63. *The Possible Significance of Fenton's Reaction in Relation to Oxidations effected by Enzyme Systems.*

By D. J. Mackinnon and William A. Waters.

Merz and Waters (*J.*, 1949, S 15) classified organic compounds R·H into two groups according to whether oxidation by Fenton's reagent involved chain or non-chain processes. It is now suggested that the classification depends on the oxidation–reduction potential $E_{(-e)}$ of the radical R· for the change $R\cdot \rightleftharpoons R^+ + e$. Substrates undergoing oxidation by the chain process have $E_{(-e)}$ well below E_0 for $Fe^{2+} \rightleftharpoons Fe^{3+} + e$. It has now been found that these same substrates also give radicals capable of reducing two triaryltetrazolium salts to coloured products.

A correlation with enzyme oxidation is suggested; the chain-reacting substrates appear to be those attacked by dehydrogenases which are linked with the pyridine-nucleotide coenzymes. A revised free-radical theory of enzyme oxidation is put forward. Except for peroxidase action it is suggested that a cyclic one-electron redistribution occurs within an enzyme–coenzyme–substrate complex, no *free* radicals being generated.

In a survey of the types of organic molecules which can be oxidised by Fenton's reagent, Merz and Waters (*J.*, 1949, S 15) showed that both chain and non-chain reactions could occur. They represented chain oxidations by the sequence (3), (4), chain initiation being by reaction (1) and chain termination by reaction (2): non-chain reactions did not involve (4) and chain termination required either (5) or (6).

$$
\begin{array}{ll}
(1) & Fe^{2+} + HO{-}OH \longrightarrow Fe^{3+} + HO\cdot + OH^- \\
(2) & Fe^{2+} + \cdot OH \longrightarrow Fe^{3+} + OH^- \\
(3) & R{-}H + \cdot OH \longrightarrow R\cdot + H_2O \\
(4) & R\cdot + HO{-}OH \longrightarrow ROH + \cdot OH \\
(5) & R\cdot + \cdot OH \longrightarrow ROH \\
(6) & 2R\cdot \longrightarrow \text{Products (dimerisation or disproportionation)}
\end{array}
$$

They developed two equations to illustrate the essential differences in stoicheiometry of the chain and non-chain processes, *viz.*:

$$
\begin{array}{ll}
(A) & \Delta[H_2O_2]/\Delta(R{-}H] = 1 + k_2[Fe^{2+}]/k_3[R{-}H] \\
(B) & \Delta[H_2O_2]/\Delta[R{-}H] = 2 + k_2[Fe^{2+}]/k_3[R{-}H]
\end{array}
$$

which were substantiated by showing that, for all the substrates tested, plots of $\Delta[H_2O_2]/\Delta[R{-}H]$ against [Initial Fe^{2+}]/[Initial R–H] had extrapolated intercepts of either 1 or 2 for the condition $[Fe^{2+}]/[R{-}H] = 0$, provided that ΔFe^{2+} was always much less than [Initial Fe^{2+}]. Later (*J.*, 1949, 2427), they pointed out that $\Delta[H_2O_2]$ and $\Delta[R{-}H]$ had to be computed in ***equivalents*** (not moles), $\Delta[R{-}H]$ being taken as $(\Delta H_2O_2 - \Delta Fe^{2+})$, to allow for the fact that the oxidising equivalent of R–H is different in reaction (6) according as the radical R· dimerises or disproportionates. This essential feature of the theory has been overlooked by Uri (*Chem. Reviews*, 1952, **50**, 403).

Kolthoff and Medalia (*J. Amer. Chem. Soc.*, 1949, **71**, 3777) and Barb, Baxendale, George, and Hargrave (*Trans. Faraday Soc.*, 1951, **47**, 462) pointed out that the chain-continuing reaction (4) is more probably a composite of (7) followed by (1):

$$
(7)\quad R\cdot + Fe^{3+} \longrightarrow R^+ + Fe^{2+};\quad R^+ + H_2O \longrightarrow R{-}OH + H^+
$$

and this is substantiated by the fact that addition of a fluoride, which prevents reduction of Fe^{3+} by (7), alters the stoicheiometry of the chain oxidations. Without prejudice to the possibility that with some substrates (4) may also be possible, we accept this view, since it affords a clear theoretical explanation of the second test proposed by Merz and Waters (*loc. cit.*) for the discrimination between chain and non-chain oxidations, *viz.*, the capability of the chain-propagating radicals to reduce both mercuric ions and molecular iodine. We now suggest that organic free radicals and their related cations and anions constitute pairs of thermodynamically reversible oxidation–reduction systems

$$
R\cdot \rightleftharpoons R^+ + e,\ E_{(-e)};\quad e + R\cdot \rightleftharpoons (R{:})^-,\ E_{(+e)}
$$

to which two different redox potentials can be assigned. Organic radicals which undergo chain oxidation in Fenton's reaction will be those for which $E_{(-e)}$ is decidedly below that of the Fe^{3+}–Fe^{2+} system (+ 0·74 v at pH 0). The redox potentials of the Hg^{2+}–$\frac{1}{2}Hg_2^{2-}$ and $\frac{1}{2}I_2$–I^- systems are sufficiently close in value to this to lead to the same classification of organic radicals by the tests hitherto used. Oxidation–reduction potentials $E_{(+e)}$ have been measured for free radicals of the triphenylmethyl series (Bent and Keevil, *J. Amer. Chem. Soc.*, 1936, **58**, 1228; Keevil, *ibid.*, 1937, **59**, 2104), but in general electrochemical measurements of this type are not practicable because ensuing association reactions, $R^+ + OH^- \rightleftharpoons R{-}OH$ and $R^- + H^+ \rightleftharpoons R{-}H$, are not sensibly reversible under appropriate experimental conditions.

In attempts to develop this concept of the oxidation–reduction potential of a free radical we have sought other oxidation–reduction systems which might be used in hydrogen peroxide–ferrous salt mixtures to demonstrate the relative reducing powers of different radicals. First, we examined oxidation by the similar system hypochlorous acid–ferrous salt, which generates atomic chlorine by reaction (1′) and initiates oxidations by (3′). However, though reaction (3′) is much less effective than (3), as shown by the great change in k_2/k_3 ratios (see Table), all the substrates which undergo chain oxidation with hydrogen peroxide also undergo chain oxidation with hypochlorous acid, because the same reduction of Fe^{3+} by the free radical (7) is involved in each case. This finding is further experimental evidence for the occurrence of reaction (7).

(1′) * $Fe^{2+} + HO{-}Cl \longrightarrow Fe^{3+} + OH^- + \cdot Cl$
(3′) $Cl\cdot + R{-}H \longrightarrow R\cdot + H{-}Cl$

Secondly, we studied the effect of adding dyes to our oxidation mixtures. Unfortunately, compounds such as methylene-blue, sodium indigosulphonate and other dyes regularly used as indicators of reductions effected by titanous chloride, and in biochemical work, do not give unequivocal results. Though they can be reduced reversibly to leuco-compounds they can be oxidised irreversibly by hydroxyl radicals, yielding, sometimes after a sequence of colour changes, colourless or nearly colourless products, and the amount of colour change may be diminished or increased by the addition of an organic substrate in a way which is not always easily explicable (compare Kauffmann, *J. Amer. Chem. Soc.*, 1947, **69**, 899; 1951, **73**, 4311). For instance, methylene-blue is irreversibly bleached by Fenton's reagent but an appreciable amount of hydrogen peroxide is required. If *n*-propanol is added to a methylene-blue–ferrous sulphate solution, and then, *in vacuo*, insufficient hydrogen peroxide to bleach the dye on its own is admixed the colour promptly disappears, but gradually returns slightly. We suggest that the radical $RCH(OH)\cdot$ reduces the dye to the leuco-base, whilst Fe^{3+}, which is also formed, slowly reoxidises this until an equilibrium is reached. This irreversible oxidation of dyes by the hydroxyl radical has vitiated attempts to use them for quantitative measurement of radiochemical changes in aqueous solution, when both H· and ·OH may be generated simultaneously (compare *Discuss. Faraday Soc.*, 1952, **12**, 133—293). However, we have found that the colourless triaryltetrazolium salts (I), which yield coloured, water-insoluble azo-compounds (II) on reduction, are almost inert to hydrogen peroxide, ferrous sulphate, and their interacting mixture, and yet do give the marked colours of the reduced form (II) when used in the presence of organic substrates, provided that an excess of fluoride is also added so as to form complexes of Fe^{3+} and eliminate competition with reaction (7). The Table shows that two of these salts (*a*) 2 : 3 : 5-triphenyltetrazolium chloride (" red salt "), which gives a red product (II), and (*b*) 3 : 3′-dimethoxydiphenyl-4 : 4′-bis-2″-(3″ : 5″-diphenyltetrazolium) dichloride (" blue salt "), which gives a much more intense blue product, lead to

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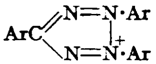
* Reaction (1′) for the course of electron transfer to hypochlorous acid is to be preferred to the reverse, $Fe^{2+} + HOCl \longrightarrow Fe^{3+} + Cl^- + \cdot OH$, since all recent investigators of Fenton's reaction (refs. above) have shown that chloride anions are marked retarders of oxidations by hydroxyl radicals. This has been ascribed to the occurrence of the reaction

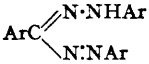
$$HO\cdot + H^+ + Cl^- \longrightarrow Cl\cdot + H_2O; \quad -F_{298} = 2{\cdot}8 \text{ kcal.}$$

first studied by Taube and Bray (*J. Amer. Chem. Soc.*, 1940, **62**, 3362), which converts active hydroxyl radicals into less active chlorine atoms. Unequivocal chemical evidence for the transient existence of free chlorine atoms in dilute aqueous solution is not yet available.

the classification of oxidisable organic substrates into the same two groups as those listed by Merz and Waters. The radicals of the chain-reaction type are evidently very powerful reducing agents.

(I)





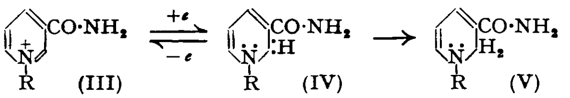
(II)

Significance in Relation to Enzyme Oxidation.—Tetrazolium salts (I) can, like methylene-blue, be used in Thunberg's technique for studying dehydrogenase processes (cf. Bertho and Grassmann, " Laboratory Methods of Biochemistry," pp. 152—160, Macmillan, London, 1938) and so it is of interest to examine the listed substrates in relation to their responses to different dehydrogenase enzymes. It can be seen that primary and secondary alcohols, lactic and formic acids, acetaldehyde, and formaldehyde, which are oxidised by the chain reaction, can be biochemically classified together as substrates which undergo biochemical oxidation by enzymes linked to the pyridine–nucleotide coenzymes (Warburg's coenzymes I and II), whereas substrates such as propionic and succinic acids, which are oxidised by the non-chain process, fall into a different biochemical group. Now, it has been established that the biochemical role of the pyridine–nucleotide coenzymes (III)

Classification of free-radical oxidations.

	Oxidation process :				Effect on tetrazolium salts : *	
	Fe^{2+}–HOCl		Fe^{2+}–H_2O_2			
Substrate	Intercept	k_3/k_2	Intercept	k_3/k_2	" red "	" blue "
Methanol	1	0·12	1	2·3	+	+
Ethanol	1	0·14	1	3·8	+	+
n-Propanol	1	0·38	1	2·6	+	+
*iso*Propanol	1	0·19	1	3·0	+	+
n-Butanol	1	0·56				
sec.-Butanol	1	0·26				
Diethyl ether	1	0·33	1	6·4		
Tetrahydropyran	1	0·49	1	4·5	+	+
Tetrahydrofuran	1	0·42	1	6·2		
Di*iso*propyl ether	1	0·18			+	+
Ethylene glycol	1	0·21	? 2	3·6	+	+
Butane-1 : 2-diol	1	0·21			—	Slight +
Butane-1 : 3-diol					+	+
Pinacol			2	1·25	—	—
tert.-Butanol	Uncertain		2	0·65	—	—
Methyl acetate	1 ?		2	0·85	—	Trace +
Ethyl acetate			2	1·10	—	Trace +
Formaldehyde			1	3·0	+	+
Chloral hydrate			2	4·7	—	—
Acetone	No oxidation		No oxidation		—	—
Acetic acid	No oxidation		No oxidation		—	—
Propionic acid			2	0·86		
*iso*Valeric acid			2	3·3	—	—
Succinic acid			—	—	—	—
Adipic acid			2	1·25	—	—
Benzoic acid	Little oxidation		2	7·0	—	—
Maleic acid			No oxidation		—	—
Sodium formate			—	—	+	+
Glycollic acid			1	2·0	+	+
Lactic acid			1	1·7	+	+
Glycine			Uncertain		—	—
Allyl alcohol			—	—	—	—

* See p. 324.



(III) (IV) (V)

involves their reduction to (V), and it may be noted that the change (III) ⟶ (V), like the change (I) ⟶ (II), is one from an ammonium salt cation to a tertiary amine.

In 1943 (*Trans. Faraday Soc.*, **39**, 140) one of us (W. A. W.) suggested that this reduction was accomplished by addition of the organic radical to the N^+ centre, but the alternative view that this reduction is like reaction (7), a single-electron transfer (III) ⟶ (IV), now seems more probable. We suggest that the biochemically measured oxidation–reduction potentials of the ethanol–acetaldehyde, lactate–pyruvate, and similar systems (cf. Anderson and Plaut in " Respiratory Enzymes," ed. by Lardy, Burgess Pub. Co., Minneapolis, 1949) are in fact the potentials $E_{(-e)}$ for the further oxidation of the substrate radicals $R{\cdot}\dot{C}H{\cdot}OH$ ($\longrightarrow R{\cdot}\overset{+}{C}H{\cdot}OH$). Their values (*ca.* $-0{\cdot}2$ v at pH 7) are comparable with those of Warburg's coenzymes, but well below those of indicator dyes (*e.g.*, methylene-blue $+0{\cdot}01$ v at pH 7) or that for the succinate–fumarate system. When the effects of pH change, and of dissociations of carboxylic acids into their anions, are both taken into account it will be seen that the same structural criteria for radical stability as those discussed by Merz and Waters (*loc. cit.*) in connection with the Fenton reaction apply to substrate–coenzyme relations in dehydrogenase action.

Now, since reaction (III) ⟶ (IV) (corresponding to 7) completes the oxidation of the organic substrate, a second one-electron transfer is needed to complete the reduction (IV) ⟶ (V) of the coenzyme. This may involve (i) another substrate radical, or (ii) (*in vivo*) the reduced form of another coenzyme, *e.g.*, riboflavin–nucleotide, but (iii) re-oxidation of a metallic ion (8) is also possible, since $E_{(+e)}$ for (IV) may well be high, though $E_{(-e)}$ may be low.

$$(8)\quad H^+ + (IV) + Fe^{2+} \begin{smallmatrix}\longrightarrow\\ \overset{e}{\dashleftarrow}\end{smallmatrix} (V) + Fe^{3+}$$

Reaction (8) may be compared with the reversible stage of conversion of quinol into benzoquinone by Fe^{3+} (Baxendale, Hardy, and Sutcliffe, *Trans. Faraday. Soc.*, 1951, **47**, 963). In attempting to gauge the values of $E_{(+e)}$ and $E_{(-e)}$ for the semiquinone radicals, analogous to (IV) of indicator dyes, we have reduced methylene-blue and sodium indigo-disulphonate, *in vacuo* at pH 7, by the powerful one-electron reduction $[Co(CN)_6]^{4-} \longrightarrow [Co(CN)_6]^{3-} + e$ in the presence of alcohols, and both succinic and maleic acids, but found that addition of each of the organic substrates was without effect. Since under these conditions the reductions of the dyes must occur in two stages, *e.g.*, $MB + H^+ + e \longrightarrow MBH{\cdot}$; $MBH{\cdot} + H^+ + e \longrightarrow MBH_2$, we infer that the forward reactions (9), (10), and (11) do not proceed at rates competitive with the further reduction (12) of the semiquinonoid forms of the dyes, though the reverse processes may well occur easily.

$$(9)\quad MBH{\cdot} + CH_3{\cdot}CH_2{\cdot}OH \longrightarrow MBH_2 + CH_3{\cdot}\dot{C}H{\cdot}OH$$

$$(10)\quad MBH{\cdot} + {}^-O_2C{\cdot}CH_2{\cdot}CH_2{\cdot}CO^- \longrightarrow MBH_2 + {}^-O_2C{\cdot}\dot{C}H{\cdot}CH_2{\cdot}CO_2{}^-$$

$$(11)\quad MBH{\cdot} + {}^-O_2C{\cdot}CH{:}CH{\cdot}CO_2{}^- \longrightarrow MB + O_2C{\cdot}\dot{C}H{\cdot}CH_2{\cdot}CO_2{}^-$$

$$(12)\quad MBH{\cdot} + H^+ + [Co(CN)_6]^{4-} \longrightarrow MBH_2 + [Co(CN)_6]^{3-}$$

Consequently the half-reduced form of the dye cannot be a sufficiently powerful oxidising agent to be capable of abstracting hydrogen very easily from an alcohol, or succinate, molecule, or a sufficiently powerful reducing agent to be able to add hydrogen rapidly to maleate. Initiating reactions, such as (1) and (3) involving hydroxyl radicals and chain-continuing reactions such as (7) and (8) therefore seem to be needed for reduction of a coenzyme (or an equivalent dye) by an oxidisable substrate.

The suggestion that free hydroxyl radicals were involved in dehydrogenase enzyme action was first made by Haber and Willstätter (*Ber.*, 1931, **64**, 2844) : free hydroxyl radicals have been found to oxidise alcohols with great speed and in the requisite manner, but no other chemical oxidisers are known which could operate under the requisite temperature and pH conditions. We therefore suggest that for the enzyme oxidation of the CH·OH group at least stage (i) involves hydroxyl transfer from an enzyme complex by a process equivalent to reactions (1) and (3) of the Fenton scheme, stage (ii) involves the reduction of the coenzyme (III) ⟶ (IV) which we have now simulated, and stage (iii) may be represented by reaction (8) in which the reduced centre of the enzyme is re-oxidised to the level requisite for attack on a further substrate molecule. A very similar reaction scheme, in which process (i) is brought about by the photochemical activation of a ferric complex, such as $(FeOH)^{2+}$ or $(FeCl_4)^-$, has been shown by Brealey and Uri (*J.*

Chem. Phys., 1952, **20**, 257) to lead to oxidation of ethanol by thionine (compare Brealey, Evans, and Uri, *Nature*, 1950, **166**, 959; Bates, Evans, and Uri, *ibid.*, p. 869). If, further, one supposes that, before reaction, enzyme and coenzyme form a binary complex, to which the substrate must come up to a specific site to form a ternary complex (Michaelis and Menten, *Biochem. Z.*, 1913, **49**, 333), then the one-electron transfers (i), (ii), and (iii) of the dehydrogenase process can all be represented as occurring within the (enzyme + coenzyme + substrate) complex so that, though organic radicals are concerned, no *free* radicals are involved.

Westheimer, Fisher, Conn, and Vennesland (*J. Amer. Chem. Soc.*, 1951, **73**, 2403), by using 1 : 1-dideuteroethanol, have shown that in the enzyme oxidation of ethanol, hydrogen is transferred directly from the carbinol group to the pyridine–nucleotide coenzyme. This strongly supports the view that the oxidation occurs by way of a cyclic electron transference within an enzyme–substrate complex from which even a proton cannot escape during the process. The deuterium transfer can easily be represented by writing stage (i) as

$$(\text{Fe–OH})^{2+} + \text{CH}_3\cdot\text{CD}_2\cdot\text{OH} \longrightarrow (\text{Fe,HOD})^{2+} + \text{CH}_3\cdot\dot{\text{C}}\text{D}\cdot\text{OH}$$

and stage (iii) (cf. equation 8) as

$$(\text{Fe,HOD})^{2+} + (\text{IV}) \longrightarrow (\text{IV—D}) + (\text{Fe–OH})^{2+}$$

Here $(\text{Fe–OH})^{2+}$ represents a co-ordinated ferric complex, with hydroxyl as one of the six co-ordinated groups, and $(\text{Fe,HOD})^{2+}$ a ferrous complex with a co-ordinated water (HOD) molecule, following current usage in depicting the corresponding photochemical activation.

Though, to illustrate the analogy with known reactions, we have represented (i) as an electron transference involving a metallic ion of variable valency, we must emphasize that our cyclic oxidation–reduction mechanism is not necessarily restricted to those enzyme systems in which the presence of a transition metal is essential. The initiating agent must have a sufficiently high redox potential to be able to abstract a hydrogen atom from a specific C–H group of the adsorbed substrate, and it is just possible that certain thiyl radicals, R–S·, may be active enough for this purpose (cf. Harris and Waters, *Nature*, 1952, **170**, 212; Bickel and Kooijman, *ibid.*).

A similar cyclic one-electron transfer to the scheme (i), (ii), (iii) was proposed by one of us (Waters, *J.*, 1942, 266) to explain the specific action of cuprous salts in the Sandmeyer reaction, and, as Cowdrey and Davies have pointed out (*J.*, 1949, S 48), it is impossible to show experimentally the actual movements of electrons within a complex in its transition state. Nevertheless, each stage of the process suggested now has an analogous simple chemical reaction that has been observed in the laboratory.

It must be emphasised that the cyclic scheme for dehydrogenase action detailed above refers only to oxidations linked with one or other of Warburg's pyridine-nucleotide coenzymes, and that the concept that organic radicals have characteristic oxidation–reduction potentials, $E_{(+e)}$ and $E_{(-e)}$, shows why each particular coenzyme system is group-specific. This redox potential concept clearly shows why, *e.g.*, the succinate–fumarate system must be linked to a coenzyme of more positive redox potential, and it may well be that the initiation of this oxidation does not require hydroxyl transfer.

Catalase, however, is another enzyme, almost certainly associated with oxidation by hydroxyl transfer since not only can catalytic decomposition of hydrogen peroxide be brought about by Fe^{3+} and Fe^{2+} ions acting in conjunction, but again catalase does oxidise those same primary and secondary alcohols, etc., which respond to the pyridine-nucleotide linked dehydrogenase. It is easily possible to depict all catalase actions by means of a cyclic electron-transfer process involving ferric ion in the enzyme portion and a molecule of hydrogen peroxide playing the part of coenzyme, and associating with the Fe^{3+} in the type of complex depicted by Chance (*Adv. Enzymology*, 1951, **12**, 153). Whether the electron switches require the possibility of lowering of valency to Fe^{2+}, as suggested in the preceding pages, or of increase in valency to Fe^{4+}, as George's results (*Nature*, 1952, **169**, 612; George and Irvine, *ibid.*, 1951, **168**, 164) may indicate, is again an indeterminable consideration for a transition complex.

Peroxidase, however, though it requires both hydrogen peroxide and an iron-containing enzyme, is evidently quite different in its action from either of the enzymes already mentioned. Its group-specificity is quite distinct, for it does not affect alcohols or aldehydes, but brings about the oxidations of monohydric phenols, aromatic amines, and enediols. As indicated by the work of Cosgrove and Waters (*J.*, 1951, 1726), the products are radical dimers and their degradation products, so that the enzyme must only effect a single-stage one-electron oxidation. This is entirely consistent with George's active ferryl form. Fieser's critical oxidation potential measurements (*J. Amer. Chem. Soc.*, 1930, **52**, 5204) show that the active agent for phenol and amine oxidation must have an oxidation–reduction potential of not less than +1·1 v, and this accords with the postulated existence of a complex containing iron with valency greater than 3.

EXPERIMENTAL

Oxidations with Hypochlorous Acid–Ferrous Sulphate Mixtures.—Hypochlorous acid solutions were prepared by Derbyshire and Waters's method (*J.*, 1951, 75), and the oxidations were carried out as follows: 10 ml. of 0·1N-ferrous ammonium sulphate in 0·5N-sulphuric acid were mixed with the appropriate volume of a standard aqueous solution of the organic substrate and the whole was diluted with distilled water to 40 ml. 10 Ml. of 0·05N-hypochlorous acid were then added with shaking. After 1 hour, or more, the Fe^{3+} content was determined by titration with 0·05N-mercurous perchlorate in the presence of 20 ml. of 10% potassium thiocyanate solution. The titration was carried out in the cold, but the last few drops were added at 60—70° (see Pugh, *J.*, 1945, 588). As in the preceding work by Merz and Waters, freshly boiled-out distilled water was used throughout so as to minimise effects due to free oxygen. Equation (A) was tested graphically for the evaluation of the intercept, and the k_3/k_2 values reported for relative oxidation rates are means of at least six measurements.

Reduction of Tetrazolium Salts.—The two salts, (*a*) 2 : 3 : 5-triphenyltetrazolium chloride ("red salt") and (*b*) 3 : 3′-dimethoxydiphenyl-4 : 4′-bis-2′′-(3′′ : 5′′-diphenyltetrazolium) dichloride ("blue salt") were pure laboratory specimens kindly given to us by Dr. R. Slack. So that the testing should be comparable with our study of Fenton's reaction, all mixtures were made up in 0·1N-sulphuric acid and contained, besides ferrous sulphate solution and an excess of the organic substrate, sodium fluoride solution in amount equivalent to 10 times the Fe^{3+} that might be produced if no oxidation of the substrate occurred. This was usually 5 times the total molar quantity of the ferrous iron. Oxidations were effected by adding sufficient N/200-hydrogen peroxide to oxidise only one-quarter of the ferrous salt. With the "red salt" there was a very slight colour production in the absence of any added organic substrate. The "chain-reacting" radicals all produced a much more intense colour, but some of those of the "non-chain" category produced even less colour than the blank. The "blue salt" produced a much more intense colour with a reactive substrate than did the "red salt," and there was no coloration in the blank. In the absence of fluoride there was no evident reduction of either of the tetrazolium salts to coloured products.

Attempted Induced Reductions with Dye Radicals.—Potassium cobaltocyanide is such a powerful reducing agent that the addition of dilute cobalt sulphate solution to a mixture of an organic dye and potassium cyanide solution brings about immediate reduction of the dye to its leuco-derivative. This great change in the oxidation–reduction potential of cobalt on formation of a complex ion was selected as being a possible model for the activation of a metal-containing enzyme when an enzyme–coenzyme complex is formed.

Thunberg's technique was used. Constant volumes of dye solution, potassium cyanide. and phosphate buffer (pH 7) were mixed in the main Thunberg tube, and after evacuation, dilute cobalt sulphate solution was admixed from the side-tube in amount just sufficient to decolorise the dye. Representative substrates, listed in the Table, were then added to the buffered dye, and tests were made to discover whether any adjustment of the quantity of cobalt salt required for complete decoloration was necessary. Both methylene-blue and sodium indigodisulphonate being used as indicator dyes, all tests gave negative results.

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