XXIV. CHEMICAL MECHANISM OF THE OXIDATION OF PROTOHAEMATIN TO VERDOHAEMATIN

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THERE are four assumptions possible for the mechanism of the reaction described in the previous paper by which ferrous haemochromogen is oxidized to verdohaemochromogen.

(1) The ferric haematin iron may oxidize the porphyrin nucleus.

(2) A peroxide of ascorbic acid may cause this oxidation. Krebs [1936] has observed a coupled oxidation of haemoglobin or haemoglobin derivatives and amino-acids in the presence of amino-acid oxidase, and has tentatively suggested an amino-acid peroxide as the first intermediate step of the oxidative deamination.

(3) The autoxidation of ferrous haemochromogen may lead directly to verdohaemochromogen. This need not be discussed however since it would still remain to be explained why neither porphyrins nor ferric haematin compounds undergo the oxidation to bile pigment compounds.

(4) Hydrogen peroxide, formed during the autoxidation of ferrous haemochromogen or of ascorbic acid, may cause oxidative scission of the porphyrin ring.

McLeod & Gordon [1922] have shown that the formation of "green pigment" from haemoglobin by certain bacteria [Schottmüller, 1903, "viridans effect"] can be brought about under certain conditions by the action of hydrogen peroxide on heated blood agar; pneumococci can themselves produce hydrogen peroxide. Hart & Anderson [1933] were however unable to transform haemoglobin into green pigment with hydrogen peroxide, nor did they observe inhibition of pigment formation by catalase; they showed later [Anderson & Hart, 1934] that the same pigment is produced by the simultaneous action of oxygen and reducing systems (ascorbic acid, cysteine, dihydroxyacetone, glucose + glycine + ammonia) on haemoglobin. From their observations and from what has been said in our previous paper, there can be little doubt that this reaction is closely related to the coupled oxidation of pyridine-haemochromogen and ascorbic acid.

Possibility (1) can be safely excluded by the following experiment: 40 mg. $Na_2S_2O_4$ were dissolved in 100 ml. of O_2-free water and amounts varying from 0·1 to 2·5 ml. filled into the bent, hollow stoppers of Thunberg tubes. Into each tube were introduced 5 ml. of haematin solution in 20% pyridine (1 mg. haematin). The tubes were then evacuated, filled with O_2-free nitrogen, and their contents mixed. Spectroscopic observation showed that the tube with the largest amount of $Na_2S_2O_4$ (2·5 ml.) contained ferrous haemochromogen only, whereas in all the other tubes (with 0·8, 0·4, 0·2 and 0·1 ml. $Na_2S_2O_4$) the reduction was incomplete and varied from about 80 to about 10%. These tubes thus contained mixtures of ferric and ferrous haemochromogens, and if ferric

haematin iron were able to oxidize the porphyrin nucleus of ferrous haemochromogen, verdohaemochromogen formation should occur. No alteration and no formation of the absorption band in the red, characteristic of verdohaemochromogen, could be observed, even when the tubes were kept for a week.

A similar experiment was carried out with ascorbic acid instead of $Na_2S_2O_4$, with the same negative result.

(2) This theory, rather improbable in itself, can also be shown to be incorrect. Ascorbic acid, under the conditions in which pyridine-haemochromogen is oxidized, is not autoxidizable in absence of haematin. When ascorbic acid is shaken with air in dilute pyridine solution, no O_2 is taken up, and when haematin is now added, its oxidation to verdohaematin is not accelerated when compared with a solution of ascorbic acid in pyridine from which O_2 has previously been exhausted. There is also no trace of the formation of the intermediate product, described in a later section (which possesses an absorption band at 639 mμ.), when a pre-shaken solution of ascorbic acid in dilute pyridine is mixed under N_2 with a solution of haematin in pyridine.

Still more conclusive is the observation, discussed in greater detail in a later section, that verdohaemochromogen is also formed when ascorbic acid is replaced by cysteine and Cu, whereas pure cysteine is not active. It is very unlikely that a cysteine peroxide should be formed only in the presence of Cu and cause the oxidation of haematin to verdohaematin.

(4) If hydrogen peroxide were to arise during the coupled oxidation of haemochromogen and ascorbic acid and cause the oxidative cleavage of the porphyrin nucleus, it should be possible to inhibit the oxidation of haematin by catalase. Catalase inhibits, indeed, the oxidation of haematin, though by no means completely, whereas it is without effect on the oxidation of ascorbic acid (Fig. 1).

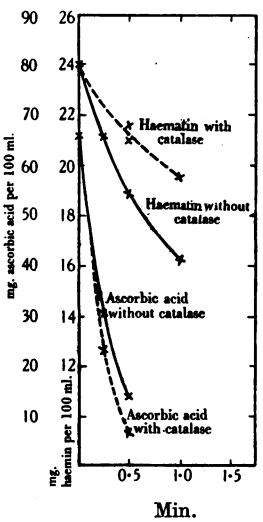


Fig. 1.

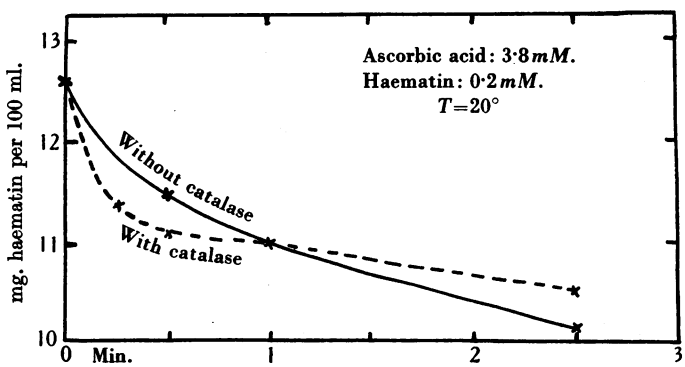


Fig. 2.

Fig. 1. Effect of catalase on the rate of oxidation of haematin and ascorbic acid. Haematin: 0·4 *mM.*; ascorbic acid: 3·8 *mM.*; $T=37°$. In experiments with catalase: 0·5 ml. catalase solution ($k=6000$) in 3 ml.

Fig. 2. Effect of catalase on the rate of haematin oxidation in the presence of $M/1000$ cyanide.

The last-mentioned fact makes it improbable that the inhibition of the haematin oxidation is caused by a non-specific effect of the catalase solution, e.g. a protein effect, since this would probably affect the oxidations of haematin and ascorbic acid to the same extent.

It can be shown, moreover, that $M/1000$ cyanide completely prevents the effect of catalase on the velocity of haematin oxidation (Fig. 2).

Since hydrogen peroxide is the only substrate upon which catalase acts rapidly, these observations make it very probable that hydrogen peroxide plays a part in the oxidation of haematin to verdohaematin.

Effect of hydrogen peroxide on pyridine-haemochromogen in the presence of ascorbic acid

Attempts to oxidize haematin in pyridine solution by H_2O_2, BaO_2 or CeO_3 in various concentrations were without success. With small amounts of peroxides haematin remained unaltered, the only alteration being that BaO_2 caused the solution to become alkaline and transformed ferric haemochromogen into alkaline haematin, and that H_2O_2 was destroyed catalytically. With larger amounts of peroxide a gradual decoloration took place. No verdohaemochromogen could be detected.

Attempts to oxidize pyridine haemochromogen by H_2O_2 in the presence of ascorbic acid seemed at first to give equally negative results. These experiments have, of course, to be carried out in nitrogen, since in the presence of oxygen verdohaemochromogen would be formed without H_2O_2.

Varying amounts of haematin in phosphate buffer *p*H 7·6, and of ascorbic acid, with a little pyridine were mixed in Thunberg tubes under N_2 with H_2O_2 (0·5–4 *mM*.). The tubes were then not examined until the following morning, when the apparent spectrum of protohaemochromogen was still strong, and in trichloroacetic acid filtrates H_2O_2 was detected by the titanic sulphate test. The conclusion from this that there had been no oxidation by H_2O_2 was invalidated by our finding that titanic sulphate gives a yellow colour with ascorbic acid, a reaction described by Ettori [1936]. The strength of the apparent haemochromogen absorption bands will be explained below.

When the experiments were repeated with BaO_2 we observed a distinct and rapid reaction, and the same could also be achieved with suitable amounts of H_2O_2. The red solution, containing ferrous pyridine-haemochromogen produced by action of ascorbic acid on ferric haemochromogen, turned brownish green on mixing with the dilute H_2O_2 solution or a few mg. of BaO_2 contained in the stoppers. A strong absorption band with the centre at 639 mμ. appeared which under suitably chosen conditions became the only band noticeable. A considerable excess of ascorbic acid was necessary. Complete disappearance of the haemochromogen and maximum formation of the band at 639 mμ. were produced when the following amounts of reagents, dissolved in 5 ml. dilute pyridine, were used: 0·4 mg. haemin, 0·5 mg. H_2O_2 and 12 mg. ascorbic acid. The molar ratio is ascorbic acid = 4·6 H_2O_2 = 113 haematin. When an excess of H_2O_2 or of ferricyanide was added after the green compound had been produced, the solution turned brown and displayed no visible absorption bands. These additions were made through the side-arms of the evacuated tubes under N_2.

The compound giving the absorption band at 639 mμ. could not be acid haematin, for in the presence of pyridine the latter was only formed when the *p*H was decreased to less than 4·5 with acetic acid, whereas the *p*H in our experiments was 7·9 with H_2O_2 and still higher with BaO_2. It was also not

verdohaemochromogen or any other haemochromogen,[1] for the absorption band at 639 mμ. disappeared on reduction with $Na_2S_2O_4$, the solution turning red again and strong haemochromogen bands reappearing apparently unaltered in position. The same occurred, though much more slowly, when the tubes were kept without adding reducer after the production of the green compound; in the course of a few hours the solution turned red again, the band at 639 mμ. disappeared and the haemochromogen bands returned. This explains why we had overlooked the reaction in our first experiments in which the tubes were not investigated at once.

The reappearance of the haemochromogen bands could be accelerated by the addition of catalase to the solution after the green compound had been produced as above.

When the tubes containing the green compound with the band at 639 mμ. were opened and shaken with air for a few minutes, the solutions turned pure green. The main absorption band was now found shifted to 652 mμ., the two other absorption bands of verdohaemochromogen being also visible, and $Na_2S_2O_4$ no longer destroyed the band in the red, but caused the solution to turn yellow. We must conclude from these experiments that the compound with the absorption band at 639 mμ., arising by the action of H_2O_2 on protohaemochromogen in the presence of ascorbic acid, is rapidly transformed into verdohaemochromogen by molecular O_2, but not by H_2O_2. The unexpected result of these experiments is that, though H_2O_2 may be essential for the reaction, it cannot replace molecular O_2 altogether.

The compound with the absorption band at 639 mμ. is formed as an intermediate product during the oxidation of haemochromogen to verdohaemochromogen by molecular oxygen

When the absorption band in the red part of the spectrum appears in this process, it is found to lie close to 640 mμ. and only gradually moves towards the red until it reaches the position at 652 mμ. When $Na_2S_2O_4$ is added in the initial stages, the band in the red disappears completely or becomes much weaker, whereas later it is intensified. These differences are particularly noticeable under conditions in which the reaction proceeds more slowly, for example, when it is carried out under diminished O_2 pressure. Cyanide does not prevent the formation of this compound by H_2O_2 or as intermediate product in the aerobic haemochromogen oxidation. It has also been found that addition of a small amount of BaO_2 or H_2O_2 accelerates haematin oxidation when haematin in pyridine is shaken with ascorbic acid in air.

Nature of the compound with absorption band at 639 mμ.

The situation of the two absorption bands of this haemochromogen were, within the limits of experimental error, the same as those of protohaemochromogen (557·4 and 526·3 mμ.; protohaemochromogen: 556·6 and 524·2 mμ.). The relative strengths of the two bands were different, however, the second band being distinctly broader and at least as strong as the first, whereas the first band of protohaemochromogen is by far the stronger.

A quantitative spectrocolorimetric estimation confirmed the results of spectroscopic observation. In a Thunberg tube haematin in the presence of excess ascorbic acid was oxidized by H_2O_2 to the compound with 639 mμ. band. This was then reduced by adding $Na_2S_2O_4$ under nitrogen. The strength of both bands

[1] Biliviolinohaemochromogens have an absorption band in this region [Lemberg & Wyndham, 1936].

was determined in a layer of the solution in the tube by spectrocolorimetric comparison with standard haemochromogen. The control giving the strength of bands of protohaemochromogen was prepared in a Thunberg tube of the same dimensions, H_2O_2 being replaced by the same volume of H_2O.

In another experiment the reduction was carried out by keeping overnight with excess ascorbic acid.

This compound is formed by the action of H_2O_2 on pyridine haemochromogen in the presence of excess ascorbic acid. It is destroyed by excess of H_2O_2 or $H_3Fe(CN)_6$, and on reduction gives a compound with absorption bands in the position of those of protohaemochromogen. On this evidence it was reported by us [1937, 1] that the substance was a ferrous haem-H_2O_2 compound.

The absorption spectrum of the substance was unlike that of known haem-H_2O_2 compounds. Several have been described [Keilin & Hartree, 1935, 1; 1936; Keilin & Mann, 1937] but all have a 2-banded spectrum totally different from that of our compound. The reactions of our compound did not accord with those described in the above literature.

It was not until we discovered that the absorption spectrum obtained on reducing the green compound with the band at 639 mμ. was not that of protohaemochromogen that we were able to discard our first view of its structure.

Table I

	Reading of haemochromogen standard	
	Absorption band I 557 mμ.	Absorption band II 525 mμ.
Control (without H_2O_2)	33	33
New haemochromogen after $Na_2S_2O_4$ reduction	12	27
New haemochromogen after reduction by ascorbic acid	12	38
New haemochromogen after reduction by ascorbic acid, $Na_2S_2O_4$ added	14	38

Table I shows that only 40% of the original strength of the first haemochromogen band can be restored after the treatment with H_2O_2, whereas the strength of the second band has hardly changed. The reduction by keeping overnight leads to the same result, showing that the reduction by the excess of ascorbic acid is slow, but complete. When the reduction is carried out in this way, the second band is somewhat stronger than in the control or after reduction by $Na_2S_2O_4$.

The haemochromogen recovered after H_2O_2 treatment was altogether different from protohaemochromogen. When the latter is shaken with air in the presence of ascorbic acid, it turns gradually green; when, however, a solution of the new haemochromogen was shaken with air, it turned green in a flash. The green solution contained at first the compound with the absorption band at 639 mμ., later verdohaemochromogen. Hence the compound with the band 639 mμ. is to be considered as the ferric haemochromogen of the pseudo-protohaemochromogen.

When a solution of pyridine-haemochromogen containing $Na_2S_2O_4$ and ascorbic acid is extracted with ether and glacial acetic acid, the haemochromogen passes largely unaltered into the ether, and can be extracted from it by Na_2CO_3. When, however, the solution of the pseudo-protohaemochromogen was treated in the same way, the ether solution was green, not red and the Na_2CO_3 solution contained verdohaematin instead of protohaematin.

Finally, in the presence of reducing substances protohaemochromogen is split by HCl to protoporphyrin. Under the same conditions the haemochromogen obtained by ascorbic acid reduction after hydrogen peroxide treatment yields neither porphyrin nor bile pigment, but a chlorin-like compound for which we suggest the constitution of an *oxyporphyrin*. We shall, therefore, call the new haemochromogen provisionally "oxyhaemochromogen" and the compound with the absorption band at 639 mμ. "oxyhaematin", though it might be ferric oxyhaemochromogen. The name "oxyhaematin" might cause confusion, since this name is used in other languages for hydroxyhaematin. The term oxyporphyrin-haematin is clumsy, but should be used where a mistake is possible. In a preliminary publication [1937, 2] we have already given a short report on these results.

These facts enable us to understand better some difficulties we had encountered in explaining the kinetics of the haematin oxidation, described in the last paper. The decrease in haematin concentration has been measured there by the decrease in strength of the first absorption band of haemochromogen. When oxyhaemochromogen is present, as it is during the initial stages of the reaction, not only the transformation of haemochromogen into verdohaemochromogen, but in addition 60 % of the transformation of haemochromogen into oxyhaemochromogen is measured in this way, since the strength of the first band of oxyhaemochromogen is only 40 % of that of haemochromogen. The actual destruction of haematin during the initial phase of the reaction is therefore still greater than it appears from these experiments, which had already given an unexpectedly high initial rate of haematin oxidation. We had already concluded that more than one process must be at work, and can now explain the phenomenon by assuming a rapid formation of oxyhaemochromogen from haemochromogen (with loss of 60 % of the extinction at 557 mμ.), followed by a somewhat slower oxidation of oxyhaemochromogen to verdohaemochromogen (with loss of the remaining 40 % of the extinction at 557 mμ.). We can also understand why the later part of the reaction does not depend on the vitamin concentration, whereas the initial reaction does. H_2O_2 arises only by the action of O_2 on ferrous haemochromogen, and also acts only on ferrous haemochromogen to give oxyhaematin, so that the action of ascorbic acid is required. Oxyhaematin, however, reacts with O_2 to give verdohaematin whether in the ferrous or in the ferric form, and ascorbic acid is therefore not required.

Preparation and properties of the oxyporphyrin

In each of ten Thunberg tubes the oxyhaematin was prepared under nitrogen as described above, and the oxyhaemochromogen obtained by keeping overnight. Through the side-arm of each tube 3 ml. of conc. HCl were sucked in under nitrogen, and the tubes were kept for 48 hr. They were then opened and the green solution filtered from a small amount of precipitate. The filtrate showed the following absorption spectrum: I, 621·5; II, 567 mμ. Intensities: I, II. There was also a very weak band in the green, and perhaps a further band in the extreme red.

With sodium acetate the pigment was brought into ether, in which it dissolved giving a blue-green colour. The spectrum showed four bands: I, 650; II, 584; III, 546; IV, 511 mμ. Intensities: I, IV, III, II. The spectrum is thus more like that of a rhodin than of a chlorin. The compound was completely extracted from ether by 1 % HCl, its Willstätter number being 0·25. In 20 % HCl the blue-green solution (brown in thick layer) showed two absorption bands (623·5 and 568·4 mμ.).

The Cu complex salt was obtained by heating the residue of the ethereal solution for a short time with alcoholic Cu acetate. Its solution displayed two bands: 573·5 and 537·5 mμ., the second being the stronger. When this absorption spectrum is compared with that of the protoporphyrin Cu complex (527·8 and 534·4 mμ., the first being the stronger) the same similarity is noticed as between the haemochromogens which we described in the previous section. This proves that the splitting of the haemochromogen with acid does not involve an alteration in the metal-free pyrrolic compound.

The properties of the complex salts show that our oxyporphyrin is different from otherwise very similar compounds obtained by Fischer and co-workers by the action of H_2O_2 on porphyrins in strong H_2SO_4 [Fischer *et al.* 1927; Fischer *et al.* 1930]. These compounds contain one or two oxygen atoms more than the corresponding porphyrins which Fischer assumes to be either on the methene groups linking the pyrrole rings or in the β-position of a hydrogenated pyrrole nucleus as in certain oxychlorins [Fischer & Lautsch, 1937]. Both, mono- and di-oxymesoporphyrin, are reported to have the same absorption spectrum, and Table II shows that this spectrum is indeed similar to that of our compound in neutral as well as in acid solutions. The difference in the absorption spectra between our compound and green porphyrin ozonides [Fischer & Deželic, 1934] is much greater. Our oxyporphyrin is, however, a much stronger base than the oxyporphyrins of Fischer (see the HCl numbers in Table II) and, while these give complex salts of the chlorin-type, our oxyporphyrin gives complex salts of the porphyrin type with the only difference that the ratio of the strengths of the two absorption bands is reversed.

Table II. *Absorption spectra of oxyporphyrin and related compounds*

	Absorption bands in mμ.				
	In ether-acetic acid	In 20% HCl	Cu salt	Pyridine haemochromogen	HCl number
Our oxyporphyrin	650, 584, 546, 511 (I, IV, III, II)	623·5, 568·5 (I, II)	573·5, 537·5 (II, I)	557, 527 (II, I)	0·25
Fischer's oxymesoporphyrin	642, (629), (613), 584, 540, 504 (I, III, IV, II)	621·5, 576, 537 (I, II, III)	620, 570, (513) (I, II)	Indistinct	14·5
iso-Aetioporphyrin ozonide	645, (614), 591, 524, 491 (I, IV, III, II)	—	—	—	—
Protoporphyrin	632, 579, 537, 503 (IV, III, II, I)	606, 558 (II, I)	573, 534 (I, II)	557, 524 (I, II)	—

A porphyrin which yields a haemochromogen with a strong second absorption band is phylloporphyrin, which carries a methyl group on one methene group of the porphyrin nucleus [Treibs & Wiedemann, 1928]. Although our oxyporphyrin has not yet been obtained in amounts sufficient for analysis, our observations make it probable that it carries a hydroxyl group on the methene group α which is eliminated when the porphyrin ring of oxyhaemochromogen is split open by oxygen with formation of verdohaemochromogen. In the complex salts this oxyporphyrin is in the form of hydroxyporphyrin, since the spectra are similar to those of porphyrins and particularly to phylloporphyrin in which the methene group also carries a substituent. In the free state the oxyporphyrin is probably present in the keto-form and the chain of conjugated double bonds of the porphyrin nucleus is thus interrupted, so that the spectrum is no longer that of a porphyrin but similar to that of chlorins or rhodins.

Experiments with cysteine

When a solution of metal-free cysteine hydrochloride is added to a solution of haematin in phosphate buffer *p*H 7·6, a peculiar type of absorption spectrum is formed. There is a strong band in the red (656mμ.) and a weaker band at 551mμ. This cannot be a verdohaematin compound, for on addition of pyridine the band in the red disappears completely and pyridine-haemochromogen is formed. This observation shows that haematin probably forms a compound with cysteine having an absorption band in the red. Haemoglobin gives a similar reaction and a compound with an absorption band at 653mμ. and two weak bands in the green is formed; this spectrum persists when the solution is shaken with air or when $Na_2S_2O_4$ is added, but it is immediately destroyed by pyridine. We shall investigate these reactions further but they have no direct bearing on the problem discussed here.

The presence of pure cysteine (Schering-Kahlbaum, freed from traces of metal according to Warburg) does not prevent the formation of verdohaemochromogen when protohaemochromogen is shaken with ascorbic acid in air; the initial formation of oxyhaematin also occurs as indicated by the appearance of the absorption band at 639mμ. in the first stages of the reaction.

Pure cysteine alone does not, however, reduce pyridine-haematin to haemochromogen, and when the mixture is shaken with air, no verdohaemochromogen is formed. On reduction with $Na_2S_2O_4$ there is seen a weak absorption band at 584mμ. besides those of protohaemochromogen. It has been reported in the preceding paper that glutathione acts in the same way. These observations are of special interest, since they show a way in which cytochrome-*a*-haematin may be formed from protohaematin compounds. The haematin-*a* (or rather haematins-*a*) obtained from cytochrome-*a* [Negelein, 1933; Roche & Bénévent, 1936] give haemochromogens with an absorption band between 587 and 580mμ.

When cysteine (B.D.H.), not specially purified, is used instead of metal-free cysteine a different result is obtained. Ferric haemochromogen is reduced, verdohaemochromogen is formed, and, when the amount of cysteine is not too large (3 mg. cysteine-HCl per 6 ml.), the presence of oxyhaematin in the first stage of the reaction is shown by the development of the absorption band at 639mμ. This band was formed for example when haematin (1·5 mg.) in 20% pyridine (5 ml.) was shaken with a solution of cysteine hydrochloride (3 mg.) in phosphate buffer *p*H 7·6 (0·6 ml.).

The addition of traces of Cu acetate to metal-free cysteine causes rapid formation of verdohaemochromogen; there is also some formation of haemochromogen-*a*. When a solution of haematin (0·4 mg.) in 20% pyridine (0·4 ml.) is shaken with a solution of cysteine hydrochloride (10 mg.), neutralized with Na_2CO_3, in the presence of Cu acetate (0·5 mg.), a considerable amount of verdohaemochromogen is formed during 1 min. shaking with air. Addition of Fe ($FeSO_4$) has the same effect, but its action is much less pronounced than that of Cu; there is a slow formation of oxyhaematin and later of verdohaemochromogen.

In contradistinction to ascorbic acid, cysteine alone is not able to produce oxyhaematin from pyridine-haematin with H_2O_2. To a solution of haematin (0·4 mg.) in 20% pyridine (3 ml.) various amounts of a neutralized solution of metal-free cysteine hydrochloride (1–10 mg.) were added in the bottom part of Thunberg tubes just before the tubes were evacuated, the stoppers of the tubes containing various amounts of H_2O_2 (0·02–0·1 ml. 0·5% solution). In none of the tubes was a band at 639mμ. formed. When $Na_2S_2O_4$ was introduced through

the side-arms of the tubes, a weak absorption band at 587 mμ. and a very weak band at 612·5 mμ. were observed in addition to the bands of protohaemochromogen.

When however the same experiment was repeated with addition of 0·5 mg. Cu acetate, there was immediate formation of a strong band at 639 mμ. This appears, indeed, to be the best method for preparation of the oxyhaematin. Hence, ascorbic acid is not specific for the formation of this compound any more than for the formation of verdohaemochromogen, and the same reaction mechanism is set in motion by ascorbic acid without metal and by cysteine and Cu and, to a lesser degree, by cysteine and Fe.

With glutathione no formation of the oxyhaematin could be obtained even in the presence of Cu, though Cu distinctly accelerated the formation of haemochromogen *a* by hydrogen peroxide.

Some further observations on the formation and role of hydrogen peroxide in verdohaemochromogen formation

We have studied the effect of catalase under conditions in which we normally obtained a quantitative formation of oxyhaematin. To the mixture of haematin and ascorbic acid was added catalase solution (half the amount which caused very incomplete inhibition of aerobic verdohaemochromogen formation): addition of H_2O_2 now failed to give a trace of the absorption band at 639 mμ. and gas development indicated the destruction of H_2O_2 by the catalase. In the presence of catalase BaO_2 was also inactive.

It is unlikely that concentrations of H_2O_2 greater than those applied in this experiment should be formed when haemochromogen is shaken in air in the presence of ascorbic acid. That this is not so was proved by the following experiment. By the use of "Luminol" (3-aminophthalic acid hydrazide), which gives with H_2O_2 in presence of small amounts of haematin and even in presence of reducing agents a blue chemiluminescence, we detected H_2O_2 in a solution containing (in 8 ml.) 0·4 mg. haematin, 1·6 mg. ascorbic acid, 5 ml. 0·1 *mM*. H_2O_2. No chemiluminescence was observed however when pyridine haemochromogen was shaken with air in the presence of ascorbic acid and luminol, though the formation of verdohaemochromogen proceeded as usual.

This seems to show that hydrogen peroxide formation does not occur in the autoxidation of protohaemochromogen. How then are we to explain the inhibition of the verdohaemochromogen formation by catalase? We have brought evidence which we consider conclusive that the effect of catalase is actually due to the enzyme, and H_2O_2 is its only known substrate. Keilin & Hartree [1936] have recently shown that the H_2O_2 formed when $Na_2S_2O_4$ is shaken with air reacts with certain catalase compounds to form H_2O_2-compounds with them. Here again, luminol does not indicate the formation of H_2O_2 in the solution, though added H_2O_2 acts on luminol in the presence of $Na_2S_2O_4$. The negative result of the luminol test therefore does not exclude the presence of H_2O_2 in a concentration small enough to react with haematin compounds, but it excludes its presence in larger concentrations.

The incomplete inhibition of the aerobic formation of verdohaemochromogen by catalase can thus not be due to the fact that more H_2O_2 is present in these experiments than in the experiment with added H_2O_2 under nitrogen. It is not impossible, though improbable, that there are two simultaneous mechanisms of aerobic verdohaemochromogen formation, one proceeding through H_2O_2 and oxyhaematin and being completely prevented by catalase, and a second mechanism in which H_2O_2 does not participate and which is, therefore, not

affected by catalase. It appears more reasonable to assume that the H_2O_2 is somehow protected against catalase under the conditions of our experiments. The most likely way in which it can be protected is by combination with the haem molecule with which it is to react to form oxyhaematin. If by the autoxidation of haemochromogen not free H_2O_2 is formed, but a haem-H_2O_2 compound, which partially dissociates liberating free H_2O_2, but is rapidly transformed into oxyhaematin, the partial inhibition of this process by catalase can be understood. When free H_2O_2 is added to a mixture of catalase and haemochromogen, it is destroyed rapidly by the former before it can combine with the haemochromogen.

If this hypothesis of the reaction mechanism is correct, we must assume that the haem-hydrogen peroxide compound is formed without free H_2O_2 being an intermediate product. This is in agreement with the second theory, mentioned in the introduction, that a haemochromogen peroxide acts as acceptor of the hydrogen from ascorbic acid but is not in agreement with the radical chain theory of Weiss [1934, 1935].

Observations on the ratio of ferrous to total protohaem iron during the reaction with ascorbic acid

In our preliminary paper [1937, 1] it was stated that the ratio of ferrous to total haemochromogen during the coupled oxidation with ascorbic acid depends on the initial ascorbic acid concentration in an unexpected manner. In some experiments it was found that increased ascorbic acid concentration apparently lowered this ratio. This phenomenon can be explained on the basis of oxyhaematin formation; the ferric form of the oxy-compound does not absorb in the 527 mμ. region, but on reduction with $Na_2S_2O_4$ this band of protohaemochromogen is considerably strengthened. Thus the figure for total haemochromogen appears higher than it actually is, and the experiments show that ascorbic acid accelerates the oxidation of ferrous haemochromogen, not to ferric haemochromogen, but to ferric oxyhaemochromogen.

Discussion

We have shown in this paper that catalase inhibits the formation of verdohaemochromogen from pyridine-protohaemochromogen, though it does not prevent it, whereas the action of free H_2O_2 or haemochromogen (in an atmosphere of N_2) is completely prevented by catalase.

Similar facts have been observed by other authors with regard to the action of H_2O_2 and catalase on the formation of "green pigment" from haemoglobin, and this is, indeed, good evidence in favour of an essential similarity of the "green pigment" formation from haemoglobin and the "green haemin" (verdohaemochromogen) formation from haemochromogen. It has been mentioned earlier that McLeod & Gordon [1922] were able to obtain "green pigment" by the action of H_2O_2 on heated blood agar (in which no catalase is present), whereas in the presence of catalase added H_2O_2 is not active, and catalase does not prevent the green pigment formation with O_2 [Hart & Anderson, 1933]. That catalase, however, inhibits the reaction is evident from the work of Schreus & Carrié [1934]. These authors incubated haemoglobin with liver-brei at 37° or higher temperatures (optimum 70°) in the presence of alkaline buffer, and obtained biliverdin from the acetic acid-ether extract. After initial difficulties (the authors did not give experimental details of their method) we have been able to confirm their results. Although the yield is very small, there can be no doubt that the pigment is biliverdin (dehydrobilirubin). The blank was completely

negative. In consideration of the strong reducing power of liver tissue it is extremely unlikely that biliverdin should be formed by secondary oxidation of bilirubin, nor does such a reaction occur during the extraction as the authors assume. It is possible that verdohaemochromogen[1] and not biliverdin is formed under these conditions and is transformed into biliverdin during extraction or treatment with acid. However that may be, verdohaemochromogen is probably an intermediate product. Heat and catalase affect the reaction in the same way as they do verdohaemochromogen formation from pyridine-haemochromogen, catalase inhibiting, but by no means completely, and rise in temperature increasing the rate of oxidation. It is therefore probable that an unstable haem-H_2O_2 compound is formed in this process as in the formation of pyridine-verdohaemochromogen. As an explanation that catalase does not prevent aerobic verdohaemochromogen formation, we have assumed that the haem-H_2O_2 compound is formed directly from a haem peroxide by hydrogen transference from the ascorbic acid:

$$(Fe) + O_2 \rightarrow (Fe)O_2;\ (Fe)O_2 + H_2A \rightarrow (Fe).H_2O_2 + A,$$

where (Fe) means haemochromogen, H_2A ascorbic acid and A dehydroascorbic acid. One might attempt to explain the observed facts by assuming "nascent hydrogen peroxide" to be formed during the aerobic haemochromogen oxidation, as Keilin & Hartree [1935, 2] have done to explain the coupled oxidation of alcohol by dehydrogenase systems. We do not believe that this is a satisfactory explanation. The meaning of the word "nascent" is not clear; nascent hydrogen peroxide may mean activated H_2O_2, or H_2O_2 constantly reformed in small concentration, or H_2O_2 held in combination. The first assumption does not explain the experiments of Keilin (activated H_2O_2 would hardly require the presence of a catalyst for a reaction and it would lose its activation energy in such a combination with the catalyst); it is also unnecessary for the explanation of our experiments in which H_2O_2 does not require activation. The slow delivery effect does not explain the experiments of Keilin, at least if catalase is supposed to be the catalyst for the oxidation of alcohol, since the formation of a catalase-H_2O_2 compound would have to precede this reaction as well as the catalytic H_2O_2 destruction. In our case it might appear to explain the observed facts, since a small amount of H_2O_2 escaping the action of catalase might be supposed to have a noticeable effect when constantly renewed. The catalase inhibition is, however, too small for such an explanation, and other peroxides which slowly liberate hydrogen peroxide and with which Keilin could replace the "nascent hydrogen peroxide" in his experiments, were in our experiment in the presence of catalase as inactive as H_2O_2. Should the third possibility be correct the term "combined" rather than "nascent" hydrogen peroxide would be suitable, and in our case the protection from catalase is best understood by assuming combination with haem, haem being the molecule with which the H_2O_2 is to react, and by assuming the formation of such a compound without intermediate formation of free H_2O_2. The mechanism described above fulfils these conditions and also explains why the reaction occurs only with substances which can be considered as hydrogen donators, but not with other reducers, as $Na_2S_2O_4$.

In pyridine-haemochromogen the six co-ordination positions of the iron atom are filled, four by the porphyrin nitrogens and two by pyridine nitrogens. O_2 must, therefore, remove one pyridine molecule from the Fe atom, as CO does in forming CO-haemochromogen. In an earlier publication it has been

[1] Verdohaematin combines with native globin to a haemochromogen, not to a verdohaemoglobin [Lemberg & Wyndham, 1936; Roche & Bénévent, 1937].

assumed that O_2 enters between the Fe atom and pyridine nitrogen forming a peroxide in which O_2, by the particular way in which it is bound in the compound, becomes a strong oxidizer. Such a formula explains less readily the second step of the oxidation of haematin which we assume in our more recent hypothesis, the hydrogenation of the peroxide to an H_2O_2-compound. To form this substance, one pyridine molecule must be removed completely, since H_2O_2 cannot be inserted between haem iron and pyridine nitrogen. This might therefore occur in the formation of the peroxide. The O_2 must be bound, however, in a different way from that in oxyhaemoglobin.

In contradistinction to oxyhaemoglobin pyridine-haemochromogen peroxide is unstable and breaks down to give ferric haemochromogen in a way which is not yet clear, but which does not require the presence of reducers. When these are present they react with the haem peroxide to give some haemochromogen-H_2O_2 compound in a reaction which proceeds simultaneously with the formation of ferric haemochromogen. There is no evidence that the oxidation of ferrous to ferric haemochromogen is accelerated by the presence of reducers, as we believed at first. The velocity of ascorbic acid oxidation which we can consider as a measure of the rate of oxidation of ferrous to ferric haemochromogen is therefore not decreased by catalase.

Quite different from the autoxidation of pyridine-haemochromogen is the oxidation of haemoglobin to methaemoglobin, since the reaction with O_2 in the absence of reducing substances or systems leads to oxyhaemoglobin, whereas in their presence methaemoglobin arises [cf. Warburg *et al.* 1931, where the earlier literature on the subject is reviewed]. The conditions under which methaemoglobin is formed are, indeed, often the same as those which cause green pigment formation (e.g. in the bacterial systems [Hart & Anderson, 1933]). Bernheim *et al.* [1936] and Bernheim & Michel [1937] have shown that dehydrogenase systems of tissues and other dehydrogenase systems oxidize haemoglobin to methaemoglobin in the presence of oxygen, and that catalase inhibits, but does not prevent the reaction. It is possible that here the H_2O_2 formed by the dehydrogenase systems oxidizes the haemoglobin and being constantly renewed escapes partly the destruction by catalase. It is also possible, however, that the activated substrates transfer their hydrogen to oxyhaemoglobin and thus form an unstable haemoglobin-H_2O_2 compound which breaks down to give methaemoglobin.

The experiments of Heubner *et al.* [1923] on the action of phenylhydroxylamine on oxyhaemoglobin leave no doubt that the two substances react. The catalytic effect of haemoglobin on the oxidation of phenylhydroxylamine and the rapid quantitative reaction of the latter with oxyhaemoglobin in the absence of free O_2 cannot be explained by the action of H_2O_2 formed by autoxidation of phenylhydroxylamine on oxyhaemoglobin, since the autoxidation of phenylhydroxylamine is much slower. The equations

$$HbO_2 + 2C_6H_6.NHOH \rightarrow Hb.H_2O_2 + C_6H_5.N_2O.C_6H_5;$$
$$Hb.H_2O_2 + HbO_2 \rightarrow 2HbOH + O_2$$

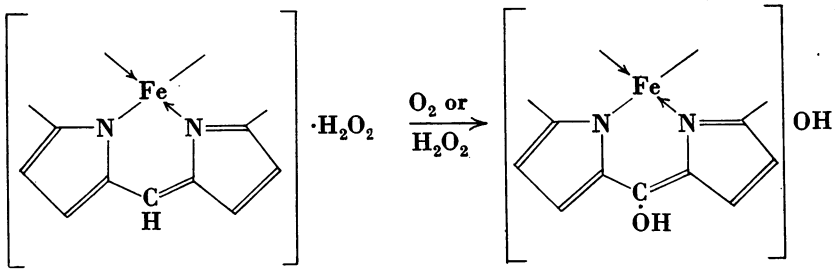
are in agreement with the observations of the authors.

It is also the only explanation applicable to the formation of green pigment from oxyhaemoglobin and ascorbic acid. The possibility had not been excluded by our experiments that the direct autoxidation of pyridine-haemochromogen without participation of H_2O_2 yields verdohaemochromogen when frequently repeated. The formation of green pigment cannot, however, be due to autoxidation of haemoglobin, since even often repeated exhaustion and oxygenation of

haemoglobin does not cause green pigment formation. If this were so, verdohaemochromogen would be formed in the normal course of events in the red cells, and there is no evidence for this. We have to assume that hydrogen donators cause the formation of green pigment by reducing oxyhaemoglobin to an unstable H_2O_2 compound.

More experimental material, however, is required to understand fully the oxidation of haemoglobin to "green pigment" and to methaemoglobin in the presence of reducing substances. For the formation of methaemoglobin the process assumed above is without doubt only one of several (formation by H_2O_2 or by oxidizing substances formed by oxidation of reducing substances).

The third step in the formation of verdohaemochromogen from pyridine-haemochromogen is the formation of the compound with the absorption band at 639mμ. which we consider to be a ferric haematin of an oxyporphyrin, a porphyrin in which oxygen, probably in the form of a hydroxyl group, has been attached to the methene bridge α. We can write:

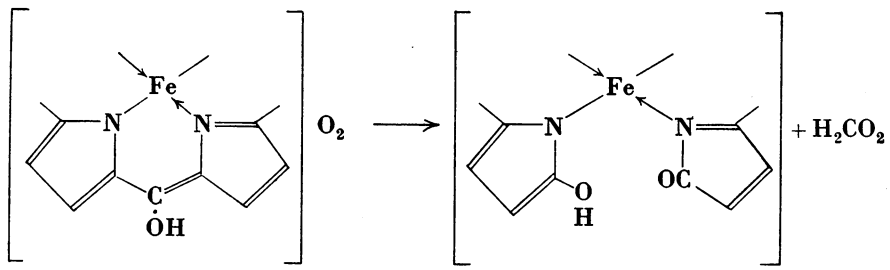


This compound is probably identical with a compound with the same absorption band, observed by Warburg *et al.* [1933] in *Bact. Pasteurianum*, when suspensions of the bacteria were shaken with air in the presence of cyanide. The absorption band at 639mμ. could not be observed under anaerobic conditions with or without cyanide or under aerobic conditions without cyanide. For these reasons Warburg considered the compound to be a cyanide compound of a ferric haematin different from both respiratory ferment and cytochrome. He assumed that it played an important part in the respiration of these bacteria, reducing the ferric form of respiratory ferment and oxidizing the ferrous form of cytochrome-*a*. He believed that in consequence of its high redox potential it should be found in the cell in the reduced form only, even under aerobic conditions, but that cyanide should shift the equilibrium to the ferric side, forming the relatively stable cyanide compound and thus inhibiting respiration. As a weak point of this theory he admitted the fact that no ferric haem-cyanide compound with an absorption band in this region is known.

If, as we believe, this compound is identical with oxyhaematin and is not a cyanide compound, it cannot be a normal constituent of the cells, since its band is not visible in the cells in the absence of cyanide. The action of cyanide in causing its formation must be more complex. Warburg has shown, that the greater the cyanide inhibition of the respiration, the stronger is the absorption band at 639mμ. Catalase, however, is very sensitive to cyanide, and its inactivation may allow the formation of hydrogen peroxide in the cells, which, as we have shown, forms oxyhaematin from protohaematin in the presence of hydrogen donators. This reaction is little inhibited by cyanide, and the absorption spectrum of the bacterial suspension makes the presence of protohaemochromogen probable. The formation of H_2O_2 in *Bact. Pasteurianum* has not been

proved owing to the presence of catalase, but Wieland & Pistor [1936] have made the presence of H_2O_2 and its reaction with alcohol probable in the similar *Acetobacter peroxidans* which is free from catalase, but contains haematin pigments.[1] In the lactic acid bacteria, which are also facultative aerobes but do not contain haematin pigments or catalase, the formation of H_2O_2 has been demonstrated. The respiration remaining in the presence of cyanide may suffice to form the small amounts of H_2O_2 necessary for the oxidation of protohaemochromogen to oxyhaematin. This will be further oxidized, but its formation is much more rapid than its further oxidation. We believe, therefore, that the compound observed by Warburg *et al.* in *Bact. Pasteurianum* is an artificial product arising in the cells only under non-physiological conditions.

The mechanism of the final step, the oxidation of oxyhaematin to verdohaematin, is not yet quite clear. Molecular oxygen is required for this reaction, whereas H_2O_2 either does not react, or destroys completely. The oxygen, already attached to the methene group α of oxyhaematin, makes it more liable to further oxidation (splitting of the porphyrin ring), but free oxyporphyrin is stable and does not break down to give bile pigments. The haem iron is, therefore, necessary for this reaction also. Since, however, the reaction appears to be independent of ascorbic acid concentration and the latter reduces oxyhaematin only slowly, the ferric form appears to be attacked by oxygen without previous reduction, and no satisfactory explanation can be given for the mechanism of such a reaction. Perhaps an oxidation product of ascorbic acid, possessing a stronger reducing power than the acid itself, causes reduction of the oxyhaematin to oxyhaemochromogen; weak absorption bands of oxyhaemochromogen remain, indeed, visible when a solution of it is shaken with air. The reaction would then be a true autoxidation of oxyhaemochromogen, leading partly to oxyhaematin and partly to verdohaemochromogen and several repetitions of this process would be required for a complete transformation of oxyhaematin to verdohaematin. This final step of the reaction we write as follows:



For the action of cysteine in forming oxyhaematin and verdohaematin Cu or Fe is necessary; they are required for the first step of the reaction, the reduction of ferric to ferrous haemochromogen. These metals form complex salts with cysteine which, by a valency exchange of their metal atom with that of haematin iron, perform the reduction of the latter, which cysteine is unable to do alone. The role which the metals play in this reaction deserves consideration. There might be a correlation of this action of the metals, particularly Cu, in blood pigment breakdown and their well-known activity in blood pigment formation. Both Cu and ascorbic acid are necessary to prevent anaemia. What

[1] This peroxidative destruction of alcohol may be similar to the action of "nascent hydrogen peroxide" on alcohol found by Keilin & Hartree.

this correlation may be, however, is still quite obscure, particularly since Cu does not enable glutathione to reduce ferric haemochromogen and to form verdohaemochromogen.

SUMMARY

As intermediate product in the formation of verdohaemochromogen from pyridine haemochromogen by O_2 in the presence of ascorbic acid, a haematin compound with an absorption band at 639mμ. is found, which is characterized as a ferric haematin compound of an oxyporphyrin.

This substance can be formed from protohaemochromogen by the action of hydrogen peroxide, and is probably identical with the compound having the same absorption band, observed by Warburg and co-workers in *Bact. Pasteurianum* under aerobic conditions in the presence of cyanide.

By action of molecular oxygen it is transformed into verdohaemochromogen.

Aerobic verdohaemochromogen formation is inhibited, but not prevented, by catalase, whereas the enzyme completely prevents the formation of the oxyporphyrin-haematin by hydrogen peroxide. The presence of free hydrogen peroxide during the aerobic formation of verdohaemochromogen cannot be proved by other means than the catalase inhibition. From these observations it is concluded that the first steps of the oxidation of protohaemochromogen to verdohaemochromogen are the formation of a haemochromogen peroxide (Warburg) and its hydrogenation (Wieland) to a haem-hydrogen peroxide compound..

The oxidation of ascorbic acid is due to ferric haemochromogen formed by autoxidation of ferrous haemochromogen, and this reaction is independent of the reaction mechanism causing the formation of verdohaemochromogen.

Ascorbic acid in these experiments can be replaced by cysteine + copper or cysteine + iron, not, however, by metal-free cysteine or by glutathione with or without metal. Glutathione and metal-free cysteine cause the formation of a haemochromogen-*a*.

The formation of "green pigment" from haemoglobin and oxygen in the presence of ascorbic acid and other hydrogen donators is discussed and its mechanism is found to be essentially the same. This mechanism is probably responsible for some instances of methaemoglobin formation.

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