ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE ELDRIDGE REEVES JOHNSON FOUNDATION FOR MEDICAL PHYSICS, UNIVERSITY OF PENNSYLVANIA]

On the Oxidation of Peroxidase by One-electron Oxidizing Agents[1]

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The stoichiometry and kinetics of the action of bromoiridate (a one-electron oxidant) on peroxidase were studied in order to shed light on the reaction mechanism. The oxidation potential of K_2IrCl_6 was found as $E^0 = -0.93$ volt, that of K_2IrBr_6 as $E^0 = -0.92$ volt. Above pH 7, hydrolysis occurs. It was found that more than 4 moles of K_2IrBr_6 is required to oxidize one mole peroxidase to complex I. Complex II was never observed to be formed from the enzyme directly but always by way of complex I. The results of this study make it seem possible that complex formation occurs by virtue of an intermediate formation of H_2O_2, possibly by a Haber–Weiss back reaction or as a product from the reaction of K_2IrBr_6 with protein groups such as histidine. The applicability of this hypothesis is discussed.

Increasingly detailed studies of the mechanisms of enzyme reactions have led to many modifications of the general principles to which these systems have formerly been supposed to adhere. One such principle has been the assumption that hydrogen peroxide is the specific substrate for the iron–porphyrin containing enzymes catalase and peroxidase. The discovery by George[2] that the enzymatically active complexes of peroxidase can also be formed when this enzyme reacts with other oxidizing agents has forced a more searching analysis of this assumption.

George has shown that peroxidase can be oxidized by a variety of one- and two-equivalent oxidants including K_2IrCl_6, $K_3Mo(CN)_8$,[3] $NaClO_2$, NaOCl, ClO_2 and O_3 to compounds similar to the enzymatically active complexes I and II[4,5] which are, respectively, two and one oxidizing equivalents higher than peroxidase itself.

The complexes of peroxidase and catalase may play an important part in the oxidative pathways of living cells. That hydrogen peroxide is produced within the bacterial cell in sufficient quantities to convert catalase completely to its complex I has been recently demonstrated by Chance.[6] Nevertheless the inference from George's work[5] that some other oxidizing agent could function in place of hydrogen peroxide needs a critical examination.[7] The purpose of this paper is to discuss this suggestion with special reference to the experiments described herein on the oxidation of peroxidase by one-electron oxidizing agents.

The following considerations were believed to afford the strongest support for the proposal that H_2O_2 is not an intermediary when the aforementioned oxidants react with peroxidase.

1. The reactions between peroxidase and HOCl, HOBr, $NaClO_2$ and O_3 leading to the formation of the complexes are very fast. If peroxide is produced by a simple non-catalytic mechanism, a bimolecular velocity constant for the initial step of 10^5 mole^{-1} × sec.$^{-1}$ may be required.

2. Peroxidatic activity tests made by measuring the rate of donor (guaiacol) oxidation suggested that peroxidase reacts more rapidly with hypochlorite than with H_2O_2.[5]

3. The rate of formation of the higher oxidation state of metmyoglobin, a compound quite different from peroxidase complex I, is much faster with the one-electron transfer oxidants K_2IrCl_6 and $K_3Mo(CN)_8$ than it is with H_2O_2.[9]

Taking up these points in turn, it suffices to say of the first that Chance's direct studies[10] of the rate of complex I formation using $NaClO_2$ as oxidant are consistent with a bimolecular velocity constant no higher than 4×10^5 mole^{-1} × sec.$^{-1}$ for the initial step; a rate constant of this magnitude would be adequate to ensure that the initial step be rate determining. Chance's unpublished data suggest that the reaction with NaOCl is not of the second order. Two-electron oxidative reactions of hypochlorite with velocity constants approaching this order of magnitude are known to occur[11] and a rapid initial reaction which proceeded by oxygen atom or hydride ion transfer might conceivably give rise to peroxide formation.

To the second point it can be stated that activity determinations based on donor oxidation can give ambiguous results unless care is taken to ensure no unknown intermediates are rate limiting and corrections are made for the non-enzymatic oxidation of donor. A recalculation of George's work shows that it suffers from some of these faults and in addition does not give a rate of reaction of hypochlorite with peroxidase that is faster than the rate with H_2O_2.

The following points of George's paper[5] need comment:

(a) Of the oxidants listed in Table II,[5] only HOCl shows a rate exceeding that of H_2O_2.

(1) This work has been supported in part by the National Science Foundation.

(2) P. George, *Nature*, **169**, 612 (1952).

(3) This oxidant apparently leads to the formation of complex II only. In addition this compound hydrolyzes very readily in the light. The cyanide ions so formed complex very readily with peroxidase and change its light absorption in those regions of the spectrum where the oxidation process is best studied. The ionization of a spectroscopically observable heme-linked group also affects the absorption in solutions of pH >10 necessary for complex formation with this compound.

(4) P. George, *Arch. Biochem. Biophys.*, **45**, 21 (1953).

(5) P. George, *J. Biol. Chem.*, **201**, 413 (1953).

(6) B. Chance, *Science*, **116**, 202 (1952).

(7) Recent reviews of the mechanisms of peroxidase and catalase reactions have been provided by P. George and D. H. Irvine, *Brit. J. Radiology*, **27**, 131 (1954), and B. Chance and R. R. Fergusson.[8]

(8) B. Chance and R. R. Fergusson in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, ed., John Hopkins University Press, Baltimore, Md., 1954, p. 389.

(9) P. George and D. H. Irvine, *Biochem. J.*, **55**, xxv (1953), and **58**, 188 (1954).

(10) B. Chance, *Arch. Biochem. Biophys.*, **41**, 425 (1952).

(11) R. E. Connick, THIS JOURNAL, **69**, 1509 (1947).

(b) Using the plots of Fig. 7,[5] we get initial rates of not more than 0.070 density unit per minute, so that, since

$$k = \frac{da}{dt} \times \frac{1}{e} \times \frac{1}{a_0} \qquad (1)^5$$

$$k' = \frac{1 \times 60}{400} \times 10^{-6} \times \frac{1}{2.6 \times 10^{-10}} \times \frac{1}{5 \times 10^{-5}} =$$

1.1×10^7 mole^{-1} l. sec.$^{-1}$ at 30° for $a_0 = 5 \times 10^{-5}$

$$k'' = \frac{1.16 \times 60}{400} \times 10^{-6} \times \frac{1}{2.6 \times 10^{-10}} \times \frac{1}{10^{-6}} =$$

0.77×10^7 mole^{-1} l. sec.$^{-1}$ at 30° for $a_0 = 10^{-6}$

(c) The values that we can compute from George's data do not exceed the velocity constant for H_2O_2 (*cf.* the second point above) as obtained from experimental data presented below. George found that guaiacol reacts with HOCl even in the absence of enzyme. Strong evidence for this is also provided by Fig. 7 where the final concentration of tetraguaiacol amounts to only about one tenth of the value calculated for a complete reaction of HOCl.

(d) The rate of formation of tetraguaiacol is not proportional to the [HOCl]. A 100% increase in HOCl concentration leads to an increase in rate of at the most 30% (*cf.* also above under (b)).

Chance and Maehly[12] have recently shown by experiment that donor (guaiacol) oxidation by peroxidase and H_2O_2 can be made to depend on k_1 (formation of complex I) or k_4 (reduction of complex II by the donor) depending on the conditions of the activity assay

$$-\frac{d\,H_2O_2}{dt} = \frac{e}{\frac{1}{k_4 a_0} + \frac{1}{k_1 x_0}} \qquad (2)$$

where

e = enzyme concn.
a_0 = initial concn. of donor
x_0 = initial concn. of substrate

if $k_4 a_0 >> k_1 x_0$, k_1 becomes rate limiting; if $k_1 x_0 >> k_4 a_0$, k_4 is rate limiting. Actual experiments at *p*H 7 gave the values $k_1 = 0.89 \times 10^7$ at 20° and $k_1 = 1.03 \times 10^7$ at 30°.

The experimental conditions of the activity measurements of George do not allow to decide which of the two rate constants are measured, since $k_4 a_0 \backsimeq k_1 x_0$. ($a_0 = 6.7 \times 10^{-3}$, $k_4 = 3 \times 10^5$, $k_4 a_0 = 2 \times 10^3$; $k_1 = 1.0 \times 10^7$, $x_0 = 10^{-4}$, $k_1 x_0 = 1.0 \times 10^3$). Chance has shown[10] that the sequence of complex formation of peroxidase by hypochlorite is very similar to that by H_2O_2 except that the formation of complex I requires considerably more oxidant. Accordingly no conclusion can be drawn about the presence of H_2O_2 from such experiments.

Concerning point three, the higher rate of oxidation of metmyoglobin by K_2IrCl_6 and $K_3Mo(CN)_8$ in solutions of *p*H greater than 6.8 and 11.0, respectively,[9] as compared with the corresponding rates with H_2O_2 is perhaps the strongest piece of evidence for the non-involvement of H_2O_2 in complex formation, and a one-step electron transfer mechanism seems plausible with this hemoprotein. But with peroxidase and K_2IrCl_6 there is evidence that complex I, having two oxidizing equivalents, precedes the formation of complex II. Moreover, the rates of complex formation by H_2O_2 and one-electron oxidants have not yet been compared in the case of peroxidase. The results of such studies together with titration data and polarographic measurements of the oxidants used are here presented.

Experimental

Materials.—The horseradish peroxidase (HRP) used in this work was generously provided by A. C. Maehly. It was prepared by a modification of Theorell's method.[13] The purity was characterized by R.Z. 2.86 and the enzymatic activity by the following values for the rate constants with H_2O_2 and guaiacol, respectively

$k_1 = 1.03 \times 10^7$ mole^{-1} l. sec.$^{-1}$ at 30°

$k_4 = 3.1 \times 10^5$ mole^{-1} l. sec.$^{-1}$ [12]

The K_2IrCl_6 and K_2IrBr_6 were prepared by standard methods and estimated by titration against $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$. Solutions were made up in 0.01 molar $HClO_4$ to prevent hydrolysis.

Procedures.—Polarographic measurements were made with a rotating (1800 r.p.m.) gold electrode chosen in preference to platinum because of its higher anodic decomposition potentials. A silver/silver chloride electrode was the reference standard.

Spectroscopic measurements were made on a sensitive spectrophotometer equipped to follow fast reactions.[14]

Figure 4 was obtained by a sensitive recording spectrophotometer designed by Yang.[15] All rate measurements were made at 25°. Chance's values[16] for the extinction coefficients of HRP, complexes I and II were used.[17] The formation of complex I was measured at 403 mμ. Corrections were made for the absorption of the oxidants at this wave length. The disappearance of K_2IrBr_6 was followed at 510 mμ.

Results

Polarographic Measurements.—The oxidation potentials of K_2IrCl_6 and K_2IrBr_6 were measured polarographically in solutions of different *p*H. There was no evidence for any *p*H dependence and the potentials were reversible. Table I gives the results for the chloride. The bromide showed the same constancy of E^0 with *p*H and the measured values of the oxidation potentials in 1 molar $HClO_4$ of K_2IrCl_6 and K_2IrBr_6 were $E^0 = -0.93$ volt and $E^0 = -0.92$ volt, respectively. The polarographic current–voltage curves are given in Figs. 1 and 2. These values lie within 0.1 volt of the values calculated from e.m.f. measurements by Dwyer, McKenzie and Nyholm.[18] In solutions more alkaline than *p*H 7, hydrolysis of the oxidants occurred as the curves of Fig. 2 show. The rate of hydrolysis increased with the *p*H of the solution. There are no indications that the hydrolyzed species were reducible at the electrode. The half-life of K_2IrCl_6 in 0.01 *M* borate buffer was of the order of ten minutes. K_2IrBr_6 solutions were found to be more resistant to hydrolysis. Because of this and because of its more

TABLE I

THE OXIDATION POTENTIAL OF K_2IrCl_6 IN SOLUTIONS OF DIFFERENT *p*H

Solution	*p*H	E^0 (volts)
1.0 *M* $HClO_4$	~0	−0.93[5]
0.01 *M* sodium borate	9.18	− .93
0.1 *M* acetate buffer	5.0	− .94
1 *M* potassium chloride	~7	− .93

(12) B. Chance and A. C. Maehly, in "Methods in Enzymology," Vol. II, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, Inc., New York, N. Y., in print.

(13) A. C. Maehly, in "Methods of Enzymology," Vol. II, S. P. Colowick, N. O. Kaplan, Ed., Academic Press, Inc., New York, N. Y., p. 801.

(14) B. Chance, *Rev. Sci. Instru.*, **22**, 619 (1951).

(15) C. C. Yang and V. Legallais, *ibid.*, **25**, 801 (1954); C. C. Yang, *ibid.*, **25**, 807 (1954).

(16) B. Chance, *Arch. Biochem. Biophys.*, **41**, 404 (1952).

(17) Recent work of C. Cecil and A. G. Ogston (*Biochem. J.*, **49**, 105 1951)) and of H. Theorell and A. Ehrenberg (*cf.* A. C. Maehly, ref. 13) has shown that the molecular weight of HRP is close to 40,000. This leads to an increase of the extinction coefficients by a factor of 1.1. This change, however, affects the data of this paper very little.

(18) F. P. Dwyer, H. A. McKenzie and R. S. Nyholm, *J. Proc. Roy. Soc. N. S. W.*, **84**, 195 (1951).

favorable extinction coefficients, K_2IrBr_6 was the preferred oxidant for most of this work.

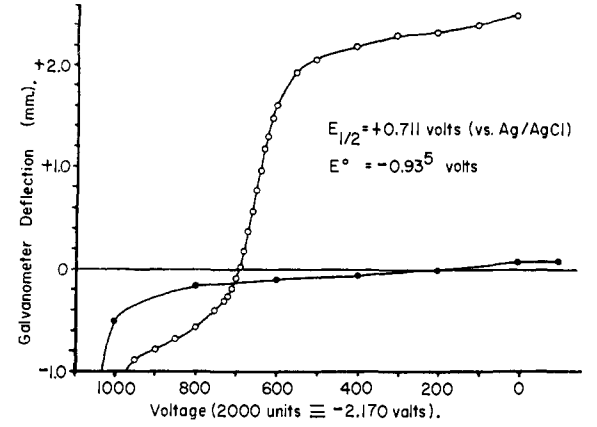

Fig. 1.—Plot of the current *vs.* voltage curve for the polarographic reduction of K_2IrCl_6 in 1.0 mole $l.^{-1}$ $HClO_4$ (pH ~0): ○, values of the experiment; ●, blank values for the supporting electrolyte.

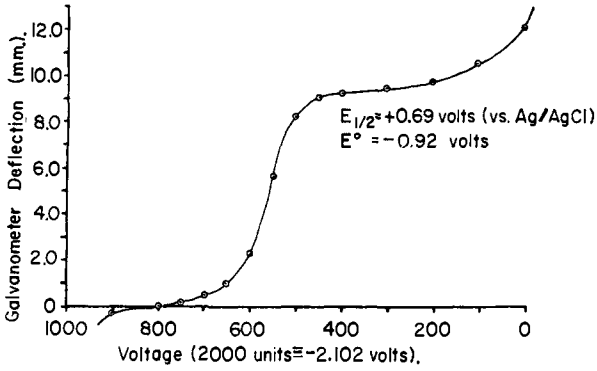

Fig. 2.—Plot of the current *vs.* voltage curve for the polarographic reduction of K_2IrBr_6 in 1.0 mole $l.^{-1}$ $HClO_4$ (pH ~0).

Formation of Complex I.—It was verified that in the reaction between K_2IrCl_6 or K_2IrBr_6 and HRP no complex II was produced by a bimolecular electron-transfer reaction. At all concentrations used, and for solutions more alkaline than the limiting pH of 6.5, complex II was preceded by complex I. In addition there was a one-to-one stoichiometric sequential transition HRP → complex I → complex II → HRP. This is shown clearly by Fig. 4 in which the isosbestic points for these transitions are seen to be essentially time invariant.

Kinetics of Complex I Formation and Oxidant Disappearance.—The results can be presented as follows: 1. The disappearance of oxidant follows first-order kinetics. At fixed pH and enzyme concentration, the first-order rate constant does not change appreciably over a range of concentrations of oxidant (see Table II). 2. The rate of formation of complex I is first order and the measured rate constants at fixed pH and enzyme concentration are independent of the concentration of oxidant over a wide range (see Table II). 3. The rates of both complex formation and oxidant disappearance increase on increasing the pH but there is no simple relationship apparent. 4. The rate of

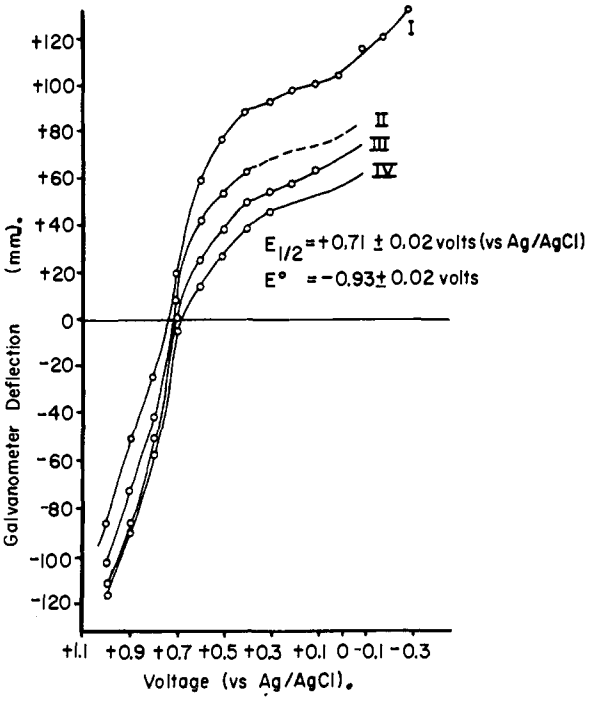

Fig. 3.—Plot of the current *vs.* voltage curves K_2IrCl_6 at pH 9.2 (0.01 mole $l.^{-1}$ sodium borate) to show the effect of hydrolysis. Curves I to IV were measured successively at 10-minute intervals.

disappearance of oxidant increases on increasing the enzyme concentration; again there is no simple relationship apparent.

TABLE II

RATE OF FORMATION OF COMPLEX I AT DIFFERENT INITIAL CONCENTRATIONS OF K_2IrBr_6

	Initial concn. of K_2IrBr_6, 10^{-6} mole $l.^{-1}$	First-order rate constant, $sec.^{-1}$
pH 9.48, borate buffer 0.01 mole	39	0.22
$l.^{-1}$, HRP, 1.25×10^{-6}, mole	39	.22
$l.^{-1}$	31	.24
	23	.25
	15.5	.21
	15.5	.24
pH 9.18, borate buffer 0.01 mole	14	0.076
$l.^{-1}$, HRP, 1.0×10^{-6} mole	20	.067
$l.^{-1}$	28	.077

RATE OF DISAPPEARANCE OF K_2IrBr_6 AT DIFFERENT INITIAL CONCENTRATIONS OF K_2IrBr_6

pH 9.18, borate buffer 0.01 mole	14	0.041
$l.^{-1}$, HRP, 1.0×10^{-6} mole	17	.037
$l.^{-1}$	17	.037
	20.5	.039
	20.5	.041
	24	.034
	28	.030

Titration Experiments.—The principal results are presented in Figs. 5–8. These measurements were all made at pH 9.2 in 0.01 molar borate buffer and they show that: 1. The amount of complex I formed is directly proportional to the amount of peroxidase present initially (see Fig. 5). 2. The amount of complex formed is not directly propor-

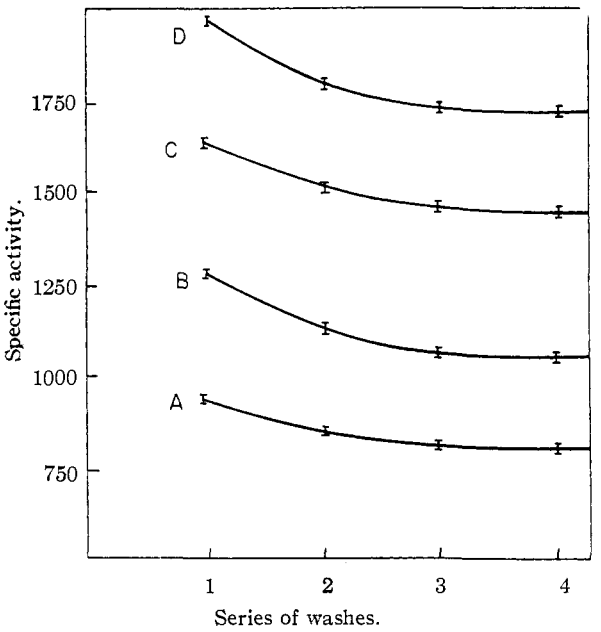

Fig. 1.—Relative specific activity (c. min.$^{-1}$ ml.$^{-1}$ × 5) as a function of series of washes for various contact times, where A, B, C, D are 21.5, 43.5, 67.5 and 89.5 hr., respectively.

131-benzene. The amount of iodine-131 was varied to investigate radiation catalysis.

Results and Discussion

Exchange Reaction.—A typical exchange study was run at a concentration of aqueous potassium iodide of 10^{-2} F at room temperature. The dependence of the specific activities of iodobenzene of Fig. 1 upon the time of contact is shown in Fig. 2.

For an extremely slow isotopic exchange reaction the usual first-order rate of approach to isotopic equilibrium[6] can be expected to be indistinguishable from a zero-order reaction for the time scale of the laboratory measurement. Thus the rate of isotopic change will be constant giving a linear relationship between the specific activity of a reactant and time from initial non-isotopic equilibrium. The expected linear relation is shown in Fig. 2.

The apparent zero-time exchange of Fig. 2 can be explained by a rapid transfer of radioiodine by reaction or exchange with a trace impurity in the iodobenzene. In Fig. 2 the extrapolation to zero time by the method of least squares corresponds to 1.63 × 10^{-3}% apparent exchange. This could be caused by as little as 2.0 × 10^{-7} mole fraction of appropriate impurity. When the iodobenzene was pretreated by shaking with a solution of inactive potassium iodide, the apparent zero-time exchange decreased. This indicated that some of the transfer of radioiodine was by chemical reaction as opposed to simple isotopic exchange with an impurity.

It was important to find out if the slow transfer of radioactivity to the iodobenzene was caused by a chemical reaction as opposed to the expected isotopic exchange reaction. In addition to the careful wash-out procedure, 20 ml. of the activated iodobenzene was fractionally crystallized as shown in Table I. It is apparent that the specific activities of the crystals and supernatants are constant with a satisfactory material balance. A similar check by fractional distillation also showed constant specific activity within experimental error.

(6) (a) H. A. C. McKay, *Nature*, **142**, 997 (1938); (b) R. B. Duffield and M. Calvin, THIS JOURNAL, **68**, 557 (1946); (c) H. T. Norris, *J. Phys. Colloid Chem.*, **54**, 777 (1950); (d) G. M. Harris, *Trans. Faraday Soc.*, **47**, 716 (1951).

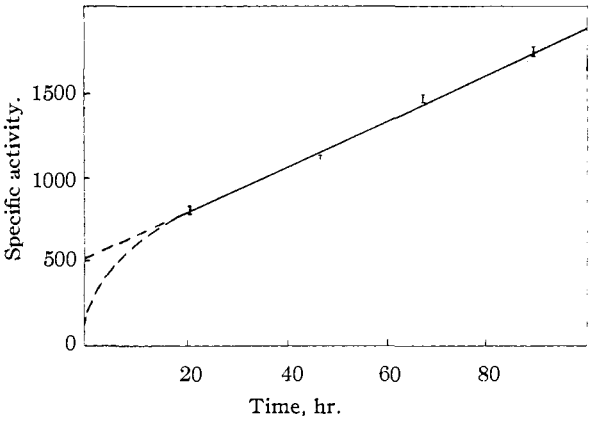

Fig. 2.—Change in the specific activity of the C_6H_5I with contact time.

TABLE I

INVESTIGATION BY FRACTIONAL CRYSTALLIZATION OF THE CHEMICAL FORM OF I^{131} TRANSFERRED TO IODOBENZENE

Fraction[a]	Fraction, ml.	Spec. act. (c./m./ml.)	Total act. (c./m.)	% Act. balance
0	20.0	2512	Av. 50,120	
		2500		
				97.6
S-1	10.4	2491	25,906	
C-1	9.6	2395	22,992	
				101.0
S-2	6.0	2495	14,970	
C-2	3.6	2459	8,852	
				100.0
S-3	1.3	2493	3,241	
C-3	2.3	2466	5,672	
			Av.	99.5

[a] O, original; S, supernatant; C, crystals.

The question of radiation catalysis in this reaction was investigated by varying the amount of iodine-131 per series by a factor of almost 14. The results are shown in Table II in which, for each

TABLE II

INVESTIGATION OF RADIATION CATALYSTS OF THE EXCHANGE REACTION

I^{131},[a] millicuries	KI, F	Isotopic exchange constant[b]	Rate of exchange[c]	Ratio of rates[d]
0.15	10^{-4}	28.8	2.88	1.5
0.41	10^{-2}	0.645	6.45	1.2
1.14	10^{-2}	0.511	5.11	1.0
1.58	10^{-4}	18.9	1.89	1.0
2.05	10^{-4}	26.7	2.67	1.4

[a] At the time of mixing. [b] Units of hr.$^{-1}$ × 10^6. [c] Units of moles liter^{-1} hr.$^{-1}$ × 10^9; last digit not significant. [d] Observed to expected (Table III).

must of necessity be produced if a peroxide is an intermediary in the formation of complex I from a one-equivalent oxidant and peroxidase.[22]

The Conversion of Complex II to Complex I.—When complex I is formed by reaction between K_2IrBr_6 and HRP and reduced to complex II by either endogenous or added donor, then a further addition of K_2IrBr_6 will reconvert complex II, apparently directly, back to complex I.

Non-specific Protein Oxidation.—The amino acids tyrosine, tryptophan and histidine have been found to react with K_2IrBr_6 and the rates of these reactions increase as the *p*H is increased. At *p*H 9.2 tyrosine reacts quite rapidly with K_2IrBr_6 ("bimolecular rate constant," 10^3 mole^{-1} l. sec.$^{-1}$). If tyrosine is added to the peroxidase solution the rate of complex I formation by the action of K_2IrBr_6 is decreased. Thus reactions between protein end groups consisting of these amino acids and the oxidant will account for the observed high ratio of oxidant consumed/complex I formed.

Discussion

Although it is very probable that the halogen atoms of K_2IrBr_6 and K_2IrCl_6 are inert to substitution in acidic solution,[23,24] it is clear from the polarographic measurement presented that hydrolysis takes place in alkaline media. For this reason the oxidation potentials of the unhydrolyzed compounds are no longer a very useful guide to the mechanism of the oxidation process; and the polarographic measurements show no indication of the oxidation potentials for the hydrolyzed species. It is worth noting that the effect of *p*H on hydrolysis parallels the rate of complex formation. For these reasons one should be cautious in drawing conclusions as to the oxidation–reduction properties of the complexes from the standard oxidation potentials of the oxidants (*cf.* ref. 10).

Clear evidence that a direct electron transfer reaction does not lead to complex formation is provided by the constancy of the first-order rate constants for both complex I formation and also oxidant disappearance. And titration data show that more than twice the theoretical amount of K_2IrBr_6 is required to form complex I. Thus neither the titration nor the kinetic data confirm George's suggestion that H_2O_2 (or HO_2^-) is not a component part of the structure of complex I and is not necessary for its formation. The extremely complicated kinetic and stoichiometric behavior makes it, unfortunately, impossible to be definite about the nature of the intermediate leading to the formation of complex I. It has frequently been suggested that powerful one-electron oxidants may lead to the formation of hydrogen peroxide by the Haber–Weiss back reaction, such as

$$Ce^{+4}OH^- + OH^- \longrightarrow Ce^{+3} + H_2O_2 \qquad (3)$$

discussed by Evans and Uri.[25] A reaction of this general type involving K_2IrBr_6 and its hydrolyzed species might be expected to proceed quite rapidly because of the favorable energetics involved.[26] An alternative theory is that protein end groups such as histidine[27] react with the oxidizing agent (K_2IrBr_6) and form an intermediate that reacts with the iron atom. It should be recognized that these possibilities are at the present time highly speculative.

It is useful to contrast the reactions of peroxidase or ferrimyoglobin with hydrogen peroxide or with one-electron transfer agents such as chloro- or bromoiridates. Hydrogen peroxide reacts with peroxidase in one of the most carefully studied second-order reactions of enzyme and substrate; the velocity constant is very large (10^7 mole^{-1} l. sec.$^{-1}$). With ferrimyoglobin, the reaction proceeds at less than $^1/_{50,000}$ of this rate and no data have been published to prove that the reaction is of the second-order.[10] This paper shows that the reaction of bromoiridate with peroxidase is not second order and is slow (the first-order velocity constant divided by the bromoiridate concentration $\leqslant 10^3$ mole^{-1} l. sec.$^{-1}$), whereas George and Irvine find that ferrimyoglobin reacts rapidly with chloroiridate ($>10^5$ mole^{-1} l. sec.$^{-1}$). The nature of the reaction products differs; peroxidase gives the very reactive complex I, ferrimyoglobin gives the relatively unreactive compound which is similar to peroxidase complex III. In view of these contrasts, the conclusion of George that ferrimyoglobin reacts with chloroiridate in a one-electron transfer reaction does not seem to be relevant to the mechanism of the peroxidase reaction.

Acknowledgments.—The author would like to express his appreciation for the help and encouragement given him by Professor Britton Chance, Dr. A. C. Maehly and the other members of the Johnson Foundation.

(22) The virtual absence of radical formation during the reaction of peroxidase with CH_3OOH as well as in lyophilized samples of complexes I and II was demonstrated by Chance and Commoner (*cf.* ref. 8) using electronic resonance absorption.

(23) L. L. Larson and C. S. Garner, THIS JOURNAL, **76**, 2180 (1954).

(24) H. Taube and H. Myers, *ibid.*, **76**, 2103 (1954).

(25) M. G. Evans and N. Uri, see "Atoms and Radicals in Aqueous Systems, in S. E. B. Symposia, V, Photosynthesis," Academic Press, New York, N. Y., 1951, p. 130.

(26) M. G. Evans and N. S. Hush and N. Uri, *Quart. Rev. Chem. Soc.*, **6**, 186 (1952).

(27) That horseradish peroxidase contains tyrosine and histidine was shown by A. C. Maehly and S. Paléus, *Acta. Chem. Scand.*, **4**, 508 (1950).