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# The Enzymes



CHEMISTRY AND MECHANISM OF ACTION

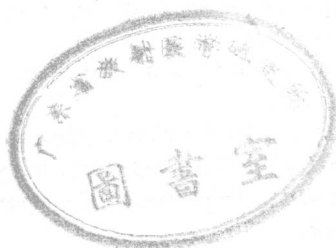
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## CHAPTER 44

### Theory of Oxidation-Reduction

By LEONOR MICHAELIS\*

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#### I. Definitions and Classification of Oxidation and Reduction

In order to understand the nature of oxidative enzymes and their catalytic action, it is first of all necessary to discuss the nature of those chemical processes designated as oxidations and reductions, irrespective of whether or not they are catalyzed by enzymes. A biochemist who wishes to enter the field of enzymatically catalyzed oxidations, with the idea in mind that the fundamental theory concerned with atomistics, kinetics, and thermodynamics of oxidation had been sufficiently prepared by physicochemists to be merely applied to biological problems, will be dis-

\* The Editors are indebted to Dr. S. Granick of the Rockefeller Institute for Medical Research, who undertook the burdensome task of correcting proof after Dr. Michaelis' death.



appointed. An essential part of the fundamental background had to be elaborated by the biochemists themselves as they were confronted with problems which did not seem important before, although such a distinction in importance will no longer be upheld at the present time.

We are here essentially interested in the oxidation of organic compounds. The behavior of organic compounds is in certain respects distinctly, though not always quite specifically, different from that of inorganic compounds. The special problem as it presents itself in organic chemistry is the concurrence of two principles which, viewed from the standpoint of routine structural chemistry, appear to be incompatible, but are not so in reality. One of these is the fact that carbon compounds are, in general, stable only when all four valences of the C atom are satisfied; the other is the principle of compulsory univalent oxidation, that is to say, electrons are transferred from one molecule to another singly, and any bivalent oxidation or reduction can be resolved into successive univalent steps.<sup>1-6</sup> To what extent exceptions to this principle may be subject to further discussion in chemistry in general will be discussed in a later chapter. For *organic* chemistry there seems to be no doubt of its general validity. The fact that these two steps usually overlap has obscured for a long time this fundamental principle. In order to maintain the quadrivalence of C, one has to think in terms of bivalent oxidations, such as leucodye  $\rightleftharpoons$  dye + 2 electrons (or H atoms), or, at least in terms of bimolecular oxidations, such as 2 cysteine minus 2 H  $\rightleftharpoons$  cystine. A univalent oxidation is incompatible with the maintenance of the quadrivalence of C, which was held the cornerstone of classical organic chemistry. It implies the formation of a free radical, a molecule with one of its atomic valences unoccupied, at least as a necessary intermediary step which may be followed by another univalent step of oxidation, thus bringing about the expected bivalent oxidation. The relative instability, in most cases, of the intermediate step, in other words, its relatively high energy content, represents a barrier to the over-all bivalent oxidation. The existence of this barrier, in its turn, causes that relatively high stability, or rather inertia, of organic compounds, without which the whole realm of organic chemistry, with its hundred thousands of well defined and seemingly stable molecular species, could not exist. This apparent stability is due solely to the fact that, although the oxidation as a whole, speaking merely thermodynamically, may involve the release of free energy, it has to pass

<sup>1</sup> L. Michaelis, *J. Biol. Chem.* **36**, 703 (1932).

<sup>2</sup> L. Michaelis, *Chem. Revs.* **16**, 243 (1935).

<sup>3</sup> L. Michaelis and M. P. Schubert, *Chem. Revs.* **22**, 437 (1938).

<sup>4</sup> L. Michaelis, *Cold Spring Harbor Symposia Quant. Biol.* **7**, 33 (1939).

<sup>5</sup> L. Michaelis, *Am. Scientist* **34**, 573 (1946); *Science in Progress*, 1947.

<sup>6</sup> L. Michaelis, in D. E. Green, ed., *Currents in Biochemical Research*. Interscience, New York, 1946, pp. 207-227.

through a state of high energy which is not likely to occur spontaneously. An energy hill has to be overcome before the process can spontaneously slide down to an energy valley. The problem of the mechanism of oxidation or reduction of organic compounds is essentially the problem of how this intermediate energy hill is to be overcome. It is, in other words, the problem of the nature of activation energy.

Oxidation may consist either in the loss of electrons, or the loss of H atoms, or the attachment of (OH) groups, or the combination with an O atom. To a certain degree, all these processes are equivalent. Reduction is the reversal of oxidation and needs no additional comment. However, this equivalence is not always perfect. So, to arrive at an unambiguous definition of oxidation one has to choose one of the processes just enumerated as the essential one and call it the one characteristic of oxidation proper, and to represent the others as secondary or corollary reactions. In order to follow the original idea of Lavoisier, namely, that oxidation is combination with O, one might be inclined to choose this criterion as the process primarily characteristic of oxidation. Such a definition, however, would very soon lead to absurdities. On the one hand,  $\text{Fe}^{3+}$  ion could not be called an oxidation product of  $\text{Fe}^{2+}$  ion; on the other hand, oxyhemoglobin should be considered as an oxidation product of hemoglobin. Although, for the latter case, this nomenclature is still sometimes used, it is fully recognized that the reversible "oxygenation" of hemoglobin is not representative of a typical, ordinary oxidation, whereas methemoglobin must be considered as a true oxidation product of hemoglobin, although it does not arise from it by the combination with O. Another suggestion, at least for the field of organic compounds, was to distinguish clearly between oxidation, meaning addition of O or OH radical, and dehydrogenation, meaning detachment of H atoms. Since Wieland's elaborate studies on the oxidation of organic compounds, the concept of hydrogenation instead of reduction, and of dehydrogenation instead of oxidation has been used abundantly. Indeed it is very appropriate to speak of the conversion of succinic acid to fumaric acid as a dehydrogenation process. This idea seemed to Wieland<sup>7</sup> even more justified as he tried to show that Pd can "oxidize," or, rather, "dehydrogenize" hydroquinone to quinone by withdrawing H from it. He thought that the combining power of Pd with H is so great that the metal simply pulls out the H from hydroquinone. However, Gillespie and Liu<sup>8</sup> have shown that Wieland's experiment was fallacious. It is easy to calculate the pressure of  $\text{H}_2$  gas with which the detachable H of hydroquinone would be in equilibrium. Biilmann<sup>9</sup> calculated that a saturated solution of quinhydrone is in thermo-

<sup>7</sup> H. Wieland, *Ber.* 45, 482 (1912).

<sup>8</sup> L. J. Gillespie and Hsien Liu Tsun, *J. Am. Chem. Soc.* 53, 3969 (1931).

<sup>9</sup> E. Biilmann, *Ann. chim.* [9], 15, 109 (1921).

dynamic equilibrium at room temperature, with  $H_2$  gas of  $10^{-24}$  atmospheres of pressure. This can readily be inferred from the oxidation-reduction potential of quinhydrone. This  $H_2$  pressure is so small that it is meaningless from an atomistic point of view. The situation is the same as in a problem often discussed some 50 years ago: If one starts from Nernst's theory, the potential of an Ag electrode depends on the concentration of free  $Ag^+$  ions in the solution with which the metal is in contact. Then, the potential of Ag against a solution of AgCN is such as to indicate a concentration, in the solution, of free  $Ag^+$  ions so low that there would be about 3  $Ag^+$  ions in a liter. This result simply shows that the potential is not established by the interaction of the metal with the free  $Ag^+$  ions, but by direct interaction of the metal with the AgCN molecule. The latter has a certain tendency to throw off  $Ag^+$  ions when in contact with an electron donor (the metal) whereby  $Ag^+$  ion will be converted to an Ag atom. This tendency can be measured in terms of a thermodynamically definable and meaningful magnitude, namely, the "activity of the  $Ag^+$  ions" in the AgCN solution. In an  $AgNO_3$  solution, the  $Ag^+$  activity is proportional to the concentration of  $Ag^+$  within a restricted range, say, between  $10^{-2}$  and  $10^{-5}$  M. If the concentration of  $Ag^+$  is below that range, as in an AgCN solution, there is no longer any reasonable correlation between the activity of the  $Ag^+$  ions and their true concentration.

In the case of the quinone-hydroquinone system, the activity of the H atoms is extremely low. On the justifiable assumption that, for a very dilute gas, activity is proportional to pressure, one finds a pressure of  $10^{-24}$  atmospheres  $H_2$  gas in a solution of quinhydrone, as stated above. This statement is meaningless, from a molecularistic point of view. Here, the H activity can no longer be correlated with any pressure in the original meaning of the word, and is just a thermodynamically definable magnitude. Gillespie indeed showed that the oxidative power of Pd is not due to its tendency to capture H from hydroquinone. He ascribed it to a contamination of the Pd with palladium oxide. A perfectly O-free Pd is not able to dehydrogenize hydroquinone to any extent detectable even by the most sensitive laboratory tests. Wieland's misinterpreted experiment has done much harm to the real understanding of oxidation-reduction equilibria. His concept of hydrogenation and dehydrogenation, however, if applied to its justifiable extent, is very useful. Its use in biochemistry should not be discouraged by these arguments.

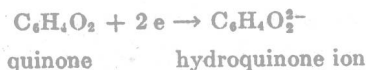
All those difficulties concerned with the definition of oxidation and the relationship of oxidation to dehydrogenation can readily be overcome if one chooses, as the primary characteristic of oxidation, the withdrawal of electrons, and as reduction its reversal, the attachment of electrons.<sup>3, 5, 10</sup>

<sup>10</sup> W. M. Clark, U. S. Pub. Health Service, Pub. Health Repts. 38, 443 (1923).

In some cases this is in fact the only process occurring:



This reaction takes place when  $\text{Fe}^{3+}$  ion, *i.e.*, an acidified solution of  $\text{FeCl}_3$ , is exposed to an electron donor (*i.e.*, an oxidizing agent) or to anodic polarization. In other cases the primary process, as in:



may be accompanied, if it occurs in a medium of sufficiently high concentration of H ions, by an addition of protons:



or:



In such a case the process as a whole is a hydrogenation, and this process may be thought of as (1) acceptance of electrons, a step characteristic of "reduction" proper, and (2) acceptance of protons, as a consequence of, but not belonging to, the reduction proper, according to this definition. It should not be asserted that the electron is necessarily added first, and the proton thereafter. If one considers as essential for oxidation, in any case of dehydrogenation, the withdrawal of an electron, and the withdrawal of a proton, or the addition of OH ion, as the concomitant reaction not belonging to the oxidation process proper, one should not postulate that the electrons always react first; the removal of the H atom as a whole is quite compatible with our definition, which implies nothing about the mechanism of the dehydrogenation and the temporal sequence of the steps into which it may be resolved.

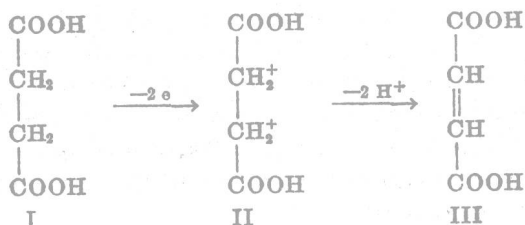
Another example of how the electron transfer can be reconciled with older ideas is this: Consider the action of  $\text{O}_2$  on Zn. The formation of ZnO is an oxidation even according to Lavoisier's original definition. If one considers the ZnO crystal, and takes into consideration that it is an ionic compound and consists of  $\text{Zn}^{2+}$  ions and  $\text{O}^{2-}$  ions, it is reasonable to describe the oxidation of Zn by saying that Zn has lost two electrons, and  $\text{O}_2$  has accepted them. Although Zn and O still stick together, and comply with the old definition of oxidation, yet two electrons are pulled from Zn nearer to O, and thus the new definition is justified. In other cases, if in an

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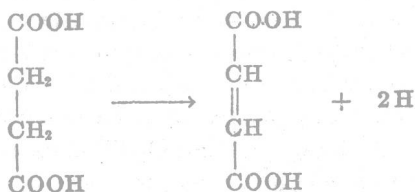
oxide of the form  $\text{M}_2\text{O}$  or  $\text{MO}$  the bond is not so much a merely ionic one, but more or less essentially a covalent one, having but little ionic character, if any, it will be more difficult to apply the analogy. So, water should by all means be considered as an oxidation product of H, yet one might speak of a "transfer" of electrons from the H atom to  $\text{O}_2$  only insofar as each chemical bond is represented by two electrons, of which

one originally belonged to an H atom and has been transferred into the electronic shell of O, without the proton's losing it entirely. In general it will be justifiable to consider the transfer of the electron as the fundamental process of oxidation or reduction and all other processes concomitant with it as secondary and not belonging to the process of oxidation proper. In any case, the reversible addition of an O<sub>2</sub> molecule, as in hemoglobin and a few other cases to be discussed later, should not be counted among the oxidation processes proper. This is usually designated as *oxygenation*, following Conant's<sup>11</sup> proposal. As will be discussed later, oxygenation may be either a process which for most purposes may be considered as reversible, as in oxyhemoglobin, or oxygenation may be followed by an intramolecular electron rearrangement by which the O<sub>2</sub> is reduced and the substrate oxidized. In such a case oxygenation is a precursor of oxidation. This seems to be a usual process when molecular O<sub>2</sub> is used as an electron acceptor, or "oxidizing agent."<sup>12</sup>

In organic compounds, and especially in those occurring as metabolites, very often oxidation may, without hesitation, be identified with dehydrogenation. In compliance with the above arguments, the conversion of succinic acid to fumaric acid should be described as follows (disregarding the fact that reaction I → II may be resolved into two steps):



This can be just as well described by going from step I directly to step III:



which implies that the concentration of the molecular species of step II might be too low to measure. In such cases, which are very common in oxidative metabolism, the use of the concept of dehydrogenation and hydrogenation, instead of oxidation and reduction, is quite legitimate and useful. Still, it is important to keep the electronic scheme in mind,

<sup>11</sup> J. B. Conant, *J. Biol. Chem.* **57**, 401 (1923).

<sup>12</sup> L. Michaelis, *Federation Proc.* **7**, 509 (1948).

because even in metabolism not every oxidation is a dehydrogenation. When cytochrome c (in its oxidized state) oxidizes succinic acid, with the aid of a specific enzyme, it can do so only by pulling out electrons; the protons are hereby necessarily released because molecule II is so labile (or in other terms the "acid" II is so strong) that it releases its protons.

In the discussion of oxidation, both with respect to its thermodynamics and to its atomistic mechanism, one has to distinguish two classes of processes, or even three. As a first case, both the oxidizing agent and the reducing agent are molecularly dispersed in a homogeneous solution. (Reactions in the gas phase would belong to this category too but are of no interest here.) The oxidation takes place due to a collision of the two molecules without the formation of a compound or complex of the two of any appreciable stability. To be sure, even when they react upon each other on mere collision by transferring an electron from the one to the other, one may speak, during the time of contact, of a "compound" in the sense of the modern theory of chemical kinetics.<sup>13</sup>

For a brief review see Polányi.<sup>14</sup> According to a personal communication from Professor Eyring the "time of contact" or the "lifetime of the activated complex," may be estimated, under ordinary conditions at room temperature, to be about  $2 \times 10^{-13}$  second. This calculation is based on the assumption that the two molecules, as they approach each other, may be said to be in contact, or to form the "activated complex" as long as their distance is  $\leq 3 \times 10^{-9}$  cm., a value which slightly depends on the "reduced mass" of the molecules, and is taken to be 10 here (on the scale in which hydrogen is 1.008). Half that distance and half that time the two molecules will be approaching the energy barrier and the other half they will be separating.

As the two molecules approach each other they will come so close as to suffer a mutual polarization or deformation. Hereby the electronic configuration is distorted in such a way as to form what may be called a common molecular unit called the transitional state, which has both a chance of undergoing a rearrangement and also a chance of flying apart again without any reaction occurring. If a reaction is to take place it must do so during the time of contact, as long as the transitional state formed by mutual deformation is in existence. No force holds the two colliding molecules together for any appreciable length of time; on collision they either do react or they do not. In our present discussion we do not speak of such intermediary compounds as "compounds." We speak of them as such only if there is an appreciable attraction which holds the molecules together after the collision has taken place thus forming a new molecular species which has an appreciable lifetime. With this distinction in mind, let us

<sup>13</sup> S. Glasstone, K. J. Laidler, and H. Eyring, *Theory of Rate Processes*. McGraw-Hill, New York, 1941.

<sup>14</sup> M. Polányi, *Nature* **139**, 575 (1937); *Endeavour* **8**, 3 (1949).

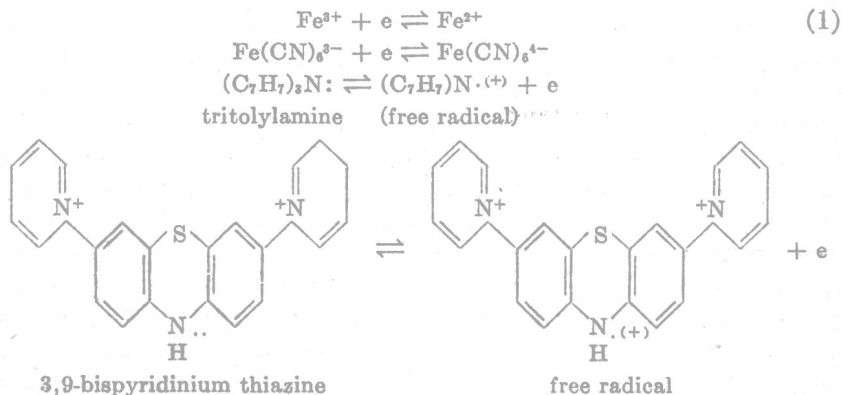
consider separately oxidations due to molecular collision without the formation of any compound or complex of the two reacting molecules, and on the other hand those oxidations which take place after the two reacting molecules have formed a compound in which, not necessarily immediately after its generation, but gradually, in the course of time, a rearrangement of electrons takes place, with the result that after the separation of the two moieties electrons eventually have been transferred from the one to the other. The latter situation is seemingly always true for enzymatically catalyzed oxidations. Enzymatic oxidations may, therefore, be considered as a special case of the second type. However, because of their singular biological importance and their singular atomistic mechanism, they may rather be considered as a third type of oxidation; it is the one in which the enzymologist eventually is interested.

The preceding arguments may be summarized as follows: Oxidation-reduction consists essentially in an electron exchange. Two cases must be distinguished, those in which the electron exchange takes place intermolecularly, and those in which it takes place intramolecularly within a molecular compound formed from the electron donor and the electron acceptor. Oxidation-reduction catalyzed by specific enzymes may be considered as a special subclass of the second type, but will be dealt with rather as a third type because of its peculiarities.

## II. Oxidation in Homogeneous Systems, by Molecular Collision, Without Complex Formation. Intermolecular Electron Exchange

### 1. UNIVALENT OXIDATION

If the essential process in oxidation, expressed in terms of electron transfer, is concerned with the transfer of a single electron, one speaks of univalent oxidation. These are examples<sup>15-17</sup>:



<sup>15</sup> S. Granick, L. Michaelis, *J. Am. Chem. Soc.* **62**, 2241 (1940).

<sup>16</sup> L. Michaelis, S. Granick, and M. P. Schubert, *J. Am. Chem. Soc.* **63**, 351 (1941).

<sup>17</sup> S. Granick, L. Michaelis, and M. P. Schubert, *J. Am. Chem. Soc.* **62**, 1802 (1940).



The two latter examples belong to those rare cases in which an organic molecule yields a relatively stable univalent oxidation product, but no stable bivalent one.

Let us consider the first example in detail. In a given mixture of a salt of  $\text{Fe}^{2+}$  and a salt of  $\text{Fe}^{3+}$ , the ratio of the two ions is, of course, constant in time. However, the equilibrium is not a static one but arises from the fact that the rate of the process (1) from left to right equals that from right to left. Process (1) when read from right to left may be visualized under the general aspect of a dissociation.  $\text{Fe}^{2+}$  dissociates into  $\text{Fe}^{3+}$  and an electron. To be sure, the free electron does not exist in any solution to any appreciable concentration. The case is analogous to the dissociation of an acid  $\text{AH}$  into  $\text{A}^-$  and  $\text{H}^+$ . Here also  $\text{H}^+$  does not exist to any really measurable extent in a free condition. What happens is that  $\text{AH}$  transfers  $\text{H}^+$  to any  $\text{H}^+$  acceptor, such as  $\text{H}_2\text{O}$ :



The tendency of  $\text{AH}$  to throw off protons may be described as a kind of proton pressure, and the tendency of  $\text{H}_2\text{O}$  to capture a proton may be described as a kind of suction. These two opposite tendencies, in general, do not cancel out. A very slight amount of protons, not measurable by methods of chemical analysis, will remain free. In spite of their extreme smallness, their "activity" is definitely established by the poise of what we called pressure and suction. The same consideration holds for the "dissociation" process (1). In comparison with the state of electroneutrality there is an immeasurably small excess (or deficiency, as the case may be) of negative charge, which may be described in terms of electron activity,  $[e]$ .

Now, the rate of the process (1) from left to right must be:

$$R_1 = k_1[\text{Fe}^{3+}][e]$$

where  $k_1$  is a constant characteristic of this process, and the rate from left to right must be:

$$R_2 = k_2[\text{Fe}^{2+}]$$

In the state of equilibrium,  $R_1 = R_2$ , or :

$$\frac{[\text{Fe}^{3+}][e]}{[\text{Fe}^{2+}]} = \frac{k_2}{k_1} = K \quad (2)$$

$$\text{or:} \quad -\ln [e] = -\ln K + \ln \frac{[\text{Fe}^{3+}]}{[\text{Fe}^{2+}]} \quad (3)$$

The meaning of equation (3) can be understood by comparing it with the customary electrode potential equation which relates the potential,  $E$ , of a

platinum electrode in contact with the solution in question to the activities of the two components of the redox system:

$$E = \text{const.} + \frac{RT}{F} \ln \frac{[\text{Fe}^{3+}]}{[\text{Fe}^{2+}]} \quad (4)$$

This is compatible with equation (3) if we put:

$$E = -\frac{RT}{F} \ln [e]$$

and:

$$\text{const.} = -\frac{RT}{F} \ln K$$

where "const." is the potential when  $[\text{Fe}^{2+}] = [\text{Fe}^{3+}]$ . In practice one never measures the absolute value of the potential  $E$ , but its difference from an arbitrarily chosen standard potential, that of the normal  $\text{H}_2$  electrode. Then, the constant of the ferric-ferrous system is designated as the "normal potential" of the ferric-ferrous system. The arbitrariness of the reference point of potential has its counterpart in the fact that the concept of activity also is determined only up to an arbitrary factor. When dealing with ordinary molecular species, the activity factor by which the analytical concentration must be multiplied in order to obtain activity will usually be chosen so that in a solution of very low, but still meaningful, concentration activity equals molarity.

Equation (4) can be experimentally verified on measuring the potentials in a series of mixtures of a  $\text{Fe}^{2+}$  salt and an  $\text{Fe}^{3+}$  salt. In order that the brackets in (4) may be equal, or at least proportional, to concentrations, the ionic strength of the solution must be kept constant. Furthermore, hydrolysis of the Fe salts must be prevented by working in an acid solution (about 0.01  $M$   $\text{HCl}$ ). This is an important matter for ions of high valence in which the activity factor depends on the concentration to an enormous extent. The desired experimental conditions are most easily fulfilled by choosing as a solvent not pure water but a rather concentrated solution of an indifferent neutral salt, say a 1  $M$  solution of  $\text{KCl}$  containing some  $\text{HCl}$  ( $10^{-2} M$ ) to prevent hydrolysis. The total concentration of the Fe, although varied over a large range, should not exceed say  $10^{-2} M$  or so. The fact that Peters<sup>13</sup> in his often quoted paper confirmed to a satisfactory degree equation (4) without taking those precautions is due to a coincidence.

Equation (4) is usually referred to as Peters' equation, not quite justly. Peters was a student in Hans Bredig's laboratory and worked under his direction. Equation

<sup>13</sup> R. Peters, *Z. physik. Chem.* **26**, 193 (1898).