigment concentration, and in these cases the water-jacketed attachment of path length 0.99 cm. was used for the region 495–640 mμ., and the observation tube of path length 0.20 cm. for the region 400–430 mμ. The reactants, methaemoglobin (metHb) and H₂O₂, were made up in buffer solutions; metHb was put in one reactant vessel, buffer in another, and H₂O₂ in the other two, such that appropriate pairs of fluids could be run together. With a particular wavelength setting the procedure was as follows: (1) H₂O₂ and buffer were run together, and the spectrophotometer set to zero extinction in the usual manner; (2) metHb and buffer were run together, and the extinction measured, equal to that of the reaction mixture at the instant of mixing; (3) metHb and H₂O₂ were run together, and the extinction of the flowing mixture measured, corresponding in these experiments to either 0.2 sec. or 0.8 sec. after mixing; flow was then stopped abruptly by closing the exit stopcock at the end of the observation tube, and the time course of the extinction was recorded with a stopwatch; (4) finally, H₂O₂ and buffer were run together again, and the solvent-setting checked. These operations were repeated at other wavelengths. From the series of plots of extinction against time, or spectrokinetic curves, so obtained, the absorption curve of the reaction mixture at any stage of the reaction could be built up.

In preliminary experiments on the overall reaction, the reactants were mixed in an optical cell, and the time course of the extinction was followed at two or three wavelengths by making measurements in rapid succession from about 1 min. after mixing. This was repeated at other wavelengths with fresh reaction mixture.

Kinetic calculations. For an isolated or reversible reaction involving only two coloured substances, reactant R and the stable, quantitative product P, which obey Beer's law, the fractional composition of the reaction mixture at time *t* after mixing may be calculated from the extinction of the mixture, *E_t*, at any suitable wavelength by the relation

$$C_t/C_0 = \frac{E_t \sim E_P}{E_R \sim E_P}, \quad (1)$$

where *C₀* = initial concentration of R, *C_t* = concentration of R at time *t*, and *E_R*, *E_P* = extinctions of R and P at concentration *C₀* and the same path length as *E_t*. Normally, both (*E_t* ~ *E_P*) and (*E_R* ~ *E_P*) would be measured directly, using calibration fluids (Dalziel, 1953). In the present experi-

ments, since the product is unstable, E_p was estimated from the spectrokinetic curves, and it will be shown that equation (1) gives reasonably accurate results for methaemoglobin-hydrogen peroxide formation in spite of the consecutive reaction. The precision of the analysis depends upon the magnitude of ($E_R \sim E_p$), the extinction change for complete reaction, and hence upon the wavelength: the maximum error in C_4/C_0 is estimated as $\pm 3\%$ C_0 when ($E_R \sim E_p$) > 0.1 .

Concentration units and extinction coefficients. It is common practice to express the concentration of a haem pigment solution in terms of the millimolar concentration of haematin, or its equivalent, and to record its spectral absorption in terms of extinction coefficients based on these units. This is appropriate to the present work. The designation of such coefficients as millimolar extinction coefficients, $\epsilon_{mM} = E/cd$, where c is the millimolar concentration of haematin (cf. Lemberg & Legge, 1949) is misleading, however, especially as some workers use this symbol to denote the true millimolar extinction coefficient of a haem pigment (e.g. catalase, Chance & Herbert, 1950). The use of the convenient haematin units would be facilitated by a suitable symbol: in this paper, the pigment concentration will be expressed as m-equiv./l., 'mEq.', where 1 mEq. metHb $\equiv$ 1 mM haematin. The corresponding extinction coefficient, the milliequivalent extinction coefficient, will be denoted by $\epsilon_{mEq.}$.

Reagents

Hydrogen peroxide. The experiments were made with AnalaR '20 vol.' reagent freshly diluted to the required concentration with Clarke & Lubs's 0.05M phosphate:NaOH, pH 6, or borate:NaOH, pH 8.5. Such solutions were stable for a few hours. The stock reagent was repeatedly analysed by Andrews's $KIO_3:As_2O_3$ titration. Other experiments with a commercial H_2O_2 sample containing stabilizer gave similar results.

Catalase-free methaemoglobin. Red cells separated from freshly drawn human blood (to which 10 mg. heparin/100 ml. was added) were washed thrice with 0.9% NaCl, and haemolysed in 5 times their volume of distilled water. The haemolysate was diluted with an equal volume of 0.4M acetate buffer, to give a pH of 5.8, and the precipitate of cell debris immediately removed by centrifuging. Catalase was removed by treating the solution with three or four successive portions of alumina suspension, allowing the mixture to stand with gentle mixing for 5 min. each time, and separating the alumina by centrifuging. The perfectly

clear solution of oxyhaemoglobin, having less than 1% of its original catalase content, was oxidized without delay by adding solid $K_3Fe(CN)_6$ to a concentration of about 10–15 mol./haematin mol. In some preparations the oxidation was carried out at pH 5.8, in others the pH was first brought to about 7, and in still others to 8.5. The product was dialysed against tap water, distilled water, and finally borate buffer of pH 8.5, and filtered. The pH at which oxidation was carried out did not significantly affect the spectrum of the product (see below) or the reaction with H_2O_2 . But oxidation at pH 8.5 gave a product less stable to extremes of pH; in dilute solution at pH 6 such samples of metHb became turbid quite quickly, and precipitated after some hours.

The concentration of metHb in the preparations was estimated spectrophotometrically by conversion into the cyanide compound, as recommended by Drabkin & Austin (1935). A suitable volume (1 or 2 ml.) was pipetted into a 10 ml. volumetric flask, 0.2 ml. of a solution of KCN containing 0.3 g./100 ml. was added, and the flask made up to the mark. The extinction was measured in 1 cm. cells at 551, 545 and 540 $m\mu$., and the cyanmethaemoglobin concentration calculated from Drabkin's values for $\epsilon_{mEq.}$ at these wavelengths, namely, 11.1, 11.5 and 11.5, respectively.

The metHb preparations were diluted as required in Clarke & Lubs's phosphate (pH 6.0) (cf. Vogel, 1939) or borate (pH 8.5) buffer solutions. In most experiments the initial metHb concentration was about 0.05 mEq., to permit accurate spectrophotometric measurements in the visible and Soret regions with 1 cm. and 0.2 cm. path lengths, respectively. In a few experiments with lower pigment concentrations, down to 0.003 mEq., measurements were confined to the Soret region with 1 cm. path length.

Spectrophotometry of methaemoglobin

The wavelengths of maximum absorption and milliequivalent extinction coefficients for two typical metHb preparations are given in Table 1. These samples were made from the same catalase-free oxyhaemoglobin solution by oxidation at pH's 5.8 and 8.5. The complete absorption curves were measured at pH 6.0 (0.05M- KH_2PO_4 :NaOH) and pH 10.0 (0.05M- H_3BO_3 :KCl:NaOH). Analysis by the micro-Kjeldahl method gave 17.3 and 17.5% for the N content of the samples, assuming 1 mEq. metHb = 16.7 g./l.; these figures and the extinction coefficients are based on Drabkin's data for cyanmethaemoglobin referred to previously.

Table 1. Absorption maxima and milliequivalent extinction coefficients, $\epsilon_{mEq.}$, of methaemoglobin

$\epsilon_{mEq.} = 1/cd \log_{10} I_0/I$; c = m-equiv. metHb/l. = m-mol. haematin/l.; d = cm.; N.B.W. = nominal band width. MetHb solutions prepared from alumina-treated haemolysates of washed human red cells, by oxidation with $K_3Fe(CN)_6$, and dialysis. Diluted for measurements with Clarke & Lubs's 0.05M phosphate (pH 6.0) or borate (pH 10.0).

pH of oxidation	$\epsilon_{mEq.}$							
	pH 6.0				pH 10.0			
	Absorption maxima ($m\mu$.)				Absorption maxima ($m\mu$.)			
	275	405	500	630	275	413	540	576
	N.B.W. ($m\mu$.)				N.B.W. ($m\mu$.)			
	2.8	1.3	1.5	6.2	2.8	1.4	1.8	2.4
5.8	29.6	167	9.3	4.0	32.2	107	10.7	9.6
8.5	29.8	164	9.3	4.0	32.2	106	10.6	9.5

RESULTS

Preliminary experiments in which metHb and H_2O_2 were mixed in optical cells, to give a pigment concentration of about 0.05 mEq. and molar ratios, R , of peroxide to haematin of from 4 to 90, showed the formation of a compound at pH's 6.0 and 8.5 with an absorption spectrum in the visible region similar to that recorded by Haurowitz (1935) and Keilin & Hartree (1951). The compound was unstable; but the subsequent changes of light absorption were relatively slow.

Spectrokinetic measurements

The spectral changes accompanying the early stages of the reaction were studied quantitatively in the rapid reaction apparatus by recording the time course of the extinction at appropriate wavelengths.

In Expt. 1 with 0.049 mEq. metHb and $R = 40$, at pH 6.0, observations were made at the wavelengths: 402, 405, 408, 415, 418, 422, 495, 500, 515, 540, 545, 550, 560, 576, 580, 590, 610, 615, 620, 630, 640 $\text{m}\mu$. Some of the spectrokinetic curves are shown in Fig. 1, and the absorption spectra of the reaction mixture at 0.2 or 0.8, 3, 15 and 300 sec. after mixing in Fig. 2. Up to 10–15 sec., the spectrokinetic curves are smooth and continuous, consistent with the transformation of metHb to a single product characterized by the integrated spectrum after 15 sec., with points of approximately constant intensity at 414, 517 and 614 $\text{m}\mu$. There is no indication of the formation in appreciable concentration of any spectroscopically distinct intermediate. At about 10–15 sec., the spectro-

kinetic curves show discontinuities: at 415–422, 540–560 and 620–640 $\text{m}\mu$, dE/dt changes sign; at 495 and 500 $\text{m}\mu$, a constant value of E succeeds the initial fall; at 615 $\text{m}\mu$, E begins to increase and at 402–408 $\text{m}\mu$ inflexions are apparent. These changes are attributed to consecutive reactions: (i) the formation of a compound with absorption maxima at 416–418 and 545 $\text{m}\mu$. (Fig. 2), followed by (ii) slow destruction of the compound accompanied by decreasing absorption in the whole Soret region and near 545 $\text{m}\mu$, and increasing absorption in the region 590–640 $\text{m}\mu$.

In a similar experiment with $R = 3$ and the same pigment concentration, again at pH 6.0, the results indicated that the reaction was incomplete, but otherwise differed only in rate from that with the higher peroxide concentration. Up to 2 min., the spectral absorption of the reacting mixture showed isosbestic points in about the same positions as before, and the spectrum at 2 min. was consistent with a mixture of metHb and methaemoglobin-hydrogen peroxide. The consecutive reaction in the period 2–5 min. was also slower. As in Expt. 1 ($R = 40$), a decline of the maximum at 545 $\text{m}\mu$ was accompanied by increasing absorption in the region 610–640 $\text{m}\mu$, but the uniform decrease of absorption in the Soret region was not reproduced with this lower peroxide concentration; in fact, the absorption at 402–408 $\text{m}\mu$ remained constant from 2 to 5 min., and then began to increase again, suggesting reversion to metHb.

Less comprehensive experiments were made with R values of 0.7, 7, 15, 28 and 110 (Expts. 3–7) and the same pigment concentration of 0.049 mEq.

Fig. 1.

Fig. 2.

Fig. 1. Changes of extinction with time for a reacting mixture of 0.049 mEq. metHb and 2.0 mM- H_2O_2 , pH 6.0 (Expt. 1).

Fig. 2. Spectral absorption of a reacting mixture of 0.049 mEq. metHb and 2.0 mM- H_2O_2 , pH 6.0, at intervals from 0 to 300 sec. after mixing (Expt. 1).

Observations were restricted to the wavelengths of the absorption maxima of the compound, 418 and 545 $m\mu$., and of metHb, 405, 500 and 630 $m\mu$. The spectrokinetic curves for 418 and 500 $m\mu$. (Fig. 3) show how the rate and the extent of the reaction increase with R . Once again the coincidence in time of the breaks in the curves for different wavelengths is consistent with essentially separate consecutive reactions. The curves for 418 $m\mu$. show that the rate of destruction of the compound also increases with R , but it is evident from the slopes that at each peroxide concentration it is too slow to significantly depress the maximum extinction value. At 500 $m\mu$., the consecutive reaction is not

accompanied by significant change of extinction, and measurements at this wavelength should yield reliable estimates of the proportion of free metHb. Both sets of curves show that with $R > 40$, the metHb is rapidly and almost completely converted into the compound, whilst with lower peroxide concentrations free metHb persists.

Fig. 4 shows the changes in light absorption of a reaction mixture at pH 8.5, with 0.046 mEq. metHb and $R = 60$ (Expt. 8). The spectrokinetic curves at eighteen wavelengths, from which the curves of Fig. 5 were constructed, are smooth and continuous up to 1 min. after mixing, and the absorption spectrum of the mixture then agrees

Fig. 3. Time course of the extinction at 418 $m\mu$. (A) and 500 $m\mu$. (B) for reacting mixtures of 0.049 mEq. metHb and various amounts of H_2O_2 , indicated as the molar ratio H_2O_2 /haematin; pH 6.0 (Expts. 1-7).

Fig. 4. Spectral absorptions of a reacting mixture of 0.046 mEq. metHb and 2.8 mm- H_2O_2 , pH 8.5, at intervals from 0 to 300 sec. after mixing (Expt. 8).

satisfactorily with the 10 sec. spectrum of Expt. 1 at pH 6·0 (Fig. 1). The destruction of the compound in the period 1–5 min. is slower than at pH 6·0, and is accompanied by decreasing or constant extinction at all wavelengths, including 610 and 630 $m\mu$. Less detailed experiments with $R=26$ and the $R=130$ and the same pigment concentration showed that the rate of compound formation increased with R but was considerably smaller than with the corresponding peroxide concentrations at pH 6·0, and did not vary significantly in extent over this range of R values.

Experiments with lower pigment concentrations and higher R values at pH's 6·0, 6·9 and 8·5, in which observations were made at ten wavelengths

from 400 to 430 $m\mu$., gave similar results, and confirmed the location of an absorption maximum at 416–418 $m\mu$. due to methaemoglobin-hydrogen peroxide. The peroxide used in these experiments (*a-e*) contained stabilizer.

Quantitative interpretation of the spectrokinetic data

The spectrokinetic observations suggest that for the purposes of approximate quantitative treatment the formation of methaemoglobin-hydrogen peroxide in the presence of excess of peroxide ($R>40$ at pH 6·0, >20 at pH 8·5) may be regarded as an isolated reaction. Although the compound is unstable, the extinction changes accompanying its subsequent reaction are at most wavelengths, and over a range of H_2O_2 concentration, slow relative to those accompanying its formation. It will be assumed that when $E\ 418$ (i.e. E at 418 $m\mu$.) and $E\ 545$ reach a maximum the metHb has been completely converted into methaemoglobin-hydrogen peroxide, and molar extinction coefficients for the latter will be estimated. The concentrations of free and combined metHb at any stage during the initial reaction may be calculated approximately from the spectrokinetic curves at any wavelength by equation (1).

Extinction coefficients for methaemoglobin-hydrogen peroxide. In Table 2, extinction ratios $E\ 418/E_0\ 408$ and $E\ 545/E_0\ 545$ are shown for experiments at pH 8·5, and $E\ 418/E_0\ 405$ and $E\ 545/E_0\ 500$ for experiments at pH 6·0. For each experiment the E_0 values are measurements on the metHb calibration fluid and the E values are the maximum extinctions reached by the reaction mixture. The ratios are constant at a given pH, independent of R , the initial metHb concentration, and the path length, supporting the view that they are reasonably accurate absorption ratios compound/metHb. Independent estimates of $\epsilon_{mEq.}$ were made for each experiment from $E\ 418$ and $E\ 545$, the optical path length, and the concentration of the compound = initial metHb concentration, the latter being calculated from the

Fig. 5. Rate of reaction of metHb + $H_2O_2 \rightarrow$ compound at pH 8·5 (Expt. 8). *A*: $\bullet$, % metHb calculated from extinction measurements at ten wavelengths; the broken lines are theoretical first-order curves for half-reaction times of 9 and 10 sec. *B*: $\log C_0/C_t$, where C_0 = initial metHb concentration, C_t = metHb concentration at time t , calculated from the best extinction measurements, $\bullet$, 405, 408 and 410 $m\mu$.; $\circ$, 418 and 422 $m\mu$.

Table 2. Spectrophotometric constants for methaemoglobin-hydrogen peroxide

		Composition of reaction mixture		Absorption ratios. Compound, $E\lambda_1$ /metHb, $E\lambda_2$				$\epsilon_{mEq.}$ λ_{max} . ($m\mu$.)	
pH	Expt.	MetHb concentration (mEq.)	Molar ratio H_2O_2 /haematin (R)	418/405	418/408	545/500	545/545	418	545
8·5	8	0·046	60	—	0·84	—	1·17	103	10·4
	9	0·048	26	—	0·86	—	1·18	109	10·7
	10	0·043	130	—	0·83	—	1·18	108	10·9
	<i>c</i>	0·0030	600	—	0·86	—	—	104	—
	<i>d</i>	0·0032	550	—	0·85	—	—	101	—
	<i>e</i>	0·016	110	—	0·86	—	—	102	—
6·9	<i>b</i>	0·0030	600	—	—	—	—	107	—
6·0	1	0·049	40	0·61	—	1·10	—	106	10·2
	7	0·049	110	0·62	—	1·12	—	106	10·4
	<i>a</i>	0·0030	600	0·63	—	—	—	104	—

concentration of the metHb reagent and the measured relative flow rates of the reactants in the kinetic apparatus. In view of the combined uncertainties of these quantities, the agreement between the values so obtained (Table 2) is satisfactory, and the spectral absorption of methaemoglobin-hydrogen peroxide is evidently not significantly affected by pH in the range 6.0–8.5.

Kinetics of methaemoglobin-hydrogen peroxide formation. For the experiments with high peroxide concentrations listed in Table 2, the spectrokinetic data at any wavelength may be transposed into approximate kinetic data by equation (1) in the form:

$$\% \text{ metHb} = 100 \frac{C_t}{C_0} = 100 \frac{(E_t \sim E_x)}{(E_0 \sim E_x)}, \quad (1a)$$

where C_0 = initial metHb concentration, C_t = metHb concentration at time t , E_0 = extinction of metHb calibration fluid, E_t = extinction of reaction mixture at time t ($< x$), and E_x = extinction of reaction mixture at time x such that $E_x 418 = \hat{E} 418$. The kinetic curves so obtained will deviate from the true kinetic curve for the reaction to varying extents at different wavelengths, depending upon the extent to which, as a result of the consecutive reaction of the compound, E_x deviates from the true extinction at complete reaction. Moreover, the deviations may be in either sense, depending upon whether the decomposition enhances or diminishes the extinction change associated with compound formation. A reasonable estimate of the true kinetic curve might therefore be obtained, in spite of the instability of the product, as a mean of the results for wavelengths subject to opposite errors.

Fig. 5 shows the plot of % metHb versus time for Expt. 8, pH 8.5, calculated by equation (1a) from the spectrokinetic measurements at the ten wavelengths for which $(E_0 \sim E_x) > 0.07$. The points fall, as closely as can be expected, on a logarithmic curve; the broken lines are the theoretical first-order curves for half-reaction times, $t_{0.5}$, of 9 and 10 sec.

Fig. 5 also shows the plot of $\log C_0/C_t$ versus time for the most precise data of the same experiment, namely the measurements at 405, 408, 410, 418 and 422 m μ . The points for 418 and 422 m μ . fall on one straight line through the origin, and those for 405, 408 and 410 m μ . on another, in accordance with the form of equation (1a) and the expected deviations discussed above: at 405, 408 and 410 m μ . destruction of methaemoglobin-hydrogen peroxide enhances the extinction change associated with its formation, whilst at 418 and 422 m μ . the reverse is true. The mean straight line through the origin should be a good approximation to the true plot, and gives $t_{0.5} = 10$ sec.

The data of other experiments at pH 8.5 with excess of peroxide yielded similar results, and therefore the initial course of the reaction in each experiment is satisfactorily described by

$$\frac{d[\text{compound}]}{dt} = k[\text{metHb}]. \quad (2)$$

Estimates of $t_{0.5}$ for the various experiments are given in Table 3. The data for Expts. c–e show that with a given excess of peroxide, $t_{0.5}$ is independent of the initial metHb concentration. It can be seen that with constant metHb concentration the reaction rate increases with the peroxide concentration (Expts. 8–10), and the function $t_{0.5} \times [\text{H}_2\text{O}_2]$ is reasonably constant, at a given temperature. The kinetics at pH 8.5 may therefore be described by

$$\frac{d[\text{compound}]}{dt} = k_2 [\text{H}_2\text{O}_2] [\text{metHb}]. \quad (3)$$

Values for the second-order constant, k_2 , are given in Table 3, and increase significantly with increase of temperature.

Table 3 also shows kinetic data for the few experiments with excess of peroxide at pH's 6.0 and 6.9. The values for $t_{0.5}$ are less reliable, especially at pH 6.0, because the reaction is faster and only two or three kinetic measurements were made at each

Table 3. *Kinetic data for methaemoglobin-hydrogen peroxide formation*

pH	Temp. (°)	Expt.	Reactant concentrations		Half-reaction time, $t_{0.5}$ (sec.)	Sp. reaction rate $k_2 = 0.69$ [H_2O_2]/ $t_{0.5}$ (mm ⁻¹ sec. ⁻¹)
			MetHb (mEq.)	H ₂ O ₂ (mm)		
8.5	23	8	0.046	2.8	10	0.025
		9	0.048	1.25	18	0.030
		10	0.043	5.6	4.7	0.027
	17.4	c	0.0030	1.8	20	0.019
		d	0.0032	1.8	22	0.018
		e	0.016	1.8	22	0.018
6.9	17.4	b	0.0030	1.8	5.0	0.077
6.0	23	1	0.049	2.0	1.15	0.30
		7	0.049	5.4	0.55	0.23
	17.4	a	0.0030	1.8	1.8	0.22

wavelength. Nevertheless, the combined data for fifteen wavelengths were consistent, in each experiment, with equation (2). The results of the three experiments at pH 6.0, which cover quite a wide range of reactant concentrations, also conform reasonably well with equation (3), as shown by the constancy of k_2 . The specific reaction rate increases markedly with increase of acidity.

The reaction with low peroxide concentration, pH 6.0. The effect of peroxide concentration on the extent of methaemoglobin-hydrogen peroxide formation at pH 6.0, with initial metHb concentrations of 0.049 and 0.163 mEq., was studied in optical cells by extinction measurements at 500 μ ., where the effect of the consecutive reaction on the light absorption is negligible. With the higher pigment concentration, 60% compound formation was obtained with $R=0.5$; this, and the kinetic data with excess of peroxide, is in accordance with the requirement of 1 mol. hydrogen peroxide per haematin group, deduced by Keilin & Hartree (1935) from spectroscopic measurements at three times this pigment concentration.

The maximum concentration of methaemoglobin-hydrogen peroxide was attained rapidly under these conditions, and in spite of the presence of traces of catalase, it seemed worth while to test the conformity of the data with the equation

(where metHb represents one haematin equivalent) on the assumption that the peroxide concentration was the initial concentration less that used in compound formation. The results are shown in Fig. 6 as a plot of $\log [\text{compound}]/[\text{metHb}]$ against $\log [\text{H}_2\text{O}_2]$. The points do not deviate significantly from a straight line of unit slope, and give an apparent $pK = 4.4$ at 23°.

A further test of the formulation of the reaction as a simple equilibrium is the ability of the equation

$$\frac{d[\text{compound}]}{dt} = k_2 [\text{metHb}] [\text{H}_2\text{O}_2] - k_1 [\text{compound}] \quad (4)$$

to describe the kinetics of the reaction with low peroxide concentrations. If a = initial peroxide concentration in mol./l., b = initial metHb concentration in equiv./l., and x = compound concentration in equiv./l., this equation leads to the relation

$$x_1 e^{k_1 t} - x_2 e^{k_2 t} = k_2 \int_{t_1}^{t_2} e^{-k_1 t} (b-x) (a-x) dt.$$

The integral can be evaluated graphically for chosen time intervals from experimental values of x and t , and if k_1 is known independently, values of k_2 may be calculated for the several intervals (Hartridge & Roughton, 1925).

The experimental values of $K = 4 \times 10^{-5} \text{ M}$ and $k_2 = 270 \text{ M}^{-1} \text{ sec}^{-1}$ give an approximate value for $k_1 = K \times k_2 = 0.011 \text{ sec}^{-1}$ at 23° and pH 6.0. From the data of Expt. 2, $R=3$, in which the maximum concentration of methaemoglobin-hydrogen peroxide (68%) was reached in about 100 sec., values of k_2 were calculated from the concentration changes in successive time intervals for several assumed values of k_1 around 0.01 sec^{-1} , and are plotted against the mean times in Fig. 7. It can be seen that for the early stages of the reaction, $k_2 = 240 \text{ M}^{-1} \text{ sec}^{-1}$, in satisfactory agreement with the value obtained with higher peroxide concentrations, and that if $k_1 = 0.010$ –0.015, reasonably constant values of k_2 are obtained throughout the reaction. Similar results were obtained with the data of Expt. 3, $R=0.7$. Within the rather large experimental error, therefore, equation (4) apparently represents the kinetics of incomplete methaemoglobin-hydrogen peroxide formation at low peroxide concentrations.

Fig. 6. Effect of H_2O_2 concentration on the extent of compound formation. $\times$, 0.163 mEq. metHb; $+$, 0.049 mEq. metHb.

Fig. 7. Test of fit of $d[\text{compound}]/dt = k_2 [\text{metHb}] [\text{H}_2\text{O}_2] - k_1 [\text{compound}]$ to the kinetics of incomplete compound formation; Expt. 2, 0.049 mEq. metHb, 0.15 mm- H_2O_2 . Values of k_2 , calculated from the measured concentration changes in successive time intervals for several assumed values of k_1 , are plotted against the mean times. The k_1 values are, $\times$, 0.005; $\bigcirc$, 0.010; $\bullet$, 0.015; $+$, 0.030 sec^{-1} .

*Reaction of methaemoglobin-hydrogen peroxide
with sodium dithionite at pH 8.5*

Solutions of methaemoglobin-hydrogen peroxide and of dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), buffered at pH 6.0, were mixed in the kinetic apparatus, and spectrokinetic curves were recorded by the stopped-flow method at several wavelengths.

Because of the instability of methaemoglobin-hydrogen peroxide, a fresh sample was prepared for each wavelength and each set of concentration conditions studied, and reaction was brought about within a few minutes. Samples of the reaction mixture were also collected from the apparatus and stored under paraffin; subsequently, the absorption spectra of the products and of denatured globin carboxyhaemochromogens prepared from them by the method of Lemberg *et al.* (1941), were recorded. In most of the experiments the initial pigment concentration in the reaction mixture was 0.02 mEq.; initial H_2O_2 concentrations of 1, 3 and 7 mm. were used with 0.8% dithionite; and with an initial H_2O_2 concentration of 1 mm., dithionite concentrations of 0.05, 0.1, 0.2, 0.4, 0.8, and 2.0% (w/v) were used.

The absorption spectra of the products, and of carboxyhaemochromogen derivatives, were consistent with mixtures of haemoglobin and choleglobin in varying amounts, and showed that protohaematin was destroyed to an extent which increased with the peroxide concentration and diminished with increase of the dithionite concentration. Correspondingly, the yield of choleglobin was greatest with high H_2O_2 concentrations but tended to increase, rather than diminish, with increase of dithionite concentration, so that the loss

of protohaematin was not entirely accounted for by choleglobin formation.

The spectrokinetic curves, recorded at the wavelengths of the absorption maxima of methaemoglobin-hydrogen peroxide, haemoglobin and choleglobin, namely 418, 430, 545, 555 and 630 $\text{m}\mu$., and also at 580 and 600 $\text{m}\mu$., showed that at least two reactions occurred. At the haemoglobin maxima, 430 and 555 $\text{m}\mu$., the extinction at first decreased for about 30 sec., and then increased slowly in accordance with haemoglobin formation. The initial fall of extinction increased both in magnitude and duration with increase of the peroxide concentration and decreased with increase of the dithionite concentration. The rate of the subsequent increase of extinction at 430 $\text{m}\mu$. increased with the dithionite concentration up to 0.8%. Further increase of dithionite concentration did not affect the rate of haemoglobin formation significantly, nor did the peroxide concentration. The effect of dithionite concentration is shown in Fig. 8 as a plot of $\log(E_\infty - E_t)$ for 430 $\text{m}\mu$. against time, where E_∞ = extinction of the final products and E_t = extinction of the mixture at time t after mixing. The graphs are linear for concentrations of dithionite greater than 0.1%. If it is assumed that after E_{430} begins to increase the reaction consists solely of the conversion of methaemoglobin-hydrogen peroxide into haemoglobin, then $(E_\infty - E_t)$ is proportional to the concentration of the former substance. The half-time for the reaction, calculated from the slopes, decreases from 152 sec. with 0.1% dithionite to 50 sec. with 0.8–2.0% dithionite at about 18°.

There was no evidence, at the few wavelengths studied, for the formation of any intermediate in the reaction.

DISCUSSION

The time course of the spectral absorption from 0.2 sec. after mixing human methaemoglobin and hydrogen peroxide suggests that at both pH's 6.0 and 8.5 the initial reaction is the formation of a single compound by an essentially isolated and quantitative reaction. With an excess of peroxide, this reaction goes rapidly to completion before significant loss by other reactions occurs; the absorption spectrum of the unstable product was quantitatively reproduced with a range of methaemoglobin and excess of peroxide concentrations, and was identical at pH's 6.0 and 8.5. In addition to a maximum at 545 $\text{m}\mu$., $\epsilon_{\text{mEq.}} = 10.5$, and a pronounced shoulder at 570–580 $\text{m}\mu$., recorded previously for methaemoglobin-hydrogen peroxide by Haurowitz (1935) and by Keilin & Hartree (1951) the compound has a sharp peak at 418 $\text{m}\mu$., $\epsilon_{\text{mEq.}} = 105$. With low peroxide concentrations at pH 6.0, the spectrokinetic curves during the initial stages of the reaction were consistent with a partial

Fig. 8. Kinetics of reduction of 0.02 mEq. methaemoglobin-hydrogen peroxide solutions, containing 1 mm. H_2O_2 , by $\text{Na}_2\text{S}_2\text{O}_4$ at various concentrations: $\odot$, 0.1; $\triangle$, 0.2; $\square$, 0.4; $\bullet$, 0.8; and, $\times$, 2.0 g./100 ml. $\text{Na}_2\text{S}_2\text{O}_4$, pH 8.5, 18°. ($E_\infty - E_t$) may be assumed to be proportional to the methaemoglobin-hydrogen peroxide concentration at time t after adding $\text{Na}_2\text{S}_2\text{O}_4$.

conversion of methaemoglobin into methaemoglobin-hydrogen peroxide, and this reaction appeared to reach an equilibrium before the consecutive reaction of the unstable compound occurred to a significant extent.

Metmyoglobin forms an apparently analogous hydrogen peroxide compound (Keilin & Hartree, 1951). George & Irvine (1952*a*), working with horse metmyoglobin, concluded that only at pH 8.0–9.0 is metmyoglobin quantitatively converted into the compound by hydrogen peroxide. Although the absorption spectrum of the compound prepared in this pH range was unaffected by subsequent change of pH, the absorption spectra of the products of the reaction at pH's 5.8 and 6.8, and of haemochromogen derivatives, showed that metmyoglobin-hydrogen peroxide was not the only product, and it was concluded that simultaneous side reactions were brought about by a transient oxidizing entity produced during compound formation. The time course of the spectral changes accompanying the reaction was apparently not studied, and the time after mixing at which the absorption curves were recorded is not stated. The molar ratio of hydrogen peroxide to haematin in these experiments was much less than in ours, and in later work George & Irvine (1953) found that with excess of peroxide the simultaneous side reactions were eliminated. The products of the side reactions apparently resembled those formed from methaemoglobin-hydrogen peroxide in the presence of excess of peroxide at pH 6.0 by a slow consecutive reaction (Dalziel & O'Brien, 1954).

The absorption spectra of the hydrogen peroxide compounds of methaemoglobin and metmyoglobin are quite analogous. The metmyoglobin compound has absorption maxima at 423 and 549 $m\mu$. (George & Irvine, 1951), 4–5 $m\mu$. nearer to the red than those of the methaemoglobin compound, which is the usual difference exhibited by corresponding myoglobin and haemoglobin derivatives; the millimolar extinction coefficients, 105 and 9.8, respectively, are in good agreement with those of methaemoglobin hydrogen peroxide. It is of interest to compare the Soret spectra of these compounds with those of the secondary hydrogen peroxide compounds of catalase and peroxidase. The shift of the maximum from 405 $m\mu$. in acid methaemoglobin to 418 $m\mu$. in methaemoglobin-hydrogen peroxide may be compared with similar changes from 402 $m\mu$. in horse-radish peroxidase to 417 $m\mu$. in compounds II and III (Chance, 1949*a*, 1952) and from 405 $m\mu$. in catalase to 410 $m\mu$. in the erythrocyte catalase compound II (Chance, 1950) and to 425 $m\mu$. in the bacterial catalase compound II (Chance & Herbert, 1950). The methaemoglobin and metmyoglobin compounds resemble the catalase compounds II and III in having less intense

Soret bands than the parent haemoprotein; this is not true of the peroxidase compounds II and III (Chance, 1952; George, 1953). On the basis of light absorption in the visible region, the methaemoglobin and metmyoglobin compounds have been classified with the type III compounds of hydroperoxidases (Keilin & Hartree, 1951). There was no evidence in our experiments for the formation of more than one compound of methaemoglobin with hydrogen peroxide, the spectrokinetic curve at each wavelength studied passing without significant discontinuity from the methaemoglobin value through the earliest point at 0.2 or 0.8 sec. after mixing (Fig. 1).

The consecutive reaction of methaemoglobin-hydrogen peroxide was slow enough to permit reasonably reproducible kinetic data for the formation of the compound to be deduced from the spectrokinetic curves at a number of wavelengths. With high peroxide concentrations at pH's 6.0 and 8.5 the data are satisfactorily described by the equation for a second-order reaction. The approximate value of $220\text{M}^{-1}\text{sec}^{-1}$ for the velocity constant at pH 6.0 and 17° may be compared with $170\text{M}^{-1}\text{sec}^{-1}$ for acid metmyoglobin (horse heart) at 18° (George & Irvine, 1953). The specific reaction rate decreases with increase of pH, and inspection of the limited data of Table 3 suggests that, even if alkaline methaemoglobin does not react with hydrogen peroxide at an appreciable rate, the decreases of the rate constant from pH 6.0 to 6.9 and to 8.5 are too great to be attributed to the acidic dissociation at the iron atom, for which $pK = 8.1$. If hydrogen ions do not participate in the reaction directly, some other haem-linked group or groups with lower pK values are apparently operative. Now that the general character of the initial reaction and the effect of the consecutive reaction on the spectrum has been made clear, it should be possible to obtain more accurate kinetic data by suitable choice of wavelength and reactant concentrations.

The freshly formed compound can be converted quantitatively into denatured globin carboxy-haemochromogen (Dalziel & O'Brien, 1954) and during the destruction of the compound, whilst the hydrogen peroxide is destroyed, part of the pigment undergoes degradation and part reverts to methaemoglobin (Keilin & Hartree, 1935; Dalziel & O'Brien, 1954). The view that the compound is a reversible complex, analogous to the azide, fluoride, hydrosulphide and cyanide derivatives of methaemoglobin (Keilin & Hartree, 1951) would appear to receive some support from equilibrium and kinetic measurements with low peroxide concentrations at pH 6.0. The results, admittedly of uncertain significance, are consistent with the formulation of the reaction as a simple reversible system

(where metHb represents one haematin equivalent) for which $pK = 4.4$ at pH 6.0. Our experiments, however, provide no direct evidence of the structure of the compound. The most obvious formulation of the reaction, accounting for its apparent reversibility, would be the replacement of the water molecule on the iron atom of acid methaemoglobin (Coryell, Stitt & Pauling, 1937; Keilin & Hartree, 1949) by a hydrogen peroxide molecule or ion, OOH^- ; in view of the greater acidic properties of hydrogen peroxide compared with water, and the fact that the spectrum of the compound is of the alkaline methaemoglobin type and unaffected by pH in the range 6.0–8.5, the hydroperoxide structure, analogous to that proposed by Chance (1949b, 1951) for the peroxidase and catalase compounds, would seem to be the more probable. George & Irvine (1951, 1952a, b) have shown, however, that such structures are unsatisfactory for metmyoglobin-hydrogen peroxide, as well as for the compound II of peroxidase (George, 1952), since these compounds are reduced to the original ferric haemoprotein by ferrocyanide in a one-equivalent step. Moreover, an apparently identical metmyoglobin complex can be formed by the action of oxidizing agents other than hydrogen peroxide (George & Irvine, 1953). George (1952) has suggested, amongst other possibilities, that the compounds contain quadrivalent iron, perhaps as the ferryl derivative, FeO . Magnetic-susceptibility measurements on metmyoglobin-hydrogen peroxide and the peroxidase compound II indicate the presence of two unpaired electrons, and therefore essentially covalent bonding of the iron atom (Theorell, Ehrenberg & Chance, 1952; Theorell & Ehrenberg, 1952). George & Irvine (1952a) also found the effect of absolute concentration on the extent of metmyoglobin-hydrogen peroxide formation to be much smaller than would be expected for a reversible equilibrium, and formulated the reaction as an irreversible one of the type

where X is a transient entity possessing one oxidizing equivalent.

There is obviously as yet insufficient experimental data on which to base a comparison of the methaemoglobin and metmyoglobin-hydrogen peroxide compounds; in particular, studies of the mechanism of the side reactions which occur during the formation of the metmyoglobin compound, and of its subsequent reaction in presence of excess of peroxide, are required on the one hand, and a determination of the effective oxidation number of the methaemoglobin compound on the other, as well as more detailed studies of the effect of concentration on the extent of compound formation in both acid and alkaline solution. Magnetic-suscepti-

bility measurements on the methaemoglobin compound have not yet been reported, but are said to be under way (Theorell *et al.* 1952). The absorption spectra of the two compounds are characteristic of analogous haemoglobin and myoglobin compounds, and it seems likely that the two compounds have the same structure, or at any rate that such differences as there may be are common to other pairs of corresponding derivatives. But it is not improbable that the two compounds should react differently, for example, with excess of hydrogen peroxide, and it may be that the apparent reversibility of the methaemoglobin compound and its slow irreversible destruction (Dalziel & O'Brien, 1954) involve intramolecular reactions between the four haems not possible for the metmyoglobin compound. It must be emphasized, however, that the human methaemoglobin used in our experiments was not crystallized or purified other than by alumina treatment, and the possibility exists that enzymes of the erythrocyte play a part in the destruction of the compound.

The transient spectrum formed when hydrogen peroxide is added to haemoglobin in dithionite solution at pH 8.5 (Dalziel & O'Brien, 1952a) shows maxima at 417 and 545 $m\mu$. of similar intensity to those of methaemoglobin-hydrogen peroxide, but in contrast to the latter there is also a distinct maximum at 582 $m\mu$., of slightly greater intensity than that at 545 $m\mu$. The reproducibility of this spectrum under a variety of conditions suggest that it characterizes a single compound, and not a mixture, and spectrokinetic measurements show that its rate of formation is greater than that of methaemoglobin-hydrogen peroxide with the same initial peroxide and pigment concentrations at pH 8.5. To obtain further evidence regarding the identity or otherwise of the two compounds, we made preliminary spectrokinetic experiments on the reaction of methaemoglobin-hydrogen peroxide with excess of dithionite at pH 8.5. In the overall reaction some protohaem is lost, partly as cholehaem and partly as unidentified products. The spectrokinetic curves show that the formation of haemoglobin is preceded by a reaction involving general loss of absorption, and the duration and extent of this initial reaction, the overall loss of protohaem and the yield of choleglobin increase with increase of the initial peroxide concentration. Increase of the initial dithionite concentration curtails the initial reaction and the protohaem destruction but does not significantly affect the yield of choleglobin. Apparently in this initial period reaction occurs between methaemoglobin-hydrogen peroxide or an intermediate formed from it, and the excess of free hydrogen peroxide in the presence of dithionite, possibly by a peroxidative mechanism as suggested by Keilin & Hartree (1935), in which some of the

pigment undergoes oxidative degradation to choleglobin and other products.

This initial reaction is followed, presumably after destruction of the free hydrogen peroxide, by the slow reduction of the remaining methaemoglobin-hydrogen peroxide to haemoglobin. The spectrokinetic curves for 430 $m\mu$. were logarithmic, and showed that the rate of haemoglobin formation increases with the initial dithionite concentration to a limiting value for the half-reaction time of 50 sec. with 0.8–2.0% (w/v) dithionite. The spectrokinetic curves for 580 $m\mu$. did not show any initial increase of extinction such as would suggest the intermediate formation of the transient spectrum observed in the reaction of haemoglobin, dithionite and peroxide. In this latter reaction, the rate of reversion of the transient compound to haemoglobin also increases with the initial dithionite concentration, to a limiting value for the half-reaction time of 25 sec. with 1.6–2.0% dithionite, and appears, therefore, to be significantly faster than the reduction of methaemoglobin-hydrogen peroxide. We hope to report experimental evidence regarding the nature of this transient compound shortly.

SUMMARY

1. The reaction between catalase-free human methaemoglobin and hydrogen peroxide has been studied spectrokinetically. With high concentrations of peroxide at pH's 6.0 and 8.5, methaemoglobin is completely converted into methaemoglobin-hydrogen peroxide, which then undergoes a relatively slow consecutive reaction. The absorption spectrum of the compound, which shows maxima at 418 $m\mu$., $\epsilon_{mEq.} = 105$ and 545 $m\mu$., $\epsilon_{mEq.} = 10.5$, is not affected by pH in the region 6.0–8.5.

2. Reasonably reliable kinetic data for the formation of methaemoglobin-hydrogen peroxide have been deduced from the spectrokinetic data of experiments in which the hydrogen peroxide was present in several excessive concentrations. The rate increases with the hydrogen-ion concentration, the velocity constant at 17° being $18M^{-1} \text{ sec.}^{-1}$ at pH 8.5 and $220M^{-1} \text{ sec.}^{-1}$ at pH 6.0. Spectrokinetic data for the reaction at pH 6.0 with low peroxide concentrations are consistent with a reversible reaction for which $pK = 4.4$ at 23°. The results are discussed in relation to recent work on the reaction of metmyoglobin with hydrogen peroxide.

3. Some observations on the reaction of methaemoglobin-hydrogen peroxide with sodium dithionite at pH 8.5 are described. The compound is mainly reduced to haemoglobin in a relatively slow reaction, but some protohaematin is degraded to cholehaem

and other products in a rapid initial reaction with the excess of hydrogen peroxide.

4. Comparative spectral and kinetic data for methaemoglobin-hydrogen peroxide and the transient compound formed by the action of hydrogen peroxide on haemoglobin in the presence of dithionite are briefly discussed.

5. The absorption maxima and extinction coefficients of human methaemoglobin in acid and alkaline solution are recorded.

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