REFERENCES

- Banaschak, H. & Jung, F. (1956). *Biochem. Z.* **327**, 515.
- Bjerrum, J., Schwarzenbach, G. & Sillen, L. G. (1958). *Stability Constants, Part II: Inorganic Ligands*. London: The Chemical Society.
- Brill, A. S., Ehrenberg, A. & Hartog, H. den (1960). *Biochim. biophys. Acta*, **40**, 313.
- Chance, B. (1952). *Arch. Biochem. Biophys.* **41**, 404.
- Chance, B. & Fergusson, R. R. (1954). In *Mechanism of Enzyme Action*, p. 389. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
- Gaines, A., Hammett, L. P. & Walden, G. H. (1936). *J. Amer. chem. Soc.* **58**, 1668.
- George, P. (1953). *Biochem. J.* **54**, 267.
- George, P., Beetlestone, J. & Griffith, J. S. (1959). *Symposium on Haematin Enzymes, Canberra*, 1959 (in the Press). Pergamon Press Ltd.
- George, P. & Hanania, G. (1952). *Biochem. J.* **52**, 517.
- George, P. & Hanania, G. (1953). *Biochem. J.* **55**, 236.
- George, P. & Irvine, D. H. (1953). *Biochem. J.* **55**, 230.
- Griffith, J. S. (1958). *Disc. Faraday Soc.* **26**, 81.
- Hartree, E. F. (1946). *Rep. Progr. Chem.* **43**, 295.
- Jillot, B. A. & Williams, R. J. P. (1958). *J. chem. Soc.* p. 462.
- Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
- Longuet-Higgins, H. C., Rector, C. W. & Platt, J. R. (1950). *J. chem. Phys.* **18**, 1174.
- Pauling, L. (1931). *J. Amer. chem. Soc.* **53**, 1367.
- Pauling, L. (1948). *The Nature of the Chemical Bond*, 2nd ed. New York: Cornell University Press.
- Perrin, D. D. (1959). *J. chem. Soc.* p. 1710.
- Scheler, W. (1960). *Biochem. Z.* **332**, 542.
- Scheler, W., Schoffa, G. & Jung, F. (1957). *Biochem. Z.* **329**, 232.
- Scheler, W., Schoffa, G. & Jung, F. (1958). *Biochem. Z.* **330**, 538.
- Theorell, H. & Ehrenberg, A. (1951). *Acta chem. scand.* **5**, 823.
- Theorell, H. & Ehrenberg, A. (1952). *Arch. Biochem. Biophys.* **41**, 442.
- Tomkinson, J. C. & Williams, R. J. P. (1958). *J. chem. Soc.* p. 2010.
- van Vleck, J. H. (1935). *J. chem. Phys.* **3**, 807.
- Williams, R. J. P. (1956). *Chem. Rev.* **56**, 299.
- Williams, R. J. P. (1959a). In *The Enzymes*. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Williams, R. J. P. (1959b). *Symposium on Haematin Enzymes, Canberra*, 1959 (in the Press). Pergamon Press Ltd.

Biochem. J. (1961) **78**, 253

Primary Compounds of Catalase and Peroxidase

BY A. S. BRILL*† AND R. J. P. WILLIAMS

The Clarendon Laboratory and the Inorganic Chemistry Laboratory, University of Oxford

(Received 9 February 1960)

The mechanisms by which many haemoproteins fulfil their roles in biological oxidation and peroxide catalysis are not yet completely defined in spite of great experimental activity in this field of research. It would be of considerable help in arriving at an understanding of those mechanisms involving the higher oxidation states of haemoproteins if we knew the structures of the higher oxidation compounds. Great effort has been put into a determination of the absorption spectra (Keilin & Hartree, 1951; Chance, 1952a; George, 1953) of the higher oxidation compounds for it is only through the absorption spectra that these compounds can be followed separately and quantitatively through their reactions. Other properties, such as the magnetic moments and number of

oxidizing equivalents of the compounds, can only be evaluated accurately after the absorption spectra have been established. The spectra themselves are useful in that they can be related to structure (see preceding paper).

Attention in this paper is directed particularly to the green primary compounds, 'compound I', of catalase and peroxidase with peroxides. Chance (1949a) has stated that alkyl hydroperoxides can convert free catalase completely into compound I. Our experiments show that this is only approximately correct, and we produce an absorption spectrum different from those arrived at in the past for primary compounds. The spectrum is not similar to any known haematin-containing protein, nor to any known porphyrin. We tentatively conclude that the oxidative event which has taken place is in part an attack on the porphyrin conjugated ring system, saturating it at one methene bridge. This conclusion is reinforced by similarities between our spectrum of compound I and the spectra of certain bile pigments.

* Donner Research Fellow, Division of Medical Sciences of the National Academy of Sciences, National Research Council.

† Present address: Department of Engineering Physics, Rockefeller Hall, Cornell University, Ithaca, New York, U.S.A.

MATERIALS AND METHODS

Catalase was prepared at the Johnson Research Foundation, University of Pennsylvania, U.S.A., from *Micrococcus lysodeikticus* (American Type Culture Collection no. 4698) produced on a large scale in submerged culture (Beers, 1955). The enzyme was extracted by the method of Herbert & Pinsent (1948) and purified by repeated precipitations with acetone from neutral 0.01 M-phosphate buffer (A. S. Brill & A. C. Maehly, unpublished work). Catalase concentration was measured by light-absorption at 406 m μ (Soret peak) in a Unicam quartz spectrophotometer. The millimolar extinction coefficient (E_{mm}) of the Soret peak is 103 mm $^{-1}$ cm. $^{-1}$ per haematin group. The Kat. f. (Euler & Josephson, 1927) for this preparation is 143 000 at 25° as determined by the method of Bonnichsen, Chance & Theorell (1947).

The maximum purity value obtainable for bacterial catalase is 0.92 where purity value is defined as the ratio $E_{406 \text{ m}\mu}/E_{280 \text{ m}\mu}$. The purity value of the enzyme solutions used in these experiments was in the range 0.86–0.88 except for one set of measurements in the visible for which it was 0.82. Low absorption in the u.v. is necessary for the success of the experiments to be described below.

In preparation for each set of experiments, a concentrated solution of enzyme was dialysed against 0.01 M-phosphate buffer, pH 7.25 $\pm$ 0.05. All dilutions were made with this type of buffer.

A Cary recording spectrophotometer was used. Preceding each set of experiments a base-line was obtained over the spectral region of interest by recording with water in both the sample and reference cuvettes. Then the diluted catalase solution was placed in the sample cuvette, and the absorption spectrum of the free enzyme was recorded as a check upon the concentration and upon the proper functioning of the spectrophotometer.

Ethyl hydroperoxide was most kindly given by the Laboratory of Laporte Chemicals Ltd. Its concentration was measured by light-absorption at 230 m μ , where E_{mm} is 0.043 mm $^{-1}$ cm. $^{-1}$ (Reiche, 1931).

The temperature at which these experiments were performed was not controlled, and varied from 24 to 26°.

In the paramagnetic resonance experiments a radio-frequency field-modulated spectrometer of the type described by Llewellyn (1957) was employed. The magnetic field was slowly swept through the region of interest, a band width of 2 cyc./sec. was employed, and the signal was traced with a recording milliammeter. The sensitivity was calibrated before each experiment with the same crystal of the Tutton salt, $\text{Zn}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, containing 1.6% of $\text{Cu}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. In this salt the bivalent ions are in two differently orientated complexes, there being about 1.3×10^{15} Cu^{2+} ions of each type in the calibrating crystal. The signal-to-noise ratio at 90° K for a hyperfine line of half-width 7 oersteds was eight.

RESULTS

Conditions for maximum formation of compound I

When ethyl (or methyl) hydroperoxide reacts with catalase a transient green compound is formed, compound I. As compound I disappears, compound II and free catalase appear. (A summary

of the peroxide compounds of catalase and peroxidase is presented in the preceding paper.) The experiment described in this section demonstrates that the maxima of concentration of compound I reached in this reaction are a non-equilibrium function of the concentrations of peroxide and enzyme.

The wavelength was fixed at 405 m μ , and the extinction of free catalase solution was recorded. Upon a signal, between 0.05 and 0.10 ml. of ethyl hydroperoxide solution was blown from a pipette into the cuvette containing 3.0 ml. of enzyme solution, and the reaction mixture stirred with a rod previously placed in the cuvette. The time which elapsed from the signal until the stirring rod was removed was 7 or 8 sec. The recording was started exactly 10 sec. after the signal. Fig. 1 shows one such recording.

Fig. 1. Recording of $E_{405 \text{ m}\mu}$ showing steady state. Final concentrations: 6.32 μM -catalase-haematin, 295 μM -ethyl hydroperoxide, 0.01 M-phosphate; pH 7.25.

Depending upon the concentration of peroxide, a steady state is reached in 35–80 sec. and lasts 15–20 sec. before E begins to change again. The change in E from that of free catalase to that of the steady-state reaction mixture is a measure of the amount of free enzyme converted into compound I. Table 1 shows the relationship between peroxide concentration and compound I formation. The latter is maximal at a peroxide concentration of about 300 μM .

Absorption spectrum of the steady-state reaction mixture

Under conditions (300 μM -ethyl hydroperoxide) of maximum conversion into compound I, the experimental routine described in the previous section was performed at 15 $\text{m}\mu$ intervals from 300 to 435 $\text{m}\mu$ and from 510 to 690 $\text{m}\mu$, and also at 460 and 485 $\text{m}\mu$. The concentrations of enzyme for the experiments in the Soret and u.v. regions were 6–8 times less than that used in the visible. The difference in E between the free enzyme solution and the steady-state reaction mixture was divided by the enzyme concentration to obtain ΔE_{mM} . The enzyme concentration used in this calculation was the average of that before and after adding the ethyl hydroperoxide (which differed at most by 3.2%). Values of ΔE_{mM} were then subtracted from the extinction coefficients for the free enzyme and plotted as curve A, Fig. 2.

Because the reaction mixture is almost entirely compound I for a period of about 2 min. during and after the steady-state period, it was possible to use the wavelength-scanning properties of the Cary spectrophotometer to assist in filling in the regions between the points determined from the steady-state measurements. Fig. 3A shows a scanning trace started at the beginning of a steady state at 550 $\text{m}\mu$ and run during 6.25 min. up to 700 $\text{m}\mu$. Fig. 3B is a trace started about 20 min. after the start of the trace in Fig. 3A and shows that compound II is now the major component of the reaction mixture.

Change in extinction on adding cyanide to a steady-state reaction mixture

Chance & Herbert (1950) have shown that cyanide combines rapidly with bacterial catalase, and have used this fact to demonstrate that,

Fig. 2. Absorption spectra of A, the steady-state reaction mixture of catalase (6.3 μM -haematin) and ethyl hydroperoxide (300 μM); B, the cyanide complex (4.1 μM -haematin, 360 μM -potassium cyanide); C, free catalase. 0.01 M-Phosphate, pH 7.25, for all curves. The crosses on curve A indicate those wavelengths at which changes in extinction coefficient were determined from steady-state measurements. D is the calculated curve for compound I.

Fig. 3. A, Recording of E scanning from 550 $\text{m}\mu$ at the beginning of the steady state to 700 $\text{m}\mu$ 6.25 min. later. B, Recording of E scanning from 490 to 600 $\text{m}\mu$, starting about 20 min. after the steady state was reached. Final concentrations, A and B: 32 μM -catalase-haematin, 610 μM -ethyl hydroperoxide, 0.01 M-phosphate, pH 7.25.

Table 1. Concentration of compound I as a function of ethyl hydroperoxide concentration

pH 7.25; 0.01 M-phosphate; 25°; spectrophotometric measurements at 405 $\text{m}\mu$ with 1 cm. optical depth.

Catalase-haematin (μM)	Ethyl hydroperoxide (μM)	Difference in E /catalase-haematin (mm^{-1})
5.04	32	-30.5
5.08	98	-49.3
5.25	286	-54.3
6.36	295	-53.0
6.32	560	-48.0

during the catalytic decomposition of hydrogen peroxide, substantially more than half of the catalase haematin is free. The Soret spectrum of the cyanide complex of their enzyme differs negligibly from ours (Fig. 2*B*) in the location and extinction coefficient of the peak at $426\text{ m}\mu$, but absorbs substantially more light below $410\text{ m}\mu$.

Chance (1949*a*) added cyanide to a mixture of horse-liver catalase and ethyl hydroperoxide and recorded the change of $E_{435\text{ m}\mu}$. Finding 'no appreciable' rapid reaction with cyanide, he concluded that ethyl and methyl hydroperoxide can bind all three of the haematin of horse-liver catalase. The difficulty in detecting the presence of free catalase at $435\text{ m}\mu$ is the same as that at $426\text{ m}\mu$ which is described in the last paragraph of this section. Without presenting further evidence, Chance & Herbert (1950) assumed that methyl hydroperoxide binds completely all four haematin of bacterial catalase. We show that free catalase is still present under the best conditions for the formation of compound I from ethyl hydroperoxide. From the absorption spectrum given by Chance & Herbert, we infer that free catalase is

also present in the reaction mixture with methyl hydroperoxide.

At $405\text{ m}\mu$ the value of ΔE_{mM} for the change from free catalase to the cyanide complex is $-49\text{ mm}^{-1}\text{ cm}^{-1}$ per haematin. Indeed, as can be seen in Fig. 2, the absorption of the cyanide complex at $405\text{ m}\mu$ is only slightly greater than that of the steady-state reaction mixture. If cyanide is added to the steady-state reaction mixture while the spectrophotometer is set at $405\text{ m}\mu$, E will drop quickly, if free catalase is present, owing to formation of the cyanide complex. E can drop only if free enzyme is present before addition of cyanide since conversions from compound I and compound II into the cyanide complex are accompanied by increases in E .

A steady-state reaction mixture was produced as described in the preceding sections. When the steady state was reached, the recorder was stopped, and 0.085 ml. of 0.013 M -cyanide solution was blown into the reaction mixture containing 3.00 ml. of $1.06\text{ }\mu\text{M}$ -catalase ($4.24\text{ }\mu\text{M}$ -haematin) solution and 0.052 ml. of 0.017 M -ethyl hydroperoxide. The recorder was started 10 sec. later. Fig. 4*A* is a record of one such experiment. The drop in E upon the addition of cyanide is apparent. However, part of this drop is due to dilution. Fig. 4*B* shows the dilution effect in an optically identical but non-reacting solution, 0.0055 extinction unit, which must be subtracted from the change observed in Fig. 4*A*. There remains a decrease of over 0.01 extinction unit, which demonstrates unmistakably the presence of free haematin groups.

The conversion of compound I back into free catalase, which is immediately complexed by the cyanide, can be seen at the end of the record of Fig. 4*A*. This conversion can be studied more readily at $426\text{ m}\mu$ where the difference in light-absorption between compound I and the cyanide complex is greatest. The conversion, as shown in Fig. 5, is accurately first-order with a rate constant of $8.6 \times 10^{-3}\text{ sec}^{-1}$. Therefore when the spectrophotometer was started 10 sec. after the cyanide was introduced, 8% of the compound I which was present had already been converted into the cyanide complex. In the record shown in Fig. 4*A*, for example, the drop in E must be increased by 0.002 . All factors taken into account, the amount of free catalase present at the steady state is at least 5% but not more than 10% of the total catalase. It should be possible to apply a rapid-flow technique to this method and achieve great accuracy in establishing the composition of the steady-state reaction mixture.

Fig. 5 shows that at $426\text{ m}\mu$ it is difficult to determine the unbound catalase accurately. Because the changes in E from free catalase and

Fig. 4. *A*, Recording of E , showing effect of addition of cyanide (0.085 ml. of 0.013 M -potassium cyanide) to the steady-state reaction mixture (3.00 ml. of $4.2\text{ }\mu\text{M}$ -catalase-haematin, 0.052 ml. of 0.017 M -ethyl hydroperoxide), 0.01 M -Phosphate, pH 7.25, $\lambda\text{ }405\text{ m}\mu$. *B*, Recording of E , showing the dilution effect; $\lambda\text{ }405\text{ m}\mu$.

from compound I to the cyanide complex have the same sign, the immediate and exponential changes are in the same direction. The later change is rapid and would have to be accurately extrapolated to zero time. However, when dilution is considered, the effect of the free catalase is apparent.

Paramagnetic resonance experiments

The growth of crystals of bacterial catalase of sufficient size for solid-state paramagnetic resonance measurements has thus far been unsuccessful. We have made measurements on solutions where the broadness of the lines of the iron makes their detection impossible, but where the presence of free radicals might be observed. In earlier paramagnetic resonance measurements on aqueous solutions and freeze-dried samples, compound I of peroxidase has not produced a free radical signal (Chance & Fergusson, 1954).

Two factors determine the concentration of free radicals which can be detected, the width of the absorption band and the saturation of the spin system. The spin-lattice relaxation times of free radicals are frequently long and saturation may occur at moderately high levels of input power. However, the intramolecular magnetic interactions among possible free radicals and the four iron atoms of catalase produce a short spin-spin relaxation time and saturation should not be reached in this particular spin system at the power used. Indeed, the intramolecular dipolar fields in haemoproteins seriously decrease the possibility of observing a signal from free radicals within the molecule because of line-broadening. A free radical in a porphyrin ring would be in the field of an iron atom distant only a few Ångström units. The line width of this radical signal would be too great for the absorption to be detectable. (The frequency of

the Brownian rotation of the catalase molecule is low compared with the frequency corresponding to the line width. Whence the orientation of the intramolecular fields with respect to the applied d.c. field are not averaged over a sufficiently short time to decrease the effect of the dipolar interactions.) However, a radical in the protein moiety might be observed. The field at a radical, due to an iron atom of unit spin at a distance of 10 Å, would produce a line-broadening of (half) width less than 30 gauss (g). This signal would be observable.

Samples of steady-state reaction mixture (0.1 ml., 65–70 μM -iron, 4×10^{15} spins) were introduced into small Pyrex tubes which were then placed in the 3 cm. cavity of the spectrometer. At room temperature the region g (Landé splitting factor) = 1.9–2.3 was examined in sections appropriate to the lifetime of the green catalase compound. The absorption was the same as with the Pyrex tube alone. Assuming a line of width (half) of about 30 g for possible free radicals in the protein moiety, their concentration was less than 20% of that of the iron. In experiments in which the reaction mixture was quickly brought to liquid-oxygen temperature, again no resonance was detected; this, for the 30 g line width, indicates that the concentration of free radicals was less than 4% of that of the iron. The absence of a signal in the $g=2$ region suggests that the protein moiety of compound I does not contain a free radical. [There is evidence (Brill, 1960) that free-radical intermediates are produced when reducible substances are present.]

DISCUSSION

Under the conditions in which potassium cyanide was added to the preformed steady state of catalase and ethyl hydroperoxide a minimum of 5% of the catalase was in the free state whereas a minimum of 90% was not free. We have calculated the 'best' spectrum for compound I assuming that the steady state contains 92.5% of compound I (Figs. 6, 7). It is not possible on the basis of our experiments to define the steady state more closely than this. The spectrum of compound I of bacterial catalase is not finally established therefore, but several new features (ii–v) appear in the following list of characteristics: (i) There is a peak at 662 $m\mu$. (ii) There is a broad absorption band from 570 to 600 $m\mu$ with a maximum at 585 $m\mu$ but not distinct α - and β -bands. (iii) There is increased absorption at 450 $m\mu$ over that observed in free catalase. (iv) The intensity of the Soret peak is considerably less than half the free-catalase value and very low compared with all other catalase complexes; its position is obscure. (v) There is increased absorption in the region 290–360 $m\mu$ over free catalase.

Fig. 5. Recording of E , showing the effect of addition of cyanide (0.085 ml. of 2.62 M -potassium cyanide) to the steady-state reaction mixture (3.00 ml. of 4.2 μM -catalase-haematin; 0.052 ml. of 0.017 M -ethyl hydroperoxide). 0.01 M -Phosphate, pH 7.25, λ 426 $m\mu$.

We have looked at the spectrum of peroxidase compound I, which is well established (Fig. 8), carefully seeking similarities with the catalase compound I spectrum. We note the following points. We have preferred the spectrum given by George (1953) for compound I in the visible and that of Fergusson (1956) in the ultraviolet for these authors give the most detailed spectra in the respective regions. Where comparisons can be made, there is little disagreement among different authors (i.e. Keilin & Hartree, 1951; Chance, 1952*a*). We have not distinguished here between the different methods of preparing compound I, either from H_2O_2 or from reagents such as K_2IrCl_6 . The reasons for not making any distinction are largely those of Chance (1952*b*) and Fergusson & Chance (1955). They will become clear in our discussion. (i) There is a band in the region of $650\text{ m}\mu$ which is weaker than the $662\text{ m}\mu$ band of

catalase. (ii) There is a relatively much stronger broad band at about $540\text{--}590\text{ m}\mu$. (iii) There is an absorption band in the region of $420\text{--}490\text{ m}\mu$. The absorption in the region $420\text{--}450\text{ m}\mu$ is higher than that of free peroxidase. (iv) There is a weak Soret band but it is more pronounced than in catalase, being a little more than half of the usual intensity of the Soret bands in peroxidase complexes. This much weakened Soret band of compound I is at a longer wavelength than that of peroxidase itself. (v) There is quite high absorption in the region of $360\text{ m}\mu$, but the absorption here is relatively less than in catalase compound I. There is no evidence as yet that peroxidase compound I absorbs more strongly than does peroxidase itself at shorter wavelengths than $360\text{ m}\mu$, although this looks possible from the spectrum of Fig. 8.

Before attempting to relate the spectra of the two compounds I, we draw attention to the following further facts about the catalase spectrum. The absorption in the region $330\text{--}500\text{ m}\mu$ is obviously not due to a single peak. It can be formulated most simply as a combination of three absorption peaks with maxima at $340\text{--}360$, $405\text{--}415$ and $450\text{--}460\text{ m}\mu$ (Fig. 7). The ratios of the heights of the assumed absorption peaks at 360 and $405\text{ m}\mu$ to that at $662\text{ m}\mu$ are about 3:1 and 4:1 respectively. The peak-height ratio $405\text{ m}\mu:662\text{ m}\mu$ is very low compared with the corresponding ratio (usually 8–15:1) of peak heights of the Soret band to any band in the visible region of the spectra of other iron protoporphyrin complexes. On the basis of these observations we regard the spectrum of compound I as being composed of two parts.

(a) A part due to a normal porphyrin-type complex with absorption in the Soret region $400\text{--}410\text{ m}\mu$, and in the visible between 440 and

Fig. 6. Absorption spectrum of catalase compound I in the visible region, corrected for the presence of 7.5% of free catalase in the steady-state reaction mixture. Points indicate the wavelengths at which changes in extinction coefficient were determined from steady-state measurements. The spectrum of free catalase is given for comparison.

Fig. 7. Solid curve: absorption spectrum of catalase compound I in the u.v. region, corrected for the presence of 7.5% of free catalase in the steady-state reaction mixture. Broken curves: analysis of this spectrum into three absorption bands.

Fig. 8. Absorption spectrum of compound I of peroxidase. Soret-band region is from Fergusson (1956). Visible region is from George (1953). The spectrum of free peroxidase is given for comparison (broken curve).

480 m μ (the second charge-transfer band, see preceding paper) and between 540 and 600 m μ (the α - and β -bands).

(b) A part due to some other type of complex which has bands in the region 340–360 m μ and at 662 m μ .

In clarification of point (b) we note that no known ferric protoporphyrin complex has such strong bands as low as 360 m μ or as high as 662 m μ . The usual δ -bands of haemoproteins are only about one-fifth of the intensity of the Soret bands. We have also examined the published spectra of compound I of erythrocyte catalase (Chance, 1952*a*) and find that, although undoubtedly partly in error, it has most of the outstanding features mentioned above.

This analysis of the spectrum of compound I of catalase can be extended to compound I of peroxidase, and shows that there is a close similarity between the two. We describe each as a combination of the two component spectra. For catalase compound I it is spectrum *b* with bands at 360 and 662 m μ which predominates, whereas for peroxidase it is spectrum *a* with bands at 410, 480 and 580 m μ which predominates.

Magnetic-susceptibility measurements on the reaction of bacterial catalase with methyl hydroperoxide (Brill, 1960) show a change in molar susceptibility on going from the free enzyme to the steady-state reaction mixture of -7800×10^{-6} e.m.u. at 20°. In order to calculate the concentration of compound I in the reaction mixture we need to know its spectrum. Rather than presume that the spectrum of catalase methyl hydroperoxide compound I is the same as that of ethyl hydroperoxide compound I, we have used the published data of Chance & Herbert (1950). We believe this spectrum to be incorrect, owing to the presence of about 10% of free catalase and the magnetic moment we calculate for catalase compound I is probably too high. Free catalase is a high-spin ferric complex of molar paramagnetic susceptibility $14\,000\text{--}14\,500 \times 10^{-6}$ e.m.u., which makes the susceptibility of compound I $6000\text{--}6500 \times 10^{-6}$ e.m.u. Theorell & Ehrenberg (1952) obtained a value of 6500×10^{-6} e.m.u. for compound I of peroxidase. These susceptibility values are close to the 'spin only' value of 6340×10^{-6} e.m.u. for a complex with average spin 3/2.

Thus the two compounds I of peroxidase and catalase are similar in magnetic moment. Table 2

shows that in those complexes where catalase and peroxidase have intermediate moments corresponding to between 1 and 5 unpaired electrons, the peroxidase complex has much the lower moment. This suggests that compound I is unlikely to be a simple complex of ferric iron which is the same for both catalase and peroxidase.

The magnetic evidence allows the possibilities that compound I is (a) a simple Fe^V complex of spin 3/2, (b) a mixture of iron complexes of average spin per iron close to 3/2, (c) a radical in the (i) porphyrin or (ii) protein moiety combined with a strong field Fe^V state (unit spin), (d) a diradical in the porphyrin (unit spin) combined with a strong field Fe^{III} state (spin 1/2). The long half-life of compound I argues against the assumption of any radical, and we therefore are inclined to dismiss (c) and (d). Additional evidence against (c ii) is provided by the paramagnetic-resonance absorption spectra of catalase and peroxidase compounds I which give no signal for a radical of $g=2$. We are unable to offer an explanation of the spectra of the peroxidase and catalase complexes on the basis of assumption (a). No metal porphyrin complex of spin 3/2 which is not a mixture of spin states is known. However, the Cr^{III} phthalocyanine complexes (spin 3/2) have been studied (Anderson, Bradbrook, Cook & Linstead, 1938). Their spectra are in no way exceptional. Phthalocyanines and porphyrins resemble one another closely in spectroscopic properties, and we would expect Fe^V porphyrin complexes to have a typical metal porphyrin spectrum. The spectrum of compound I is very different from this. We can offer the following explanation of the spectrum on assumption (b) that compound I is a mixture of two components. We shall attempt to show that they are the ferric complexes given in Fig. 9.

Component *A*, which we shall call 'POR', appears to retain the porphyrin ring intact. POR could be a simple ferric porphyrin complex, either

with a proton attached elsewhere to the protein. It is highly probable that the initial step in the peroxide reaction with peroxidase (or catalase) is the direct attack of the peroxide on the iron atom. The evidence is that peroxidase is oxidized to compound I by peroxides at pH 7.0, but not at pH 11.0 (Chance, 1949*b*), when peroxidase is present as the Fe^{III}-OH⁻ complex. The latter is a

Table 2. *Magnetic moments in Bohr magnetons of complexes of catalase and of peroxidase (Hartree, 1946)*

	F ⁻	H ₂ O*	N ₃ ⁻	SH ⁻	CN ⁻	OH ⁻
Catalase	5.89	5.89	5.86	4.20	4.02	—
Peroxidase	5.90	5.48	—	2.40	2.67	2.66

* The H₂O column refers to catalase and peroxidase themselves.

strong-field complex (low-spin) which exchanges ligands slowly and cannot form a complex $\text{Fe}^{\text{III}} \leftarrow \text{HO}_2\text{Et}$ rapidly. Catalase, which undergoes no corresponding acid dissociation, reacts with peroxides independently of pH (Chance, 1952*c*). On the other hand, K_2IrCl_6 and $\text{K}_3\text{Mo}(\text{CN})_8$

oxidize peroxidase to compound I only in alkaline solution (George, 1953). Strong-field complexes are more readily oxidized by electron-transfer reagents such as K_2IrCl_6 and $\text{K}_3\text{Mo}(\text{CN})_8$. The reaction with chloroiridate would proceed as in Fig. 10. The final complex is identical with the component B of

Fig. 9. The suggested components (A and B) of compound I. P, Protein.

Fig. 10. Proposed reaction of peroxidase with chloroiridate in alkaline solution. P, Protein.

Table 3. *Properties of the primary peroxide compounds*

The approximate fractions of enzyme present at equilibrium as the ROX and POR components have been estimated from the spectra of the compounds I. The magnetic moments of POR of catalase and ROX of peroxidase have been taken as 5.9 and 2.1 Bohr magnetons respectively, and the moments of ROX of catalase and POR of peroxidase computed from $\mu_{\text{meas.}}^2 = f\mu_{\text{ROX}}^2 + (1-f)\mu_{\text{POR}}^2$, where f is the estimated fraction of enzyme present as ROX and $\mu_{\text{meas.}}$ is the measured magnetic moment.

	Absorption maxima (m μ)		Approx. fraction	Magnetic moment (β)
Catalase	ROX	360, 662	2/3	2.3
	POR	405, 450, 585	1/3	5.9
Peroxidase	ROX	360, 650	1/3	2.1
	POR	410, 480, 580	2/3	4.5

compound I (Fig. 9) which we expect to be formed by H_2O_2 . It is also a tautomer of component A. The proportion of A to B will be a function of the relative stability of these components.

Component B, which we shall call 'ROX' (for oxidized ring), is not a porphyrin but a bile-pigment type of compound. It has a system of conjugated bonds like that of glaucobilin (Stern & Pruckner, 1937), or hydroxyporphyrin haematin (Lemberg, Cortis-Jones & Norrie, 1938). Glaucobilin has absorption bands at 360 m μ and 650–680 m μ and, unlike porphyrins, the ratio of the intensities of the bands is roughly 3.0. Hydroxyporphyrin haematin also has a spectrum with bands at 360 m μ and above 600 m μ . These spectra bear marked similarities to that of part B of the compound I spectrum.

The magnetic moment of compound I demands that this ferric complex (assuming that it is a ferric complex) should have an average spin of about 3/2. We expect ROX, as a ferric-hydroxyl type of complex to have a moment less than that for spin 5/2 and probably closer to spin 1/2. POR is probably also of spin less than 5/2, but certainly of greater spin than ROX. The moments of the POR and ROX components of peroxidase should be lower than those of catalase to be consistent with known complexes (Table 2). The order of magnetic moments is very likely

$$\text{POR}_{\text{cat.}} > \text{POR}_{\text{per.}} > \text{ROX}_{\text{cat.}} > \text{ROX}_{\text{per.}}$$

so that the average moment of compound I of peroxidase (which is mostly POR) can well be the same as that of compound I of catalase (which is mostly ROX).

The changes in the absorption spectrum of ferric haemoproteins which occur as they are changed from the high-spin (5/2) type to types of intermediate spin (between 5/2 and 1/2) such as POR are known from studies of, for example, the influence of pH changes on the spectrum and paramagnetic susceptibility of metmyoglobin (Theorell & Ehrenberg, 1951). As the magnetic moment falls we expect (a) a movement of the Soret band to

longer wavelengths, (b) increased absorption between 530 and 590 m μ , and (c) decrease in absorption at 630 m μ , and between 500 and 530 m μ . The same correlation, discussed in detail in the preceding paper, between changes in the absorption spectrum and magnetic moment has been shown by varying the ligand in methaemoglobin complexes (Scheler, Schoffa & Jung, 1957). All these changes but one occur in the peroxidase reactions with hydroperoxides and are due to POR. The exception is the increase in absorption at 630 m μ which we have already attributed to the presence of some of the oxidized ring structure, ROX. For catalase, the Soret band does not shift perceptibly to the red because the POR component of catalase compound I should be of quite high spin. Also ROX is the major component and has a greater influence on the spectrum, tending to shift the Soret band to shorter wavelengths.

Table 3 summarizes our views about the nature of compound I. It will be seen that the two molecules suggested as components of compound I are such that migration of a group changes one to the other. If this is so it is pointless to discuss whether there is an enzyme-substrate complex or a chemical compound. Our theory is open to test for it requires that the ferric ion in complexes like ROX should have a low spin.

SUMMARY

1. A study has been made of the properties of the primary compound formed between ethyl hydroperoxide and bacterial catalase. The compound is incompletely formed under the experimental conditions and its physical properties have been deduced from an examination of the steady-state reaction mixture.

2. The absorption spectrum obtained for compound I differs from that reported in the literature. It has a very weak Soret band, a band at approximately 360 m μ and two bands in the visible at approximately 580 and 660 m μ . This spectrum is unlike that of any other iron porphyrin complex.

3. Consideration of the physical and chemical properties of compounds I of bacterial catalase and of peroxidase has led us to suppose that these compounds are mixtures of two components. One of the components is a simple ferric porphyrin complex with the hydroperoxide. The other is the result of an attack of the hydroperoxide upon the porphyrin ring.

The authors wish to thank Professor Sir Hans Krebs, F.R.S., for providing laboratory facilities for preparing the solutions and for the use of the Cary spectrophotometer.

REFERENCES

- Anderson, J. S., Bradbrook, E. F., Cook, A. H. & Linstead, R. P. (1938). *J. Chem. Soc.* p. 1151.
 Beers, R. F., jun. (1955). *Science*, **122**, 1016.
 Bonnichsen, R. K., Chance, B. & Theorell, H. (1947). *Acta chem. scand.* **1**, 685.
 Brill, A. S. (1960). *Symposium on Free Radicals in Biological Systems, Stanford University*, 1960 (in the Press). New York: Academic Press Inc.
 Chance, B. (1949a). *J. biol. Chem.* **179**, 1311.
 Chance, B. (1949b). *Arch. Biochem.* **22**, 224.
 Chance, B. (1952a). *Arch. Biochem. Biophys.* **41**, 404.
 Chance, B. (1952b). *Arch. Biochem. Biophys.* **41**, 425.
 Chance, B. (1952c). *J. biol. Chem.* **194**, 471.
 Chance, B. & Fergusson, R. R. (1954). In *Mechanism of Enzyme Action*, p. 389. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
 Chance, B. & Herbert, D. (1950). *Biochem. J.* **46**, 402.
 Euler, H. von & Josephson, K. (1927). *Leibigs Ann.* **452**, 158.
 Fergusson, R. R. (1956). *J. Amer. chem. Soc.* **78**, 741.
 Fergusson, R. R. & Chance, B. (1955). *Science*, **122**, 466.
 George, P. (1953). *Science*, **117**, 220.
 Hartree, E. F. (1946). *Rep. Progr. Chem.* **43**, 295.
 Herbert, D. & Pinsent, J. (1948). *Biochem. J.* **43**, 193.
 Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
 Lemberg, R., Cortis-Jones, B. & Norrie, M. (1938). *Biochem. J.* **32**, 149, 171.
 Llewellyn, M. (1957). *J. sci. Instrum.* **34**, 236.
 Reiche, A. (1931). *Alkylperoxyde und Ozonide*. Dresden: Steinkopff.
 Scheler, W., Schoffa, G. & Jung, F. (1957). *Biochem. Z.* **329**, 232.
 Stern, K. & Pruckner, F. (1937). *Z. Phys. Chem.* **178** A, 420.
 Theorell, H. & Ehrenberg, A. (1951). *Acta chem. scand.* **5**, 823.
 Theorell, H. & Ehrenberg, A. (1952). *Arch. Biochem. Biophys.* **41**, 442.

Biochem. J. (1961) **78**, 262

Studies in Detoxication

85. THE METABOLISM OF *m*-DINITRO[¹⁴C]BENZENE IN THE RABBIT*

By D. V. PARKE

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 25 July 1960)

m-Dinitrobenzene is used industrially as a dye-stuff intermediate and as a constituent of mining explosives. Animals poisoned with *m*-dinitrobenzene develop methaemoglobinaemia, anaemia, liver damage, convulsions and cerebral paralysis (Kunz, 1942; Kiese, 1949). The tissues of rabbits dosed with *m*-dinitrobenzene have been shown to contain *m*-nitroaniline (Belaborodova, 1945) and *m*-nitrophenylhydroxylamine (Lipschitz, 1920), and reduction of *m*-dinitrobenzene to *m*-nitrophenylhydroxylamine has been demonstrated *in vitro* in rabbit muscle (Comel, 1931) and blood (Lipschitz, 1948). In the presence of fermenting yeast, *m*-dinitrobenzene is converted into *m*-nitroaniline and 3:3'-dinitroazoxybenzene (Neuberg & Reinfurth, 1923) which is probably an artifact of *m*-nitrophenylhydroxylamine formed by atmospheric oxidation. The formation of dinitroazoxybenzene is evidence of the probable stepwise reduction of *m*-

dinitrobenzene to *m*-nitrosonitrobenzene, *m*-nitrophenylhydroxylamine and *m*-nitroaniline.

The present work with *m*-dinitro[¹⁴C]benzene has made it possible to account for the greater part of an oral dose, most of which is quickly excreted in the urine as *m*-nitroaniline, *m*-phenylenediamine, 2-amino-4-nitrophenol and 2:4-diaminophenol, together with traces of 2:4-dinitrophenol, 4-amino-2-nitrophenol, *m*-nitrophenylhydroxylamine and artifacts derived from this, and unchanged *m*-dinitrobenzene.

EXPERIMENTAL

Melting points are corrected.

Preparation of m-dinitro[¹⁴C]benzene. *m*-Dinitro[¹⁴C]benzene, m.p. 91°, randomly labelled with ¹⁴C in one carbon atom, was obtained as a by-product of the preparation of nitro[¹⁴C]benzene (Parke, 1956). Unlabelled *m*-dinitrobenzene (2 g.) was added to the solid residue which remained after distillation of the nitro[¹⁴C]benzene, and the material was recrystallized from ethanol until it was shown

* Part 84: Parke (1960).