Spectrophotometric Studies of the Reaction of Methaemoglobin with Hydrogen Peroxide

2. THE DEGRADATION OF METHAEMOGLOBIN BY HYDROGEN PEROXIDE

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Keilin & Hartree (1935) showed spectroscopically that methaemoglobin-hydrogen peroxide, formed by the addition of hydrogen peroxide to catalase-free solutions of horse methaemoglobin at pH 5·8–6·5, decomposed on standing with the liberation of acid methaemoglobin, the peroxide being destroyed without oxygen evolution, presumably in true oxidative degradation of that part of the methaemoglobin which was lost during the reaction. Later, more detailed observations of the formation and decomposition of the compound were reported (Keilin & Hartree, 1951). In presence of an excess of peroxide in the pH range 5·5–9·2, the formation of oxyhaemoglobin was detected, followed by decomposition of the hydrogen peroxide with oxygen evolution, and the eventual destruction of the pigment to a colourless solution. In solutions more acid than pH 5, with smaller peroxide concentration, the formation of oxyhaemoglobin was not observed, and the reaction yielded a red-green solution with a wide absorption band at 590 mμ.

According to Lemberg, Legge & Lockwood (1941) hydrogen peroxide reacts with methaemoglobin, oxyhaemoglobin and haemoglobin to form choleglobin, a green haem pigment first characterized in the products of the coupled oxidation of oxyhaemoglobin and ascorbic acid (Lemberg, Legge & Lockwood, 1938, 1939). These workers suggested a compound of haemoglobin and hydrogen peroxide to be the precursor of choleglobin. In the reaction of hydrogen peroxide with haemoglobin in the presence of sodium dithionite at pH 8·5, which also yields choleglobin, we have observed the conversion of haemoglobin into an unstable intermediate compound (Dalziel & O'Brien, 1951, 1952*a*). The similarity between the spectral absorption of this transient compound and that of methaemoglobin-hydrogen peroxide, led to the experiments on the formation and breakdown of the latter compound described in this and in the preceding paper.

In this paper, detailed spectrophotometric studies of the reaction of methaemoglobin with hydrogen peroxide are described. At pH 6, with the peroxide in moderate excess, a stable green pigment distinct from choleglobin is formed, and yields a carbon monoxide derivative in the presence of sodium dithionite. The formation of the green pigment is accompanied by the progressive transformation of the protohaematin into a new haem which will form stable haemochromogen derivatives. Complete conversion of protohaematin into the green haem was not achieved: with low peroxide concentrations, the reaction was apparently quantitative although incomplete, and with higher peroxide concentrations both the protohaematin and the green haematin were destroyed. This work has already been published in the form of an Abstract (Dalziel & O'Brien, 1952*b*).

EXPERIMENTAL

Spectrokinetic measurements

Measurements of spectral absorption were made with a Beckman quartz photoelectric spectrophotometer, model D.U., and the results are plotted as the extinction, $E = \log I_0/I$. The reactants were mixed in optical cells of 1 cm. or 0·5 cm. path length, and the time course of the extinction at two or three wavelengths was followed by making measurements in rapid succession from about 1 min. after mixing. This was repeated at other wavelengths with fresh reaction mixtures. The complete absorption spectrum of the mixture at any time after mixing could be built up from the plots of extinction against time for many wavelengths. In the later stages of the reaction, when the light absorption was changing but slowly, the whole spectrum was rapidly scanned back and forth, so that the means of the pairs of extinction measurements corresponded to the same time after mixing.

Reagents and derivatives

Hydrogen peroxide. A.R. '20 vol.' H_2O_2 was diluted as required with Clarke & Lubs's buffer solutions (cf. Vogel, 1939), 0·05M phosphate:NaOH, pH 6·0, or 0·05M borate:NaOH pH 8·5. The stock solution was repeatedly standardized against $KMnO_4$.

Methaemoglobin (metHb). This was prepared from haemolysates of washed human red cells by oxidation with $K_3Fe(CN)_6$, after removal of the catalase by adsorption on alumina; the concentration of metHb solutions was estimated by conversion into the cyanide (Drabkin & Austin, 1935), and is expressed as m-equiv./l. (mEq.), where 1 mEq. metHb = 1 mM haematin. Details of the preparation and analysis were given previously (Dalziel & O'Brien, 1954).

Denatured globin carboxyhaemochromogen derivatives. A very slight modification of the procedure described by Lemberg *et al.* (1941) was used. Coal gas was rapidly passed into 5 ml. metHb solution, or metHb and H_2O_2 mixture, in a test tube for 15 sec., and approx. 50 mg. $Na_2S_2O_4$ were then added; the tube was inverted twice, and whilst coal gas was again passed slowly into the mixture, 0·5 ml. 5 N-NaOH was added down the wall of the tube. The mixture was quickly rolled round the wall of the tube, and finally coal gas was passed again for 1 min. The method gave fairly reproducible results, and tests of its validity in the presence of free H_2O_2 are described below. For brevity, such preparations will be referred to as CO-haemochromogens.

RESULTS

Spectrokinetic observations

The changes of light absorption accompanying the overall reaction of metHb and H_2O_2 at pH's 6·0 and 8·5 were recorded in optical cells. The reaction mixtures were initially about 0·06 mEq. with respect to metHb and various peroxide concentrations were used. The formation of methaemoglobin-hydrogen peroxide and the early stages of its subsequent reaction were described previously (Dalziel & O'Brien, 1954).

At pH 8·5 with initial peroxide/haematin ratios (R) of 4·5, 18 and 44, the spectrum of methaemoglobin-hydrogen peroxide first formed, with a maximum at 545 mμ. and a pronounced shoulder at 570–580 mμ., slowly decreased in intensity, and the absorption maximum at 545 mμ. gradually shifted back to 540 mμ. The final products with the lower peroxide concentrations showed depressed maxima at 408, 546 and 576 mμ., consistent with partial reversion to metHb, and on addition of dithionite gave a haemoglobin spectrum. The spectra of CO-haemochromogen derivatives of the products were those of CO-protohaemochromogen with a very small additional maximum at 625–630 mμ. The extents of protohaematin and metHb destruction during the reaction, calculated from the absorption spectra, were about the same, and increased with the initial peroxide concentration. Evidently the decomposition of the compound in presence of excess of peroxide under these conditions is accompanied by disintegration of part of the protohaematin, without the formation of appreciable quantities of stable intermediate pigments.

In weakly acidic conditions, with not too great an initial concentration of peroxide, at least one stable pigment other than metHb is formed, and the remainder of this paper is devoted to the reaction at pH 6·0. In Fig. 1, absorption curves are shown for three 0·059 mEq. metHb solutions containing various amounts of peroxide. After 2 min., the spectrum of each reaction mixture is qualitatively that of methaemoglobin-hydrogen peroxide (Keilin & Hartree, 1951), although with the lowest peroxide concentration, $R=4·5$ (Fig. 1*a*), compound formation is incomplete. The subsequent course of the reaction depends upon the peroxide concentration. With $R=91$, Fig. 1*c*, the specific absorption is gradually destroyed, and the spectrum of the compound persists throughout the degradation. With $R=4·5$, the colour of the mixture slowly reverts from red to brown; the changes of spectral absorption (Fig. 1*a*) in the region 500–550 mμ. are broadly consistent with partial reversion to metHb, but the changes at longer wavelengths, and the spectrum of the stable products (85 min.), show that one or more other substances with significant absorption at 520–630 mμ. are also formed. With $R=18$, Fig. 1*b*,

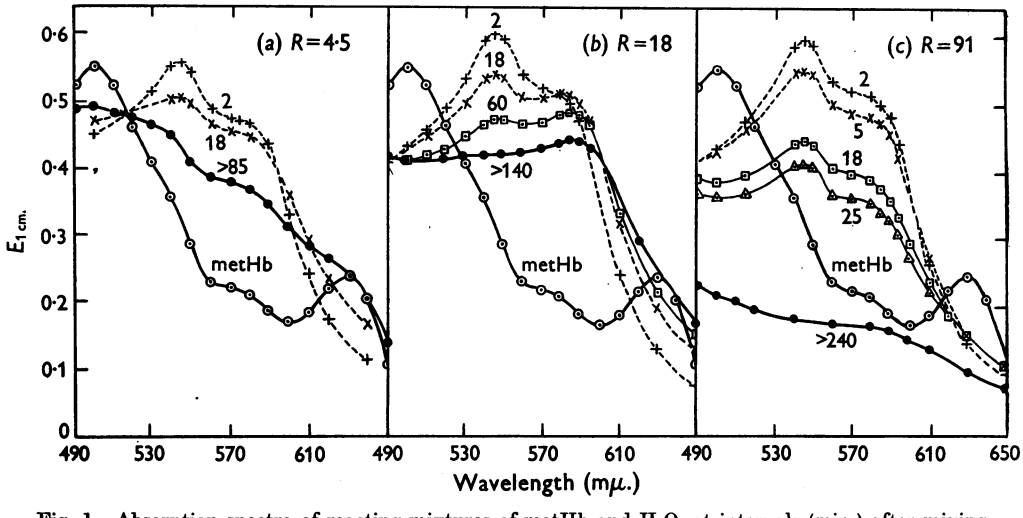

Fig. 1. Absorption spectra of reacting mixtures of metHb and H_2O_2 at intervals (min.) after mixing. MetHb: 0·059 mEq. H_2O_2: (*a*) 0·265 mM, (*b*) 1·06 mM, (*c*) 5·37 mM, pH 6·0.

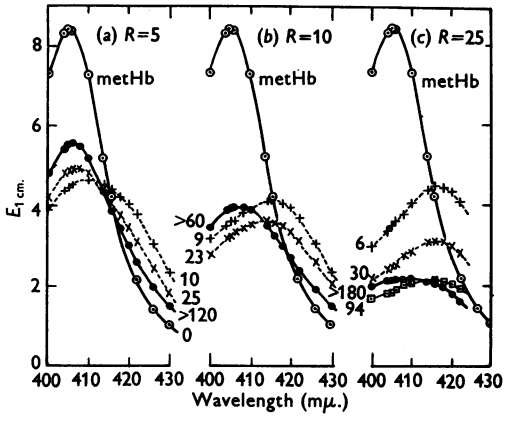

Fig. 2. Spectral changes with time (min.) in the Soret region during the later stages of the reaction of 0·05 mEq. metHb with H_2O_2: (*a*) 0·25 mM-H_2O_2, (*b*) 0·50 mM-H_2O_2, (*c*) 1·25 mM-H_2O_2. pH 6·0.

there are no clear indications of metHb formation, and whilst the maximum at 545 mμ. is destroyed, a broad peak at 585 mμ. emerges; the reaction yields a green-brown solution.

In Fig. 2, the corresponding changes in the Soret region are shown for 0·050 mEq. metHb solutions with $R = 5$, 10 and 25. The early stages of the reaction, the partial transformation of the metHb into the compound, $\lambda_{max.}$ 418 mμ., have been described previously (Dalziel & O'Brien, 1954) and are not shown. With the lowest peroxide concentration partial reversion to metHb is apparent. With the higher peroxide concentrations, destruction of methaemoglobin-hydrogen peroxide is accompanied by decreasing absorption from 400–430 mμ., followed by increasing absorption near 405 mμ., again consistent with some reversion to metHb in the final stages of the reaction.

Reaction of the protohaematin

After treatment with dithionite in the presence of carbon monoxide, the green products of the reaction with $R = 18$, described above, showed a typical carboxyhaemoglobin spectrum with an additional pronounced maximum at 613 mμ.; further treatment with alkali and carbon monoxide gave a CO-protohaemochromogen spectrum with an additional maximum at 617 mμ. The absorption intensities in both cases indicated loss of protohaematin during the reaction with peroxide. Similar derivatives were obtained from the reaction products with $R = 4·5$, but the new maxima were less intense and the protohaematin losses smaller. These observations suggested that part of the protohaematin had been converted into a derivative of the choleglobin type.

The progress of the reaction of the prosthetic group was followed by withdrawing samples from reacting mixtures of metHb and peroxide at intervals, converting into CO-haemochromogens by treatment with coal gas, dithionite and alkali, and recording the absorption spectra. This technique was used by Lemberg *et al.* (1941) in quantitative studies of the conversion of protohaem into cholehaem in mixtures of oxyhaemoglobin and ascorbic acid; the formation of choleglobin during the haemochromogen preparation, by the rapid reaction of haemoglobin with hydrogen peroxide formed by autoxidation of the dithionite, was prevented by saturating the test samples with coal gas before adding the dithionite. The validity of the method in its present application to mixtures of metHb and peroxide was confirmed by the following observations: (1) Carboxyhaemoglobin solutions to which peroxide was added ($R = 18$ and 91) yielded quantitative CO-haemochromogen spectra. (2) The absorption spectra of CO-haemochromogens prepared from metHb solutions immediately after the addition of peroxide ($R = 4·5$) by treatment with dithionite, alkali and coal gas, in that order, showed a significant loss of protohaematin and a pronounced CO-cholehaemochromogen maximum at 630 mμ. When the metHb and peroxide solutions were saturated with coal gas before mixing, similar treatment yielded a quantitative CO-haemochromogen spectrum. (3) Treatment of a reaction mixture of metHb and peroxide ($R = 18$) with coal gas immediately after mixing had no effect on the slow reaction involving the protohaematin described below, as judged by CO-haemochromogen spectra of samples withdrawn at intervals up to 20 min. after mixing. Accordingly, in order to study the reaction during the first minute or two after mixing, the reactants were separately saturated with coal gas before mixing, and the reaction was quenched at the desired time by the addition of dithionite. This last observation suggests that oxyhaemoglobin is not an intermediate in the formation of the green pigment.

The absorption spectra of CO-haemochromogen derivatives prepared from the three reaction mixtures whose spectral absorptions were given in Fig. 1, are shown in Fig. 3. With $R = 45$ and $R = 18$, Fig. 3*a*, *b*, the decline of the CO-protohaemochromogen maxima at 539 and 568 mμ. is accompanied by the progressive elevation of a peak at 617 mμ. Since there is an isosbestic point at 585 mμ. (and at 510 mμ.) in both sets of curves, and the increases of $E617$ (i.e. E at 617 mμ.) for given decreases of $E568$ are about equal, the partial conversion of protohaematin into a new, stable haematin apparently occurs without much loss by other reactions. In the mixture with $R = 91$, however, the haem structure is evidently rapidly destroyed: the spectra, Fig. 3*c*, do not show an isosbestic point, and a limited increase of absorption around

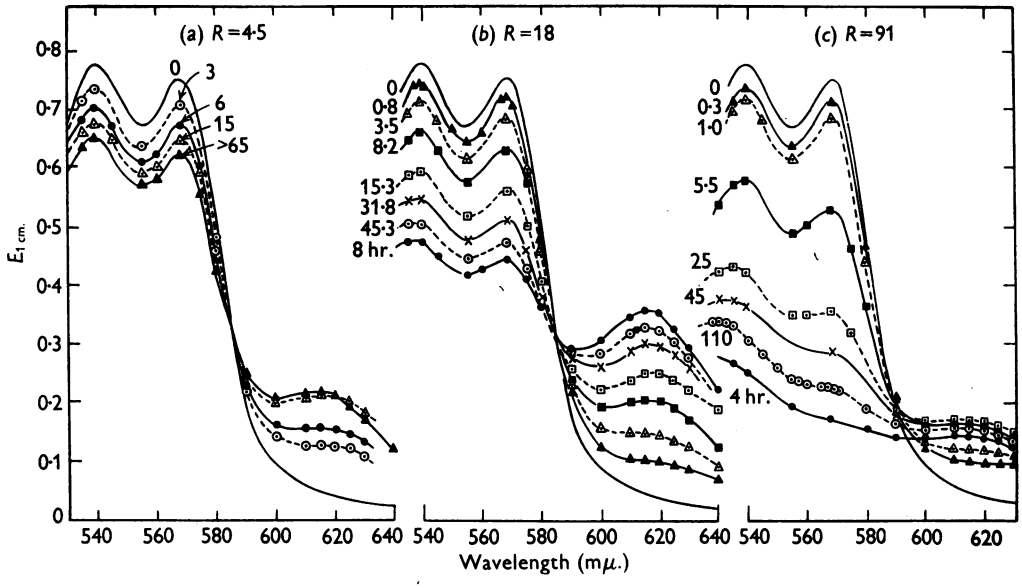

Fig. 3. Absorption spectra of CO-haemochromogen derivatives prepared at intervals (min.) during the reaction of 0·059 mEq. metHb with H_2O_2: (*a*) 0·265 mM-H_2O_2, (*b*) 1·06 mM-H_2O_2, (*c*) 5·37 mM-H_2O_2. pH 6·0.

617 mμ. in the first few minutes is partly destroyed subsequently.

The time course of the protohaematin reaction (Fig. 3) throws some light on the concurrent, more complex changes in the absorption spectra of the reaction mixtures (Fig. 1). With $R = 91$, it is evident that during the degradation the haem exists mainly as protohaematin in the form of methaemoglobin-hydrogen peroxide. With $R = 18$, the reaction involving the protohaematin lasts about 1 hr., and is associated with the emergence in the pigment spectrum (Fig. 1*b*) of the maximum at 585 mμ. and also with decreasing absorption in the Soret region (Fig. 2*b*, *c*). The more uniform subsequent decline of the pigment spectrum in the region 500–600 mμ., ultimately quite extensive, is not accompanied by significant changes in the CO-haemochromogen spectrum (Fig. 3*b*), and is doubtless in part due to reversion of the remaining methaemoglobin-hydrogen peroxide to metHb; the terminal changes in the Soret spectrum (Fig. 2*b*, *c*) support this interpretation. Similarly, with $R = 4·5$ the changes in the pigment spectrum are satisfactorily accounted for by the reaction involving the protohaematin, occurring mainly in the first 15 min. (Fig. 3*a*), accompanied and followed by reversion of the compound to metHb.

The effect of hydrogen peroxide concentration on the nature of the products

The complexity of the reaction at pH 6·0 is apparent when the absorption spectra of the final products of the reaction of 0·059 mEq. metHb with

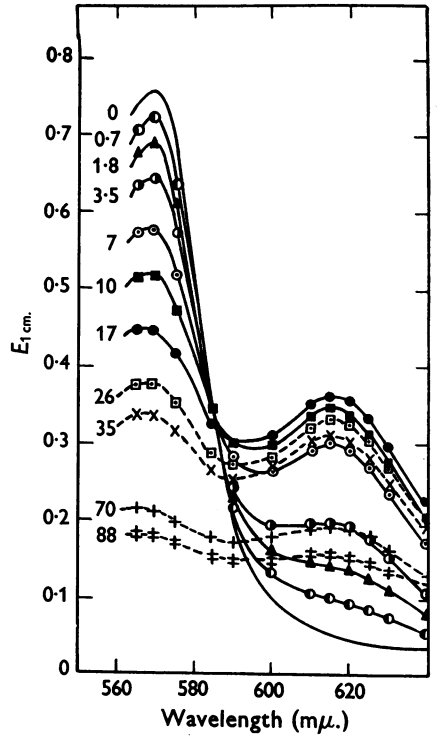

Fig. 4. Absorption spectra of CO-haemochromogens prepared from the products of the reaction of 0·059 mEq. metHb with various amounts of H_2O_2, expressed as R = initial peroxide/haematin molar ratio. Full lines, R = 0–17; broken lines, $R > 17$.

various amounts of peroxide and of the corresponding CO-haemochromogen spectra are examined. The group of CO-haemochromogen curves (Fig. 4) for peroxide concentrations from $R = 0{\cdot}7$ to $R = 10$ shows an isosbestic point at 584 mμ. and is consistent with a series of mixtures of constant total concentration of protohaemochromogen, $\lambda_{max.} = 568$ mμ., and the haemochromogen responsible for the peak at 617 mμ. With R values greater than 17, the CO-haemochromogen spectra have progressively smaller specific absorption, and the residual maxima at 539 and 568 mμ. and the new maximum at 617 mμ. are affected to about the same extent. The Soret spectrum of the derivatives from the products of the reaction with $R = 17$ and $R = 88$ were also recorded, and showed peaks at 419 mμ. consistent with decreasing amounts of residual CO-protohaemochromogen.

The absorption spectra of the products themselves, some of which are shown in Fig. 5, form a more complicated series, but a discontinuity at $R = 10$–17 can again be distinguished. As R is increased from 0·7 to 10 the metHb maximum at 500 mμ. is progressively destroyed and a new peak emerges at about 580 mμ. There are no isosbestic points, however, and since the haemochromogen spectra suggest that there is no significant total haem destruction in these mixtures, the metHb is evidently transformed into more than one product in this range of peroxide concentration; a small peak at 530 mμ. in the products with $R = 7$ is noteworthy in this connexion. In the Soret region the intensity decreases rapidly with increase of peroxide concentration and the maximum shifts slightly towards the red. The products with $R = 17$ show maxima at 585 and 408 mμ., the latter having about two-fifths the intensity of the original metHb Soret band, which bands persist with decreasing intensity in the products with higher peroxide concentrations as the specific absorption is destroyed.

It is concluded that in the overall reaction the protohaematin of metHb is partially transformed by peroxide into a new haem in the form of a green pigment which has an absorption maximum at 585 mμ. and much smaller absorption than metHb in the Soret region. With peroxide concentrations up to $R = 10$ the reaction is apparently almost quantitative, but with higher peroxide concentrations haem destruction occurs to an increasing extent. The yield of green haem is greatest with $R = 10$–17, but even with higher initial peroxide concentrations unchanged protohaematin apparently persists, and might account for the residual Soret band observed in the spectra of the products and derivatives.

The effect of repeated reaction with hydrogen peroxide

The complete conversion of protohaematin into the green haem was attempted by successive treatment of 0·059 mEq. metHb with three portions of 7 equiv. each of peroxide, added as a concentrated solution to avoid appreciable dilution of the pig-

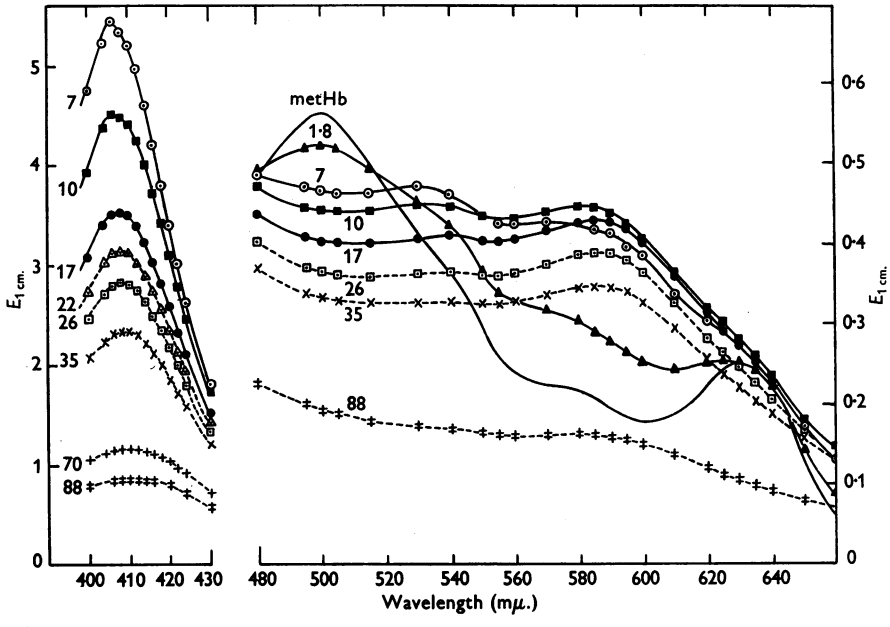

Fig. 5. Absorption spectra of the products of the reaction of 0·059 mEq. metHb with various amounts of H_2O_2, expressed as R = initial peroxide/haematin molar ratio. Full lines, $R = 0$–17; broken lines $R > 17$.

ment solution. After each addition of peroxide the reaction was followed spectrokinetically at 500–630 mμ. until the spectrum became stable. A sample was then withdrawn before the next addition of peroxide, and converted into CO-haemochromogen derivatives.

The second 7 equiv. of peroxide caused immediate changes in the spectrum of the pigment consistent with the formation of some methaemoglobin-hydrogen peroxide, the increase of E 545 and E 576 corresponding to the conversion of about 25% of the original metHb into this compound. When stable, the absorption spectrum had a distinctly sharper and more intense maximum at 585 mμ. than that obtained by a single reaction with 15 equiv. of H_2O_2. The CO-haemochromogen spectrum showed a corresponding enhancement of the 617 mμ. maximum at the expense of the CO-protohaemochromogen maxima, but there was also a small decrease of intensity at the isosbestic point, 584 mμ., and the ratio of the decrease of E 568 to the increase of E 617 was significantly greater than in the reaction with the first 7 equiv., showing that some protohaematin had been lost in the second stage as products other than the green haem. Residual maxima still remained, however, at 539 and 568 mμ. The third 7 equiv. of peroxide produced only small immediate changes in the pigment spectrum, which corresponded to the conversion of about 2% residual metHb into methaemoglobin-hydrogen peroxide and the reaction resulted ultimately in small, uniform decreases of intensity in both pigment and CO-haemochromogen spectra, without change of shape, which indicated some destruction of total haem, including green haem.

In a similar experiment, three successive reactions with 5 equiv. of peroxide were carried out. In this case, the third reaction also produced a significant amount of methaemoglobin-hydrogen peroxide and ultimately an increase in the yield of green pigment. The final pigment and CO-haemochromogen spectra agreed closely with those obtained after two reactions with 7 equiv. of peroxide.

Properties of the products

The reaction of 0·059 mEq. metHb with two successive portions of 7 equiv. of peroxide seemed to give the maximum yield of green pigment, and apparently there was little unchanged metHb. The green-brown solution was allowed to stand for several hours at room temperature and then overnight in the cold store. The absorption spectrum in the visible region is shown in Fig. 6. The Soret band at 408 mμ. had about one-third the intensity of the original metHb peak at 405 mμ. The effect of various reagents on the spectrum were studied and the absorption curves of some of the derivatives are given in Figs. 6 and 7.

Carbon monoxide produced no change in the absorption spectrum other than a small uniform decrease of intensity.

Cyanide and fluoride. The addition of 0·1 ml. 0·3% potassium cyanide to 5 ml. pigment solution produced only a slight change in the spectrum; the increase of E 545 indicated a residual metHb content of 8% of the original. The products of the reaction with a single 7 equiv. of peroxide when similarly

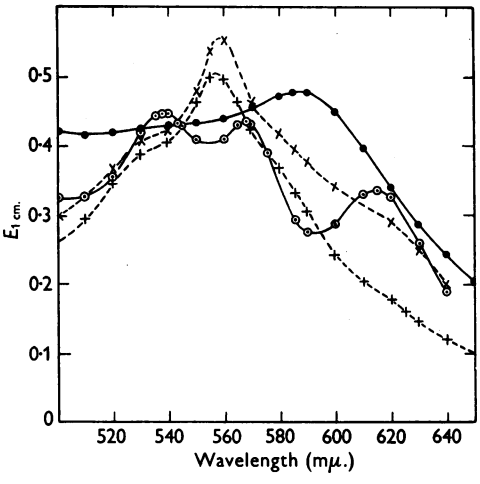

Fig. 6. Effect of $Na_2S_2O_4$ and $CO + Na_2S_2O_4$ on the absorption spectrum of the products of the reaction of 0·059 mEq. metHb successively with two portions of 7 equiv. each of H_2O_2: ●—●, reaction products; × - - - ×, 2 min., and + - - - +, 2 hr., after addition of $Na_2S_2O_4$; ⊙—⊙, $CO + Na_2S_2O_4$.

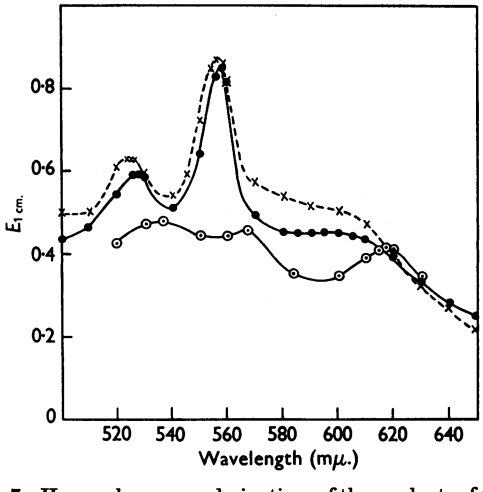

Fig. 7. Haemochromogen derivatives of the products of the reaction of 0·059 mEq. metHb successively with two portions of 7 equiv. each of H_2O_2: ●—●, denatured globin haemochromogen; ⊙—⊙, denatured globin CO-haemochromogen; × - - - ×, pyridine hemochromogen.

treated yielded a spectrum with a broad maximum at 540–550 mμ., consistent with the presence of 33% residual metHb, and the crossing-points with the pigment spectrum were in good agreement with those of metHb and its cyanide derivative, confirming that the green pigment does not react with cyanide. With fluoride similar results were obtained; the extinction changes indicated the presence of 32 and 4% residual metHb in the products after the first and second reaction, respectively.

Haemochromogens. Pyridine haemochromogens were prepared by diluting 2·5 ml. pigment solution with 1·4 ml. phosphate buffer, pH 6, and adding 0·1 ml. 5N-NaOH, 1 ml. pyridine and 50 mg. dithionite. Denatured globin haemochromogens were prepared by diluting 2·5 ml. pigment solution with 2 ml. buffer and adding 50 mg. dithionite and 0·5 ml. 5N-NaOH. Denatured globin carboxyhaemochromogens were prepared as described previously. The absorption spectra were recorded in 1 cm. cells sealed with liquid paraffin and a cover glass, and were stable for at least an hour. The results, corrected for dilution, are given in Fig. 7. Corresponding to the maximum at 617 mμ. in the CO-haemochromogen spectrum, the haemochromogen spectra each have a shoulder at about 610 mμ.: the direct conversion of the denatured globin haemochromogen spectrum into that of the carbon monoxide derivative was confirmed. All three spectra show distinct residual maxima in the green corresponding to those of protohaematin derivatives.

Reduction in presence of carbon monoxide. The absorption curve obtained on addition of 50 mg. dithionite to 5 ml. pigment solution after saturation with coal gas is shown in Fig. 6. The bands in the green correspond with those of carboxyhaemoglobin, and that in the red is centred at 613 mμ. When dithionite was added to the pigment in absence of carbon monoxide, a slow reaction occurred which is described below. Carbon monoxide and CO-haemochromogen derivatives prepared 30 min. or more after the addition of dithionite showed no peaks in the red, but the two-banded spectrum in the green remained unchanged.

Reaction with dithionite. When a little dithionite was added to the green-brown pigment solution, the brown tinge was destroyed, and spectroscopic observation showed that the broad, diffuse band at 592 mμ. was replaced by a sharper band at 560 mμ. After exposure to air with gentle shaking, the colour of the solution changed from green to pale red-brown, and the band at 560 mμ. was replaced by two bands at 544 and 578 mμ. This reaction was reversed by addition of more dithionite, and ferricyanide also destroyed the two-banded spectrum.

These reactions were studied more fully with the spectrophotometer. Approximately 12 mg. dithionite were weighed into a dry 1 cm. cell, 3 ml. pigment solution pipetted in, and a layer of paraffin added. After being mixed with a fine glass stirrer, the cell was completely filled with paraffin and sealed with a cover slip. The extinction was recorded at three or four wavelengths in rapid succession and later at more wavelengths as the reaction slowed down. Similar measurements were made at other wavelengths with fresh preparations. The integrated spectral absorption of the mixture after 2 min. (Fig. 6), shows the immediate formation of a maximum at 558–560 mμ. and a marked inflexion at 530 mμ., which persists for longer than 2 hr. whilst the intensity of absorption gradually decreases, especially in the region 560–640 mμ. The carbon monoxide and CO-haemochromogen derivatives no longer show a band in the red. Evidently the green haem is destroyed in this reaction.

Reduction of the pigment solution without destruction of the green haem was effected by the addition of the minimum quantity of dithionite and oxidation of the excess by aeration. The pH of the pigment solution was first raised to about 7, and traces of dithionite were added until spectroscopic observation showed that the band at 592 mμ. had been replaced by one at 560 mμ. The absorption spectrum of a sample was briefly checked in the spectrophotometer, and the remainder was exposed to air with gentle agitation. The spectrum of the red-brown solution obtained, with peaks at 539 and 576 mμ., was quite stable. On treatment with coal gas the peak at 576 mμ. shifted to 569 mμ. and a distinct inflexion appeared at 610–615 mμ. The addition of dithionite restored the peak at 558 mμ. and the inflexion at 530 mμ., and the absorption spectra of denatured globin haemochromogen and CO-haemochromogen derivatives were similar to those obtained directly from the products of the reaction of metHb with peroxide, showing bands in the red. It appears that the green haem has not been completely destroyed, but now reacts with carbon monoxide.

DISCUSSION

In the overall reaction of human methaemoglobin with hydrogen peroxide at pH's 6·0 and 8·5, protohaematin was lost to an extent which increased with the initial hydrogen peroxide concentration. With a great excess of peroxide, the reaction ultimately yielded colourless products, but with smaller initial peroxide concentrations part of the methaemoglobin was recovered. Spectrokinetic studies suggested that the prosthetic group of the methaemoglobin-hydrogen peroxide first formed, which yields protohaem derivatives in the presence of sodium dithionite, underwent a slow reaction, presumably with the excess of hydrogen peroxide,

and that if the initial peroxide concentration were not too great, the remaining methaemoglobin-hydrogen peroxide reverted to methaemoglobin. These findings are in accord with those of Keilin & Hartree (1935, 1951) for horse methaemoglobin. At pH 8·5, neither the reaction mixtures nor their CO-haemochromogen derivatives gave spectrophotometric evidence for the occurrence of significant amounts of any haem compounds other than methaemoglobin-hydrogen peroxide and methaemoglobin. The reaction at pH 6, however, yielded a stable green pigment with a broad absorption maximum at about 585 mμ., and the spectra of derivatives of the products showed that part of the protohaematin had been converted into a new haem distinct from cholehaem.

Comparison of the absorption spectra of reacting mixtures with those of CO-haemochromogen derivatives prepared at intervals during the reaction (Figs. 1–3), and of the spectra of the products obtained with a range of initial peroxide concentrations with those of CO-haemochromogen derivatives (Figs. 4, 5) established the connexion between the green pigment and the new haem, and threw some light on the course of the reaction. With an initial methaemoglobin concentration of 0·059 m-equiv./l., the yield of green pigment increased with the initial peroxide concentration up to a peroxide/haematin ratio of about 15. Within this range the presence of an isosbestic point in the haemochromogen spectra suggested that protohaematin was progressively transformed into the green haem without appreciable loss by destruction of the haem nucleus or by other reactions, but a source of protohaemochromogen apparently remained in the products. The latter could account for the residual Soret bands, and it was evident from the rapid fall of absorption during the reaction that the green pigment and its CO-haemochromogen have relatively small absorption in the Soret region. There was evidence, particularly in the time course of the spectrum in the Soret region (Fig. 2), that when the excess of hydrogen peroxide had been used up in green pigment formation, some methaemoglobin was regenerated. There was evidence also, in the spectrum of the products with low peroxide concentrations, of the formation of at least one pigment other than methaemoglobin and green pigment which also yields the CO-haemochromogen of either protohaem or green haem. With peroxide/haematin molar ratios greater than 15, disintegration of the haem structure occurred to an increasing extent, and the yield of green pigment decreased. During the degradation to colourless products which occurs with great excess of peroxide, the intact haem was present very largely as the prosthetic group of methaemoglobin-hydrogen peroxide (Figs. 1 and 3, $R=91$); the intermediate formation of oxyhaemoglobin, observed by Keilin & Hartree (1951) with horse methaemoglobin in much greater concentration, was not detected.

Successive reaction of methaemoglobin with hydrogen peroxide gave a slightly greater maximum yield of green pigment than could be obtained in a single reaction, but again, haemochromogen spectra showed distinct protohaemochromogen bands which could not be destroyed by further addition of peroxide to the products without concomitant destruction of green haem. From the effects of cyanide, fluoride and hydrogen peroxide on the spectrum of the products, it was estimated that about 5% of the original methaemoglobin remained. In the absorption spectra of pyridine and denatured globin haemochromogens, the pronounced residual bands in the green attributed to protohaem were apparently undistorted, and on the reasonable assumption that the absorption spectra of the green haem derivatives were flat in this region, it was estimated from the height of the maxima above the central minimum that about 25% of the initial protohaem was recovered, an amount significantly greater than the estimated methaemoglobin recovery.

Carbon monoxide alone had no specific effect on the spectrum of the products, but sodium dithionite in the presence of carbon monoxide yielded a stable spectrum comprising typical undistorted carboxyhaemoglobin maxima at 539 and 568 mμ., and a maximum at 613 mμ. due to the green pigment derivative; from the height of the bands in the green above the saddle it was estimated that about 25% of the initial methaemoglobin was thus recovered as carboxyhaemoglobin. In the absence of carbon monoxide the green pigment was slowly destroyed by dithionite, as shown by the disappearance of the bands in the red in the haemochromogen and carbon monoxide derivatives, but the peaks in the green were unaffected. After reaction with dithionite, the spectrum of the products resembled that of haemoglobin, but in addition to a maximum at 560 mμ., there was a pronounced inflexion at about 530 mμ. These observations suggest that oxyhaemoglobin may be the source of the residual protohaem in the products, but further evidence is required on this point.

The prosthetic group of the green pigment yields stable pyridine and denatured globin haemochromogens with bands in the red, which appear in the absorption spectra of derivatives of the reaction products as distinct shoulders at 600–610 mμ. (Fig. 7); the denatured globin haemochromogen forms a carbon monoxide derivative with a maximum at 617 mμ. The green pigment itself is more difficult to characterize because of the presence of other pigments in the products and because it undergoes a reaction with dithionite in which the

green haem is probably destroyed; it has an absorption maximum in the neighbourhood of 585 mμ., and relatively small absorption in the Soret region. Its most definite characteristic is the formation of a stable derivative on treatment with dithionite in the presence of carbon monoxide, with an absorption maximum at 613 mμ. Carbon monoxide alone has little effect on the spectrum of the green pigment. It is probable, therefore, that the pigment is a ferric compound, although it did not appear to react with cyanide or fluoride. There were indications that a stable form which would react with carbon monoxide could be obtained by treatment with a trace of dithionite.

The mode of preparation and the spectral characteristics of the green pigment and its derivatives suggest that it is yet another green haemoglobin of the choleglobin type. We hope to investigate its relationship to other members of the group and to verdohaem and biliverdin in future work. Comparison with the results of other workers also reveals some anomalies which require resolution. Lemberg *et al.* (1941) reported that choleglobin was formed in the reaction of methaemoglobin with hydrogen peroxide, but experimental details were not given; it was suggested that methaemoglobin was reduced to haemoglobin by the peroxide, which was then converted into a haemoglobin-hydrogen peroxide compound the precursor of choleglobin (Lemberg *et al.* 1941; cf. Dalziel & O'Brien, 1952*a*). Since we have found that carbon monoxide does not inhibit the formation of 'green haem' from methaemoglobin, the view that only ferrous compounds are oxidized by hydrogen peroxide to 'bile pigment haem' derivatives (Lemberg & Legge, 1943) may require revision. Keilin & Hartree (1951) found a green pigment very similar in spectral characteristics to that which we have described, in the products of the reactions of recrystallized horse methaemoglobin and metmyoglobin and hydrogen peroxide at pH 4·5, with similar reactant concentrations to ours. At pH values greater than 5·0, however, with greater pigment concentration, green pigment formation was not reported; the formation of oxyhaemoglobin and oxymyoglobin was observed to precede the destruction of the haem nucleus. George & Irvine (1952), on the other hand, found that the formation of metmyoglobin-hydrogen peroxide at pH < 7·0 was accompanied by side reactions which appeared to yield a product similar to that which we have described, and the spectra of carboxyhaemochromogen derivatives showed a pronounced peak at 615 mμ. in addition to protohaemochromogen peaks. They concluded, however, that the side products were formed rapidly during the initial compound formation, and not by a slow, consecutive reaction of the compound, such as our experiments with methaemoglobin indicate; this difference may be due to the much greater peroxide/haematin molar ratios used in our experiments (George & Irvine, 1953).

The mechanism by which green pigment is formed is of interest in relation to peroxidatic activity. It is not clear why the conversion of protohaem into green haem, which appears to use hydrogen peroxide quite efficiently and is apparently the only reaction involving the prosthetic group at low peroxide concentrations, does not go to completion, and why, beyond a certain limit, further oxidation of protohaem is accompanied by destruction of the green haem. If the residual protohaem is indeed in the form of oxyhaemoglobin, it might be expected to yield methaemoglobin and subsequently more green pigment on addition of more hydrogen peroxide. There is, of course, the possibility of the formation of molecules containing both green haem and protohaem, and an attractive hypothesis is that smooth conversion of protohaem into green haem, presumably by oxidative attack at a methine linkage, is a form of intramolecular peroxidative reaction requiring at least one intact protohaem per molecule. The mechanism of the reaction and the nature of the residual protohaem deserve more detailed study.

SUMMARY

1. The reactions of human methaemoglobin and hydrogen peroxide at pH's 6·0 and 8·5, which yield initially methaemoglobin-hydrogen peroxide, have been studied throughout their course by spectrokinetic observations and by conversion into haemochromogen derivatives. With low peroxide concentrations, the compound reverted, in part, to methaemoglobin, but with high peroxide concentrations the haem structure was ultimately destroyed.

2. At pH 8·5, no evidence was obtained for the occurrence, in significant amount, of haem compounds other than methaemoglobin and methaemoglobin-hydrogen peroxide. At pH 6·0, a green pigment was formed, which, in the presence of sodium dithionite, yielded stable carbon monoxide and haemochromogen derivatives showing bands in the red. The pigment itself was not stable towards sodium dithionite.

3. The green pigment was formed by partial oxidative degradation of the prosthetic group of methaemoglobin-hydrogen peroxide, which yields protohaem derivation in the presence of sodium dithionite. The green pigment has not been obtained free from protohaem compounds; the yield increased with the initial peroxide concentration up to a certain limit, but beyond this point, even on successive reaction of the products with small quantities of peroxide, simultaneous destruction of the residual protohaem and the green haem occurred. The residual protohaem was not entirely

in the form of methaemoglobin, but was apparently accounted for as carboxyhaemoglobin after the addition of carbon monoxide and dithionite.

4. The green pigment is distinct from choleglobin, and its formation is not prevented by carbon monoxide, showing that oxyhaemoglobin is not an intermediate in the reaction.

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Bile Pigment Formation *in vitro* from Haematin and Haem Derivatives

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In a previous study it was observed that haematin in aqueous medium can undergo coupled oxidation *in vitro* with ascorbic acid to yield a product which, on treatment with 66 % acetic acid, was decomposed to bile pigments (Kench, Gardikas & Wilkinson, 1950). The formation of bile pigment *in vitro*, therefore, contrary to the views of Lemberg, Cortis-Jones & Norrie (1938), was not dependent on the iron of haem being in the ferrous state and coupled with pyridine or protein.

Other conclusions reached in the previous *in vitro* investigations were: (1) Ferrihaemoproteins, such as methaemoglobin and methaemalbumin, were as suitable sources of bile pigment as haemoglobin. There was no evidence that reduction to the corresponding ferrous compounds occurred during the reaction. (2) No bile pigments could be detected in experiments with free protoporphyrin, nor was any porphyrin found in the breakdown products of any haem derivative. (3) Coupling of haematin with protein improved the yield of bile pigment, but the effect was non-specific in that oxyhaemoglobin and methaemalbumin, with different proteins and types of linkage with the prosthetic group, were equally good sources. More recently, cytochrome *c* has proved completely resistant on incubation with ascorbic acid (Gardikas & Kench, 1952), horse-liver catalase and horse-radish peroxidase were good sources of bile pigment (Kench, 1952) and metmyoglobin provided the highest yield obtained from any haem derivative (Kench & Gardikas, 1952).

It is possible that such *in vitro* studies only remotely resemble bile pigment formation *in vivo* where enzymes are able to produce such profound effects. Even London's (1950) demonstration of the convertibility *in vivo* of injected haematin into bile pigments, however, still leaves open the questions whether methaemalbumin was formed as an intermediate, and to what extent this mechanism actually operates in the catabolism of haemoglobin.

To obtain more information on chemical factors which influence the degradation of natural haem derivatives, further experiments have been made on the *in vitro* conversion of haematin and haemoproteins into bile pigments, in which special attention has been paid to the spectroscopic detection of bile pigment intermediates. Evidence of such compounds was secured for myoglobin and haematin, and, tentatively, for horse-radish peroxidase. The final reaction mixture from haematin appeared to contain both verdohaematin and cholehaematin, possessed respectively of open and closed porphyrin structure (Lemberg & Purdom, 1949). The latter observations supported the conclusion that degradation of free haematin followed a course similar to that of haem combined in oxyhaemoglobin, in which the comparable intermediates were verdohaemoglobin and choleglobin.

EXPERIMENTAL AND RESULTS

Experiments with haematin. Haematin was prepared by the method of Nencki & Zaleski (1900) with purification as suggested by Duesberg (1934). The following experiments were made with aqueous haematin solutions in an attempt to detect spectro-