Side Reactions in the Deoxygenation of Dilute Oxyhaemoglobin Solutions by Sodium Dithionite

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(Received 27 February 1957)

Sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) is the commonest and most convenient reagent for the deoxygenation and reduction of haem pigments. The danger of undesirable side reactions between haem pigments and $\text{Na}_2\text{S}_2\text{O}_4$ or its oxidation products has been remarked by several workers (Hartridge & Roughton, 1923; Keilin, 1926, 1927; Zeile, 1932; Lemberg, Legge & Lockwood, 1941; Lemberg & Legge, 1949), and is particularly important in connexion with accurate kinetic studies of the deoxygenation of oxyhaemoglobin (HbO_2) in an excess of $\text{Na}_2\text{S}_2\text{O}_4$. Hartridge & Roughton (1923) studied the deoxygenation of HbO_2 by $\text{Na}_2\text{S}_2\text{O}_4$ in their rapid-flow apparatus, and concluded that the $\text{Na}_2\text{S}_2\text{O}_4$ did not react directly with the HbO_2 , but simply removed the oxygen from the solution as fast as it was liberated, and that the direct and quantitative conversion of HbO_2 was by a simple first-order dissociation. These conclusions seemed to be confirmed by the more accurate and comprehensive measurements of Millikan (1933, 1936). Recently, however, evidence of a more complicated state of affairs was obtained by Legge & Roughton (1950), both in a re-inspection of Millikan's results and in new experiments on sheep HbO_2 by a similar method. First, it appeared that the first-order velocity coefficient increased significantly during the first third of the reaction, as was indeed to be expected on the basis of the intermediate-compound theory of haemoglobin (Hb) reactions (Roughton, 1949). Secondly, in some experiments at neutral pH the last stages of the deoxygenation seemed to be extremely slow, and to be associated, at least in part, with the formation of pigments in side reactions brought about by $\text{Na}_2\text{S}_2\text{O}_4$ oxidation products, which would invalidate the colorimetric method of analysis. The matter was not fully worked out, but it was suggested that a precursor of choleglobin might be the interfering substance. An initial increase of the first-order velocity coefficient has also been recorded for the deoxygenation of human HbO_2 , but no slow terminal stage was observed (Dalziel, 1954). During incidental measurements, however, it was also noted that a perfectly stable Hb spectrum was not at once obtained on the addition of excess of $\text{Na}_2\text{S}_2\text{O}_4$ to a dilute, slightly alkaline HbO_2 solution in the absence of air, and

that similar slow spectral changes in the absorption spectrum of a dilute Hb- $\text{Na}_2\text{S}_2\text{O}_4$ solution were induced by brief exposure to air. These observations were consistent with the conclusions of Legge & Roughton (1950) that oxidation products of $\text{Na}_2\text{S}_2\text{O}_4$ could bring about side reactions with the pigment.

The purpose of the experiments described here was to obtain evidence of the nature of these side reactions by a closer examination of the slow spectral changes which followed the deoxygenation of HbO_2 by $\text{Na}_2\text{S}_2\text{O}_4$, and the action of oxygen on a Hb- $\text{Na}_2\text{S}_2\text{O}_4$ solution, under various conditions of concentration and pH.

EXPERIMENTAL

Measurements of light absorption were made with a Beckman quartz photoelectric spectrophotometer (model DU) and are generally recorded as the extinction

$$(E = \log I_0/I).$$

Spectrokinetic measurements

In most of the experiments solid $\text{Na}_2\text{S}_2\text{O}_4$ was weighed into a dry 1 cm. optical cell, 3 ml. of buffered HbO_2 solution was quickly pipetted in and a layer of liquid paraffin at once added. The mixture was stirred under the paraffin layer with a fine glass rod, and more paraffin was added to fill the cell, which was sealed with a cover slip. In some experiments, 1.5 ml. of buffered $\text{Na}_2\text{S}_2\text{O}_4$ solution (stored under paraffin) was pipetted under the surface of 1.5 ml. of buffered HbO_2 solutions in a cell; in others, contact with air was completely eliminated by adding solid $\text{Na}_2\text{S}_2\text{O}_4$ in a sealed-glass capillary, which was broken under the surface of the HbO_2 solution and its protective layer of paraffin. Qualitatively similar results were obtained by all these methods. The time course of the extinction of the mixture was followed by making measurements at two or three wavelengths in rapid succession, or sometimes at a single wavelength, from about 1 min. after mixing until constant values were obtained. Similar measurements were made at other wavelengths on fresh preparations. The action of O_2 was studied on the same preparations; after a stable spectrum had been obtained, some of the paraffin was removed, and O_2 was bubbled through the solution from a capillary tube at 2-3 bubbles/sec. for 30 sec. The cell was then sealed and the extinction measured again from about 30 sec. later.

Control experiments, under all the test conditions, showed that the reaction between $\text{Na}_2\text{S}_2\text{O}_4$ and O_2 alone did not

cause significant changes of extinction in the region 400–650 $m\mu$, and there were no visible signs of turbidity or denaturation in the experiments. The changes of pH, due to acidic oxidation products of the $\text{Na}_2\text{S}_2\text{O}_4$, were small in the buffered solutions.

Reagents and derivatives

Haemoglobin. The HbO_2 solutions were haemolysates of washed human red cells; saponin was used to give complete haemolysis. The Hb concentrations were estimated from extinction measurements and the extinction coefficients of HbO_2 (Lemberg & Legge, 1949).

Sodium dithionite. Ordinary commercial samples were used.

Hydrogen peroxide. A.R. '20 vol.' reagent was diluted in buffer solution as required. The stock solution was repeatedly analysed by permanganate titration.

Buffer solutions. These were Clarke and Lubs buffer 0.05M- KH_2PO_4 -NaOH (pH 6.0–8.0) and 0.05M- H_3BO_3 -KCl-NaOH (pH 8.0–10.0) (Vogel, 1939).

Reactant concentrations. Initial concentrations in the reaction mixtures are referred to throughout. Hb concentrations are expressed in m-equiv./l.; the expression mEq.-Hb is equivalent to mM-haem, and m-equiv. extinction coefficients, $\epsilon_{\text{mEq.}}$, refer to these concentration units (Dalziel & O'Brien, 1954).

RESULTS

Side reactions in the deoxygenation of oxyhaemoglobin by dithionite

The curves of Fig. 1 built up from duplicate or triplicate measurements on two series of mixtures represent the spectral absorption of mixtures of

Fig. 1. Spectral absorption of solutions of HbO_2 to which 0.1 g. of $\text{Na}_2\text{S}_2\text{O}_4$ /100 ml. has been added, recorded 1 min. (▲) and 15 min. (○) after addition of $\text{Na}_2\text{S}_2\text{O}_4$. Concentration of HbO_2 solutions for measurements between 400 and 450 $m\mu$; 8×10^{-3} mEq.; between 450 and 650 $m\mu$, 8×10^{-2} mEq.

HbO_2 and $\text{Na}_2\text{S}_2\text{O}_4$ recorded 1 and 15 min. or more after mixing. The spectral absorption 1 min. after mixing is a typical Hb curve, λ_{max} 430, 555 $m\mu$, but during the next few minutes the intensity of absorption increased over both the Hb maxima (420–445 and 540–575 $m\mu$), and decreased over the saddle, beyond 585 $m\mu$, and also in the region 400–415 $m\mu$. The crossing points of the curves at which the intensity remained constant are at about 417, 448, 536 and 579 $m\mu$. Spectrokinetic curves for four wavelengths, obtained with two HbO_2 concentrations, pH 8.5, are shown in Fig. 2. Similar results were obtained in experiments at pH 5.9 and 9.5; in the acid solutions the slow changes were distinctly smaller in magnitude and shorter in duration.

The effects of variation of the reactant concentrations and the pH on the magnitude of the slow increase of extinction at 430 $m\mu$ were systematically studied (Fig. 3). The variation of the final stable extinction coefficient with the conditions shows that HbO_2 is lost in the overall reaction as products other than Hb. Both the 1 min. and the final, 20 min., values of the extinction coefficient become greater with (i) fall of pH, (ii) increase of $\text{Na}_2\text{S}_2\text{O}_4$ concentration and (iii) increase of pigment concentration. Thus the percentage yield of Hb is smallest with dilute HbO_2 solutions, small $\text{Na}_2\text{S}_2\text{O}_4$ concentrations and high pH, and under these conditions the slow increase of extinction at the Hb maximum is most marked.

Fig. 2. Changes of extinction with time at four wavelengths 1 min. after addition of 0.2 g. of $\text{Na}_2\text{S}_2\text{O}_4$ /100 ml. to HbO_2 solutions of pH 8.5. HbO_2 concentrations: 4×10^{-3} mEq. for 415, 417, and 430 $m\mu$, and 3×10^{-2} mEq. for 460 $m\mu$.

Fig. 3. Time course of the extinction coefficient at $430\text{ m}\mu$ from 1 min. after the addition of $\text{Na}_2\text{S}_2\text{O}_4$ to HbO_2 , with various reactant concentration and pH values. (A) pH 8.5 and various reactant concentrations: $\bullet$, 0.2% $\text{Na}_2\text{S}_2\text{O}_4$ and 5×10^{-3} mEq.- HbO_2 ; $\circ$, 0.2% $\text{Na}_2\text{S}_2\text{O}_4$ and HbO_2 concentrations (1) 1.25 , (2) 2.5 , (3) 10 , (4) 20×10^{-3} mEq.; $\blacktriangle$, 5×10^{-3} mEq.- HbO_2 , and $\text{Na}_2\text{S}_2\text{O}_4$ concentrations (g./100 ml.): (a) 0.05 , (b) 0.1 , (c) 0.4 . (B) 0.2% $\text{Na}_2\text{S}_2\text{O}_4$ and 5×10^{-3} mEq.- HbO_2 at various pH values.

No evidence of side reactions had been noted in kinetic studies of the deoxygenation in a rapid-reaction apparatus (Dalziel, 1954), when relatively high pigment concentrations had been used. In accordance with the results of earlier workers, the deoxygenation had apparently been complete in less than a second; when the flow was stopped the extinction of the products had immediately assumed a value close to that of a calibration fluid consisting of fully reacted Hb and had then remained unchanged for at least a minute. More careful and prolonged observation, however, showed that slow changes of the kind described did in fact occur; the extinction of the products increased slightly at $430\text{ m}\mu$ and decreased very slightly at $460\text{ m}\mu$ during a period of 5 min. Confirmation of these small changes was sought by stopped-flow measurements in an attachment to the observation tube of optical-path length 1 cm., which permitted accurate measurements on more dilute pigment solutions. Buffered solutions of $\text{Na}_2\text{S}_2\text{O}_4$ and HbO_2 at pH 8 were mixed by flow, and when steady conditions were attained the flow was suddenly stopped and the extinction of the mixture was recorded against time. During flow through the observation tube the extinction at $430\text{ m}\mu$ increased rapidly as Hb was formed, but immediately the flow was stopped it began to decrease and continued to fall for about 30 sec., and then increased slowly until a constant value was attained after about 10 min. (Fig. 4). The reality of these small changes was established by control experiments; when a Hb- $\text{Na}_2\text{S}_2\text{O}_4$ solution

Fig. 4. Changes of extinction at $430\text{ m}\mu$ after deoxygenation of HbO_2 (4×10^{-3} mEq.) by $\text{Na}_2\text{S}_2\text{O}_4$ (0.2 g./100 ml.) at pH 8.0, recorded in the rapid-reaction apparatus by the stopped-flow method.

was substituted for the HbO_2 solution, a perfectly constant value of the extinction was recorded under similar conditions. The results suggest that two reactions follow the rapid deoxygenation.

The addition of $\text{Na}_2\text{S}_2\text{O}_4$ to dilute methaemoglobin solutions was followed by similar slow spectral changes. When red-cell suspensions were deoxygenated by $\text{Na}_2\text{S}_2\text{O}_4$, however, a practically stable spectrum was obtained at once. It seemed

therefore that the side reactions were not directly connected with the deoxygenation of HbO_2 , but arose from the action of an oxidation product of $\text{Na}_2\text{S}_2\text{O}_4$ on Hb (cf. Legge & Roughton, 1950). This conclusion was strengthened by the observation that exposure of a $\text{Na}_2\text{S}_2\text{O}_4$ solution to air immediately before mixing with HbO_2 enhanced the side reactions. The effect was not permanent, and disappeared when the $\text{Na}_2\text{S}_2\text{O}_4$ solution was allowed to stand in the absence of air for a few minutes.

*Spectral changes induced by oxygen in
haemoglobin-dithionite solutions*

After the slow spectral changes which follow deoxygenation had been recorded in the experiments described previously (Figs. 1, 2), O_2 was passed into the products for 30 sec. Measurements showed that during the action of O_2 the intensity of absorption had fallen at both the Hb maxima, and risen at the minimum and beyond 585 $\text{m}\mu$, and during the next few minutes the original spectrum was largely restored by changes in the reverse direction at most wavelengths. These slow changes appeared to be identical throughout the spectrum in magnitude, direction and rate with those which had followed the initial deoxygenation (Fig. 1). When a stable spectrum was again obtained, it differed from the initial Hb spectrum (Fig. 1) only in having smaller maxima and greater absorption in the red. Some Hb had therefore been destroyed in the overall reaction. No distinct spectral changes were associated with this destruction except perhaps a suggestion of a small maximum near 630 $\text{m}\mu$.

Fig. 5. Effect of oxygen on the extinction of haemoglobin-dithionite solutions. HbO_2 and $\text{Na}_2\text{S}_2\text{O}_4$ were mixed at zero time (see Fig. 2) and oxygen was passed for 20 sec. at times indicated by arrows. Concentrations of reactants are given in Fig. 2.

Spectrokinetic curves of the slow changes following deoxygenation (see also Fig. 2) and the subsequent action of O_2 are given in Fig. 5, and bring out the similarity between the two reactions. The curve for 430 $\text{m}\mu$ is representative of the changes at both the Hb maxima; the considerable decrease of absorption which occurred during the passage of O_2 was largely but not completely reversed in the subsequent slow reaction. A reverse sequence of smaller changes occurred at 460 $\text{m}\mu$ and at other wavelengths near the minimum and beyond 580 $\text{m}\mu$. From 400 to 417 $\text{m}\mu$, both the immediate action of O_2 and the subsequent slow reaction were accompanied by small decrease of extinction.

DISCUSSION

It has been known for some time that the action of oxygen on Hb in $\text{Na}_2\text{S}_2\text{O}_4$ solution results in the formation of choleglobin-like oxidation products, manifest by increased light absorption in the red and decreased absorption at shorter wavelengths, notably in the Soret region (Lemberg *et al.* 1941; Lemberg & Legge, 1949). The present experiments show that an unstable derivative of Hb is also formed under conditions which favour the formation of an oxidation product of $\text{Na}_2\text{S}_2\text{O}_4$, and is slowly reconverted, in part, into Hb in the presence of excess of $\text{Na}_2\text{S}_2\text{O}_4$. These reactions also accompany the conversion of HbO_2 and methaemoglobin into Hb by $\text{Na}_2\text{S}_2\text{O}_4$ in dilute solution. To account for the observed spectral changes, the unstable derivative, unlike the stable degradation products, must have (a) absorption in the Soret region about equal to, or slightly greater than, that of Hb at 400–420 $\text{m}\mu$ and considerably less than that of Hb around 430 $\text{m}\mu$; (b) a greater absorption in the regions 450–530 $\text{m}\mu$ and 580–650 $\text{m}\mu$; (c) isosbestic points with Hb at about 417, 448, 536 and 579 $\text{m}\mu$ (Fig. 1). Neither HbO_2 nor methaemoglobin satisfies these conditions.

Both the formation of the unstable derivative and the irreversible degradation of the Hb were favoured by low Hb and $\text{Na}_2\text{S}_2\text{O}_4$ concentrations and by slightly alkaline conditions, consistent with the view that the unstable compound is an intermediate in the degradation. The effect of $\text{Na}_2\text{S}_2\text{O}_4$ concentration also suggests the origin of the reactions. If $\text{Na}_2\text{S}_2\text{O}_4$ itself were involved, it would be expected that the extent of the reactions would either increase with the $\text{Na}_2\text{S}_2\text{O}_4$ concentrations or be independent of it. If a stable oxidation product of $\text{Na}_2\text{S}_2\text{O}_4$ were involved, the extent of the reactions should also be independent of the $\text{Na}_2\text{S}_2\text{O}_4$ concentration, with constant total oxygen concentration. The observed inhibitory effect of $\text{Na}_2\text{S}_2\text{O}_4$ agrees with the assumption that an intermediate product of the reaction of $\text{Na}_2\text{S}_2\text{O}_4$ with oxygen, which is ultimately destroyed by the excess of $\text{Na}_2\text{S}_2\text{O}_4$, is

responsible for these reactions. This would also explain why the activation brought about by exposure of the $\text{Na}_2\text{S}_2\text{O}_4$ solution to air is only temporary and disappears when the $\text{Na}_2\text{S}_2\text{O}_4$ is allowed to stand in absence of air.

An unstable oxidation product of $\text{Na}_2\text{S}_2\text{O}_4$ is hydrogen peroxide (Jellinek, 1912; Conant & Scott, 1926), which reacts with several haem pigments, including methaemoglobin, to form unstable compounds with well-defined spectral characteristics, and with Hb to form choleglobin (Lemberg *et al.* 1941). An unstable Hb-peroxide compound was postulated by Lemberg *et al.* (1941) as the precursor of choleglobin. It has been shown (Dalziel & O'Brien, 1957) that the unstable compound detected and characterized spectrophotometrically in the experiments described here is also formed on the addition of hydrogen peroxide to Hb in $\text{Na}_2\text{S}_2\text{O}_4$ solution.

The results underline the dangers of the use of $\text{Na}_2\text{S}_2\text{O}_4$ in quantitative experiments with Hb derivatives in the presence of oxygen. The losses involved in the conversion of HbO_2 into Hb by $\text{Na}_2\text{S}_2\text{O}_4$ in dilute, slightly alkaline solution may be of a size that may produce considerable errors in spectrophotometric measurements made during the life of the unstable intermediate. Advantage may sometimes be taken of the stability of the carbon monoxide derivatives to avoid such errors (cf. Lemberg *et al.* 1941); if HbO_2 is reduced by $\text{Na}_2\text{S}_2\text{O}_4$ and then converted into carboxyhaemoglobin the absorption spectrum of the product shows greater absorption in the red than that of a similar preparation in which the HbO_2 solution was first saturated with carbon monoxide and then treated with $\text{Na}_2\text{S}_2\text{O}_4$. It is to be expected that considerable irreversible degradation may accompany the re-conversion of Hb into HbO_2 by oxygenation of $\text{Na}_2\text{S}_2\text{O}_4$ solution. For sheep HbO_2 prepared in this way Beznak (1948) found $\lambda_{\text{max.}} = 412 \text{ m}\mu$, $\epsilon_{\text{mEq.}} = 72$, which may be compared with the generally accepted values of $\lambda_{\text{max.}} = 415 \text{ m}\mu$, $\epsilon_{\text{mEq.}} = 128$ (Lemberg & Legge, 1949), and for sheep myoglobin, similarly prepared $\lambda_{\text{max.}} = 410 \text{ m}\mu$, $\epsilon_{\text{mEq.}} = 130$. In contrast, other pairs of analogous Hb and myoglobin derivatives gave extinction coefficients in the Soret region in close agreement with one another. Beznak devised a spectrophotometric method for the analysis of mixtures of Hb and myoglobin on the basis of the decrease of extinction in the Soret region which accompanied reoxygenation of $\text{Na}_2\text{S}_2\text{O}_4$ solutions. The method probably depends, not upon any real difference between the molecular extinction coefficients of HbO_2 and oxymyoglobin, but upon a greater susceptibility of Hb to oxidative degradation, during the oxygenation, to choleglobin and other products with little specific absorption in the Soret region. There is direct evidence that myo-

globin is more resistant than Hb to degradation by hydrogen peroxide in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ (Dalziel & O'Brien, 1957).

Sodium dithionite is evidently an unsatisfactory reagent for accurate kinetic studies of the deoxygenation of HbO_2 , but no suitable alternative has yet been found. The conclusion of Legge & Roughton (1950) that the occasional slow terminal stage recorded by colorimetric analysis was an artifact is substantiated by the present work, but the fact that the increasing first-order velocity coefficient in the early stages of the reaction is found in spectrokinetic measurements at both 415 and 430 $\text{m}\mu$ (Dalziel, 1954) makes it likely that this is a true kinetic feature of the deoxygenation. It must be added, however, that discrepancies have been discovered between the rate at which carbon monoxide displaces oxygen from combination with Hb and the rate of dissociation of HbO_2 in the presence of $\text{Na}_2\text{S}_2\text{O}_4$, which may indicate that $\text{Na}_2\text{S}_2\text{O}_4$ has a direct action on the pigment and modifies the kinetics of the dissociation, at least in neutral solution (Gibson & Roughton, 1955; Gibson, 1955).

SUMMARY

1. The addition of an excess of sodium dithionite to dilute solutions of oxyhaemoglobin or methaemoglobin does not give a quantitative yield of haemoglobin. Side reactions occur in which part of haemoglobin is degraded to products with relatively low specific absorption. Similar reactions are induced by the brief oxygenation of dilute haemoglobin solutions containing an excess of sodium dithionite.

2. Evidence has been obtained that these reactions are brought about by an oxidation product of sodium dithionite, and involve the temporary formation of an unstable intermediate compound, which may be degraded or slowly reconverted into haemoglobin.

3. The significance of these results in relation to quantitative spectrophotometric and kinetic measurements with oxyhaemoglobin and haemoglobin is discussed.

We wish to thank Mr B. A. Collett for technical assistance. One of us (K.D.) is grateful to the Nuffield Haematology Fund for financial assistance.

REFERENCES

- Beznak, M. (1948). *Acta chem. scand.* **2**, 333.
- Conant, J. B. & Scott, N. D. (1926). *J. biol. Chem.* **69**, 575.
- Dalziel, K. (1954). *Disc. Faraday Soc.* no. 17, p. 128.
- Dalziel, K. & O'Brien, J. R. P. (1954). *Biochem. J.* **56**, 648.
- Dalziel, K. & O'Brien, J. R. P. (1957). *Biochem. J.* **67**, 124.
- Gibson, Q. H. (1955). *Proc. Roy. Soc. B*, **143**, 334.
- Gibson, Q. H. & Roughton, F. J. W. (1955). *Proc. Roy. Soc. B*, **143**, 310.

Hartridge, H. & Roughton, F. J. W. (1923). *Proc. Roy. Soc. A*, **104**, 395.
 Jellinek, K. (1912). *Samml. chem. Vortr.* **18**, 1.
 Keilin, D. (1926). *Proc. Roy. Soc. B*, **100**, 129.
 Keilin, D. (1927). *C.R. Soc. Biol., Paris*, **97**, (Réunion plénière, Mai), p. 39.
 Legge, J. W. & Roughton, F. J. W. (1950). *Biochem. J.* **47**, 43.
 Lemberg, R. & Legge, J. W. (1949). *Haematin Compounds and Bile Pigments*, 1st ed. New York: Interscience.

Lemberg, R., Legge, J. W. & Lockwood, W. H. (1941). *Biochem. J.* **35**, 339.
 Millikan, G. A. (1933). *J. Physiol.* **79**, 158.
 Millikan, G. A. (1936). *Proc. Roy. Soc. B*, **120**, 366.
 Roughton, F. J. W. (1949). *Haemoglobin*, p. 83. London: Butterworths Scientific Publications.
 Vogel, A. I. (1939). *Quantitative Inorganic Analysis*, 1st ed., p. 810. London: Longmans Green and Co.
 Zeile, K. (1932). *Hoppe-Seyl. Z.* **207**, 35.

Spectrokinetic Studies of the Reaction of Hydrogen Peroxide with Haemoglobin in Dithionite Solutions

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(Received 27 February 1957)

It was shown (Dalziel & O'Brien, 1957) that the action of oxygen on haemoglobin in dithionite solution converts part of the haemoglobin into an unstable derivative which, with excess of dithionite, largely reverts to haemoglobin. Small amounts of stable, choleglobin-like products were formed. These reactions were attributed to the action of hydrogen peroxide formed in the autoxidation of dithionite (cf. Lemberg, Legge & Lockwood, 1941*b*; Legge & Roughton, 1950).

In this paper studies of the reaction of hydrogen peroxide with haemoglobin in an excess of dithionite are described.

EXPERIMENTAL

Buffered solutions of H_2O_2 and of haemoglobin (Hb) containing $\text{Na}_2\text{S}_2\text{O}_4$ were studied in the rapid-reaction apparatus (Dalziel, 1953, 1954; Dalziel & O'Brien, 1954*a*). The solutions were mixed in the absence of air and the extinction was recorded 0.2 or 0.8 sec. after mixing. Stopped-flow measurements were also made. The extinction at zero time was obtained by flowing the Hb-dithionite reactant with a dithionite solution instead of H_2O_2 . Spectrokinetic curves were drawn from such measurements at different wavelengths.

Samples of the reaction mixture, and control samples of the Hb reactant mixed with $\text{Na}_2\text{S}_2\text{O}_4$ solution instead of H_2O_2 , were collected from the apparatus, covered with a layer of liquid paraffin and their absorption spectra subsequently measured.

Reagents and derivatives

Hydrogen peroxide. A.R. '20 vol.' reagent was diluted in buffer solution as required. The stock reagent was repeatedly analysed by titration with KMnO_4 .

Haemoglobin. Except where otherwise stated, the experiments were made with Hb solutions free from catalase, prepared from haemolysates of washed human red cells by treatment with alumina. The Hb concentration was estimated spectrophotometrically after conversion into cyan-

methaemoglobin (Drabkin & Austin, 1935-36; Dalziel & O'Brien, 1954*a*). Solid $\text{Na}_2\text{S}_2\text{O}_4$ (a fresh dry commercial sample stored in a sealed bottle) was put in the reactant vessel of the apparatus, and oxyhaemoglobin (HbO_2) solution poured in and immediately covered with paraffin.

Metmyoglobin (MetMb). Crystalline horse metmyoglobin (Theorell & Ehrenberg, 1951) was dissolved in water, dialysed free from $(\text{NH}_4)_2\text{SO}_4$, and finally dialysed against borate buffer (pH 8.5). The concentration of MetMb in the stock solution was determined spectrophotometrically after conversion into cyan-metmyoglobin (Drabkin, 1945).

Buffer solutions. These were Clarke and Lubs buffer 0.05 M- KH_2PO_4 -NaOH (pH 6.0-8.0) and 0.05 M- H_3BO_3 -KCl-NaOH (pH 8.0-9.5) (Vogel, 1939).

Denatured globin carboxyhaemochromogens. These were prepared by the method of Lemberg *et al.* (1941*b*) and will be referred to as CO-haemochromogens.

Reactant concentrations. Initial concentration in the reaction mixture is referred to throughout. Hb concentrations are expressed in m-equiv./l.; mEq.-Hb is equivalent to mm-haem, and m-equiv. extinction coefficients, $\epsilon_{\text{mEq.}}$, refer to these concentration units; the range of Hb concentrations used was 0.02-0.50 m-equiv./l. The concentration of $\text{Na}_2\text{S}_2\text{O}_4$ was varied from 0.05 to 2.0% (w/v). The H_2O_2 concentrations were in the range 0.5-20 m-moles/l.

RESULTS

Products of the overall reaction

The reaction between Hb in $\text{Na}_2\text{S}_2\text{O}_4$ solution and H_2O_2 was studied spectrophotometrically at pH 5.9, 7.0, 7.7, 8.0, 8.4, 9.0 and 9.5. A comparison of the absorption spectra at pH 5.9 and 7.7 with that of Hb in $\text{Na}_2\text{S}_2\text{O}_4$ solution illustrates the main features of the overall reaction (Fig. 1*A*). Depending upon the pH, there is a variable loss of Hb and the formation of substances absorbing in the orange part of the spectrum; these changes are confirmed by the spectra of the reaction products after addition of alkali and CO (Fig. 1*B*).