Reactions of Haematins with Peroxides

By JOAN KEILIN*
Biochemical Department, University of Cambridge

(*Received* 12 *October* 1951)

It is well known that hydrogen peroxide reacts with haemoproteins such as methaemoglobin, catalase and peroxidase, giving a series of compounds which can be distinguished by their absorption spectra. In recent years much work has been done on the mechanism of these reactions, and it was found that catalase and peroxidase are each capable of forming at least three distinct complexes with hydrogen peroxide. The properties of these complexes and the literature on the subject of their formation has recently been reviewed by Theorell (1947), Lemberg & Legge (1949), and Keilin & Hartree (1951). Since in all these complexes it is the haematin prosthetic group which reacts directly with the peroxide, it was of interest to find whether free haematins can combine with peroxide. So far the only attempt to study this problem was made by Haurowitz (1937*a*, *b*), whose observations on the reaction between protohaematin and hydrogen peroxide in pyridine will be discussed later in the light of the findings reported here.

In this paper it is proposed to examine the conditions under which free haematins react with hydrogen peroxide and ethyl hydroperoxide and the nature of the products of these reactions, the experiments being carried out both with urohaemin and protohaemin. Urohaemin was selected for this purpose because, being much more soluble than protohaemin on account of its eight carboxyl side chains, it had been found to react with many substances such as sodium hydroxide and amino-acids with much greater ease than protohaemin (Keilin, J., 1949, 1950).

EXPERIMENTAL

Protohaemin. This was prepared from ox or horse blood by the method of Schalfejew (1885).

Urohaematin. Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The uroporphyrin was esterified, and urohaemin was prepared from the octamethyl ester according to the method of Fischer & Orth (1934). A $6{\cdot}1 \times 10^{-4}$M solution of urohaematin in 0·02N-NaOH was obtained by dissolving the urohaemin in 5 ml. 0·1N-NaOH, and the volume was made up to 25 ml. with distilled water.

Hydrogen peroxide. The molarity of H_2O_2 in a solution containing 1 ml. perhydrol in 250 ml. distilled water was determined by titration with standard $KMnO_4$ in the usual way. Further dilutions of the H_2O_2 solution were made as required daily. A fresh stock solution was prepared and standardized every few days.

Ethyl hydroperoxide. This was prepared and standardized according to the method of Baeyer & Villiger (1901).

Spectroscopic methods. The direct observations of the reactions were carried out with a microspectroscope. The absorption spectra in the visible and violet regions were determined with either the Beckman photoelectric spectrophotometer or the Hilger Uvispek photometer; the latter instrument was used for measuring the high absorption densities in the region of the Soret band. The molecular absorption coefficient ϵ is defined as follows: $\epsilon = E/cl$, where c = molarity of the haematin solution, l = optical depth in cm., and E (extinction) = $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light respectively.

RESULTS

The absorption spectrum of the urohaematin-H_2O_2 complex

When 0·5 ml. of a $6{\cdot}1 \times 10^{-4}$M solution of urohaematin in 0·2N-NaOH is treated with an equal volume of 0·27M-H_2O_2 in the presence of 2 ml. N-NaOH, the colour of the solution at once changes from reddish brown to salmon-pink. At the same time, on direct spectroscopic examination, it is seen that the absorption band of urohaematin at 594 mμ. is replaced by two well defined bands in the green region of the spectrum, of which the β-band is a little stronger than the α-band. This absorption spectrum is, however, of very short duration, and within 45–60 sec. the urohaematin band at 594 mμ. begins to reappear as a shading in the yellow region of the spectrum. This band gradually becomes stronger, and the two bands in the green region diminish in intensity as the complex reverts to free urohaematin. These changes, although they begin to take place very soon after the complex is formed, slow down after about 1·5–2 min. and thereafter progress more slowly so that the peroxide complex takes several hours to revert completely to free urohaematin (Fig. 1). At the same time some molecular oxygen is liberated from the H_2O_2, and there is also some peroxidatic destruction of the urohaematin itself.

In view of the rapid onset of changes in the absorption spectrum of the complex, a modified technique had to be adopted for recording the spectrophotometric curve and the urohaematin-peroxide complex was freshly made for each point of the curve. The following procedure was found to

* Beit Memorial Research Fellow.

be the most convenient: 0·5 ml. of a $6{\cdot}1 \times 10^{-4}$M solution of urohaematin was placed in a 1 cm. cell and diluted with 2 ml. N-NaOH (final concentration of NaOH = 0·7N). After adjusting the instrument at the selected wavelength with the control cell in position, 0·5 ml. of a 0·27M solution of H_2O_2 (450 mol. H_2O_2/mol. urohaematin) was added at zero time by means of an all-glass syringe fitted with a stainless steel needle and the contents of the cell were stirred with a glass rod. Three to four readings of the extinction were taken within the first 2 min.; readings were then taken at 1 min. intervals for the next 5 or 10 min., so long as O_2 bubbles in the solution did not obscure the optical surfaces of the cell or could be dislodged from them by tapping the cell between readings. The changes of extinction were plotted against time and the spectrophotometric curves were constructed from the extinction obtained by extrapolating back to zero time, assuming the reaction to take place instantaneously on the addition of the hydrogen peroxide to the urohaematin. To determine the absorption spectrum of the Soret band, 0·25 ml. H_2O_2 (750 mol. H_2O_2/mol. urohaematin) was added to 0·15 ml. urohaematin diluted with 2·6 ml. of the appropriate alkali. The rest of the operation was carried out as described for the visible region of the spectrum.

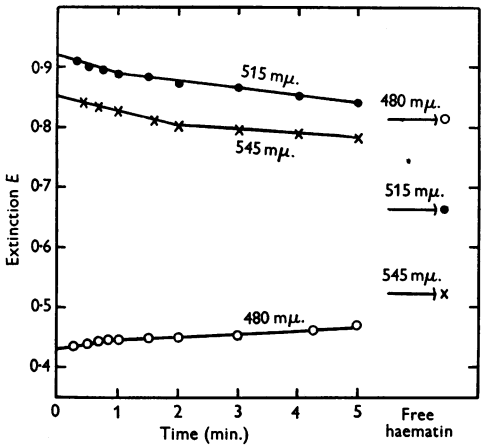


Fig. 1. Reaction of urohaematin with H_2O_2 in 0·7N-NaOH. Typical changes in extinction with time at three wavelengths (515, 545 and 480 mμ.) showing the gradual reversion of the absorption spectrum of the compound to that of free haematin, indicated by the separate points on the right of the figure. The values of $\epsilon \times 10^{-4}$ used for Fig. 2 were obtained by extrapolating back to zero time. (ϵ as defined in text.)

The spectrophotometric curves of the urohaematin-peroxide compound obtained in this way show that the positions of the two bands in the visible region of the spectrum are α—547·5 mμ. and β—521 mμ., while the values of $\epsilon \times 10^{-4}$ are 0·87 and 0·95 respectively. In the violet region of the spectrum the Soret band of the urohaematin at 395 mμ. is replaced by the single symmetrical band of the peroxide compound at 413 mμ. ($\epsilon \times 10^{-4} = 7{\cdot}9$) (Figs. 2 and 3).

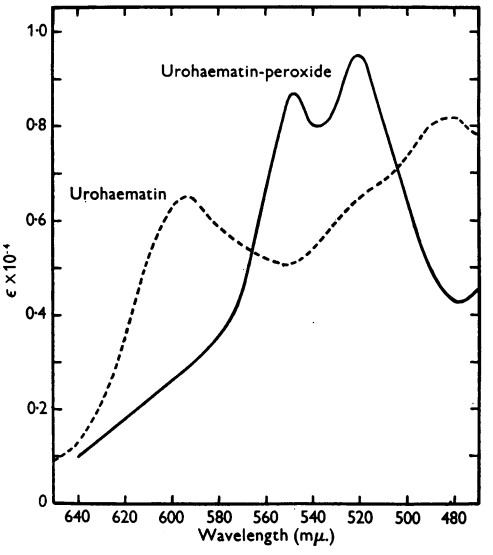


Fig. 2. Absorption bands in the visible region of the spectrum of urohaematin and urohaematin-peroxide in 0·7N-NaOH. (Urohaematin = $1{\cdot}02 \times 10^{-4}$M; $H_2O_2 = 4{\cdot}5 \times 10^{-2}$M; $l = 1$ cm.)

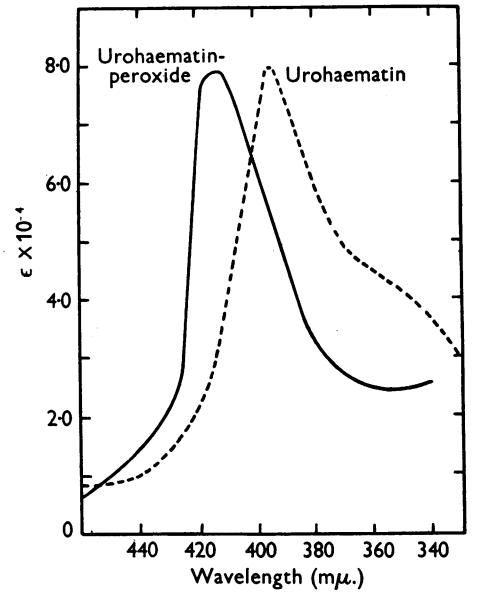


Fig. 3. Absorption spectra showing the Soret bands of urohaematin and urohaematin-peroxide in 0·7N-NaOH. (Urohaematin = $3{\cdot}05 \times 10^{-5}$M; $H_2O_2 = 2{\cdot}25 \times 10^{-2}$M; $l =$ 1 cm.)

Stoicheiometric relationships

In order to determine the number of molecules of H_2O_2 combined with each molecule of urohaematin, the urohaematin was treated with various concentrations of H_2O_2 in the presence of 0·7 N-NaOH under standard conditions and the percentage formation of the urohaematin-peroxide compound formed in each case was determined as follows. To 0·5 ml. of a $6{\cdot}1 \times 10^{-4}$ M solution of urohaematin in 0·02 M-NaOH in a 1 cm. optical cell, were added 2 ml. M-NaOH and the calculated amount of distilled water required to bring the total volume of the solution to 3 ml. after the addition of the H_2O_2. A dilute solution of H_2O_2 was introduced into the cell at zero time by means of a syringe so as to provide concentrations of H_2O_2 in the solution ranging from $2{\cdot}05 \times 10^{-4}$ to $4{\cdot}65 \times 10^{-2}$ M, i.e. from 2 to 465 mol. H_2O_2/mol. urohaematin. The extinction at 594 mμ. was then measured at intervals during the first 2 min. and the value at zero time was obtained by extrapolation as described in the previous section. From these values the percentage formation (x) of the peroxide compound at each concentration of H_2O_2 was calculated by means of the formula $x = 100(a-c)/(a-b)$, where a = extinction of free urohaematin, b = extinction of the fully formed urohaematin-peroxide (465 mol. H_2O_2/mol. urohaematin), and c = extinction of the solution under investigation, all the extinctions being measured at the same wavelength (594 mμ.).

If the reaction is represented by the following equation:

$$\text{Urohaematin} + n H_2O_2 \rightleftharpoons \text{Urohaematin } (H_2O_2)_n,$$

then by the law of mass action

$$K = \frac{[\text{Urohaematin}]\,[H_2O_2]^n}{[\text{Urohaematin } (H_2O_2)_n]}.$$

Hence if x represents the percentage of the total urohaematin which is combined with H_2O_2, then

$$\frac{1}{K} = \frac{x}{(100-x)\,[H_2O_2]^n},$$

whence

$$\log [x/(100-x)] = n \log [H_2O_2] - \log K.$$

By plotting $\log [x/(100-x)]$ against $\log [H_2O_2]$ a straight line can be obtained which has a slope of n. When $x = 50$,

$$\log [x/(100-x)] = 0 \quad \text{and} \quad \log K = n \log [H_2O_2].$$

Thus the value of $\log H_2O_2$ when

$$\log [x/(100-x)] = 0, \quad \text{equals} \quad 1/n \log K.$$

Since n can be calculated from the slope of the line, $\log K$ can be obtained. The position of the line and the values of n and K were obtained by applying the method of least squares to the experimental results after a correction had been made for H_2O_2 combined with urohaematin (Table 1, Fig. 4). It was found that 1 molecule of peroxide is combined per molecule of urohaematin ($n = 1{\cdot}085$) and the dissociation constant $K = 3{\cdot}34 \times 10^{-4}$ M at 20°.

Table 1. *The relation between the concentration of* H_2O_2 *and percentage formation of urohaematin-peroxide as determined experimentally*

Molecular ratio H_2O_2 to urohaematin	Formation of complex (%) (x)	Free urohaematin (%) ($100-x$)	$\log\left(\frac{x}{100-x}\right)$	Total H_2O_2 concn. (M)	Free H_2O_2 concn. (M)	Free H_2O_2 concn. (log M)
2	22·8	77·2	−0·5287	$2{\cdot}046 \times 10^{-4}$	$1{\cdot}811 \times 10^{-4}$	−3·7423
5	38·0	62·0	−0·2125	$5{\cdot}115 \times 10^{-4}$	$4{\cdot}707 \times 10^{-4}$	−3·3273
10	55·8	44·2	+0·1011	$1{\cdot}023 \times 10^{-3}$	$9{\cdot}66 \times 10^{-4}$	−3·0150
15	77·1	22·9	+0·5250	$1{\cdot}534 \times 10^{-3}$	$1{\cdot}455 \times 10^{-3}$	−2·8371

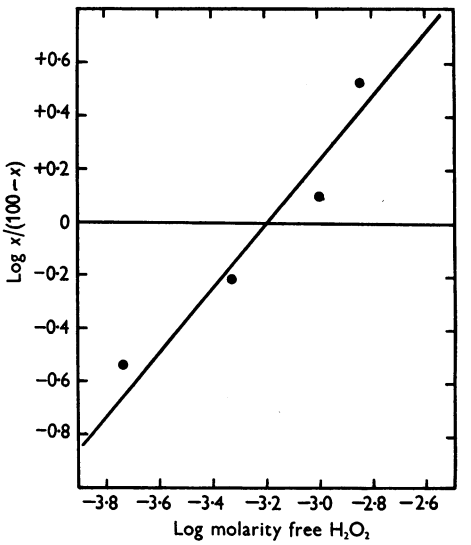


Fig. 4. Relationship between log molarity H_2O_2 and $\log [x/(100-x)]$, where x = percentage formation of urohaematin-peroxide, and $(100-x)$ = percentage of free urohaematin. The experimental results are indicated by dots. The position of the line was obtained by applying the method of least squares to the experimental results. Correction has been made for the H_2O_2 combined with the urohaematin (Table 1).

Conditions required for the formation of the urohaematin-H_2O_2 complex

If urohaematin in 0·2N-NaOH is treated with H_2O_2 in the presence of 0·1N-NaOH instead of N-NaOH as described above, the complex formation never proceeds to completion and a shadow due to the persistence of the free urohaematin band is always seen in the yellow region of the spectrum. A spectrophotometric curve constructed under these conditions (total concentration of NaOH = 0·07N) showed that both bands were nearer the blue end of the spectrum and their relative intensities were reversed (α—544·5 mμ., $\epsilon \times 10^{-4} = 0{\cdot}9$; β—516 mμ., $\epsilon \times 10^{-4} = 0{\cdot}845$). The urohaematin band persisted as a shoulder about 594 mμ. and from the extinction readings at this wavelength it was calculated that only 83% of the urohaematin had reacted with H_2O_2. On the addition of more H_2O_2 to such a solution in an attempt to produce 100% formation of the complex, the colour of the solution turned to greenish brown and the absorption bands disappeared, indicating that the urohaematin was destroyed.

Similar results are observed using the microspectroscope when the total concentration of NaOH is 0·016N or when 0·1 or 0·28M-Na_2CO_3 is used as diluting fluid instead of NaOH. If, however, under these conditions a few drops of N-NaOH are added the complex formation proceeds to completion.

Urohaematin dissolved in 0·1M-phosphate buffer, pH 7·3, fails to react with H_2O_2 and is, instead, rapidly destroyed. The same results are obtained when the solution is made more alkaline by the addition of disodium phosphate or Na_2CO_3 (0·1, 0·28 or 1·4M), but the addition of NaOH to urohaematin in phosphate buffer allows the peroxide compound to be formed as already described.

When urohaematin is dissolved in 10% (v/v) ammonia, the crimson solution of ammonia uroparahaematin shows a very broad and diffuse absorption band in the green region of the spectrum extending from 580 to 510 mμ. with a sharper component centred at 540 mμ. On the addition of H_2O_2 (50 mol. H_2O_2/mol. urohaematin) the colour of the solution changes to a scarlet red and the two bands of the peroxide compound appear, only to give way in a few minutes to the absorption band of the parahaematin. If sodium dithionite ($Na_2S_2O_4$) is added to the urohaematin-peroxide compound in ammonia, the intense absorption bands of the ammonia haemochromogen immediately appear, whereas reduction of urohaematin-peroxide in NaOH gives rise to dihydroxyl-haem (Keilin, J., 1949).

Reactions of pyridine uroparahaematin with H_2O_2

Urohaemin is not readily soluble in pyridine, and the pyridine parahaematin is only obtained in aqueous solution at approximately neutral or slightly acid pH. The red solution then shows the characteristic two-banded spectrum of a parahaematin in which the β-band is stronger and broader than the α-band (α—555 mμ., $\epsilon \times 10^{-4} = 0{\cdot}69$; β—525 mμ., $\epsilon \times 10^{-4} = 0{\cdot}92$). On the addition of H_2O_2 to the parahaematin at about pH 7 the red

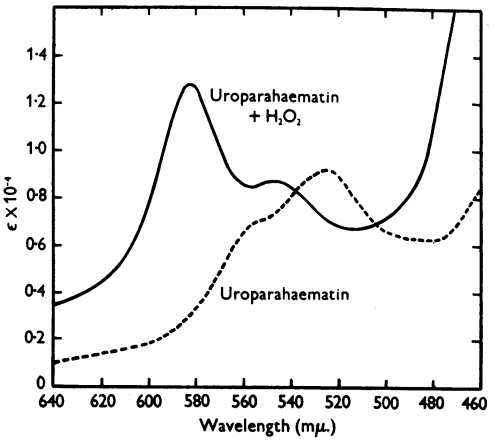


Fig. 5. Absorption bands in the visible region of the spectrum of pyridine uroparahaematin and the green compound given by it on the addition of H_2O_2. (Uroparahaematin = $1{\cdot}045 \times 10^{-4}$M; $H_2O_2 = 4{\cdot}5 \times 10^{-3}$M; l = 1 cm.)

solution quickly turns brown and appears green when viewed in a thin layer. Direct spectroscopic observation shows that this colour change is accompanied by the disappearance of the parahaematin absorption bands. After a slight lag of about 15 sec. during which no absorption bands are visible, two bands appear and gradually grow in intensity. The α-band at 582 mμ. becomes very strong while the β-band at 547 mμ. remains weak (Fig. 5). This absorption spectrum is stable and, so long as excess H_2O_2 is not present to bleach the solution, it may remain for at least 24 hr. At pH 4 these absorption bands appear more intense, while the addition of NaOH causes them to become weaker and finally to disappear. The addition of $Na_2S_2O_4$ to the fully formed green compound at either acid or alkaline pH does not result in the formation of any pyridine haemochromogen, and there is no spectroscopic evidence that the compound combines with CO (either before or after the addition of $Na_2S_2O_4$) or is affected by potassium ferricyanide.

The spectrophotometric curve of the green peroxide-treated parahaematin ('green compound')

was determined as follows: to 0·5 ml. of $1·045 \times 10^{-4}$ M-urohaematin in 0·02 N-NaOH were added 0·75 ml. of 0·02 M-acetic acid, 0·5 ml. pyridine and 0·5 ml. of 0·027 M-H_2O_2 and the volume of the solution (containing 50 mol. H_2O_2/mol. haematin) was brought up to 3 ml. with distilled water; for pyridine uroparahaematin the H_2O_2 was replaced by distilled water. The spectrophotometric curves were determined in the usual way in the visible region, but in the case of the green compounds it was found that,

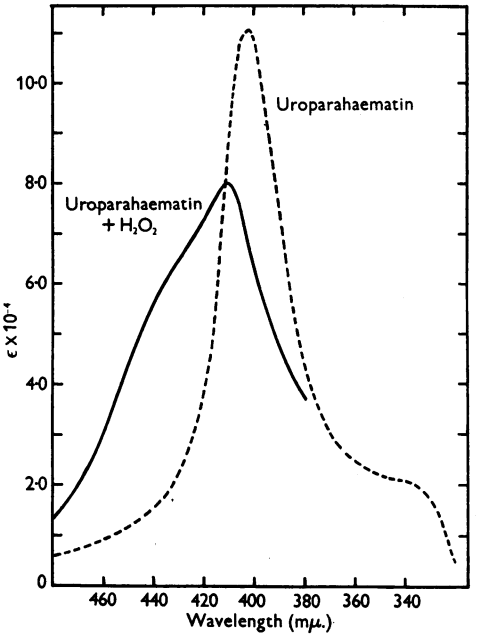


Fig. 6. Absorption spectra showing the Soret bands of pyridine uroparahaematin and the green compound given by it on the addition of H_2O_2. (Uroparahaematin = $1·016 \times 10^{-4}$ M; $H_2O_2 = 2·97 \times 10^{-3}$ M; $l = 1$ cm.)

at the much greater dilution required for measuring the Soret band, this band was very evanescent. A fresh solution therefore had to be prepared for measuring each point of the curve when determining the height of the Soret band and the peroxide was added last to the cell by means of a syringe as described earlier in this paper. Even with these precautions it was not possible to obtain reliable extinction readings. As seen in Fig. 6, the asymmetric Soret band of the 'green compound' has a main peak at 410 mμ. ($\epsilon \times 10^{-4} = 8·0$ approx.) and a shoulder at 435 mμ. which may be due to some intermediate compound.

Reactions of protohaemin and H_2O_2 in pyridine

In view of the differences between the reactions of urohaematin with H_2O_2 described above and the results obtained by Haurowitz (1937*a*, *b*) which will be fully discussed later, it was decided to re-examine the effect of H_2O_2 on protohaematin in pyridine under various conditions.

Protohaemin dissolves readily in pyridine giving a red parahaematin with the usual two-banded absorption spectrum (α—560 mμ., β—530 mμ.). On the addition of H_2O_2 (perhydrol diluted as required with pyridine), the solution becomes greenish brown and a diffuse absorption band appears at about 605 mμ. while the α-band of the

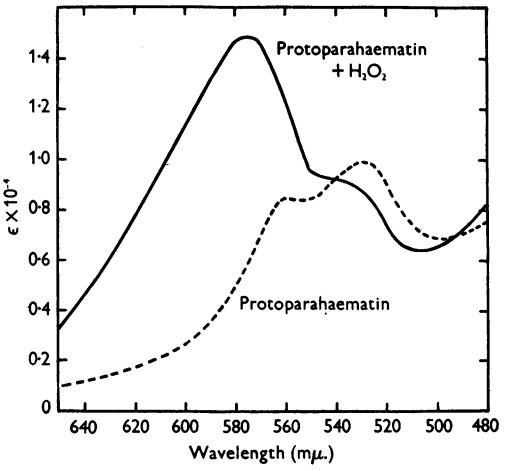


Fig. 7. Absorption bands in the visible region of the spectrum of pyridine protoparahaematin and the green compound obtained on treating it with H_2O_2. (Protoparahaematin = $1·32 \times 10^{-4}$ M; $H_2O_2 = 1·86 \times 10^{-2}$ M; $l =$ 1 cm.)

parahaematin becomes stronger and the β-band at 530 mμ. becomes weaker. The band in the red then tends to become stronger and broader and to move towards the blue end of the spectrum so that its centre lies at about 583 mμ. The general appearance is therefore of a two-banded spectrum with a broad, strong band at 583 mμ. and a weaker band at 560 mμ., but the exact positions of these bands depend on both the pH of the solution and the amount of H_2O_2 added. Thus at pH 6·5 the solution is green with red dichroism; the α-band is markedly asymmetric and appears to extend from 570 to 600 mμ. on direct spectroscopic examination, though the spectrophotometric curve shows that the peak lies at 574 mμ. (Fig. 7). A weak β-band lies at 535 mμ., while in the violet region the Soret band of the parahaematin at 403·5 mμ. is replaced by the band of the 'green compound' at 426 mμ. On reduction with $Na_2S_2O_4$ a haemochromogen (α—558 mμ., β—530 mμ.) is formed by that part of the protohaem which has not been irreversibly altered by the peroxide. With greater concentrations of H_2O_2 the bands at 583 and 560 mμ. appear more

directly and soon disappear due to the destruction of the protohaematin. Under such conditions, therefore, no haemochromogen is obtained on the addition of $Na_2S_2O_4$.

Reactions between ethyl hydroperoxide and haematins

Ethyl hydroperoxide (EtOOH) reacts with haemoproteins giving, in most cases, complexes which are analogous to those obtained with H_2O_2, so it was of interest to examine the effect of EtOOH on uro- and proto-haematin.

With urohaematin in NaOH, EtOOH gave an unstable red compound with two absorption bands in the visible region of the spectrum in the same positions as those of the urohaematin-OOH compound. This spectrum could already be observed in solutions containing 0·02 N-NaOH, but the concentration of NaOH in the solution had to be raised to 1·5 N before the compound was completely formed, as shown by the disappearance of the band of free strong, wide β-band at 546 mμ. The reduction of urohaematin-OOEt to dihydroxyl-urohaem can, of course, be brought about at once by the addition of $Na_2S_2O_4$ to the compound.

With pyridine uroparahaematin, EtOOH reacted somewhat differently from H_2O_2. The scarlet colour of the parahaematin gave way to a greenish brown; at the same time the absorption bands of the parahaematin became diffuse, disappeared and were replaced by two bands, a strong α-band at 575 mμ. and a weak β-band at 537 mμ. After standing a few minutes (or immediately on the addition of $Na_2S_2O_4$) these bands were replaced by a wide asymmetric α-band lying at 560 mμ. and a weak β-band at 527 mμ. belonging to a reduced compound. This absorption spectrum remained unchanged in the presence of CO thus indicating that it was not a true haemochromogen, and it reverted to the oxidized form on the addition of potassium ferricyanide (Table 2).

Table 2. *Positions and extinction coefficients of the α-, β- and γ-bands of the compounds of urohaematin and protohaematin with H_2O_2 and ethyl hydroperoxide*

	α-Band		β-Band		γ-Band	
Compound	Wavelength (mμ.)	$\epsilon \times 10^{-4}$	Wavelength (mμ.)	$\epsilon \times 10^{-4}$	Wavelength (mμ.)	$\epsilon \times 10^{-4}$
Urohaematin in 0·7 N-NaOH	594	0·65	483	0·82	395	8·0
Urohaematin in 0·7 N-NaOH + H_2O_2	547·5	0·87	521	0·95	413	7·9
Urohaematin in 1·5 N-NaOH + EtOOH	547·5*	—	521*	—	—	—
Reduction compound of urohaematin in 1·5 N-NaOH + EtOOH	578*	—	546*	—	—	—
Dihydroxyl urohaem†	578	0·76	546	1·4	435	11
Pyridine uroparahaematin	555	0·69	525	0·92	402	11·05
Pyridine uroparahaematin + H_2O_2	582	1·27	547	0·87	410	8·0
Pyridine uroparahaematin + EtOOH	575*	—	537*	—	—	—
Reduction compound of pyridine uroparahaematin + EtOOH	560*	—	527*	—	—	—
Pyridine urohaemochromogen (pH 7)	550*	—	520*	—	—	—
Pyridine protoparahaematin	(560	0·84	530	0·99	403·5	6·55
Pyridine protoparahaematin + H_2O_2 (pH 8 approx.)	605)* 583*	—	560	—	—	—
Pyridine protoparahaematin + H_2O_2 (pH 6·5)	574	1·48	535	0·91	426	5·0
Pyridine protoparahaematin + EtOOH (pH 7·8)	(605)* 562*	—	530*	—	—	—
Pyridine protohaemochromogen	558*	—	530*	—	—	—

* Positions of bands determined with microspectroscope. † Keilin, J., 1949.

urohaematin at 594 mμ. Increasing the amount of EtOOH at lower concentrations of NaOH merely led to the destruction of the urohaematin.

When the red urohaematin-OOEt compound was formed under optimal conditions and allowed to stand, the following changes took place within about 3 min. The absorption bands of the compound faded, the solution becoming orange-pink in colour, and they were then gradually replaced by the characteristic absorption spectrum of the red ferrous compound, dihydroxyl-urohaem, which consists of a narrow, weak α-band at 578 mμ. and a

Since ethyl hydroperoxide decomposes spontaneously to give acetaldehyde and water, it was necessary to examine the action of acetaldehyde on various urohaematin compounds before attributing to EtOOH the spontaneous reductions of urohaematin-OOEt and the green compound given by the parahaematin. Acetaldehyde was therefore added to alkaline urohaematin and to pyridine uroparahaematin but no changes were observed in the absorption spectra of these compounds.

In the case of protohaematin no reaction was observed between EtOOH and alkaline protohaematin,

although various concentrations of NaOH and both aqueous and ethanolic solutions of protohaematin were examined. Ethanolic solutions were investigated, since it appeared likely that a haematin requires for its reaction with peroxide the same conditions as are necessary for it to give a dihydroxyl haem in its reduced state (protohaematin can only react with NaOH to give its dihydroxyl compound in the presence of a solvent such as ethanol). This point will be discussed more fully later.

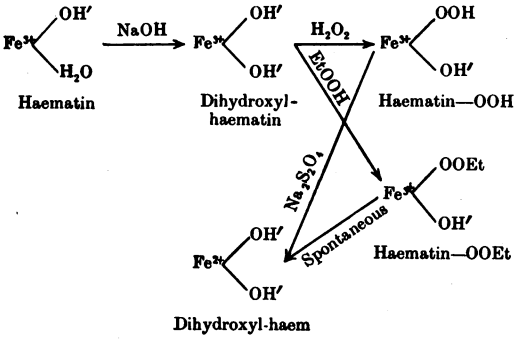
Ethyl hydroperoxide did, however, react with pyridine protoparahaematin. At pH 7·8 the parahaematin solution became greenish brown on the addition of EtOOH and an absorption band was seen for about 30 sec. at 605 mμ. Meanwhile, the band at 560 mμ. became stronger and the parahaematin spectrum was spontaneously replaced by that of a fairly stable green compound with α- and β-bands at 562 and 530 mμ. respectively, the change being complete within a few minutes. This compound, which failed to react with CO and was not affected by $Na_2S_2O_4$, is probably analogous to the reduction compound given by pyridine uroparahaematin and EtOOH. If less EtOOH were used, the reaction was complicated by the final appearance of a haemochromogen (α—558 mμ., β—530 mμ.) given by some of the haematin which had not been altered by EtOOH. This haemochromogen reacted normally with CO.

DISCUSSION

Urohaematin, although very freely soluble in all dilute alkalis and alkaline buffer solutions, was found to react with H_2O_2 and with ethyl hydroperoxide to form reversible compounds only when excess hydroxyl ion was present as in sodium hydroxide solutions. A well defined, though unstable, compound was then formed with two absorption bands in the visible region of the spectrum (α—547·5 mμ., β—521 mμ.). This absorption spectrum was of relatively short duration, and in the case of the H_2O_2 compound it reverted to that of free urohaematin. Titration of urohaematin with H_2O_2 showed that one molecule of H_2O_2 is combined per molecule of urohaematin.

It is now generally accepted that in alkaline haematins the iron atom is co-ordinated in positions 5 and 6 with one hydroxyl group and one molecule of water (Davies, 1940; Shack & Clark, 1947), whereas in haems (ferroporphyrins) these places are occupied by 2 molecules of water. It was recently shown (Keilin, 1949) that under certain conditions in the presence of NaOH, haems react with the hydroxyl ion giving dihydroxyl-haems in which two hydroyxl groups are co-ordinated per atom of haem iron.

Since, for the formation of the peroxide compound, urohaematin also requires the presence of excess hydroxyl ion, and reduction of the urohaematin-peroxide compound with $Na_2S_2O_4$ gives dihydroxyl-urohaem, it is suggested that a dihydroxyl-urohaematin may be an intermediate in the reaction between urohaematin and H_2O_2, one molecule of H_2O_2 replacing a hydroxyl group on the urohaematin iron. As ethyl hydroperoxide gives a compound with alkaline urohaematin analogous to that given by H_2O_2 and under the same conditions, one may assume that the mechanism of formation of the peroxide compound is the same in each case. It was found, however, that urohaematin-OOEt is spontaneously reduced to dihydroxyl-urohaem in a few minutes. Since acetaldehyde, the decomposition product of ethyl hydroperoxide, cannot cause this reduction one must conclude that it is probably due to the reducing action of ethyl hydroperoxide itself. As there is no ferrous form of urohaematin-peroxide a change in the valency of the urohaematin iron must result in the formation of dihydroxyl-urohaem. In the following scheme, which summarizes these reactions, the lines joining the urohaematin iron to the reacting groups do not indicate the nature of the valency bonds and the four valencies directed towards the pyrrol nitrogen atoms are omitted.



So far a protohaematin-peroxide compound analogous to that given by urohaematin has not been obtained, but this does not preclude its existence and it is possible that the conditions for its formation may yet be found.

Haurowitz (1937*a*, *b*), in his important investigations on the reactions between protohaematin and H_2O_2 in pyridine, described a transient two-banded absorption spectrum (α—582 mμ. and β—573 mμ. approx.) which was replaced by a single asymmetrical band in the visible region at 575 mμ. On the basis of these absorption bands Haurowitz postulated the existence of two protohaematin peroxide compounds in which a molecule of H_2O_2 replaced in turn each of the 2 molecules of pyridine of the parahaematin, thus giving a 'primary' and 'secondary' compound respectively.

It was mentioned earlier that in alkaline solution the addition of H_2O_2 to pyridine-protoparahaematin caused an absorption band to appear at about 583 mμ. while the parahaematin α-band at 560–565 mμ. was reinforced and, together with the new band, presented the appearance of a two-banded spectrum. It is probably this absorption spectrum which was observed by Haurowitz but ascribed by him to the 'primary compound'. The band at 575 mμ., which he attributed to the 'secondary compound', corresponds to that given best by proto-parahaematin with H_2O_2 in neutral or slightly acid conditions (Fig. 7); the fact that this band is asymmetrical and is shifted nearer to the blue end of the spectrum than that of the green compound given by uroparahaematin with H_2O_2 may indicate that it is due to a reduction compound similar to that given by ethyl hydroperoxide with both uro- and proto-parahaematins.

The actions of H_2O_2 on the pyridine-parahaematins of uro- and proto-haemins resulting in the formation of the green compounds are completely different from their actions on urohaematin in NaOH. The non-reversibility of formation of the green compounds, together with the fact that when fully formed they fail to give typical pyridine haemochromogens on the addition of $Na_2S_2O_4$, suggests that the haematin has been irreversibly modified in some way, although the presence of a Soret band indicates that the ring has not actually been opened. These green compounds therefore cannot be considered as haematin-peroxide compounds in which the peroxide is co-ordinated with the haematin iron, as was suggested by Haurowitz, but more probably belong to the oxyporphyrin class of pigments.

The reducing action of a peroxide was first described by Kuhn & Wassermann (1933), who showed that ferric salts can be reduced by H_2O_2 to ferrous salts which are detected by their ability to combine with 2:2′-dipyridyl to give the well known red complex. In the course of their work on azide catalase, Keilin & Hartree (1945) showed that H_2O_2 can reduce azide catalase to a well defined ferrous compound, and recently they have shown that the methaemoglobin-peroxide complex may, by further treatment with H_2O_2, be reduced to haemoglobin which combines with O_2 liberated during the reaction to form oxyhaemoglobin (Keilin & Hartree, 1951). However, on treating methaemoglobin with very small concentrations of H_2O_2 below pH 5, the absorption bands of methaemoglobin disappeared and were replaced by a wide band at 590 mμ. belonging to a new compound showing red-green dichroism, which could be reduced by $Na_2S_2O_4$ to haemoglobin. From a consideration of its spectroscopic properties, Keilin & Hartree concluded that it was an oxidation product of haemoglobin in which the haematin ring, though still intact, was modified, probably at one of the methine bridges. This compound bears a strong resemblance to that obtained by treating pyridine protohaematin with H_2O_2. Thus H_2O_2 can be both a reducing agent and an oxidizing agent in the same system, acting on different parts of the molecule according to the conditions of the experiment.

So far most work has been done on the complex reactions between the haemoproteins catalase, peroxidase and methaemoglobin with H_2O_2, but free protohaematin, the prosthetic group of these haemoproteins, was not known to react with H_2O_2 in any way except when co-ordinated with pyridine. It has now been shown that the compound formed in this reaction is merely an oxidation product of haematin and is not a true haematin peroxide compound such as it is necessary to visualize in the biological systems mentioned. Urohaematin, however, probably by virtue of being fully dispersed in solution, forms a true compound with H_2O_2 and with ethyl hydroperoxide and so provides a model for a free haematin peroxide compound where the peroxide is co-ordinated with the iron while the porphyrin ring remains unaltered.

SUMMARY

1. Urohaematin is shown to combine with hydrogen peroxide and ethyl hydroperoxide in the presence of excess hydroxyl ion. For complete formation of the complex the total concentration of sodium hydroxide required was 0·7 N in the case of hydrogen peroxide and 1·5 N for ethyl hydroperoxide. The effect of hydrogen peroxide on urohaematin in other alkalis is discussed.

2. The urohaematin-peroxide complex has a characteristic absorption spectrum (α—547·5 mμ.; β—521 mμ.; γ—413 mμ.) which is of short duration. In the case of hydrogen peroxide the absorption bands of free urohaematin begin to replace it within a few seconds while with ethyl hydroperoxide the compound is reduced to dihydroxyl-haem.

3. In the urohaematin-peroxide compound one molecule of hydrogen peroxide is combined per molecule of urohaematin. The dissociation constant $K = 3{\cdot}34 \times 10^{-4}$ M at 20°.

4. It is suggested that dihydroxyl-urohaematin may be an essential intermediate for the subsequent formation of the urohaematin-peroxide compound, one molecule of hydrogen peroxide or ethyl hydroperoxide replacing one hydroxyl group on the urohaematin iron atom.

5. A protohaematin-peroxide compound has not so far been obtained with either hydrogen peroxide or ethyl hydroperoxide.

6. The pyridine parahaematins of both uro- and proto-haematin react with hydrogen peroxide and

ethyl hydroperoxide to give 'green compounds' with two-banded absorption spectra. The exact positions of these bands depend on the pH and the amount of peroxide added. In the case of protoparahaematin additional transient absorption bands are observed under certain conditions.

7. The 'green compound' given by pyridine uroparahaematin and ethyl hydroperoxide reduces spontaneously and reversibly to a compound which differs from pyridine urohaemochromogen in that the positions of its absorption bands are nearer the red and in that it does not combine with carbon monoxide. The green compounds given by pyridine protoparahaematin with hydrogen peroxide (pH 6·5) and ethyl hydroperoxide are probably of the same nature.

8. It is suggested that these 'green compounds' correspond to those given by pyridine protoparahaematin and hydrogen peroxide described by Haurowitz, but that they are oxidation products of the haematins and not compounds of the haematins with hydrogen peroxide.

9. Urohaematin-peroxide is so far the only example of a free haematin-peroxide compound where the porphyrin ring is unaltered.

I wish to thank Dr E. F. Hartree for a sample of ethyl hydroperoxide and Dr E. C. Slater for helpful discussions.

REFERENCES

Baeyer, A. & Villiger, V. (1901). *Ber. dtsch. chem. Ges.* **34**, 738.
Davies, T. H. (1940). *J. biol. Chem.* **135**, 597.
Fischer, H. & Orth, H. (1934). *Die Chemie des Pyrrols*, 2. Leipzig: Akademische Verlagsgesellschaft.
Haurowitz, F. (1937*a*). *Enzymologia*, **2**, 9.
Haurowitz, F. (1937*b*). *Enzymologia*, **4**, 139.
Keilin, J. (1949). *Biochem. J.* **45**, 448.
Keilin, J. (1950). *Nature, Lond.*, **165**, 151.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 148.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Kuhn, R. & Wassermann, A. (1933). *Liebigs Ann.* **503**, 203.
Lemberg, R. & Legge, J. W. (1949). *Haematin compounds and Bile Pigments*. New York: Interscience.
Schalfejew, M. (1885). *J. Soc. phys.-chim. russe*, p. 30; from *Ber. dtsch. chem. Ges.* **18** (Referat Bd.), 232.
Shack, J. & Clark, W. Mansfield (1947). *J. biol. Chem.* **171**, 143.
Theorell, H. (1947). *Advanc. Enzymol.* **7**, 265.

A New Amino-acid Amide in the Groundnut Plant (*Arachis hypogaea*): Evidence of the Occurrence of γ-Methyleneglutamine and γ-Methyleneglutamic Acid

By J. DONE and L. FOWDEN*
Human Nutrition Research Unit, Medical Research Council Laboratories, London, N.W. 3

(*Received* 20 *October* 1951)

A brief account of preliminary studies of a new metabolite detected in groundnut plants has already been given (Done & Fowden, 1951). The substance was isolated and found to be an unsaturated amino-acid amide. This earlier work is now described in more detail, together with subsequent investigations of the structure of the new compound.

The first material examined was the 'sap' produced when the turgid stems of young seedlings were incised between the cotyledons and the first leaf. About 20 μl. of liquid exuded from the cut surfaces. It was found by paper chromatography that the main ninhydrin-reacting component of the exudate gave an orange-brown spot ($R_F = 0{\cdot}66$ on chromatograms run in phenol-ammonia). The colour of the ninhydrin spot varied somewhat on different occasions, but always it was clearly outside the range of colours obtained with known α-amino-acids.

A large number of exudates produced in the manner described have been examined, and the new substance (I) has invariably been the dominant ninhydrin-reacting component. When amounts of the order of 20 μl. were chromatographed, much smaller spots due to asparagine, glutamine, aspartic acid, glutamic acid, alanine and other amino-acids sometimes appeared.

(I) has also been detected in homogenates of stems, leaves, hypocotyls and roots, but in these many other ninhydrin-reacting compounds were also present. The new compound could not be

* Present address: Department of Botany, University College, London, W.C. 1.