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

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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*).

Fig. 1. (A) Absorption spectra of the products of the reaction of 0.02 mEq.-Hb, 0.1% $\text{Na}_2\text{S}_2\text{O}_4$ and 2 mM- H_2O_2 at pH 5.9 (●) and pH 7.7 (○). (B) Absorption spectra of CO-haemochromogen derivatives of the products of reaction of 0.02 mEq.-Hb, 0.1% $\text{Na}_2\text{S}_2\text{O}_4$ and 2 mM- H_2O_2 at pH 5.9 (●) and pH 7.7 (○). Curves without experimental points are for 0.02 mEq.-Hb in 0.1% $\text{Na}_2\text{S}_2\text{O}_4$ and CO-protohaemochromogen prepared therefrom.

At pH 5.9, where the destruction of Hb is small, there is a maximum at $620\text{ m}\mu$, which is largely destroyed upon the addition of alkali and CO. The only known pigment with properties consistent with these results is sulphaemoglobin (Lemberg & Legge, 1949).

At pH 7.7, and in more alkaline solutions, Hb is partly destroyed, and a band at $630\text{ m}\mu$ appears. This band was unaffected by treatment with alkali and CO (Fig. 1B) or by CO alone. Addition of alkali alone, however, resulted in a shift of this band to $617\text{ m}\mu$. Of the known pigments, choleglobin has the properties which best fit these facts (Lemberg & Legge, 1949). The results at pH 7 were consistent with a mixture of choleglobin and sulphaemoglobin. In all reactions, the bulk of the Hb was apparently unchanged. The yield of choleglobin decreased with increase of pH above 8.4, although the extent of Hb and protohaem destruction remained fairly constant. Detailed study of the reaction of Hb with H_2O_2 in a reducing medium showed that it was a complex one and composed of a number of stages, which are described below.

Formation of intermediate compound

The time course of the changes in light absorption during the reaction at pH 8.4 from 0.2 sec. after mixing is shown in Figs. 2A, 2B and 3. The occurrence of simultaneous discontinuities in the spectrokinetic curves of Fig. 2 shows that at least three reactions occur. The first is a rapid one, complete in 5–10 sec., in which the spectrum of the mixture changes completely, and is succeeded by a slow decline of the extinction at all wavelengths. Finally, after about 60 sec., a third slow reaction begins which results in partial reversal of the initial

changes at all wavelengths except those near $630\text{ m}\mu$. The significance of these changes is brought out in Fig. 3, which gives the integrated absorption spectra at intervals after mixing. At 20 sec. the spectrum of Hb has been replaced by a different one, with absorption maxima at 417, 545 and $582\text{ m}\mu$. This persists up to 60 sec. without significant change of shape, but with slowly decreasing intensity, and then slowly reverts to a Hb spectrum of reduced intensity with an additional maximum at $630\text{ m}\mu$, which may be attributed to choleglobin. This spectrum is referred to as the intermediate spectrum.

Similar spectrokinetic studies were made under various conditions: at pH 8.4 with reactant concentrations covering the ranges 0.02–0.1 mEq.-Hb, 0.05–2.0% $\text{Na}_2\text{S}_2\text{O}_4$ and 0.5–5.0 mM- H_2O_2 ; at pH 9–10 with up to 0.5 mEq.-Hb and 20 mM- H_2O_2 (cf. Dalziel & Ehrenberg, 1955); and at pH 5.9, 7.0 and 7.7 with 0.02 mEq.-Hb, 0.1% $\text{Na}_2\text{S}_2\text{O}_4$ and 2 mM- H_2O_2 . In every experiment an excess of $\text{Na}_2\text{S}_2\text{O}_4$ over H_2O_2 was used, and the bulk of the Hb was apparently recovered unchanged. Spectrokinetic curves were recorded at the absorption maxima of Hb, choleglobin and the intermediate spectrum and in many cases at other wavelengths. The same sequence of extinction changes occurred in all the experiments at pH 7.7–10. The same intermediate spectrum was obtained, and the $\epsilon_{\text{mEq.}}$ values were reproducible: 90 ± 3 at $417\text{ m}\mu$, 50 ± 4 at $430\text{ m}\mu$, 9.0 ± 0.4 at $545\text{ m}\mu$, 9.6 ± 0.4 at $582\text{ m}\mu$. With increase of pH from 7.7 to 10: (i) the life of the intermediate spectrum increased markedly; (ii) the rate at which it declined during its life decreased; (iii) the rate at which it was finally displaced by an Hb spectrum decreased. At pH 7 similar results

were obtained, but the intermediate spectrum had a lifetime of only a few seconds. At pH 5.9, the extinction changes were in the same sense as but smaller than those at higher pH values, and there was no 'steady state' in which a characteristic spectrum persisted.

Fig. 2. (A) and (B) Plots of extinction against time after adding H_2O_2 to Hb and $Na_2S_2O_4$. Wavelengths shown on the curves are in mμ. The reaction mixture contained initially 0.02 mEq.-Hb, 0.1 % $Na_2S_2O_4$ and mM- H_2O_2 , at pH 8.4.

In these experiments the decrease of the extinction at 430 mμ to a reproducible level shows that Hb is completely displaced in the initial reaction. The symmetry, persistence and reproducibility of the maximum at 417 mμ under varied conditions justifies the conclusion that this intermediate spectrum is largely due to a single Hb derivative, some of which undergoes degradation to products with little specific absorption, but the bulk is slowly reconverted into Hb.

Side reaction accompanying formation of intermediate compound. There are, however, indications that more than one reaction occurs in the initial stage of the reaction. In Fig. 2A, the extinction at 410–416 mμ increases between 0 and 0.2 sec. and then decreases to the level of the intermediate spectrum at 2–3 sec. These changes can be understood by the following experiment. A flowing-reaction mixture (0.2 mEq.-Hb, 0.1 % $Na_2S_2O_4$, 3 mM- H_2O_2 , pH 8.4) was observed spectroscopically in the rapid-flow apparatus; 0.09 sec. after mixing the Hb- $Na_2S_2O_4$ and H_2O_2 solutions, the bands of HbO_2 ($\alpha = 579$ mμ; $\beta = 542$ mμ) were seen; from 0.3 to 0.5 sec., the single broad band of Hb was present but, on stopping the flow, the spectrum of the intermediate compound ($\alpha = 585$; $\beta = 545$ mμ) at once appeared.

The extinction changes in the Soret region 415 mμ (λ_{max} HbO_2), 430 mμ (λ_{max} Hb) and 421 mμ in the early stages of the reaction are shown in Fig. 4. Hb and HbO_2 have the same molar extinction at 421 mμ; Hb and the intermediate compound have about equal extinctions at 415 and 421 mμ, but at 430 mμ that of the transient compound is much smaller (cf. Figs. 2A, 3). Thus the results of Fig. 4

Fig. 3. Absorption spectrum of a reacting mixture of 0.02 mEq.-Hb, 0.1 % $Na_2S_2O_4$ and mM- H_2O_2 , pH 8.4, at intervals after adding the H_2O_2 : ●, initial spectrum (Hb); ▲, 20 sec.; ■, 60 sec.; ○, 10 min.

are consistent with the sequence of reactions: (1) $\text{Hb} \rightarrow \text{HbO}_2$, complete within 0.01 sec.; (2) $\text{HbO}_2 \rightarrow \text{Hb}$, from 0.01 to 0.25 sec.; (3) $\text{Hb} \rightarrow$ transient compound, overlapping (2) and becoming apparent as a decrease of extinction at 430 $\text{m}\mu$ after 0.3 sec.

The overall decrease of $\epsilon_{\text{mEq.}}$ of the reaction mixture at 430 $\text{m}\mu$ from 0 to 0.2 sec. may be taken as a measure of the proportion of Hb converted into the intermediate compound in this time. The values in Table 1 for reaction mixtures of different composition suggest that at pH 8.4 this proportion increases with the H_2O_2 concentration, but is independent of the Hb or $\text{Na}_2\text{S}_2\text{O}_4$ concentrations.

Lifetime of the intermediate compound. The life of the compound [measured by the time taken for the extinction at 430 $\text{m}\mu$ to reach a minimum (cf. Fig. 2A)], varies directly with the H_2O_2 concentration and inversely with the $\text{Na}_2\text{S}_2\text{O}_4$ and pigment concentrations, and is shortened by catalase (Table 2).

The rate at which the intermediate spectrum declined during its lifetime was not affected by a fivefold change of H_2O_2 concentration at pH 8.4, but increased with the $\text{Na}_2\text{S}_2\text{O}_4$ and pigment concentrations.

Haemoglobin regeneration and choleglobin formation. Except near 630 $\text{m}\mu$, the initial changes of spectral absorption are largely reversed in the final stages of the reaction. At 630 $\text{m}\mu$ the extinction increases during the formation of both the intermediate compound and Hb, showing that choleglobin accumulates. The effects of varying the reactant concentrations, at pH 8.4, are represented in Table 3, where the amounts of Hb destroyed are expressed in terms of the percentage decrease of extinctions, $100\Delta E/E$, at the Hb (or CO-haemochromogen) maxima, and the amounts of choleglobin formed in terms of the increase of light absorption of haemochromogen at 630 $\text{m}\mu$. The data show that Hb destruction and choleglobin formation do not change in a parallel manner.

Fig. 4. Plots of extinction at 415, 421 and 430 $\text{m}\mu$ against time after the addition of H_2O_2 to Hb and $\text{Na}_2\text{S}_2\text{O}_4$: 0.02 mEq.-Hb, 0.1% $\text{Na}_2\text{S}_2\text{O}_4$, mm- H_2O_2 , pH 8.4, showing the early stages of the reaction.

Table 1. Extinction coefficients ($\epsilon_{\text{mEq.}}$) after the addition of hydrogen peroxide to haemoglobin-dithionite solutions

Extinction coefficients ($\epsilon_{\text{mEq.}}$) were measured at 430 $\text{m}\mu$, 0.2 sec. after the addition of hydrogen peroxide to haemoglobin-dithionite solutions, at pH 8.4. The rate of formation of the transient compound ($\epsilon_{\text{mEq.}} = 50$) from haemoglobin ($\epsilon_{\text{mEq.}} = 135$) with various reactant concentrations is shown.

Composition of reaction mixture			$\epsilon_{\text{mEq.}}$
Hb (m-equiv./l.)	$\text{Na}_2\text{S}_2\text{O}_4$ (g./100 ml.)	H_2O_2 (m-moles/l.)	
0.10	0.10	1.0	102
0.02	0.10	1.0	95
0.02	0.20	1.0	100
0.02	0.20	5.0	64
0.02	0.80	5.0	66

Table 2. Effect of reactant concentrations and catalase on the life of the transient spectrum at pH 8.4

Composition of reaction mixture				Life of transient spectrum (sec.)
Hb (m-equiv./l.)	$\text{Na}_2\text{S}_2\text{O}_4$ (g./100 ml.)	H_2O_2 (m-moles/l.)	Catalase	
0.02	0.1	0.5	Absent	28
0.02	0.1	1.0	Absent	60
0.02	0.1	2.0	Absent	160
0.02	0.2	1.0	Absent	30
0.02	0.2	5.0	Absent	111
0.02	0.8	5.0	Absent	33
0.02	0.05	1.0	Absent	210
0.02	0.8	1.0	Absent	15
0.05	0.1	1.0	Absent	24
0.10	0.1	1.0	Absent	15
0.02	0.1	1.0	Present	15
0.02	0.1	2.0	Present	21
0.10	0.1	2.0	Present	6

Table 3. *Extinction changes (ΔE) accompanying the overall reaction of haemoglobin, dithionite and hydrogen peroxide in various concentrations at pH 8.4*

Composition of reaction mixture			Products (100 $\Delta E/E$ at 430 m μ)	CO-haemo- chromogens (100 ΔE at 630 m μ)
Hb (m-equiv./l.)	Na ₂ S ₂ O ₄ (g./100 ml.)	H ₂ O ₂ (m-moles/l.)		
0.02	0.1	0.5	15	3
0.02	0.1	1.0	20	2
0.02	0.1	2.0	29	3
0.02	0.2	1.0	16	5
0.02	0.2	5.0	37	5
0.02	0.05	1.0	31	2
0.02	0.4	1.0	16	6
0.02	0.8	1.0	16	7
0.02	1.6	1.0	18	8
(100 $\Delta E/E$ at 555 m μ)				
0.02	0.1	1.0	18	2
0.05	0.1	1.0	13	9
0.10	0.1	1.0	10	20
0.10	0.1	2.0	17	21

Fig. 5. Effect of Na₂S₂O₄ concentration on the rate of reversion of the transient compound to Hb at pH 8.5. $E_{\infty} - E_t$ is approximately proportional to the concentration of the transient compound; c = initial Hb concentration (mEq.) and a = Na₂S₂O₄ concentration (%); H₂O₂ concentration was mM in all experiments.

In the Soret region, where choleglobin does not absorb appreciably (Lemberg, Holden, Legge & Lockwood, 1942), the final extinction changes are consistent with the reconversion of a single compound, characterized by the 417 m μ maximum, into Hb (Figs. 2A, 3). The rates of this reaction with various reactant concentrations at pH 8.4 are shown in Fig. 5. The plots are linear except with the lowest Na₂S₂O₄ concentrations. The slope is independent of the pigment concentration and increases with the Na₂S₂O₄ concentration up to 1.6%. The half-reaction time calculated from the slopes de-

creases from 113 sec. with 0.1% Na₂S₂O₄, to 25 sec. with 1.6% Na₂S₂O₄.

In Fig. 6A, B, C, the extinction changes at the visible maxima of Hb, the intermediate compound and choleglobin during the later stages of the reaction are shown. At different pH values the curves for 545 and 555 m μ accurately reflect the time course of the concomitant extinction changes at 430 m μ (not shown). The end of the life of the intermediate spectrum is indicated by simultaneous minima in the curves for these three wavelengths, i.e. 2.8 min. at pH 8.5, 1.3 min. at pH 7.8 and 0.5 min. at pH 7.0, after which the extinction increases again. These final increases to stable values occur at about the same rate and are complete in the same times at all three wavelengths. These results support the conclusion that the Soret maximum at 417 m μ and the maximum at 545 m μ in the intermediate spectrum are due to a single derivative, and that the final extinction changes in these spectral regions represent the reconversion of this substance into Hb.

At 580 m μ the formation of the intermediate spectrum is associated with a sharp increase of extinction. In the experiment at pH 8.5 (Fig. 6A) the extinction at this wavelength subsequently decreases at the same rate as at other wavelengths, whilst the intermediate spectrum persists undistorted but with diminishing intensity. There is an inflexion at 3 min. and the subsequent decrease of extinction occurs at the same rate as the increases at 545, 555 and 430 m μ . At pH 8.5 therefore the intermediate spectrum apparently behaves as a single entity. At pH 7.8 and 7 the extinction change at 580 m μ follow the same general course as at pH 8.5 at an increased rate. At pH 7 (Fig. 6C) the initial rise in extinction is less and this may be due to the instability of the intermediate at this pH.

Fig. 6. Spectrokinetic curves for 545, 555, 580 and 630 $m\mu$, for the reaction of 0.02 mEq.-Hb, 0.1% $\text{Na}_2\text{S}_2\text{O}_4$ and 2 mM- H_2O_2 . (A) pH 8.5; (B) pH 7.8; (C) pH 7.0.

A discrepancy does arise in that a stable extinction at 580 $m\mu$ is reached in 2 min. at pH 7.8 and in 1 min. at pH 7, although at other wavelengths the extinction is still rapidly increasing at these times. A stable Hb spectrum is not obtained until after 5 min. at pH 7.8 and 3 min. at pH 7. Therefore the regeneration of Hb cannot be wholly responsible for the extinction changes at 580 $m\mu$ and pH 7-7.8, and the intermediate spectrum cannot be said to behave as an entity throughout the reaction, as it appears to do at pH 8.5.

There is a correlation between extinction changes at 630 and 580 $m\mu$ at all three pH values. At 630 $m\mu$ the extinction reaches a maximum in 6 min. at pH 8.5, 2 min. at pH 7.8 and 1 min. at pH 7, and the extinction at 580 $m\mu$ falls to a constant value in the same times. These results suggest that the maximum at 582 $m\mu$ in the intermediate spectrum may be due partly to a choleglobin precursor.

The final decrease of extinction at 630 $m\mu$ in the reaction at pH 7 (Fig. 6C), which also occurs to a small degree at pH 7.8, may represent the further degradation of choleglobin or the regeneration of Hb.

Effect of carbon monoxide on the reaction. Hydrogen peroxide does not affect the spectrum of carboxyhaemoglobin (HbCO) in $\text{Na}_2\text{S}_2\text{O}_4$ solution at pH 6 and 8.5, and saturation of H_2O_2 solution with CO largely inhibited its reaction with Hb in $\text{Na}_2\text{S}_2\text{O}_4$. The passing of CO into a Hb- $\text{Na}_2\text{S}_2\text{O}_4$ solution immediately after the addition of H_2O_2 during the life of the intermediate spectrum was studied.

Solutions of 0.02 mEq.-Hb in 0.05% $\text{Na}_2\text{S}_2\text{O}_4$ and mM- H_2O_2 , both buffered at 8.5, were mixed by passage through the rapid-flow apparatus; under these conditions the lifetime of the intermediate

Fig. 7. Effect of carbon monoxide on the reaction of H_2O_2 with Hb in dithionite solution. Hydrogen peroxide was added at time 0 and carbon monoxide passed from 10 to 25 sec. Initial concentrations: 0.02 mEq.-Hb, 0.05% $\text{Na}_2\text{S}_2\text{O}_4$, mM- H_2O_2 ; pH 8.4. (A) Spectrokinetic curves at 418 and 430 $m\mu$ with and without CO; (B) absorption spectra of reacting mixture in Soret region: O, 10 sec. (before CO); $\blacktriangle$, 2 min.; $\blacksquare$, 5 min.; $\bullet$, 10 min.; $\triangle$, 1 hr.

compound was 3 min. A portion of the mixture was treated with CO during the period 10-25 sec. after mixing, and transferred to an optical cell which was sealed with liquid paraffin and a coverslip. Change of the extinctions at three wavelengths with time was followed in the sample; similar measurements were made on other samples at other wavelengths. The features of the reaction described below have also been observed in the spectroscope with 0.1 mEq.-Hb, 0.1% $\text{Na}_2\text{S}_2\text{O}_4$ and 2 mM- H_2O_2 at pH 9.5.

The spectrokinetic curves at 418 and 430 $m\mu$, with and without CO treatment (Fig. 7A), show

that on the introduction of CO a new reaction accompanied by an increase of extinction at $418\text{ m}\mu$ begins at once. During this reaction, the Soret maximum of the intermediate spectrum (Fig. 7B) is gradually converted into a much more intense maximum at $419\text{ m}\mu$. These changes can be accounted for by a single reaction, namely the conversion of a derivative characterized by the maximum at $417\text{ m}\mu$ into HbCO, $\lambda_{\text{max.}} 419\text{ m}\mu$, with a half-period of about 2.5 min.

The corresponding changes in the visible region up to 10 min. are shown in Fig. 8A and B. The initial data in these diagrams correspond to the intermediate spectrum obtained in control experiments. At $535\text{--}575\text{ m}\mu$, the extinction changes after the introduction of CO occur at about the same rate as those in the Soret region, and are again consistent with the formation of HbCO ($\lambda_{\text{max.}} 539, 568\text{ m}\mu$) from the intermediate compound. Another reaction also occurs; a pronounced peak appears at $600\text{ m}\mu$, reaches its greatest intensity in 2 min. and then declines. The subsequent spectral changes at $580\text{--}640\text{ m}\mu$, from 10 min. onwards, are shown in Fig. 8C. In the course of several hours, the maximum at $600\text{ m}\mu$ declines whilst a new peak develops at $628\text{ m}\mu$. Throughout this slow reaction, the absorption spectrum at shorter wavelengths remains almost unchanged.

These results show that in the presence of CO a new unstable intermediate, $\lambda_{\text{max.}} 600\text{ m}\mu$, is formed in considerable amount during the first 2 min., and is then very slowly converted into carboxycholeoglobin, $\lambda_{\text{max.}} 628\text{ m}\mu$, and the regenerated Hb converted into HbCO, $\lambda_{\text{max.}} 419, 539, 568\text{ m}\mu$. The spectral absorption of the mixture at intervals during this latter reaction (Fig. 8C) shows an

isosbestic point at $614\text{ m}\mu$ which suggests that the conversion of the new intermediate into carboxycholeoglobin is quantitative. At the start of this reaction, that is when the maximum at $600\text{ m}\mu$ has reached its greatest intensity, $E_{600\text{ m}\mu}$ is 0.200 and $E_{630\text{ m}\mu}$ is 0.060. The differences between these values and the measured extinctions E_t , at various later times (Table 4), are the same for both wavelengths, and therefore the molar-extinction coefficients of the two substances at their respective maxima are about equal. Since the reaction is not accompanied by significant spectral changes in the regions $536\text{--}580$ and $400\text{--}430\text{ m}\mu$, where typical carboxyhaemoglobin maxima persist unchanged, it also follows that both substances have little specific absorption in these regions.

At intervals during this slow formation of CO-choleoglobin from the new intermediate, samples of the reaction mixture were converted into the CO-haemochromogen derivatives. From 16 min. after the introduction of CO the absorption spectra of these derivatives remained constant, and indicated a mixture of proto- and cholehaemochromogen.

Table 4. Extinction changes at 600 and $630\text{ m}\mu$ during the formation of carboxycholeoglobin (cf. Fig. 8C)

Time (hr.)	$(0.200 - E_t), 600\text{ m}\mu$	$(E_t - 0.06), 630\text{ m}\mu$
0.85	0.026	0.025
1.75	0.046	0.044
2.75	0.058	0.056
3.75	0.074	0.072
18.0	0.113	0.111
24.0	0.115	0.114

Fig. 8. Effect of carbon monoxide on the reaction of H_2O_2 with Hb in dithionite solution; conditions were as for Fig. 7. (A) Spectrokinetic curves from 10 sec. after addition of peroxide; CO was passed during the period 10–25 sec.; (B) absorption spectra of reacting mixture: O, 10 sec. (before CO); ▲, 2 min.; ■, 5 min.; ●, 10 min.; (C) absorption spectra of reacting mixture: ●, 10 min.; ▲, 52 min.; ■, 105 min.; ○, 225 min.; ○, 18 and 24 hr.

Comparison of the absorption spectra of the final products of the reactions with and without CO treatment, and of CO-haemochromogen derivatives of the products, showed that CO greatly increased the choleglobin yield, but did not significantly affect the amount of Hb destroyed.

Effect of hydrosulphide upon the reaction. The reaction of Hb in $\text{Na}_2\text{S}_2\text{O}_4$ solution with H_2O_2 at acid pH produced a pigment with properties akin to those of sulphaemoglobin. It is known that H_2O_2 and sulphides react with Hb in $\text{Na}_2\text{S}_2\text{O}_4$ to form sulphaemoglobin in amounts greater in acid conditions than in alkaline (Michel, 1938). In the reaction under investigation, H_2O_2 is one of the reactants and the other, $\text{Na}_2\text{S}_2\text{O}_4$, is a possible source of H_2S (Bassett & Durrant, 1927). The effect of hydrosulphide on the reaction was therefore studied.

Experiments were made with 0.1 mEq.-Hb solutions in 0.1% $\text{Na}_2\text{S}_2\text{O}_4$ at pH 6, 8.5 and 9.5, and hydrosulphide (NaSH), which was a 0.5N-NaOH solution saturated with H_2S . The following observations were made with the reversion spectroscope.

(i) At either pH 8.5 or 9.5 (a) 0.1 ml. of H_2O_2 was added to 10 ml. of Hb solution. The two bands of the intermediate spectrum, at 588 and 548 $\text{m}\mu$, appeared at once, persisted for 1 min. and then

gradually fused; the products showed a broad haemoglobin band at 560 $\text{m}\mu$ and a faint band at 630 $\text{m}\mu$. (b) When 0.1, 0.3 or 0.5 ml. of NaSH was added to 10 ml. of Hb solution a faint band appeared at once at 620 $\text{m}\mu$, but the Hb band remained unaltered. (c) When 0.1 ml. of H_2O_2 was added to 10 ml. of Hb solution, followed immediately by 0.1 ml. of NaSH, a strong band appeared at once at 620 $\text{m}\mu$ and distinct bands at 575 and 541 $\text{m}\mu$. There was no change in 10 min. and addition of 30 mg. of $\text{Na}_2\text{S}_2\text{O}_4$ caused no change in 5 min. The solution was red-brown by transmitted light, green-brown by reflected light. (d) In a similar experiment, the NaSH was added 90 sec. after the H_2O_2 , when the intermediate spectrum was giving way to a Hb spectrum. Immediate observation showed a Hb band and a fairly strong band at 620 $\text{m}\mu$, weaker than that obtained in (c), much stronger than that obtained in (b). (e) 0.1 ml. of NaSH was added to 10 ml. of Hb solution, and after rapid observation of the band formed at 620 $\text{m}\mu$, cf. (b), 0.1 ml. of H_2O_2 was added. Immediate observations showed that the band at 620 $\text{m}\mu$ had become broader and stronger, and its centre had shifted to 623 $\text{m}\mu$; and that the haemoglobin band had been replaced by bands at 575 and

Fig. 9. Absorption spectrum of the products and derivatives of the reaction of 0.1 mEq.-Hb, 0.1% $\text{Na}_2\text{S}_2\text{O}_4$, mm- H_2O_2 and 5 mm-NaSH at pH 8.5. The ordinates are milliequivalent extinction coefficients referred to the initial Hb concentration. —, Hb; ●, products; ○, products treated with CO; ▲, CO-haemochromogen derivatives of products.

541 $m\mu$. There was no further change after several minutes.

(ii) At pH 6. (a) 0.1 ml. of H_2O_2 was added to 10 ml. of Hb solution. A distinct band appeared at once at 626 $m\mu$, but the Hb band remained unchanged: the intermediate spectrum was not observed. (b) The addition of 0.1 ml. of NaSH to the products of (a) 1 min. after the original addition of H_2O_2 had no effect. (c) The addition of 0.1 ml. of NaSH to 10 ml. of Hb solution had no effect.

The absorption spectra of products obtained as in (i) (c) above consistently showed a pronounced peak at 622 $m\mu$, shifted to 616 $m\mu$ by CO (Fig. 9), but its intensity varied by about 30 % in different preparations under apparently identical conditions. Two maxima at 545 and 570 $m\mu$ were usually formed, but were replaced in the course of 30 min. or more by a broad maximum at 555 $m\mu$. In some preparations a single broad maximum at 555 $m\mu$ was obtained at once; there was no correlation between the appearance of the two bands in the green and the intensity of the maximum at 622 $m\mu$. There was no sign of an inflexion at 630 $m\mu$ in the product spectrum, such as would be expected if appreciable amounts of choleglobin were present, yet in the absorption spectrum of CO-haemochromogen derivatives prepared from the fresh products (Fig. 9) a pronounced maximum at 630 $m\mu$ suggests the presence of cholehaem, and the depression of the CO-protohaemochromogen maxima (initial values $\epsilon_{mEq.} = 14.4$ at 539 $m\mu$, 14.0 at 569 $m\mu$) shows that some protohaem destruction has occurred.

Reaction of hydrogen peroxide with myoglobin in dithionite solution

The reaction was studied by spectrokinetic measurements at 13 wavelengths in the region of

Fig. 10. Spectral absorption of a reacting mixture of 0.04 mEq.-myoglobin, 0.1% $Na_2S_2O_4$ and 2 mM- H_2O_2 , pH 9.5, at intervals after the addition of peroxide: ○, myoglobin, $t=0$; ▲, 1 min.; ■, 10 min.; ●, products.

536–640 $m\mu$ from 1 min. after the addition of peroxide until a stable spectrum was obtained. Within 1 min. of the addition of H_2O_2 , the myoglobin curve was replaced by a new spectrum with a maximum at 548 $m\mu$, $\epsilon_{mM} = 10.5$, and a pronounced shoulder at 585 $m\mu$, $\epsilon_{mM} = 9.0$, which persisted without change of shape but with very slowly decreasing intensity for 10 min. and then gradually gave way to a myoglobin curve of slightly reduced intensity (Fig. 10). The intermediate spectrum is indistinguishable from that of metmyoglobin- H_2O_2 , and the extinction coefficients indicate that all the myoglobin is converted into this derivative (cf. George & Irvine, 1951). There is no evidence of the presence of cholemyoglobin or any other coloured degradation product. The absorption spectrum of CO-haemochromogen derivatives of the products confirmed that a small proportion of the myoglobin was destroyed in the overall reaction by degradation of the protohaem, but there was no evidence of the presence of any other haem derivatives.

The effect of passing CO into a reacting mixture of myoglobin, dithionite and peroxide, immediately after the addition of peroxide and during the life of the intermediate spectrum, was observed in the spectroscope. The two bands shifted slightly, to 579 and 541 $m\mu$, consistent with the formation of carboxymyoglobin, but there was no evidence of the formation of any other pigment.

DISCUSSION

The results of the experiments confirm the mechanism proposed for the action of oxygen on solutions of Hb containing $Na_2S_2O_4$ (Dalziel & O'Brien, 1957). They point to the formation of an unstable derivative of Hb on the addition of hydrogen peroxide, the existence of which is substantiated by magnetokinetic measurements (Dalziel & Ehrenberg, 1955). The substance with a Soret band at 417 $m\mu$ and low paramagnetic susceptibility appears to be formed directly from Hb at a rate which grows with the hydrogen peroxide concentration. It has a lifetime which varies directly with the peroxide concentration and inversely with the dithionite concentration, and is shortened by catalase; it is largely reconverted into Hb at a rate increasing with the $Na_2S_2O_4$ concentration up to a certain limit. It is probably formed by oxidation of, or co-ordination of an oxidizing group with, the iron atoms of Hb and survives during the life of hydrogen peroxide in the reaction mixture.

Known derivatives of haemoglobin which might be formed in the reaction are oxyhaemoglobin (HbO_2), methaemoglobin (Meth), methaemoglobin-hydrogen peroxide (Meth- H_2O_2) and methaemoglobin-hydrosulphide. The last of these may be

dismissed because hydrosulphide did not prolong the life of the intermediate spectrum. For the other three and for the intermediate spectrum, the wavelengths of the absorption maxima and the corresponding $\epsilon_{mEq.}$ are given in Table 5 (Lemberg & Legge, 1949; Dalziel & O'Brien, 1954*a*). Neither HbO₂ nor Meth accounts satisfactorily for the intermediate spectrum. The Soret band of Meth at pH 8.5 is at a distinctly different wavelength, and moves from 405 m μ ($\epsilon_{mEq.} = 165$) at pH 6 to 413 m μ ($\epsilon_{mEq.} = 106$) at pH 10. If the intermediate spectrum is attributed to HbO₂, it must also be assumed, to account for the low extinction coefficients, that 40% of the Hb is simultaneously converted into products with little specific absorption, and to account for the fact that under some conditions 80% of the Hb is eventually recovered (Table 3), that one of these products is also reconverted into Hb. Moreover, since the extinction coefficients of the intermediate spectrum are reproducible to within about 5%, the high proportion of these other products formed must be approximately constant over a wide range of reactant concentrations and pH. These conditions are improbable; and a complicated mechanism would be required to explain the persistence and slow deoxygenation of HbO₂ in the presence of excess of dithionite.

The intermediate spectrum differs significantly from that of Meth-H₂O₂ only in that it shows a maximum at 582 m μ , consistently higher than that at 545 m μ . It could be accounted for by the assumption that in the initial rapid reaction with hydrogen peroxide about 90% of the Hb is converted into Meth-H₂O₂ and the rest into a derivative with little specific absorption in the Soret region and a maximum at about 580–590 m μ , $\epsilon_{mEq.} \approx 10$. The degree of reproducibility of the broad, shallow maximum at 582 m μ permits considerable variation of such a small proportion of a secondary product formed under different conditions. A precursor of choleglobin might well exhibit such spectral properties and be formed either simultaneously with Meth-H₂O₂ or very rapidly from it, and persist in a stationary state during the life of peroxide. There was some spectrokinetic evidence that the extinction changes near 580 m μ were associated with chole-

globin formation, and in the presence of carbon monoxide a precursor of carboxycholeoglobin, λ_{max} 600 m μ , was detected.

Meth-H₂O₂ has properties consistent with the observed features of the reaction. It has been characterized as an unstable intermediate in the reaction between Meth and H₂O₂ at pH 6–9.5, which results ultimately in the oxidative degradation of part of the protohaem, and the formation at pH 6 of a green pigment distinct from choleglobin (Keilin & Hartree, 1951; Dalziel & O'Brien, 1954*a, b*). Until recently, it was assumed to be a ferric peroxide complex (Keilin & Hartree, 1935; Haurowitz, 1935), and if this were so the fact that its formation from methaemoglobin at pH 8.5 is slower than that of the compound from Hb in the presence of dithionite, with the same peroxide concentration, would be difficult to reconcile with the identity of the two compounds. There is reason to believe, however, by analogy with MetMb-H₂O₂ (George & Irvine, 1952, 1954; Theorell & Ehrenberg, 1952) that Meth-H₂O₂ may be a ferryl derivative: its formation from Meth might be represented for example by Fe³⁺ ion + H₂O₂ $\rightarrow$ FeO²⁺ ion + OH + H⁺ ion, and from Hb by Fe²⁺ ion + H₂O₂ $\rightarrow$ FeO²⁺ ion + H₂O, and there is no reason why the latter reaction should not be faster than the former. Again, the addition of Na₂S₂O₄ to a solution of Meth and hydrogen peroxide at pH 8.5 is followed by a sequence of spectral changes similar to those observed in the Hb-Na₂S₂O₄-hydrogen peroxide reaction: there is first a slow reaction associated with a general decline of specific absorption, and the Meth-H₂O₂ spectrum is then slowly displaced by a Hb spectrum with a small choleglobin maximum at 630 m μ (Dalziel & O'Brien, 1954*a*). Judged by the rate of increase of extinction at 430 m μ , the rate of Hb regeneration is slower than in the present experiments, but this difference is of uncertain significance in view of the complexity of the reactions.

The life of the intermediate spectrum is associated with a molar paramagnetic susceptibility value of 4500 ± 500 c.g.s.e.m.u. (Dalziel & Ehrenberg, 1955). Values of this order (3000–5000) are common to MetMb-H₂O₂ and -methyl H₂O₂ and the peroxide compounds of types II and III of peroxidase and catalase (Theorell & Ehrenberg, 1952). The susceptibility of Meth-H₂O₂ has not yet been measured, but is likely to be of the same order, since it resembles the myoglobin compound quite closely in spectral and other properties. The reaction between MetMb, Na₂S₂O₄ and peroxide at pH 8–9 is accompanied by a sequence of spectral changes similar to those observed with Hb, but differs from the latter in the following respects: (1) the intermediate spectrum in the visible region, λ_{max} 548 m μ , $\epsilon_{mM} = 10.5$, λ_{max} 585 m μ , $\epsilon_{mM} = 9.0$, agreed with

Table 5. Wavelengths of absorption maxima and milliequivalent extinction coefficients (in parentheses) for some haemoglobin derivatives and the intermediate spectrum at pH 8.5

	Absorption maxima (m μ)			
	415 (128)	541 (15)	576 (16)	
Oxyhaemoglobin	415 (128)	541 (15)	576 (16)	
Methaemoglobin	408 (120)	541 (8.5)	576 (8.0)	
Methaemoglobin-hydrogen peroxide	418 (105)	545 (10.5)	582 (8.6)	
Intermediate spectrum	417 (90)	545 (9.0)	582 (9.6)	

that reported for MetMb-H₂O₂, $\lambda_{\max}$ 549 m μ , $\epsilon_{\text{mM}} = 9.8$, $\lambda_{\max}$ 585 m μ , $\epsilon_{\text{mM}} = 8.4$ (George & Irvine, 1951); (2) no detectable amounts of cholemyoglobin were formed in the overall reaction; (3) the proportion of the pigment destroyed by haem degradation was smaller than with Hb under similar conditions; (4) carbon monoxide did not reveal a new intermediate or promote the formation of cholemyoglobin. It is much more likely that myoglobin and Hb differ in the rates or mechanism of haem degradation by hydrogen peroxide in the presence of Na₂S₂O₄ (cf. Dalziel & O'Brien, 1954*b*) than that they form different derivatives of intact haem under similar conditions.

Although identification of a transient derivative of Hb formed in a complex reaction mixture is not easy, and analogy between Hb and myoglobin must not be over-stressed, the evidence indicates that the intermediate spectrum is due to Meth-H₂O₂ together with a small amount of a substance ultimately converted into choleglobin.

Degradation products

The main bulk of the Hb in the reaction mixture escapes destruction as a consequence of the presence of excess of Na₂S₂O₄, and the rest is changed into definable and indefinable products. In alkaline conditions choleglobin is the main recognizable degradation product. But other products with little specific absorption are probably formed, as there is no correlation between the choleglobin formed and the Hb destroyed, a conclusion supported by the fact that passage of carbon monoxide into the reaction mixture increased carboxy-choleglobin formation but not haemoglobin destruction. Oxidative break-up of haem is therefore likely and would explain the decline of absorption intensity over the whole spectrum, including 630 m μ , during the life of the intermediate compound.

Formation of choleglobin

The experimental evidence gives but a sketch of the mode of formation of choleglobin. Since HbCO in Na₂S₂O₄ solutions did not yield the intermediate spectrum with hydrogen peroxide nor undergo degradation, it may be concluded that reaction of hydrogen peroxide at the iron atoms to form Meth-H₂O₂ conditions oxidative attack at a methine linkage. From the rate of decline of the Soret maximum of Meth-H₂O₂ during its lifetime the rate of oxidative degradation varies directly with

Na₂S₂O₄ concentration but is independent of hydrogen peroxide concentration. The yield of choleglobin is similarly affected. These facts may be explained by an oxidation-reduction cycle in which Meth-H₂O₂ is mainly reduced to Hb and partly degraded to choleglobin. The choleglobin undergoes further oxidation by hydrogen peroxide and accumulates only when the latter has been largely destroyed.

After the introduction of carbon monoxide into the reacting mixture, during the life of the intermediate spectrum, the presence of a compound closely related to choleglobin was demonstrated. Spectrokinetic measurements were consistent with the following reactions: (a) Meth-H₂O₂ $\rightarrow$ HbCO, half-time approx. 2½ min.; (b) X ($\lambda_{\max}$ 580–590 m μ) $\rightarrow$ XCO ($\lambda_{\max}$ 600 m μ), complete in 2 min.; (c) XCO $\rightarrow$ CO-choleglobin, lasting several hours. Reaction (c) is too slow to involve peroxide.

The tentative mechanism in Scheme I relates these reactions. The greatly increased yield of (carboxy)-choleglobin brought about by carbon monoxide is attributed to the rapid conversion of X into its carbon monoxide derivative, and the resistance of the latter to further oxidative degradation; it may be that X and choleglobin, like Hb, only undergo oxidative degradation in a higher oxidation state or as peroxide derivatives. The conversion of XCO into carboxycholeglobin is quantitative. Foulkes, Lemberg & Purdon (1951) have suggested that cholehaem is derived from pseudohaem, with the grouping $\gg\text{C}\cdot\text{CH}(\text{OH})\cdot\text{C}(\text{OH})\langle$ which could arise by the addition of hydrogen peroxide at a methine linkage of protohaem, and have shown that it is reduced to protohaem by boiling alkali and dithionite. The intermediate XCO rapidly yields cholehaemochromogen with cold alkali and dithionite, and might be an 'oxidized pseudohaem' derivative, with the grouping $\gg\text{C}\cdot\text{CO}\cdot\text{C}(\text{OH})\langle$ which Foulkes *et al.* (1951) believe to be the next stage in the oxidative degradation of Hb.

Sulphaemoglobin

The introduction of hydrosulphide into a reaction mixture of Hb, Na₂S₂O₄ and hydrogen peroxide at pH 8.5–9.5 during the life of the intermediate spectrum caused the immediate disappearance of the latter and the formation of large quantities of a green pigment, $\lambda_{\max}$ 622 m μ shifting to 616 m μ on treatment with carbon monoxide. These results

Scheme I

suggest that sulphaemoglobin is formed by the intervention of hydrosulphide at some intermediate stage in the oxidative fission of the methine bridge. Foulkes *et al.* (1951) suggested a structure for the prosthetic group of sulphaemoglobin closely related to that of cholehaem, with the grouping

in place of a methine bridge. Lemberg & Legge (1949) have postulated the formation of sulphaemoglobin and choleglobin from the same precursor, a peroxide derivative of Hb. The rapidity of the reaction which follows the introduction of hydrosulphide probably accounts for the variations in the sulphaemoglobin yield under apparently identical conditions. Although complete conversion of Hb into sulphaemoglobin has never been achieved, Drabkin & Austin (1935-36) estimated $\epsilon_{\text{mEq.}} = 10-11$ at 620 m μ for pure sulphaemoglobin, and Lemberg *et al.* (1942) give $\epsilon_{\text{mEq.}} = 16$ at 617 m μ for carbon monoxide-sulphaemoglobin. On the basis of these figures, it would appear that the sulphaemoglobin yield in some of our experiments was close to 100 %. This conclusion cannot be accepted, since the spectrum of the product showed a pronounced Soret band, $\lambda_{\text{max.}} = 424$, $\epsilon_{\text{mEq.}} = 97$, and according to Lemberg *et al.* (1942) carbon monoxide-sulphaemoglobin, like choleglobin, has relatively weak absorption in the Soret region. Moreover, with alkali and dithionite the products apparently yielded a mixture of proto- and chole-haemochromogen, although there was no evidence of the presence of choleglobin in the products themselves. There is evidence that sulphaemoglobin yields protohaemochromogen derivatives (Nijveld, 1943; Lemberg *et al.* 1942).

Choleglobin, sulphaemoglobin and other 'green haemoglobins' such as pseudohaemoglobin (Barkan & Schales, 1937, 1938) and cruoralbin (Holden, 1943, 1945), have generally been obtained by the relatively slow oxidation of Hb or HbO₂ under different conditions. The present work shows that two such derivatives can be obtained in quantity in rapid reactions which follow the successive addition of peroxide and a second reagent to Hb in dithionite solution, provided the second reagent is added during the lifetime of an unstable peroxide derivative of Hb. The results support the general mechanism advanced by Lemberg and his co-workers for these reactions (Lemberg & Legge, 1949; Foulkes *et al.* 1951), although they favour the view that the so-called Meth-H₂O₂, rather than a ferrous peroxide complex (Lemberg, Legge & Lockwood, 1938, 1939, 1941*a*, *b*), is the intermediate involved, and emphasize the importance of the time factor and the use of dilute solutions, in which the life of the peroxide derivative is longest. More detailed

studies by means of the compound rapid-flow method of Hartridge & Roughton (1924), whereby a second reagent, e.g. carbon monoxide, hydrosulphide or cyanide, can be introduced at various intervals after the addition of peroxide may clarify the relationship between the various green haemoglobins. The difference between the reactions of Hb and myoglobin with peroxide and dithionite, and the relationship between these reactions and products and those of Meth with peroxide, in which a green pigment distinct from choleglobin is formed (Keilin & Hartree, 1951; Dalziel & O'Brien, 1954*b*), also deserve further study, and may bear upon the mechanism of peroxidase activity.

SUMMARY

1. The reaction between haemoglobin, dithionite and hydrogen peroxide has been investigated by spectrokinetic measurements. The results show that by reaction with the peroxide, haemoglobin is largely converted into an unstable derivative, which persists for a definite period, and is then largely reduced to haemoglobin by the excess of dithionite. In the overall reaction, part of the haemoglobin is destroyed, and small amounts of choleglobin are formed at pH 7-10 and of sulphaemoglobin at pH 6.

2. The intermediate compound is probably methaemoglobin-hydrogen peroxide, which exists in a mixture with a small proportion of a substance subsequently converted into choleglobin.

3. A new unstable intermediate has been detected in the reaction at pH 8.5-9.5 in the presence of carbon monoxide, which is characterized by a maximum at 600 m μ , and is slowly but quantitatively converted into carboxycholeglobin in the presence of excess of dithionite.

4. The addition of hydrosulphide to a reacting mixture of haemoglobin, dithionite and peroxide during the life of the peroxide intermediate at pH 8.5-9.5 at once gives a large yield of a derivative with the spectroscopic properties of sulphaemoglobin.

5. A mechanism is discussed for these reactions.

6. Experiments indicate that myoglobin is temporarily but completely converted into metmyoglobin-hydrogen peroxide on treatment with hydrogen peroxide in the presence of excess of dithionite. Small amounts of pigment were destroyed in the overall reaction, but there was no evidence of the formation of cholemyoglobin or other green pigments.

7. The explanation previously advanced for the action of oxygen on haemoglobin solutions containing dithionite is confirmed.

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The Fractionation of Urinary Iodine

1. METHOD OF ANALYSIS*

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The metabolism of radioactive iodine has been the subject of intensive investigation in recent years. This has been concentrated mainly on the production of hormone by the thyroid gland and its subsequent fate in the plasma and tissues, and little has been published about the excretion of the hormone in the urine. It is, however, generally agreed that the greatest proportion of the iodine in urine is present as inorganic iodide, and only a small percentage exists in an organic form. It was to investigate this organic fraction that the present work was undertaken, both to estimate the amount of organically combined iodine and to discover the chemical nature of the products. This present paper describes a method for the investigation of the problem, and subsequent papers will describe the results obtained from the application of this method

to the urine of patients receiving large amounts of radioactive iodine for the treatment of disease of the thyroid gland.

Several methods were used in an attempt to separate the organic iodine from the iodide, and most were abandoned. The chief cause of the difficulties encountered was the ease with which iodide is oxidized in an acid solution, leading to the formation of free iodine and the production of artifacts. Acland (1952) has pointed out that biological solutions of iodide can readily produce many artifacts when acidified, and this observation has been amply confirmed. The method finally adopted was the removal of iodide from the urine at an alkaline pH by a column of silver chloride, and the subsequent concentration of the organic iodine by an ion-exchange resin.

EXPERIMENTAL AND RESULTS

Paper chromatography. Throughout this work chromatography was carried out on Whatman 3MM paper. This thick paper was chosen since all the material examined contained

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