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.

We wish to thank Mr B. A. Collett for technical assistance during a part of the work.

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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-

scopically intermediates in the catabolism of haematin to bile pigment.

Haemin (14 mg.) was dissolved in 10 ml. 5% (w/v) Na_2CO_3 and the pH brought to 8·0 by addition of 50 ml. 0·1 N HCl. This aqueous solution was incubated at 37° with ascorbic acid and continuously oxygenated by a vigorous stream of O_2. The effect on bile pigment formation of varying the incubation time and ascorbic acid concentration were studied in a series of experiments (Fig. 1).

Opening of the haem molecule, indicated by the diminution of optical density in the Soret region, was accompanied by increased spectral absorption in the red, not, however, proportional to the yield of bile pigment. The absorption band of ferrohaem at 595 mμ. was never observed. The solution was unbuffered, and it was observed that in the experiment in which 1000 mg. ascorbic acid were added continuously during 2 hr., 60 μg. free biliverdin (16% total yield), extractable with chloroform alone, were present. The final pH of the solution, measured by glass electrode, was 4·54. Experiments in which 100 mg. ascorbic acid only were added gave final pH 7·02, and no free bile pigment could be detected.

That the increased red absorption was not due to free biliverdin was clear from the experiment recorded in Fig. 3, in which 14 mg. haematin were incubated for 2 hr. following addition of 100 mg. ascorbic acid. The reaction products which had pH 5·5 gave a yield of 9·5 μg. biliverdin/ml. An aqueous solution of pure biliverdin of this concentration and pH was prepared, and its optical density in the red region measured. It was evident that the increased red absorption of the haematin reaction products could not be due to liberated biliverdin, even if all were free.

When the haematin: ascorbic acid products were treated with redistilled pyridine, two maxima rapidly appeared in the absorption curve, at 650 and 560 mμ. (Fig. 2). The latter is, undoubtedly, that of pyridine (ferro) haemochromogen, formed from residual ferrihaem in the presence of ascorbic acid. The peak at 650 mμ. corresponds with the maximum at 655 mμ. observed by Lemberg *et al.* (1938) for verdohaemochromogen, and supports the conclusion that one product in the coupled oxidation of free haematin and ascorbic acid must be similar to verdohaematin, which has been prepared in crystalline form by removal of pyridine from verdohaemochromogen (Lemberg & Legge, 1949), and shown to contain an opened porphyrin structure (Lemberg & Purdom, 1949). Verdohaematin dissolves in dilute

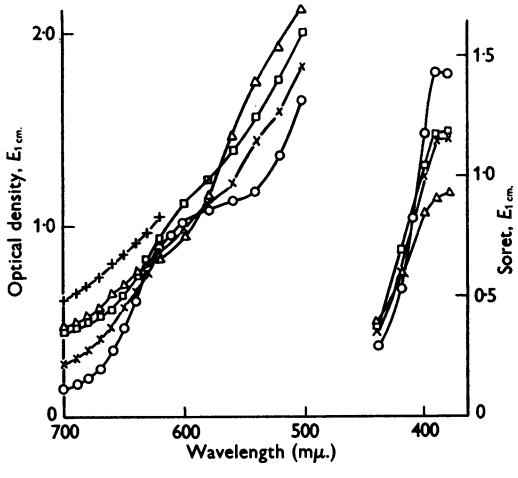

Fig. 1. Changes in the absorption spectrum of haematin on incubation with ascorbic acid. Haemin (14 mg.) was dissolved in 10 ml. 5% (w/v) Na_2CO_3, and the pH brought to 8·0 by addition of 50 ml. of 0·1 N-HCl: incubated at 37° with continuous rapid stream of O_2: ascorbic acid and incubation time varied as follows: O—O, haemin alone, 120 min., 0 μg. bile pigment. ×—×, +100 mg. ascorbic acid, 60 min., 237 μg. bile pigment. □—□, +100 mg. ascorbic acid, 120 min., 277 μg. bile pigment. △—△, +1000 mg. ascorbic acid, 120 min., 99 μg. bile pigment. +—+, +1000 mg. ascorbic acid added dropwise continuously, 120 min., 385 μg. bile pigment. The reaction mixture was examined undiluted for visual spectrum but diluted ×10 with water for Soret region.

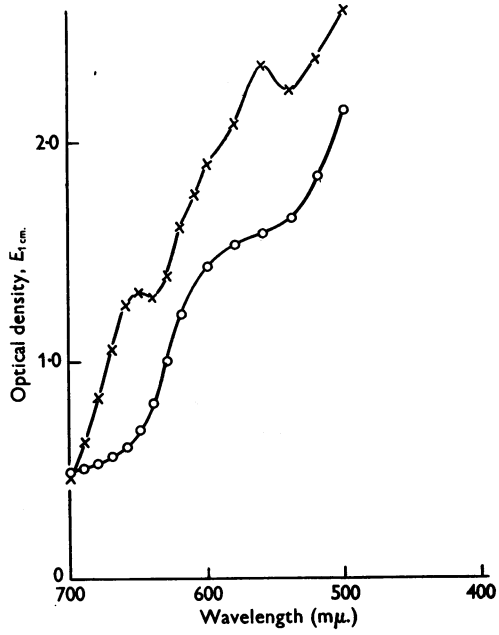

Fig. 2. Changes in the absorption spectrum of haematin: ascorbic acid reaction products following addition of pyridine. O—O, absorption spectrum of aqueous haematin (14 mg./60 ml. soln. pH 8·0) incubated 120 min. with 100 mg. ascorbic acid at 37° with passage of O_2. ×—×, 2 ml. final reaction mixture + 1 ml. redistilled pyridine. (*E* corrected for dilution with pyridine.)

sodium carbonate or phosphate buffer giving a dark-olive solution which absorbs light in the red, but shows no distinct absorption bands (cf. Fig. 1). Protein-free green haematins of the closed-ring 'chole' type have also been prepared (Lemberg & Purdom, 1949), but since choleglobin itself has not been prepared pure, no exact spectroscopic data are available for its prosthetic group, cholehaematin. Pyridine cholehaemochromogen has a maximum absorption at 619 mμ. (Lemberg, Legge & Lockwood, 1941*a*).

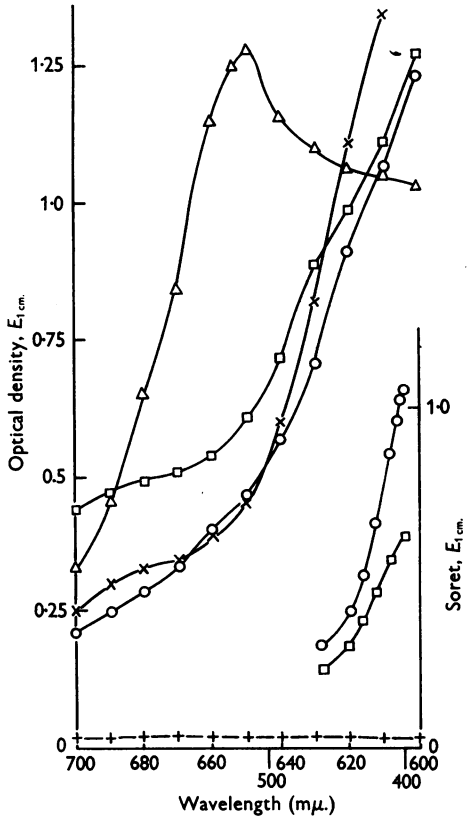

Fig. 3. Changes in the absorption spectrum of haematin: ascorbic acid reaction products following addition of pyridine. Experiments on the formation of a compound reacting with pyridine to give absorption at 650 mμ. with controls to check *de novo* formation of verdohaemochromogen, after addition of pyridine, by the action of residual ascorbic acid; latter determined by iodometric titration. O—O, 14 mg. haematin incubated alone in O_2 for 120 min. then 0·5 mg. ascorbic acid added and absorption measured immediately. ×—×, 14 mg. haematin incubated alone in O_2 for 120 min. then 1 mg. ascorbic acid added per ml. and finally 1 ml. pyridine added to 2 ml. solution. Absorption curve measured immediately. □—□, 14 mg. haematin incubated with 100 mg. ascorbic acid at 37° for 120 min. with passage of O_2. △—△, 14 mg. haematin incubated for 120 min. at 37°, with O_2, and 600 mg. ascorbic acid added in first 60 min. Final solution treated with pyridine, and examined spectroscopically immediately. +—+, absorption of aqueous biliverdin 9·5 μg./ml. pH 5·5. Solutions were undiluted for visual spectrum, but diluted × 10 for the Soret region.

Control experiments (Fig. 3) have shown that the small amount of ascorbic acid remaining at the end of the experiment was not responsible for the production of verdohaemochromogen *de novo* from unchanged ferrihaem dissolved in pyridine. More vigorous treatment of haematin through addition of larger quantities of ascorbic acid during incubation, caused the absorption maximum with pyridine at 650 mμ. to become more prominent.

Treatment of the mixture, following addition of pyridine, with 66% acetic acid led to quantitative recovery of bile pigments, which as in the absence of pyridine, comprised a mixture of biliverdin and bilipurpurin. A number of trials were now made in an attempt to convert all the ferrihaem into 'verdohaematin', following the change by measurement of red absorption and diminution of the Soret band. Unfortunately, it was found that, irrespective of the conditions of time, pH or ascorbic acid concentration, the 'verdohaematin' concentration could be increased only slightly over that achieved in earlier experiments. Optical density in the Soret region progressively declined, but when approximately 60% of haematin had been disrupted, further prolonged treatment with ascorbic acid and oxygen led to decrease in red absorption. It appeared impossible, therefore, to achieve complete conversion of haematin into 'verdohaematin' in this system.

Further information on the haematin reaction products was sought through coupling experiments with native human globin. Haematin: ascorbic acid reaction products containing an optimum yield of 'verdohaematin' as judged by increased optical density in the red region of the spectrum were treated with native human globin prepared by the method of Anson & Mirsky (1930) as modified by Jope, Jope & O'Brien (1949). Haematin reaction products (11 ml., equivalent to 86 μg. biliverdin) were incubated at 37° with 11·5 ml. solution containing 126 mg. native globin. The absorption spectrum of the final solution exhibited a plateau in the range of 660–670 mμ. (Fig. 4). This solution (4 ml.) was saturated in the spectrophotometer cell with carbon monoxide, when the maximal absorption was displaced to 620 mμ. The solution was rendered alkaline by addition of 0·4 ml. 20% sodium hydroxide solution and reduction effected by a few fine granules of sodium hydrosulphite. The cell was stoppered, inverted twice and the absorption in the range 600–700 mμ. immediately measured. A minimum quantity of sodium hydrosulphite, saturation with carbon monoxide and rapid optical measurement precluded peroxidative

effects of alkaline hydrosulphite on haemoglobin derivatives derived from residual haematin. A broad maximum was observed at 620–630 mμ. These spectroscopic changes were reminiscent of the behaviour of choleglobin (Lemberg, Legge & Lockwood, 1941*b*). Ferricholeglobin exhibited an absorption band at 670 mμ., which on treatment with coal gas was displaced to 628 mμ. due to carboxycholeglobin. Further treatment with sodium hydroxide and sodium hydrosulphite led to the formation of denatured globin carboxycholehaemochromogen with peak absorption still at 628 mμ., but with increased optical density.

In contrast, verdohaematin combined with native globin to give olive-green verdohaemiglobin with indistinct absorption in the red, reduced by hydrosulphite to verdohaemoglobin which had a sharp band at 665 mμ. (Lemberg & Legge, 1949). Treatment of these compounds with alkaline hydrosulphite produced a yellow pigment and no evidence of conversion into cholehaemochromogen, which absorbs light at 616–618 mμ. (Lemberg *et al.* 1941*a*).

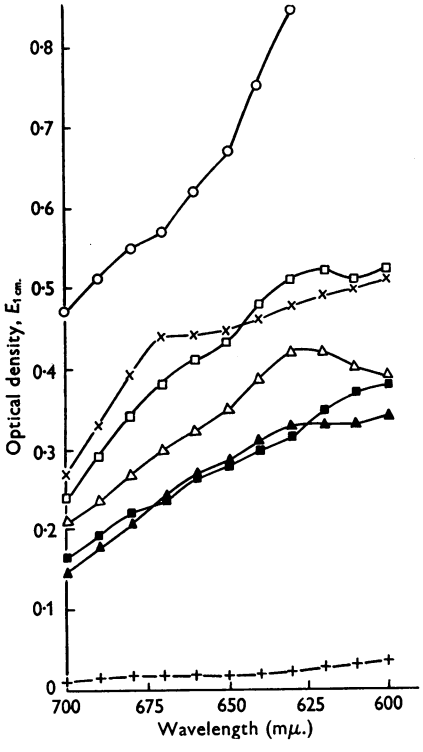

Fig. 4. Reaction of human globin with a product of the haematin-ascorbic acid reaction. O—O, 14 mg. haematin incubated at 37° for 120 min. with 100 mg. ascorbic acid + O_2. ×—×, 11 ml. final haematin solution (≡ 86 μg. biliverdin) incubated at 37° with 11·5 ml. native human globin solution containing 126 mg. protein. □—□, haematin reaction products-globin saturated with CO. △—△, haematin reaction products-globin + CO + $Na_2S_2O_4$ + NaOH. ■—■, haematin reaction products (5 ml. contains 43 μg. biliverdin) coupled with 100 mg. denatured human globin, and saturated with CO. ▲—▲, haematin reaction products—denatured globin + CO + $Na_2S_2O_4$ + NaOH. +—+, native globin solution, as used for coupling with haematin reaction products, incubated alone at 37°.

When the main bulk of the solution of haematin reaction products which had been coupled with globin was subjected to treatment with 66 % acetic acid in the usual way, no trace of bile pigment could be recovered, in contrast to the liberation of 86 μg. biliverdin before coupling with globin. This behaviour was also that of choleglobin, rather than of a verdohaem derivative (Lemberg & Purdom, 1949).

A similar experiment was made with denatured human globin, in which the pH of the globin solution before addition to the haematin reaction products was necessarily raised to keep the protein dissolved. The spectrum of the solution remained, however, without any localized absorption in the 600–700 mμ. range. The optical density of the native globin solution itself was almost uniformly low in the 600–700 mμ. region of the spectrum.

Experiments with metmyoglobin. Metmyoglobin was prepared as described by George & Stratmann (1952), the final solution in 3 M phosphate buffer, pH 6·8, being dialysed against distilled water, and concentrated at 40° to 0·43 mM, measured as pyridine haemochromogen (Rimington, 1942). The solution exhibited the characteristic absorption bands of metmyoglobin at 630 and 505 mμ. with very weak bands of oxymyoglobin at 583 and 547 mμ. To 50 ml. of 0·43 mM metmyoglobin solution (≡ 14 mg. combined ferrihaem) contained in a Petri dish were added 100 mg. L-ascorbic acid and the mixture was incubated in air at 37° for 2 hr. The reaction was followed spectroscopically using a Beck Hartridge Reversion Spectroscope (Table 1).

Rapid reduction of metmyoglobin occurred, and, after 5 min. incubation, the absorption bands at 634 and 505 mμ. were very weak and diffuse, whilst those of oxymyoglobin at 581 and 547 mμ. were correspondingly intensified. As the reaction proceeded, oxymyoglobin disappeared, and there was progressively increased absorption in the red region of the spectrum, corresponding to the recorded values of 670 and 640 mμ. for cholemyoglobin (Kiese & Kaeske, 1942). There was no evidence of reduced myoglobin with absorption maxima at 595–590 and 560–550 mμ. The red absorption finally extended down to 622 mμ., and was not due to re-formed metmyoglobin, since an absorption band at 504 mμ. was very weak in intensity. Residual unchanged haemoprotein occurred as oxymyoglobin, without autoxidation to metmyoglobin, the concentration of such pigment, however, being greatly diminished as compared with early

Table 1. *Spectroscopic changes in* 0·43 mM *metmyoglobin solution on incubation with ascorbic acid*

50 ml. 0·43 mM metmyoglobin solution (≡14 mg. ferrihaem) + 100 mg. L-ascorbic acid incubated in air at 37° for 120 min.

Incubation time (min.)	Absorption bands (mμ.)				
0	647–622 (630 Intense)	583. Narrow, very weak	—	505. Wide, intense	433. End of Soret band
5	634. Almost disappeared	581. Sharply defined, intense	547. Sharply defined, intense	505. Weak, diffuse	447. End of Soret band
35	676. Increased absorption 643. Weak 634. Disappeared	582. Almost disappeared	548. Weak	505. Very weak, diffuse	453. End absorption
60	666. Increased absorption 642–620. Diffuse	—	547. Weak	505. Very weak, diffuse	450. End absorption
90	Diffuse absorption in red down to 622	—	546. Weak	505. Very weak	—
120	Diffuse absorption in red down to 622	581. Becoming more intense	545. Becoming more intense	504. Very weak	—

stages of the reaction. The solution remained clear and bright red in colour throughout the experiment, with no evidence of the marked denaturation which obtained with haemoglobin under similar conditions.

Bile pigments were liberated and extracted by the method of Lemberg, Lockwood & Legge (1941), with quantitative determination as fluorescent zinc-bilipurpurin (Kench *et al.* 1950). Biliverdin was the sole product, with yields in four experiments of 4·10, 4·42, 5·54 and 4·47, mean 4·70 mg. This yield (36 % theoretical) was the highest observed from any haematin derivative in this series of *in vitro* experiments. A control experiment showed no cholemyoglobin (by acetic acid treatment) nor free bile pigment admixture in the original metmyoglobin solution. Neither autoxidation during preparation nor incubation at 37° in air, therefore, contributed to biliverdin production. George & Stratmann (1952) were also unable to detect either choleglobin or denatured protein during oxidation of myoglobin to metmyoglobin.

Experiments with horse-liver catalase. Horse-liver catalase of Kat. F. 17500 (von Euler & Josephson, 1923), haematin content 0·76 % of dry weight, and containing 0·65 mg. combined ferrihaem/ml. solution, was prepared by the method of Keilin & Hartree (1945). 21·6 ml. samples of the final catalase solution were diluted to 50 ml. with distilled water, the total haematin content being 14 mg. The solution was incubated in Petri dishes in air at 37° with addition, in different experiments, of varying quantities of ascorbic acid.

The course of the reaction was followed spectroscopically using a Unicam Quartz Spectrophotometer S.P. 500. Fig. 5 presents data for an experiment in which 100 mg. ascorbic acid were added at the commencement of incubation, 100 mg. after 30 min. and subsequently 50 mg. at 30 min. intervals until 120 min. A generalized increase in spectral absorption was observed, without evidence of localized increased optical density in any region. Subsequent analysis gave a bile pigment yield

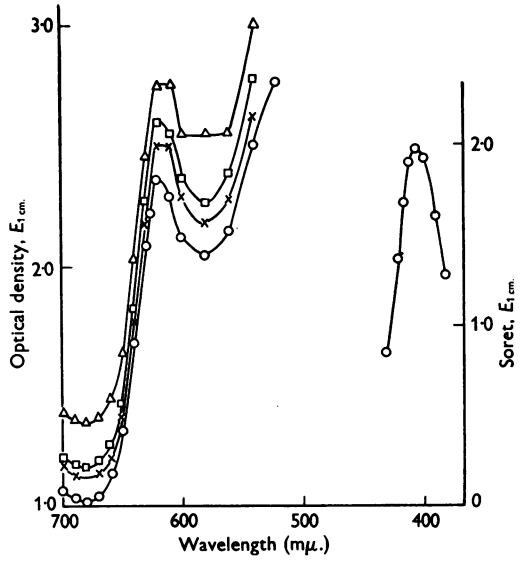

Fig. 5. Changes in absorption spectrum of horse-liver catalase on incubation with ascorbic acid. 50 ml. catalase solution containing 0·28 mg. haematin/ml. (total content 14 mg. combined haematin) were incubated in air at 37°. 100 mg. ascorbic acid added at 30 min. intervals. ○—○, 0 min.; ×—×, 10 min.; □—□, 26 min.; △—△, 87 min. The solution was diluted × 13 for measurement in the Soret region. No increase in bile pigment yield was observed.

equivalent to 22 % of the original haematin content, as compared with 27 % in a control solution, incubated without added ascorbic acid. The negative spectroscopic findings are therefore in conformity with the bile pigment production. In other concurrent experiments, unfortunately not followed spectroscopically, bile pigment formation with yields of 28 and 39 %, respectively, of original haem were found, the latter augmented production arising by treatment of catalase (≡ 7 mg. ferrihaem) with 300 mg. ascorbic acid during 2 hr. incubation, that is, the concentration of ascorbic acid was doubled as compared with the experiment giving least yield of bile pigment. During the course of the experiment a dark greenish brown, floccular precipitate separated, the supernatant remaining dark-green. Biliverdin was the only bile pigment detected among the products derived from horse-liver catalase.

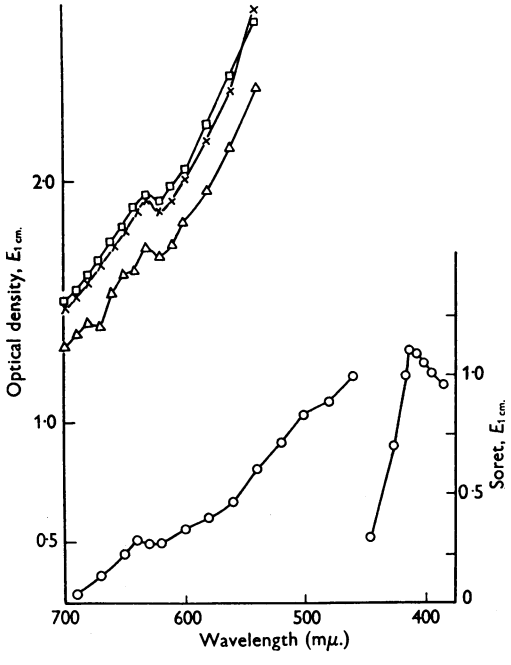

Fig. 6. Changes in the absorption spectrum of horse-radish peroxidase on incubation with ascorbic acid. ○—○, peroxidase solution in 0·25 M phosphate buffer pH 5·6 before addition of ascorbic acid. Spectroscopic readings were made on a solution diluted × 5 with buffer for the visual spectrum, but the data have been calculated on basis of × 4 dilution for comparison with observations made during subsequent incubation with ascorbic acid. 19 ml. peroxidase solution containing 0·26 mg. peroxidase haematin/ml. incubated in air at 37°. 50 mg. ascorbic acid added. For measurement of optical density, 1 ml. reaction mixture diluted × 4 with 0·25 M phosphate buffer pH 5·6. × — ×, 27 min.; □—□, 60 min.; △—△, 120 min. 143 μg. biliverdin were produced.

Experiments with horse-radish peroxidase. Horse-radish peroxidase was prepared from 20 kg. of the root by the method of Keilin & Hartree (1951), and obtained in a solution having Purpurogallin Number 225 (Willstätter & Stoll, 1918), total enzyme units 517, and containing 0·26 mg./ml. peroxidase haem. The solution (1 vol.) was diluted with 0·25 M phosphate buffer pH 5·6 (4 vol.) for measurement of the visual spectrum (Fig. 6). Peroxidase solution (19 ml., equivalent to 0·26 mg. peroxidase haem/ml.) was incubated in a Petri dish in air at 37° for 120 min. following the addition of 50 mg. ascorbic acid. Incubation with ascorbic acid caused a greatly increased general spectral absorption, and the maximal absorption of peroxidase at 640 mμ. was displaced to 630 mμ. A sample of reaction mixture (1 ml.) was removed at 27, 60 and 120 min., and diluted with 3 ml. 0·25 M phosphate buffer, pH 5·6, and the solution centrifuged to remove an interfering brownish precipitate which separated out especially during later stages of the incubation. Very little change in the absorption was observed during the period 27–60 min., but there was a general fall in optical density at 120 min., probably related to the removal of denatured pigmented material which had taken place during this phase of the reaction. The peak at 630 mμ. remained, with an interesting additional localized absorption at 675 mμ. No evidence was obtained of the bands of reduced peroxidase at 594 and 558 mμ.

At the end of the reaction, the products were treated with 66 % acetic acid in the usual way, with liberation of 143 μg. biliverdin, equivalent to 2·9 % original peroxidase haemin. Bilipurpurin could not be detected. No trace of bile pigment could be

Table 2. *Bile pigment formation* in vitro *from haematin and other haem derivatives*

	State of combined iron	Bile pigment yield* (μg.)
Protoporphyrin		0
Ferrohaem	Fe^{2+}	21†
Ferrihaem	Fe^{3+}	385
Haemoglobin	Fe^{2+}	422
Ferrihaemoglobin	Fe^{3+}	532
Metmyoglobin	Fe^{3+}	4700
Ferrohaemalbumin	Fe^{2+}	91†
Ferrihaemalbumin	Fe^{3+}	938
Cytochrome *c*	Fe^{3+}	0
Catalase	Fe^{3+}	1567
Peroxidase	Fe^{3+}	399

* Optimum yield of bile pigments (biliverdin + bilipurpurin) from haem derivative containing 14 mg. haem.

† Ferrohaem and ferrohaemalbumin were prepared from the corresponding ferric compounds by reduction with 0·5 % (w/v) $TiCl_3$. To avoid re-oxidation during the course of the experiment, the reaction time was shortened to 60 min., without supplying extra O_2. Bile pigment yields from ferro-derivatives were slightly less than those from ferric compounds under these conditions.

found when 8 ml. peroxidase solution were incubated in a similar way without addition of ascorbic acid.

The yields of bile pigment derived from metmyoglobin, horse-liver catalase and horse-radish peroxidase are presented for comparison with those obtained from other haem derivatives (Table 2).

DISCUSSION

The primary reaction in the coupled oxidation of oxyhaemoglobin with ascorbic acid has been envisaged by Lemberg & Purdom (1949) as leading to formation of choleglobin by addition of hydrogen peroxide to the α methene linkage, thus lowering the optical density in the Soret region without disrupting the porphyrin system. Further secondary reactions follow, including partial oxidation of the prosthetic group to verdohaem present in verdohaemoglobin, and only the latter type of reaction leads to bile pigment (Callaghan & O'Hagan, 1949; Lemberg & Purdom, 1949). The 10% bile pigment yield on treating the reaction mixture with acid is due to this secondary oxidation having accompanied the primary reaction.

In vitro conversion of haematin into bile pigments has been studied at length, as a simple system likely to provide information of value applying to the breakdown of haemoproteins, in which side reactions were more likely to occur. Spectroscopic examination showed that increased optical density occurred in the red region of the spectrum, and concurrent diminution in the Soret region provided an index of the destruction of the haem. Complete conversion of haematin into bile pigment precursor could not be achieved, and the maximum was reached when 60% of haematin had been disrupted. Addition of pyridine to the final reaction mixture led to the detection therein of verdohaematin, a ready source of bile pigments on treatment with acid.

No bile pigment could be recovered from the haematin reaction products coupled with globin, probably owing to some alteration in the mode of linkage of verdohaematin to globin brought about by acetic acid treatment, leading to a firm bond, resistant to acid and possibly involving the vinyl side chains as suggested by Callaghan (1949) for the similarly resistant cruoralbin. Such changes could account for the non-liberation of bile pigment, but not for the formation of denatured globin-carbon monoxide-cholehaemochromogen, characteristic of cholehaem compounds, which have been shown to possess a closed porphyrin ring (Lemberg & Purdom, 1949). The probable explanation appears to be the presence of both verdohaematin and cholehaematin in the haematin reaction products.

The yield of bile pigment from free haematin in aqueous solution at pH 8·0 was similar to that from oxyhaemoglobin, but the optimum conditions for the conversion differed in that bile pigment formation from oxyhaemoglobin was favoured by low partial pressure of oxygen (Lemberg *et al.* 1941*b*), whilst that from haematin was augmented by vigorous oxygenation and continuous addition of ascorbic acid. As *in vitro* degradation of haematin to bile pigment led to both cholehaematin and verdohaematin, the pathway was probably similar to that of haem combined with protein in oxyhaemoglobin, although here the reactions were complicated by formation of protein derivatives, such as denatured globin cholehaemochromogen ('green pigment').

Regarding the mechanism of bile pigment formation, a coupled oxidation was unlikely to be operative in the case of free haematin, since although ferrohaem was autoxidizable in air, the yield of bile pigment from it was much less than from haematin. No spectroscopic evidence of ferrohaem was obtained during the experiments with aqueous haematin. It was not necessary to assume reduction of haematin in this reaction. Free ferrihaem combined readily with hydrogen peroxide, and behaved as a peroxidase to activate hydrogen peroxide, which brought about the destruction of other haem molecules, or even protoporphyrin (Granick & Gilder, 1947). Autoxidation of ascorbic acid alone must have sufficed to bring about oxidation of haematin. Vigorous oxygenation, continuous addition of ascorbic acid to replace that irreversibly oxidized, and the catalytic effect of haem itself were factors which operated to increase the rate of hydrogen peroxide production. Ferrihaem would combine readily with hydrogen peroxide, and this activated peroxide compound would be responsible for oxidative destruction of more haem molecules. Such a protohaematin peroxide compound has not yet been isolated, although a peroxide complex with free urohaematin I has recently been described by J. Keilin (1952).

Of other haem derivatives studied, only two pigments, protoporphyrin and cytochrome *c*, failed to undergo breakdown to bile pigment in the presence of ascorbic acid and oxygen under the conditions of the experiments (Table 2). The negative result with protoporphyrin confirmed the important role of iron in the reaction with ascorbic acid, and in the autodestruction of haem and its derivatives. All other haem derivatives examined were reactive, and convertible into bile pigment, whether the iron was in the ferrous or ferric condition. The yields of bile pigment from haematin, ferrihaemalbumin and ferrihaemoglobin were all greater than those from the corresponding ferrous compounds, evidence which militates against the ferrous state being an obligatory condition for bile pigment formation.

The optimum conditions for haematin—vigorous oxygenation and continuous addition of ascorbic acid—were most suitable for ferrihaemalbumin, whilst of no advantage compared with incubation in air for methaemoglobin, metmyoglobin and catalase. Under similar conditions to those optimum for conversion of oxyhaemoglobin into choleglobin, metmyoglobin was even more readily disrupted. Myoglobin has been shown to react almost five times more rapidly with oxygen than does haemoglobin (Millikan, 1936), and in consequence of autoxidation is changed almost entirely to metmyoglobin during preparation. Also, in contrast to the slow reduction of methaemoglobin by ascorbic acid *in vitro*, metmyoglobin was observed to undergo almost complete reduction to oxymyoglobin during the first 5 min. incubation. The high yield of bile pigment could be ascribed to increased reactivity with oxygen, associated with minimal denatured by-products.

Catalase and peroxidase were two haemoproteins of particular interest from the point of view of breakdown of their constituent ferrihaem to bile pigment, since they were able to decompose hydrogen peroxide, the active agent in the ascorbic acid reaction. Interaction of hydrogen peroxide with horse-radish peroxidase and with horse-liver catalase leading to formation of complexes has been investigated by Chance (1950) and Keilin & Hartree (1951), and the latter workers have shown that the changes in colour and absorption spectrum of catalase that were brought about by ascorbic acid were due to the formation of a catalase-hydrogen peroxide complex, not to a compound between catalase and ascorbic acid. Horse-liver catalase itself contains a bile pigment haemoprotein in varying proportion (Lemberg, Norrie & Legge, 1939: Sumner, Dounce & Frampton, 1940), which Bonnichsen (1948) has recently shown to arise mainly as an artifact during the course of preparation. Lemberg & Legge (1943) have demonstrated that ascorbic acid can cause inhibition of catalase by accelerating the destruction of the enzyme by added hydrogen peroxide with production of bile pigment haemoprotein. In the present study prolonged incubation of ascorbic acid with horse-liver catalase in one instance augmented bile pigment-haematin from 27 to 39 % of its total haem, and horse-radish peroxidase experienced disruption of its prosthetic group to yield biliverdin to the value of 2·9 % of original haematin content. Considerable denaturation was observed in the peroxidase experiment and also a small localized increase in optical density at 675 mμ.

Cytochrome *c* provided the single negative result for haem–bile pigment conversion in this series of studies of haem derivatives. This might be ascribed to the fact that it was not autoxidizable and did not combine with oxygen. On the other hand, the prosthetic group is bound very firmly to its protein by two thioether linkages through the two side-groups in the 2 and 4 positions, respectively, and it is possible to remove the iron atom without breaking the attachment of haem to protein (Paul, 1951). Thus, it might be possible for reaction with ascorbic acid to occur with oxidation of the haem to form a verdohaemoprotein, which would lose iron readily, but in which the bile pigment-protein linkage would be resistant to acetic acid. In fact, the final absorption spectrum of incubated cytochrome contained, besides the bands of ferro- and ferri-cytochrome *c*, a weak absorption at 623 mμ. (Gardikas & Kench, 1952) not ascribable to any form of cytochrome *c*, and possibly due to formation of a verdohaemochromogen (Bigwood & Thomas, 1940). This 623 mμ. band has been ascribed by Lemberg & Wyndham (1937) to an artifact, probably derived from altered, autoxidizable cytochrome *c*. However, if this absorption were due to such a compound, it proved resistant to acid, and no bile pigments were recovered in any one of several tests.

From these *in vitro* investigations it is clear that the incorporation of the reactive iron atom into the porphyrin ring, whilst conferring diverse biological activity on many haemoproteins, leads to vulnerability of the porphyrin in coupled oxidation with agents, such as ascorbic acid, which liberate hydrogen peroxide. Only cytochrome *c* appeared resistant, and with this exception, there appears no chemical reason why free haematin or any of the haem derivatives here studied, if exposed to the conditions operating in the reticulo-endothelial system in which oxyhaemoglobin is catabolized to bile pigments, should not undergo a similar degradation. Moreover, the present findings concerning the haematin reaction products indicate that linkage with globin does not greatly influence the course of breakdown of the haem prosthetic group. London (1950) has observed that, in the dog, injected [^{15}N]haematin was rapidly converted into stercobilin, whilst in normal man at least 11% of stercobilin was derived from labelled precursors other than the haemoglobin of circulating erythrocytes (London, West, Shemin & Rittenberg, 1950). These authors suggested that myoglobin, or haem not utilized for haemoglobin production could be the source. Similarly, Gray & Neuberger (1952) have postulated that excess of haematin may have been responsible for bile pigment formation in congenital porphyria. Many aspects of the conversion of haematin and haem derivatives into bile pigment both *in vitro* and *in vivo* remain obscure, however, and resolution of these may depend on complementary studies in tissue and enzyme preparations.

SUMMARY

1. Spectroscopic studies have been made on the *in vitro* conversion of haematin and some other haem derivatives into bile pigments through the action of ascorbic acid and oxygen.

2. The conversion of haematin into bile pigments is associated with increased optical density in the range 630–700 mμ. and evidence was obtained for the presence of verdohaematin and cholehaematin in the reaction products.

3. The yield of bile pigment derived from metmyoglobin was approximately eleven times that from oxyhaemoglobin under similar experimental conditions. Cholemyoglobin was observed spectroscopically.

4. Horse-radish peroxidase was converted into bile pigment in 2·9 % yield of its haem content, and the bile pigment haematin content of horse-liver catalase was increased from 27 to 39 %.

5. With the exception of cytochrome *c*, all haem derivatives in the series suffered, in varying degree, oxidative breakdown to bile pigment in the presence of ascorbic acid and oxygen.

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The Measurement of the Components of the Plasminogen–Plasmin System in Biological Fluids

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Streptokinase, a product of haemolytic streptococcal metabolism, is known to activate a serum enzyme precursor, plasminogen, into a proteolytic enzyme, plasmin; plasmin causes the lysis of fibrin and the liquefaction of fibrinous exudate. The action of plasmin can be inhibited by antiplasmin or antiplasmins (nomenclature of Christensen & MacLeod, 1945; Fig. 1). Mullertz & Lasen (1953) have suggested that the activation of plasminogen by streptokinase is not a direct reaction, but is mediated through an intermediary component.

Certain human infections result in the local production of fibrin and fibrinous exudates, which may prove harmful to the patient, and streptokinase has been employed therapeutically in man to disperse such exudates. Fletcher (1954) has shown that the