Spectrophotometric Titrations involving the Higher Oxidation State of Ferrimyoglobin.

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According to spectrophotometric titrations with ascorbic acid (AH_2), ferromyoglobin ($MbFe^{2+},H_2O$), and oxymyoglobin [$MbFe(II)O_2^{2+}$] as reducing agents, the intermediate compound formed when ferrimyoglobin [$MbFe^{3+},H_2O$] reacts with hydrogen peroxide is one oxidation equivalent above the ferric state of the hæmoprotein, in conformity with the previous result obtained by using potassium ferrocyanide. Hence this higher oxidation state has properties characteristic of a quadrivalent iron compound [MbFe(IV)] and the reactions can be represented : *

$$2MbFe(IV) + AH_2 \longrightarrow 2MbFe^{3+},H_2O + A$$
$$MbFe(IV) + MbFe^{2+},H_2O \longrightarrow 2MbFe^{3+},H_2O$$
$$MbFe(IV) + MbFe(II)O_2^{2+} \longrightarrow 2MbFe^{3+},H_2O + O_2$$

SPECTROPHOTOMETRIC titration of the intermediate compound which is formed when ferrimyoglobin reacts with peroxides, with potassium ferrocyanide as reducing agent, showed that this compound is a single equivalent oxidation product (George and Irvine, *Nature*, 1951, **168**, 164; *Biochem. J.*, 1952, **52**, 511; 1953, **55**, 230). Since in ferrimyoglobin the iron is in the ferric state, the intermediate compound has the oxidation–reduction characteristics of a quadrivalent iron derivative. Its relation to ferrimyoglogin, represented by $MbFe^{3+},H_2O$, can thus be summarised :

$$MbFe^{3+},H_2O \longrightarrow MbFe(IV) + e^- \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where MbFe(IV) denotes the higher oxidation state.

Recent experiments (George and Irvine, Symp. on Co-ordination Chemistry, Copenhagen, 1953, Danish Chem. Soc., in the press) are in accord with a "ferryl ion" type of structure for MbFe(IV), which, if further substantiated, would require reaction (1) to be amplified as follows :

$$MbFe^{3+},H_2O \longrightarrow MbFe(IV)O^{2+} + 2H^+ + e^-$$

But further confirmation of the single equivalent oxidation is desirable because the stoicheiometry of the oxidation process is the basis of all further work and gives the intermediate compound its particular significance. MbFe(IV) would be the first compound

* The following conventions have been adopted : $MbFe^{2+},H_2O$ and $MbFe^{3+},H_2O$ denote ferro- and ferri-myoglobin respectively in which the superscripts 2+ and 3+ indicate that the iron is ionically bound, and the sixth co-ordination position is occupied by a water molecule. In these and the other compounds the five remaining positions are occupied by four bonds in a plane to pyrrole-nitrogen atoms and one bond to the protein. $MbFe(II)O_2^{2+}$ denotes oxymyoglobin, the symbol (II) indicating a covalently bonded complex of ferrous iron. MbFe(IV) denotes the higher oxidation state which also has covalently bonded iron, but the formula cannot be completed because the structure has not yet been established with certainty. The equations in which MbFe(IV) occurs cannot therefore be balanced, and are only to be taken as a correct representation of the interchange of oxidation equivalents : there is evidence in some cases that hydrogen ions are also involved, and it is through their participation that the ionic charges on reactants and products become equal. This usage is somewhat similar to that current in the U.S.A. where the roman superscripts are occasionally used to denote generalised reactions between various oxidation states.

of its type to be well authenticated, and, from the point of view of its oxidation–reduction behaviour, it appears to be analogous to the second of the two intermediate compounds formed by the hæmoprotein enzymes, peroxidase and catalase, with peroxides. The action of these enzymes has until recently been considered almost exclusively in terms of enzyme–substrate complex formation, *i.e.*, $E + S \rightleftharpoons [ES]$, etc. (see, *e.g.*, Chance, "Modern Trends in Physiology and Biochemistry," Academic Press Inc., New York, 1952, p. 25), but now a new mechanism with two single equivalent reduction steps linking the two intermediate compounds takes its place (George, *Adv. Catalysis*, 1952, **4**, 367; *Biochem. J.*, 1952, **53**, xix; 1953, **54**, 267).

The intermediate compound was formed as in the previous experiments (George and Irvine, *loc. cit.*, 1952) by adding the minimal quantity of hydrogen peroxide to ferrimyoglobin in borate buffer of pH 8·6. At this pH no side reactions occur. At other pH values, where this would not be the case, the intermediate compound was first made in very weak buffer of pH 8·6 and then transferred to a stronger buffer of the required pH. The results of spectrophotometric titrations with ascorbic acid as reducing agent are given in the Table. The titrations at pH 8·6 were carried out in the absence of oxygen to over-

Reduction of the intermediate compound, present in excess, with ascorbic acid at ~18°.

		At pH 5·9 in air				At pH 8·6, O_2 absent		
Concn. (10^{-6}M) of ascorbic acid present initially (*a*)	4·5	9·0	9·0	15·0	15·0	9·0	15·0	15·0
Concn. (10^{-6}M) of intermediate compound present initially	32·0	32·0	32·0	32·0	32·0	26·3	33·8	37·5
Intermediate compound (10^{-6} mole) reduced by ascorbic acid (*b*)	10·7	18·2	17·7	30·1	27·2	17·5	27·9	25·8
Ratio: *a*/*b*	0·42	0·49	0·51	0·50	0·55	0·51	0·54	0·59
		(Mean, 0·49 ± 0·03)				(Mean, 0·55 ± 0·03)		

come the difficulties caused by autoxidation of the ascorbic acid. Not only is ascorbic acid destroyed by autoxidation, but also the hydrogen peroxide so formed oxidises some ferrimyoglobin. At pH 5·9 reduction was much faster than at pH 8·6 so that the effect of autoxidation was negligible, particularly when quartz-distilled water, free from traces of copper, was used. It will be seen from the Table that approximately 0·5 mole of ascorbic acid (AH_2) reduces one mole of the intermediate compound to ferrimyoglobin in an overall reaction showing the following stoicheiometric relation:

$$2MbFe(IV) + AH_2 \longrightarrow 2MbFe^{3+},H_2O + A$$

Experiments were also carried out with ferromyoglobin [$MbFe^{2+},H_2O$] and oxymyoglobin [$MbFe(II)O_2^{2+}$] as reducing agents. Even though the latter contains an oxygen molecule bonded to the iron, oxymyoglobin still reacts as a ferrous complex; for instance, addition of potassium ferricyanide oxidises it to the ferric state (ferrimyoglobin) and the oxygen is liberated as such. In these experiments equimolar quantities (5×10^{-5}M) of the intermediate compound and the reducing agent were mixed, then when reduction was complete sodium azide was added. The spectrum of the azide complex thus obtained was then compared with that of the azide complex made from an equivalent concentration of fresh ferrimyoglobin. With ferromyoglobin the experiments were conducted in the absence of oxygen to eliminate the formation of oxymyoglobin. The results are illustrated in the Figure, which shows that equimolar quantities of the intermediate compound and ferromyoglobin or oxymyoglobin give almost solely two equivalents of ferrimyoglobin. These reactions can thus be written

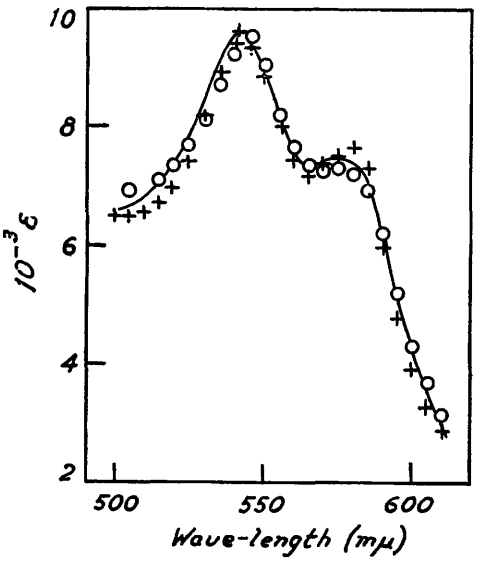
$$MbFe(IV) + MbFe^{2+},H_2O \longrightarrow 2MbFe^{3+},H_2O \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

$$MbFe(IV) + MbFe(II)O_2^{2+} \longrightarrow 2MbFe^{3+},H_2O + O_2 \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

Both reactions were relatively slow, requiring 20—30 minutes for completion with 5×10^{-5}M-solutions at pH 8·6. As an approximation, if 97% reaction occurred in 20 minutes, the bimolecular velocity constant would be about 500 l. mole^{-1} sec.$^{-1}$. In more acid solutions the reactions were faster. The slow rate must be attributed either to the way in which the intermediate compound acts as an oxidising agent or to the fact that

reaction is between two hæmoprotein molecules, for both ferromyoglobin and oxymyoglobin react with electron-transfer oxidising agents, *e.g.*, potassium chloroiridate, with velocity constants greater than 10^5 l. mole^{-1} sec.$^{-1}$ (George and Irvine, *J.*, 1954, 587). The first is the more likely explanation since the comparable reaction of ferrocytochrome-*c* with the higher oxidation state of cytochrome-*c* peroxidase (two other hæmoproteins) is much faster, reaching completion in less than 15 seconds with about 10^{-6}M-solutions (George, *Biochem. J.*, 1953, **54**, 267). A lower limit of about 3×10^6 l. mole^{-1} sec.$^{-1}$ is obtained for the velocity constant on the assumption that 97% reaction occurs in 10 seconds.

These titrations thus confirm the view that ferrimyoglobin undergoes single equivalent oxidation when it reacts with peroxide. The participation of higher oxides of iron, Fe_2O_4 and Fe_2O_5, as intermediates in the reactions of iron salts with peroxide, was often proposed before free-radical mechanisms were advanced (see, *e.g.*, Hale, *J. Phys. Chem.*, 1929, **33**, 1633), and later Polonovski, Jayle, and Glotz (*Bull. Soc. Chim. biol.*, 1939, **21**, 48) suggested the formation of a quadrivalent iron intermediate in the ferrihæmoglobin–peroxide reaction. Polonovski, Jayle, and Fraudet (*Compt. rend.*, 1941, **213**, 740)



Absorption spectra of the azide complex formed from the products of the reduction of the intermediate compound with an equimolecular concentration of ferromyoglobin (○) *and oxymyoglobin* (+), *and that formed from an equivalent concentration of fresh ferrimyoglobin* (*smooth curve*).

attempted to confirm its formation by potentiometric titration; but we have shown (George and Irvine, *Biochem. J.*, 1954, **58**, 188) that this evidence for the formation of the higher oxidation state is not unequivocal.

The reduction of the intermediate compound with ferromyoglobin and oxymyoglobin (reactions 2 and 3), together with the similar reaction of ferrocytochrome-*c* cited above, provide examples of disproportionation between various oxidation states of a transition element which are very familiar for some transition elements but have hitherto not been demonstrated for iron : reactions between ferrous or ferric salts and the ferrates [Fe(VI) in FeO_4^{2-}] cannot be studied because the former are soluble only in acid solution and the latter stable only in alkaline solution, decomposing with the liberation of oxygen if the solution is acidified.

Experimental

Ferrimyoglobin, Oxymyoglobin, and Ferromyoglobin.—The preparation of ferrimyoglobin has been described (George and Irvine, *loc. cit.*, 1952). The oxygen complex of ferromyoglobin was obtained by reducing a solution of ferrimyoglobin at the required pH with sodium dithionite (hydrosulphite) in slight excess, then shaking the solution in air until all the dithionite was oxidised. The formation of oxymyoglobin was indicated by the change from brown to pink, and identified by the characteristic absorption bands at 542 and 580 mμ. Ferromyoglobin was prepared by deoxygenating oxymyoglobin in the apparatus described below.

Ascorbic Acid.—Analytical-grade L-ascorbic acid was dissolved in quartz-distilled water immediately before use, in flasks which had been thoroughly flushed with nitrogen.

Buffer Solutions.—The buffer solution of pH 8·6 was prepared from 0·3M-solutions of boric acid and sodium hydroxide according to the procedure of Clark and Lubs. The phosphate buffer of pH 5·9 was made from 0·04M-solutions of sodium dihydrogen phosphate and sodium hydroxide.

Experiments in the Absence of Oxygen.—The apparatus was a boat-shaped vessel of about 10 ml. capacity, having a side-arm with a ground-glass joint into which a tube containing the second reactant could be fitted. The vessel had also two outlets which could be closed by taps, one of which was attached to a high-vacuum pump. There was a lead from the apparatus to a source of oxygen-free nitrogen, obtained by passing the highest grade of cylinder nitrogen over copper at 400°.

One solution was placed in the vessel and the other in the tube attached to the side-arm; both solutions were evacuated with constant shaking, and the apparatus was then flushed with nitrogen. This process was repeated 2—3 times and after a final evacuation the solutions were mixed and allowed to react *in vacuo*. To minimise losses due to evaporation the vessel was immersed in ice–salt during evacuation.

In the experiments with ferromyoglobin, oxymyoglobin was placed in the side-arm, and deoxygenation and reduction were carried out in the same apparatus.

Spectrophotometric Measurements.—Measurements were made with a Unicam Quartz Spectrophotometer. In the reduction experiments with ascorbic acid the measurements of optical density were made at 549 mμ, with 1 cm. cuvettes. From the optical density of a ferrimyoglobin solution of a specified concentration and the maximum change produced on addition of hydrogen peroxide, which can be taken to correspond to complete conversion into the intermediate compound, the concentration of intermediate compound reacting with added ascorbic acid was calculated.

For reductions with ferromyoglobin and oxymyoglobin, the extinction coefficients of the azide complex shown in the Figure were obtained by assuming that the reduction product was entirely ferrimyoglobin.

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