complex from compound II and from free catalase are identical.

8. The anions have no observable effect on the reduction produced by added donors.

9. The actions of anions on catalase, peroxidase and metmyoglobin peroxide compounds are compared, and the relevance of the phenomena to the questions of the mode of action of catalase, and the nature of the endogenous donor, is discussed.

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The Formation and Properties of Sulphmyoglobin and Sulphcatalase

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The preceding paper (Nicholls, 1961) dealt with the reactions of catalase peroxide compounds with various complex-forming ligands compared with the analogous reactions of peroxidase and metmyoglobin. Of covalent ligands, only cyanide was mentioned in that paper; the present paper describes the anomalous behaviour of another substance, hydrogen sulphide, which normally forms covalent complexes with haemoproteins.

The reactions of haemoglobin with hydrogen sulphide are complicated. At least two distinct derivatives occur: sulphaemoglobin and sulphide-methaemoglobin. The former is a green–purple (dichroic) compound produced when solutions of oxyhaemoglobin are allowed to stand in the presence of hydrogen sulphide. Sulphide–methaemoglobin is a red compound produced by the reversible reaction of methaemoglobin and hydrogen sulphide. This latter reaction is of the usual kind involving replacement of a water molecule at the sixth co-ordination site of the iron (Keilin, 1938), and further reactions can occur only if the sulphide is displaced. Sulphaemoglobin can, however, undergo reactions in its own right; thus it forms a complex with carbon monoxide, and its absorption band can be destroyed by ferricyanide (Nijveld, 1943). These reactions suggest that sulphaemoglobin is a ferrous derivative; the compound produced by ferricyanide would then be metsulphaemoglobin. No distinct spectral bands have been reported for

this compound, although Nijveld's figures suggest that acid and alkaline forms may occur.

The question of the reactants involved in the formation of sulphaemoglobin from oxyhaemoglobin has been the source of some controversy. Michel (1938) claimed that hydrogen peroxide is necessary, but Nijveld (1943) found that hydrogen peroxide gave rise to much choleglobin instead. Dalziel & O'Brien (1957) re-affirmed the need for peroxide, and showed the rapid formation of sulph-compounds in systems containing methaemoglobin peroxide; their systems, however, also contained dithionite, and several haemoglobin derivatives other than the peroxide compound, and their results do not therefore admit of one definitive interpretation. The chemical nature of sulphaemoglobin is also unknown. Keilin's (1938) study showed that both sulphaemoglobin and its ferric derivative differed from the normal reversible sulphide–methaemoglobin complex, i.e. its structure involved the modification of something other than the iron. Nijveld (1943) proposed an improbable structure with an allene carbon in the porphyrin ring. Lemberg & Legge (1949) suggested an even more improbable thiolhistidine structure; Foulkes, Lemberg & Purdom (1951) proposed replacement of the methene bridge $\geqslant C\cdot CH{=}C<$ by the grouping $\geqslant C\cdot CH(SH)\cdot C(OH)<$ but offered no evidence in favour of this idea.

The action of hydrogen sulphide on the haem enzyme catalase has also been the subject of some disagreement. Zeile, Fawaz & Ellis (1940) reported the reduction of a preparation of liver catalase to the ferrous form under the action of hydrogen sulphide and dithionite. Keilin & Hartree (1945) could not repeat this observation although they had found a light-sensitive inhibition of catalase by carbon monoxide in the presence of thiols such as cysteine and glutathione (Keilin & Hartree, 1938). Beers & Sizer (1954) demonstrated that the pattern of inhibition of catalase by hydrogen sulphide is more complicated than that produced by other ligands. These facts suggest the occurrence of more than one reaction between catalase and hydrogen sulphide.

With peroxidase fewer peculiarities have been reported. There was a claim that thiols were inhibitory, but Randall (1946) showed that they act as hydrogen donors for the enzymic activity of peroxidase.

Thus it is known that some unusual interactions between hydrogen sulphide and haemoproteins can occur. But the nature of the intermediates formed and their chemical relationships with each other and with the parent haemoprotein are unknown. And it is also not known whether the phenomena observed in catalase are analogous in any way to those occurring with haemoglobin.

This paper will show that all these observations can be interpreted in terms of the chemistry of haemoprotein peroxide compounds, and that the reactions involved may throw light upon the nature of those compounds. The existence of sets of ferrous and ferric haem derivatives analogous to those produced with the parent protohaem enzymes will be demonstrated for the sulph-compounds.

EXPERIMENTAL

The materials and methods used were as described in the preceding paper (Nicholls, 1961). Most of the observations were carried out with a Zeiss bench microspectroscope, and the major points checked quantitatively in a Hilger Uvispek spectrophotometer with a glass prism giving high dispersion in the visible region.

Sodium sulphide and ammonium sulphide (both AnalaR) were used as sources of hydrogen sulphide. The ammonium sulphide was preferred for spectrophotometric experiments, as the sodium sulphide contained small quantities of polysulphides which tended to precipitate at neutral pH.
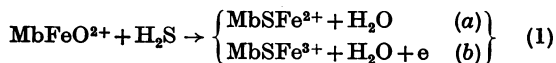
The hydrogen sulphide content of neutralized solutions prepared from these sources was estimated by adding the sulphide solution to a known excess of iodine solution in dilute sulphuric acid. The iodine which remained was titrated against standard sodium thiosulphate, with starch as indicator at the end point.

RESULTS

Formation and properties of sulphmyoglobin

The addition of hydrogen sulphide in stoicheiometric quantities to a solution of metmyoglobin peroxide compound (approx. $30\,\mu\text{M}$) at pH 8 (0·05 M-borate–potassium chloride buffer) resulted in the rapid formation of a mixture of metsulphmyoglobin and small quantities of sulphmyoglobin, with the disappearance of the peroxide compound spectrum. [At the lowest sulphide concentrations employed ($\sim 10\,\mu\text{M}$, see Table 1), the reaction was complete within seconds, indicating a second-order velocity constant greater than 1 mM^{-1} sec.$^{-1}$.] Sulphmyoglobin was recognized spectroscopically by its intense absorption band at 617 mμ; metsulphmyoglobin was recognized by the bands at 595 and 715 mμ. Excess of peroxide had been removed, after the formation of the metmyoglobin peroxide compound, by the addition of small amounts of catalase; this was necessary to prevent secondary reactions such as (4) below. When excess of sulphide was added, all the metsulphmyoglobin was reduced to the ferrous form in a secondary reaction.

If metmyoglobin peroxide is written as $MbFeO^{2+}$ (cf. ferryl ion or FeO^{2+}, George & Irvine, 1955), the reaction can be given as

$$MbFeO^{2+} + H_2S \rightarrow \begin{cases} MbSFe^{2+} + H_2O & (a) \\ MbSFe^{3+} + H_2O + e & (b) \end{cases} \quad (1)$$

The $MbSFe^{3+}$ in reaction (b) can be reduced by a second molecule of H_2S

$$MbSFe^{3+} + H_2S \rightarrow MbSFe^{2+} + H^+ + SH^{\cdot} \quad (2)$$

The major overall reaction is then:

$$MbFeO^{2+} + 2H_2S \rightarrow MbSFe^{2+} + H_2O + [H^{\cdot}] + [SH^{\cdot}] \quad (3)$$

(The fate of the extra reducing equivalent, designated by $H^{\cdot}$, is unknown.) Table 1 gives the results of a spectrophotometric experiment to demonstrate the stoicheiometry of the reaction. Table 2 gives some of the spectroscopic data obtained for the ferrous sulphmyoglobin compared with ordinary ferrous myoglobin, and shows the remarkable intensity of the absorption band in the red, which is comparable with that of the α-band of a haemochromogen.

Like sulphaemoglobin (Nijveld, 1943), sulphmyoglobin combines with carbon monoxide (bubbled through the cuvette) but not with oxygen. As Table 2 shows, the extinction coefficients of the Soret bands of the sulph-compounds are less than those of the normal derivatives. The carbon monoxide derivative of sulphmyoglobin in particular has a rather low band compared with carbon monoxide–myoglobin; but a millimolar extinction coefficient of 105 does not justify Lemberg & Legge's (1949) statement that 'carboxysulphaemoglobin has only a weak absorption (in the Soret region)'.

Like other ferrous haem compounds, sulphmyoglobin fails to react with anionic ligands (fluoride, azide, sulphide) to form complexes. It does, however, undergo a very slow reaction with sodium azide, in which the product is reduced myoglobin. This reaction requires high azide concentrations ($\geqslant 0.1\,\text{M}$), but is not due to the change in ionic strength, since chloride produces no such effect. The azide apparently reacts with the haem to remove the modifying grouping.

Oxidizing agents, such as ferricyanide, ethyl hydroperoxide and molecular oxygen, convert sulphmyoglobin into the ferric form, metsulphmyoglobin. That this change involves only one electron was shown for haemoglobin by Nijveld (1943). Hydrogen peroxide, however, unlike the other oxidizing agents, destroys the sulphmyoglobin spectrum and produces oxymyoglobin. Table 3 gives the results of a typical experiment of this nature, with the calculated concentrations of

Table 1. *Titration of metmyoglobin peroxide with hydrogen sulphide*

$60\,\mu\text{M}$-Metmyoglobin in 2·6 ml. of borate buffer, pH 8 (0·156 μmole of total metmyoglobin); peroxide compound produced by addition of 1 μmole of ethyl hydroperoxide; sulphide added as ammonium sulphide. 20°. Changes of E were measured at 580 mμ, millimolar extinction coefficients of 8·1 for the peroxide compound and 2·5 for metmyoglobin at this wavelength being used (cf. Keilin & Hartree, 1955; George & Irvine, 1952).

	H_2S added (μmole)	Metmyoglobin peroxide destroyed (μmole)	Haem/H_2S ratio
1	0·032	0·023	0·72
2	0·08	0·077	0·96
3	0·032	0·029	0·91
Total	0·144	0·129	0·90

(Further addition of 1 μmole of H_2S reduced the metsulphmyoglobin to sulphmyoglobin.)

Table 2. *Absorption bands of myoglobin and sulphmyoglobin*

Extinction coefficients for myoglobin were obtained experimentally, for reference purposes the value for the α-band of myoglobin itself given by Keilin & Hartree (1955) being used; values for sulphmyoglobin were obtained by adding excess of sulphide to solutions containing known amounts of metmyoglobin peroxide and assuming 100% conversion (cf. the titration data of Table 1).
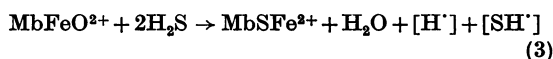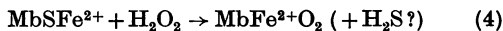
Compound	α-Band λ (mμ)	α-Band $10^{-3}\epsilon$	Soret band λ (mμ)	Soret band $10^{-3}\epsilon$
Myoglobin	558	11·0	433	110
Carboxymyoglobin	576	12·0	422	160
Sulphmyoglobin	617	17·5	420	90
Carboxysulphmyoglobin	613	19·0	422	105

Table 3. *Action of hydrogen peroxide on sulphmyoglobin*

Borate buffer, pH 8·5; 20°; sulphmyoglobin formed by addition of 0·3 mM-H_2S (as ammonium sulphide) to 85 μM-metmyoglobin peroxide. Extinction coefficients for sulphmyoglobin (SMb) were taken from Table 2 and Fig. 1; millimolar extinction coefficient of oxymyoglobin (MbO$_2$) at 580 mμ is taken as 13.

Conditions	E 618 mμ	E 580 mμ	$10^5 \times$ Calculated concn. (M) SMb	$10^5 \times$ Calculated concn. (M) MbO$_2$
(1) Sulphmyoglobin as formed	1·18	0·54	7·5	0·9
(2) Sulphmyoglobin with 1 mM-H_2O_2	0·50	0·61	2·5	3·8

sulphmyoglobin and oxymyoglobin before and after the addition of hydrogen peroxide. This back reaction is responsible for the variability in yield of sulphmyoglobin when excess of hydrogen peroxide is present.

As this reaction does not occur with ethyl hydroperoxide, it must involve the reducing action of hydrogen peroxide, and may be formulated

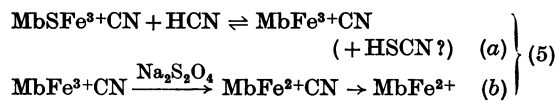$$MbSFe^{2+} + H_2O_2 \rightarrow MbFe^{2+}O_2 \; (+ H_2S?) \quad (4)$$

We therefore have two ways of re-forming myoglobin from ferrous sulphmyoglobin: (a) by a reaction with sodium azide; (b) by a reaction with hydrogen peroxide.

The metsulphmyoglobin, formed either directly (equation 1) or by oxidation of sulphmyoglobin (above), was found to have properties which clearly established the independence of the haem modification concerned from the iron atom. First, it is reversibly oxidized and may be reduced back to sulphmyoglobin by agents such as dithionite and hydrogen sulphide itself. Secondly, it forms a series of complexes with anions, of which Nijveld (1943) mentions the one with cyanide.

Complex-formation occurs with cyanide, azide and fluoride added in approximately 10 mM concentration at pH 8·0 (but not with sulphide, which acts as reductant). An ionization occurs between pH 8·5 and 9·0, giving an alkaline form. Hydrogen peroxide gives rise to a peroxide compound. The spectra of all these derivatives show analogies to those of the corresponding compounds of normal metmyoglobin, but the absorption bands (other than the Soret band) are displaced far into the red end of the spectrum (Fig. 2).

With the cyanide derivative, the relative intensities of the bands at 590 and 550 mμ were found to be variable, which is only comprehensible if these two bands belong to different compounds, the former to the true cyanmetsulphmyoglobin, the latter to cyanmetmyoglobin.

In fact, cyanide regenerates metmyoglobin from metsulphmyoglobin, and complete reversal can be achieved by adding dithionite to the mixture. In the latter reaction the spectrum of the unstable cyanmyoglobin as an intermediate could be observed, but no absorption bands attributable to a cyansulphmyoglobin complex were seen.

$$MbSFe^{3+}CN + HCN \rightleftharpoons MbFe^{3+}CN \\ (+ HSCN?) \quad (a)$$

$$MbFe^{3+}CN \xrightarrow{Na_2S_2O_4} MbFe^{2+}CN \rightarrow MbFe^{2+} \quad (b)$$

\right\} (5)

This represents a third way of re-forming myoglobin from sulphmyoglobin.

The metsulphmyoglobin peroxide compound behaves in a similar fashion to metmyoglobin peroxide (George & Irvine, 1955). It is produced rapidly by hydrogen or alkyl hydrogen peroxides, although even at pH 8·0 it is unstable compared with the normal metmyoglobin peroxide compound. The velocity constants for its decomposition by ferrocyanide or by p-cresol were similar to those for the metmyoglobin peroxide compound.

Fig. 1 shows the absorption spectra, in the visible and Soret regions, of sulphmyoglobin and met-sulphmyoglobin compared with their normal counterparts. Fig. 2 shows the visible spectra of some of the derivatives of metsulphmyoglobin, and demonstrates the analogy with and differences from the same derivatives of metmyoglobin. The nature of the compounds responsible for these spectra, their mode of formation and their properties, which may throw light upon the problem of the nature of the haemoprotein peroxide compounds, are discussed later on, after the description of the corresponding catalase compounds.

Formation and properties of sulphcatalase

The behaviour of catalase towards hydrogen sulphide is in many ways analogous to that of metmyoglobin, and this behaviour can account for many of the peculiarities of catalase in the presence of thiols.

In those systems in which cysteine was found to accelerate the decomposition of compound II (the secondary peroxide compound of catalase, in which the haem iron is effectively in a quadrivalent oxidation state), the transitory formation of a compound with an absorption band at 635 mμ could be observed (Keilin & Nicholls, 1958a). The following evidence indicates that this effect was due to hydrogen sulphide, which was present as an impurity in the cysteine and reacted with compound II in a manner analogous to its reaction with metmyoglobin peroxide.

Hydrogen sulphide reacts rapidly with compound II of catalase to give a derivative with an intense absorption band at 635 mμ. This reaction is illustrated by Fig. 3, which indicates its main features, namely: (a) rapid decrease in absorption at 565 mμ; (b) concomitant increase in absorption at 635 mμ; (c) slow subsequent decrease in the 635 mμ band.

Fig. 4 shows the major changes in the characteristics of the final spectrum, and demonstrates the further important features of the overall reaction:

(i) The final disappearance of the 635 mμ band is in at least two phases and there is no common isosbestic point.

(ii) The 635 mμ compound reacts with carbon monoxide (bubbled through the spectrophotometer cuvette) and is thereby stabilized against its subsequent disappearance.
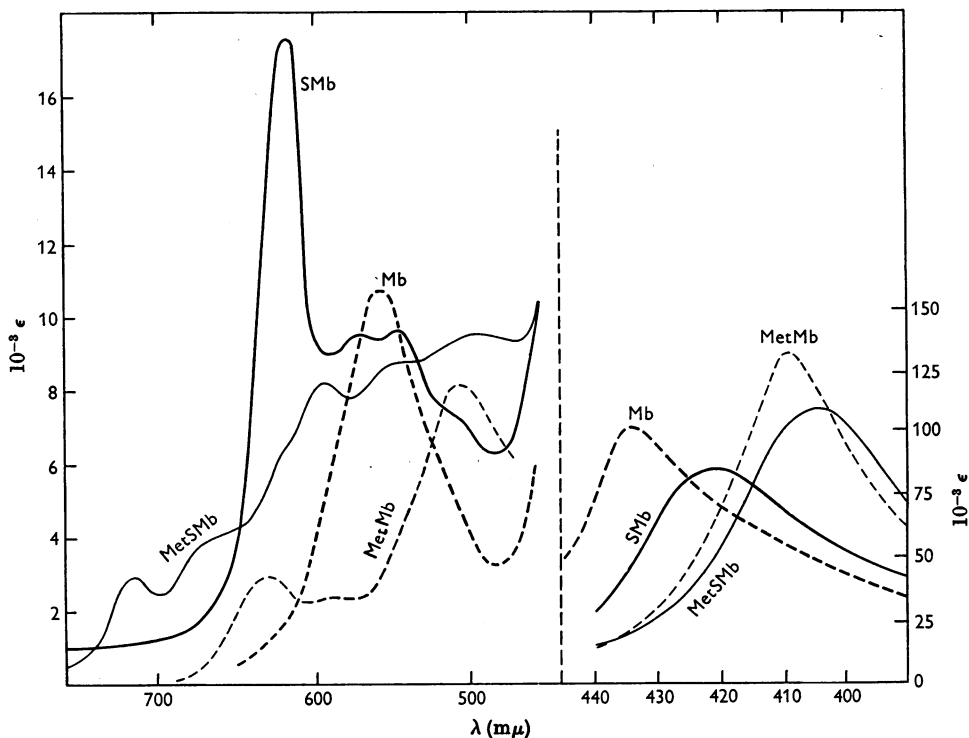
Fig. 1. Visible and Soret spectra of sulphmyoglobin and metsulphmyoglobin compared with normal myoglobin and metmyoglobin. Myoglobin and metmyoglobin in phosphate buffer, pH 6·0. Sulphmyoglobin and metsulph-myoglobin in borate buffer, pH 8·0. Mb, Myoglobin; MetMb, metmyoglobin; SMb, sulphmyoglobin; MetSMb, metsulphmyoglobin. Absolute extinction coefficients were calculated from measurements on 40 μM-myoglobin solutions in 1 cm. glass cuvettes at 20°.
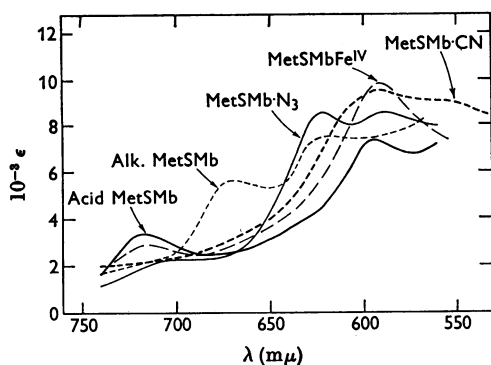
Fig. 2. Visible absorption spectra of metsulphmyoglobin and some of its derivatives. Alkaline metsulphmyoglobin (Alk. MetSMb) in borate buffer, pH 9·4. Other derivatives: acid (Acid MetSMb), azide (MetSMb N₃), cyanide (Met-SMb CN), peroxide (MetSMbFe^{IV}), in borate buffer, pH 8·0. Absolute extinction coefficients were calculated from measurements on 40 μM-metsulphmyoglobin solutions in 1 cm. glass cuvettes at 20°.
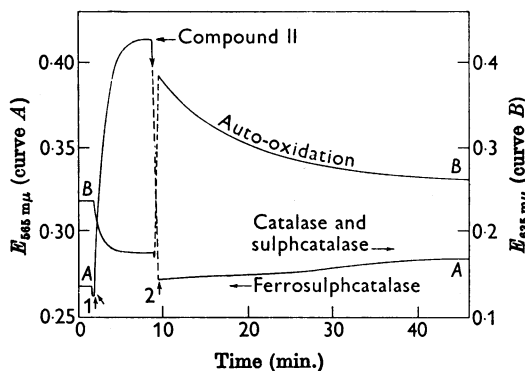
Fig. 3. Effect of hydrogen sulphide on catalase peroxide compound II. 20 μM-Catalase (haem concentration) in phosphate buffer, pH 6, at 20°; compound II was formed by addition of ethyl hydroperoxide (1 μmole) as indicated at arrow 1, and destroyed by addition of sodium sulphide (5 μmoles) at arrow 2. Curve A: measurements at 565 mμ (left-hand ordinate); curve B: measurements at 635 mμ (right-hand ordinate).

The above empirical facts are interpreted in terms of reactions of the following type:

Catalase compound II + H_2S → catalaseS·Fe^{2+}
$$+ H_2O \quad (6)$$

CatalaseS·Fe^{2+} $\xrightarrow{O_2}$ catalaseS·Fe^{3+} $\quad (7)$

CatalaseS·Fe^{3+} $\xrightarrow{O_2 \text{ (or } H_2O_2)}$ catalase·Fe^{3+}
$$(+ \text{oxidized S?}) \quad (8)$$

Reaction (6) is faster than that with metmyoglobin peroxide, equation (1); unlike the last-mentioned reaction, the resulting compound (ferrosulphcatalase) is entirely ferrous, there being no analogues of (1) *b*. Table 5 shows that the stoicheiometry of reaction (6) is the same as that with metmyoglobin.

Reaction (7) indicates the auto-oxidation of the ferrosulphcatalase. Fig. 3 shows this to be a reaction first-order with respect to ferrosulph-

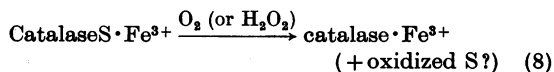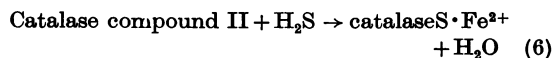Fig. 4. Decomposition of ferrosulphcatalase in air, in the presence and the absence of carbon monoxide. 17 μM-Catalase compound II in phosphate buffer, pH 6·1, at 20° under aerobic conditions; 5 μmoles of sodium sulphide were added at zero time. Fe^{2+}SCat: Ferrosulphcatalase; CO·Fe^{2+}SCat: carboxyferrosulphcatalase. Spectra for product at 0·5, 3, 12, 24, 45, 70 min. are given, together with the corresponding spectra for solutions initially saturated with carbon monoxide, at 2, 15 and 35 min.

catalase, with a velocity constant of about 2×10^{-3} sec.$^{-1}$ (10 M^{-1} sec.$^{-1}$ for dissolved oxygen concentration of 0·2 mM). This oxidation is not promoted by potassium ferricyanide, nor is ferro-sulphcatalase re-formed by the addition of dithionite or excess of sulphide; this behaviour is similar to that of normal catalase (Keilin & Hartree, 1936).

Reaction (8), indicated by the gradual appearance of an absorption band at 622 mμ (Fig. 4) due to free catalase, is discussed in the section on 'reversibility' below.

Table 5 shows the spectroscopic data obtained for catalase derivatives in the visible region of the spectrum. Sulphcatalase forms complexes with cyanide, sulphide, fluoride and azide. Sodium dithionite does not reduce it to ferrosulphcatalase, but does have some effect on the attached sulphur (see below). The catalatic activity of residual free catalase prevents observation of the action of hydrogen peroxide; but ethyl hydroperoxide gives an intermediate with a spectrum identical with that of compound II (bands at 540 and 570 mμ), and this intermediate produces free catalase on decomposition. Table 6 gives the positions of the absorption bands of the ferric derivatives, which may be compared with those of metsulphmyoglobin in Table 7.

The sulphide complex, sulphide–sulphcatalase, is an inhibited catalase derivative in which the same inhibitor, hydrogen sulphide, has acted in two ways upon the same haem prosthetic group. One molecule of the sulphide has formed a thermodynamically irreversible compound with the porphyrin ring, while a second has produced a normal reversible complex with the central iron atom.

Reversibility of sulphcatalase formation

The light-reversible inhibition of catalase by carbon monoxide obtained by Keilin & Hartree (1938) in the presence of thiols such as cysteine, and the partial reversibility of the sulphide inhibition (Beers & Sizer, 1954), indicates the possibility of the regeneration of free catalase from sulphcatalase.

Table 4. *Titration of catalase compound II with hydrogen sulphide*

20 μM-Liver catalase; 0·08M-phosphate buffer, pH 5·0; 20°; peroxide compound formed by addition of 1 μmole of ethyl hydroperoxide; sulphide was added as ammonium sulphide. Final volume: 2·5 ml. Changes of E were measured at 565 mμ and 635 mμ. Assuming: ΔE at 565 mμ of 0·025 ≡ 10^{-2} μmole of haem (compound II). ΔE at 635 mμ of 0·036 ≡ 10^{-2} μmole of haem (ferrosulphcatalase).

	H_2S added (μmole)	Compound II titrated (μmole)	Ferrosulph-catalase formed (μmole)	Haem/H_2S ratio
1 (*a*)	0·016	0·012	0·021	0·69
1 (*b*)	0·016	0·011		
2	0·032	0·022	0·018	0·62
3	0·031	0·028	0·029	0·92

As with metmyoglobin, some ways of reversing the formation of the sulph-compound do exist. In fact, the substituent in sulphcatalase is more labile than that of sulphmyoglobin. Catalase is regenerated under the following conditions:

(a) As indicated in Fig. 4, and expressed by equation (7), the substituent group is labile to oxidizing agents, including oxygen, ethyl hydroperoxide and possibly hydrogen peroxide, when the iron is in the ferric state. Sulphcatalase therefore differs from metsulphmyoglobin in that it does not form a stable peroxide derivative.

$$\text{CatalaseS} \cdot \text{Fe}^{3+} \xrightarrow{\text{oxidants}} \text{catalase} \cdot \text{Fe}^{3+}$$
$$(+ \text{oxidized sulphur?}) \quad (9)$$
$$(\text{or derivative, e.g. compound II})$$

(b) The ligands cyanide, azide and fluoride form stable compounds with sulphcatalase but they accelerate the regeneration of free ferric catalase from ferrosulphcatalase (cf. the production of myoglobin from sulphmyoglobin in the presence of azide or cyanide). The transition is not promoted by all ligands for ferric catalase, as in this respect formate is inactive. Carbon monoxide inhibits the reaction by combining with ferrosulphcatalase.

$$\text{CatalaseS} \cdot \text{Fe}^{2+} \xrightarrow[+\text{oxidant?}]{\text{F}^-,\ \text{CN}^-\ \text{or}\ \text{N}_3^-} \text{catalase} \cdot \text{Fe}^{3+} \quad (10)$$

(or derivative, e.g. catalase–anion complex)

(experiments carried out at pH 5, 20°, with 10 mM-anion).

Table 5. *Spectroscopic data for ferrosulphcatalase*

The sulph-compounds were prepared by adding excess of sulphide to a solution containing a known concentration of compound II, and the extinction coefficients were calculated assuming 100% conversion.

Compound	α-Band $\lambda_{max.}$ (mμ)	$10^{-3}\epsilon$
Catalase*	622	10·5
Catalase compound II	568	20·0*
Ferrosulphcatalase	635	21·0
Carboxyferrosulphcatalase	627	24·0

* Liver catalase; pH 5; 20°.

(c) Sodium dithionite reacts with sulphcatalase and ferrosulphcatalase; in the former case ferric catalase is produced, in the latter a mixture of ferric and ferrous catalase. Both reactions seem to involve the destruction of a certain amount of catalase.

$$\text{CatalaseS} \cdot \text{Fe}^{3+} \xrightarrow{\text{Na}_2\text{S}_2\text{O}_4} \text{catalase} \cdot \text{Fe}^{3+}$$
$$\uparrow \quad (+\text{reduced S?})$$
$$\mid \text{O}_2 \text{ (rapid)}$$
$$\text{CatalaseS} \cdot \text{Fe}^{2+} \xrightarrow{\text{Na}_2\text{S}_2\text{O}_4} \text{catalase} \cdot \text{Fe}^{2+}$$
$$(+\text{reduced S?})$$
$$(11)$$

(experiment carried out at pH 6, 20°, a few milligrams of sodium dithionite/ml. being used). The formation of ferrous catalase in the second reaction is indicated by the disappearance of the band at 635 mμ without the production of the 620 mμ band, but a broad absorption in the green; the latter may be converted into two bands at 545 and 575 mμ by passing carbon monoxide into the mixture. This phenomenon probably accounts for the claim of Zeile et al. (1940) to have obtained reduced catalase by the action of hydrogen sulphide followed by dithionite.

Reactions of sulphide with other peroxide compounds

Of the five known haemoprotein peroxide compounds which can be reduced by hydrogen donors (catalase and peroxidase compounds I and II, metmyoglobin peroxide), only metmyoglobin peroxide and catalase peroxide compound II seem to react with hydrogen sulphide to give sulph-compounds.

Catalase compound I, which might be expected to give rise to ferric sulphcatalase directly, does not appear to do so. When hydrogen sulphide was added to catalase solutions and ethyl hydroperoxide added subsequently, the first observable derivative was ferrosulphcatalase. Either the transition of compound I to II must be faster than the reaction of compound I with hydrogen sulphide, or the latter reaction must be such as not to

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Table 6. *Spectroscopic data for sulphcatalase derivatives*

Position of visible absorption bands (mμ)

Derivative	Catalase	Sulphcatalase
Acid form (H$_2$O)	622, 535, 500	710, (635)*, 585
Alkaline form (OH$^-$)	Unknown	Unknown
Cyanide	(585), 555	645
Fluoride	595	680
Azide	620	~700
Sulphide	(640)*, 585, 550	645
Ethyl hydroperoxide	568, 535	(568, 535)†

* Possibly due to sulphide–sulphcatalase contamination.   † Apparently identical with normal compound II.

Table 7. *Spectroscopic data for metsulphmyoglobin derivatives*

	Position of visible absorption bands (mμ)	
Derivative	Metmyo-globin	Metsulphmyoglobin
Acid form (H_2O)	635, 505	715, 595
Alkaline form (OH^-)	590, 540	670, 622
pK	8·9	Between 8·5 and 9·0
Cyanide	(580), 545	590, 550*
Fluoride	610	680
Azide	570, 545	622, 590
Hydrogen peroxide	580, 550	590
Sulphide	585, 545	Does not exist

* Probably due to CN–metmyoglobin re-formed from the sulphmyoglobin by action of cyanide (see text).

involve the production of an inhibitory sulph-compound.

The addition of ammonium sulphide to solutions of peroxidase compound I and II caused the disappearance of the absorption spectra of the peroxide compounds and the reappearance of the band of free peroxidase at 640 mμ. Peroxidase appears to act as oxidant towards sulphide as hydrogen donor:

Peroxidase compound II + $H_2S \rightarrow$
$$\text{peroxidase} \cdot Fe^{3+} + SH \cdot \quad (12)$$
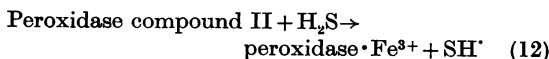
Reactions with complex thiols and with selenide

The reaction in which sulph-compounds are formed is only produced by hydrogen sulphide. As shown by Keilin & Nicholls (1958*a*), complex thiols such as cysteine do not bring it about. Furthermore, an analogous reaction does not occur with hydrogen selenide. The generation of hydrogen selenide from aluminium selenide in a solution of metmyoglobin peroxide resulted in the formation of oxymyoglobin, and eventually reduced myoglobin, owing to the removal of oxygen by auto-oxidation.

$$MbFeO^{2+} + H_2Se \rightarrow MbFe^{2+}H_2O + Se \quad (13)$$

No bands attributable to a 'selenomyoglobin' were observed.

DISCUSSION

The above results bear upon two theoretical issues: (*a*) the nature and properties of sulph-compounds as a general problem in haem chemistry, and (*b*) the structures and properties of the normal haemoprotein peroxide compounds.

Michel (1938) formulated the sulphaemoglobin-producing reaction as:

$$Hb + H_2S + H_2O_2 \rightarrow HbS + 2H_2O \quad (14)$$
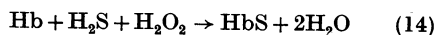
Nijveld (1943), however, thought that oxyhaemoglobin was directly involved, thus:

$$HbO_2 + H_2S(Hb) \rightarrow (Hb-2H)S + 2H_2O + Hb \quad (15)$$
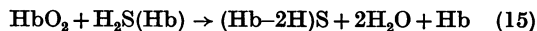
According to the results given above, equation (14) states the reactants and oxidation state of sulphmyoglobin correctly (apart from the oxidation state of initial haem) whereas equation (15) does not. The production of large amounts of choleglobin in Nijveld's experiments can be attributed to the presence of reducing agents such as ascorbic acid (Lemberg, Legge & Lockwood, 1941). But it cannot be stated that a reaction involving oxyhaemoglobin does not occur, because equation (4) may represent a reversible system; thus the sulphmyoglobin in Table 3 is not completely destroyed by peroxide. Nijveld's view that sulphaemoglobin may be reconverted into haemoglobin is supported by the present data for myoglobin; azide and hydrogen peroxide can now be added to cyanide as reagents bringing this about.

In the absence of such reagents the sulph-compounds remain quite stable and the various derivatives here described may provide a new way of investigating the relations between porphyrin, iron and protein in the reactions promoted by haemoproteins. Thus it would be interesting to study the equilibria between metsulphmyoglobin and ligands more quantitatively and compare the results with those for metmyoglobin. Nijveld, unaware of these possibilities, did attempt to measure the redox potential of the sulphaemoglobin/metsulphaemoglobin system, obtaining a value of +0·12 to +0·14v at pH 7. Preliminary experiments in this laboratory, in which the haemoproteins were equilibrated under nitrogen with the ferric–ferrous oxalate system (Michaelis & Friedheim, 1931), indicate a potential of +0·13v for the sulphmyoglobin–metsulphmyoglobin system, compared with +0·04v for myoglobin–metmyoglobin (pH 6·5, 0·024m-phosphate, 20°). This may help to explain the greater reducibility of metsulphmyoglobin by hydrogen sulphide, and suggests that the strange spectrum of the reduced form may be associated with some stereochemical or electronic stabilization.

The absence of an oxygenated form of sulphmyoglobin suggests a comparison between the formation of carbon monoxide complexes in the protohaem and sulphaem derivatives. The kinetics of sulphmyoglobin auto-oxidation might give other information on the formation of oxygen complexes than that available by direct spectroscopic examination.

The reactions between sulphmyoglobin and azide or cyanide show that considerable caution must be employed in concluding from an effect of such agents that a ferric haem compound is

involved. This becomes even more evident in the behaviour of catalase where fluoride also reacts in some way with the ferrous sulph-compound (equation 10 above). The latter case is somewhat reminiscent of the effect of anions on catalase peroxide compounds (Nicholls, 1961), but its kinetics have not yet been examined.

For catalase, the results explain the disagreement about the reduction of catalase in the presence of hydrogen sulphide, and suggest the identity of the anomalous 640 mμ band reported for sulphide–catalase complex (Zeile & Hellstrom, 1930; Keilin & Hartree, 1936) with that of sulphide–sulphcatalase. And, incidentally, they provide a new method of reducing the iron of catalase to the ferrous state. Free catalase is inert towards dithionite unless partially denatured (freeze-dried catalase). Compound I can be reduced to nitric oxide ferrocatalase by azide, a comparatively weak reductant (cf. Keilin & Hartree, 1945). Likewise, the weak reducing agent hydrogen sulphide is now shown to reduce compound II to ferrosulphcatalase. Conversely, metmyoglobin is readily reduced by dithionite but its peroxide compound is not so susceptible to reduction by azide or sulphide. The reversibility of the sulphcatalase formation permits an analysis of the effects reported by Keilin & Hartree (1938) and by Beers & Sizer (1954). Unlike the carbon monoxide inhibition of the catalatic reaction in the presence of azide, the inhibition produced in the presence of thiols is only slowly reversed by light (a lag phase of about 1 min. is shown in Keilin & Hartree's 1938 paper). In Beers & Sizer's experiments, sulphide was the only inhibitor whose effect decreased in some cases after the addition of peroxide (indicating a reversal of the inhibition by the oxidant).

Although therefore many problems in kinetic behaviour can be interpreted formally by reference to the sulph-compounds, exact chemical formulation of most of the reactions is not possible. This is largely because the chemical structure of the sulph-compounds themselves is unknown. Some conclusions may, however, be drawn. Thus the molecular structure indicated seems to be the same in sulphmyoglobin and sulphcatalase, each containing one more sulphur atom than the normal haemoprotein. The sulphur cannot be bound to the iron atom, as ligands combine with the latter, both in ferric and ferrous forms. Possibilities include:
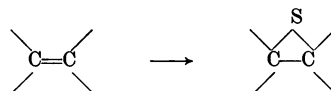
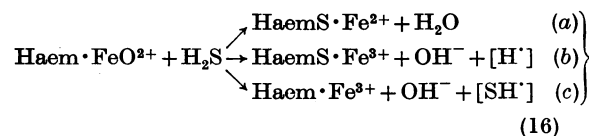(1) Methene-bridge thio ketone:
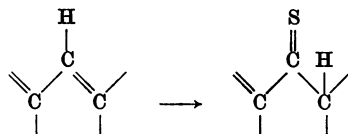
(2) Methene-bridge thiol:
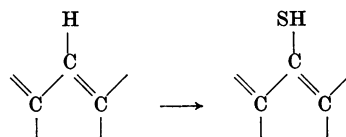
(3) Methene-bridge or pyrrole-ring cyclic thio ether:

The comparatively strong Soret bands support the thiol structure (conjugate double bonds remain), but the absence of effect of thiol reagents (lead acetate and iodoacetate) supports structures 1 or 3. The thio ketone form (1) is perhaps the most likely.

In any case it is difficult to account for the spectra. Tables 6 and 7 show that for metsulphmyoglobin the absorption bands of ionic complexes are shifted 80 mμ, those of covalent complexes about 40 mμ towards the infrared, whereas for sulphcatalase the bands of all complexes are moved some 90 mμ towards the infrared. The bands of the ferrous compounds (Tables 2 and 5) are shifted to a similar extent, but the spectral pattern is changed.

Some information about the secondary peroxide compounds is obtained from their reactions with hydrogen sulphide. There are three types of reaction:

$$\text{Haem} \cdot \text{FeO}^{2+} + \text{H}_2\text{S} \nearrow \begin{array}{l} \text{HaemS} \cdot \text{Fe}^{2+} + \text{H}_2\text{O} \quad (a) \\ \text{HaemS} \cdot \text{Fe}^{3+} + \text{OH}^- + [\text{H}^\cdot] \quad (b) \\ \text{Haem} \cdot \text{Fe}^{3+} + \text{OH}^- + [\text{SH}^\cdot] \quad (c) \end{array}$$

$$(16)$$

(a) and (b) metmyoglobin reaction; (a) catalase reaction; (c) peroxide reaction.

The similar behaviour of metmyoglobin peroxide and catalase compound II is evidence of some chemical similarity, despite the spectroscopic differences. Indeed, unlike the situation described for the reactions with donors (Keilin & Nicholls, 1958b) and anions (Nicholls, 1961), there appears here to be a closer relation between catalase compound II and metmyoglobin peroxide than between catalase compound II and peroxidase compound II.

However, there is also an important difference. Myoglobin, which forms the Fe^{4+} oxidation state directly from Fe^{3+}, forms a peroxide compound (Fe^{4+}) from the ferric sulph-derivative. Catalase, which forms the Fe^{4+} state (compound II) via a Fe^{5+} state (compound I), forms no peroxide compounds in the sulphcatalase form. This raises the question whether compound I involves interaction with the porphyrin ring in the region modified by

addition of the sulphur. This possibility will be discussed in a later paper.

The derivatives described in this paper are mainly of interest for the light they may throw upon haemoprotein chemistry in general. Similar compounds may, however, sometimes occur naturally. Sulphaemoglobin is found *in vivo* after some kinds of drug therapy, in cases of septicaemia and in poisoning with 2:4:6-trinitrotoluene (Lemberg & Legge, 1949). According to Lemberg & Legge, the hydrogen sulphide always arises from intestinal bacterial activity.

Sulphcatalase has not previously been reported but observations of inhibition by carbon monoxide in crude extract of liver catalase (Califano, 1934) may have been due to the formation of ferrosulphcatalase in such preparations.

The most interesting possibility is represented by myeloperoxidase. Its absorption spectrum, derivatives and reported behaviour show a close analogy with sulphmyoglobin and sulphcatalase (Agner, 1941). Agner (1958) has now crystallized this enzyme, and more evidence should soon be forthcoming.

SUMMARY

1. Sulphmyoglobin is formed stoicheiometrically by the reaction of hydrogen sulphide and metmyoglobin peroxide. It combines with carbon monoxide, but not with oxygen, and is oxidized to metsulphmyoglobin by ferricyanide.

2. Metsulphmyoglobin behaves like metmyoglobin. Its iron atom is co-ordinated with a water molecule dissociating under alkaline conditions; it forms fluoride, cyanide and azide complexes, and a metsulphmyoglobin peroxide compound.

3. Myoglobin is re-formed from sulphmyoglobin by the action of azide or hydrogen peroxide; metsulphmyoglobin is slowly converted into metmyoglobin in the presence of cyanide.

4. Ferrosulphcatalase is formed by the action of hydrogen sulphide on catalase compound II. It combines with carbon monoxide and is oxidized to (ferric) sulphcatalase by oxygen but not by ferricyanide.

5. Sulphcatalase forms complexes with fluoride, cyanide, sulphide and azide; it is not reducible by dithionite, does not give an alkaline form and does not produce peroxide compounds. In the sulphide-sulphcatalase complex, the sulphide is inhibiting the same haem group in two ways: by irreversibly modifying the porphyrin and by forming a reversible complex with the iron.

6. Catalase is regenerated from sulphcatalase by oxidizing agents such as oxygen and alkyl peroxides; its aerobic formation from ferrosulphcatalase is accelerated by cyanide, azide and fluoride.

7. Sulph-compounds cannot be produced from any peroxidase peroxide compounds, nor from catalase compounds I or III. Hydrogen selenide does not have an analogous effect to that of sulphide.

8. The results are discussed with reference to the problems of the chemical nature of both sulphcompounds and peroxide compounds and the possibility of the natural occurrence of sulphcompounds.

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