A Quantitative Hardness Tester for Food Products[1]

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A pressure-hardness tester has been designed by the writer and fabricated by machinists of the Division of Industrial Research to supply a quantitative method for testing hardness of fruits and other food products, replacing the qualitative "thumbnail" or "finger pressure" method. At present the device (Fig. 1) is being used to test hardness of pears in conjunction with a research project jointly sponsored by the Northwest Canners Association, Washington State Soft Fruit Commission and the Division of Horticulture, State College of Washington.

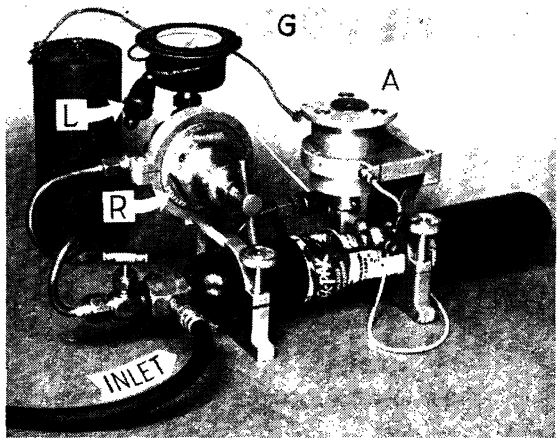

FIG. 1. Photograph of a quantitative hardness tester. Small tank not necessary when tester is connected to external source of gas pressure. Corresponding parts lettered as in Fig. 2.

The principle of the tester is the determination of that gas pressure necessary to force the blunt end of a piston a very small but fixed distance into the test material.[2] The tester now in use forces a rounded brass tip 5/32" in diameter 1/32" into the pear. The top plate serves both as a stop, restricting penetration to 1/32", and as an electrical contact, completing a circuit which lights an indicator lamp when maximum penetration is reached. Pressures found necessary to effect this penetration into normal green pears have been observed to vary from 50 to 65 pounds per square inch. Abnormally hard pears were found to test above 65. Tips of others sizes and penetrations of different depths may be used for other food products. Any convenient and suitable gas source may be used, such as compressed air or nitrogen.

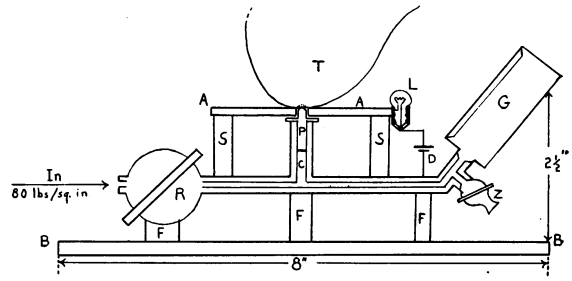

FIG. 2. Sketch of device shown in photograph. A—top plate; B—base plate; C—cylinder; D—dry cell battery; F—metal frame; G—pressure gauge; L—indicator amp; P—piston; R—regulator valve; S—insulated support; T—test fruit; Z—release petcock.

Fig. 2 shows a simplified sketch of the tester, illustrating its basic operating principles. Future models will be constructed from this design.

No injury to the fruit is apparent or expected from this test. Pears are held firmly against top plate during the test, a barely visible indention being the only effect.

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[2] A tester utilizing mechanical pressure from a spring was also designed and may be fabricated for later work. The writer is grateful to Prof. N. S. Golding for supplying a metal hypodermic cylinder body and piston and for suggestions concerning its use.

The Properties of the Enzyme-Substrate Compounds of Horse-Radish and Lacto-Peroxidase[1]

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Peroxidases are iron-containing enzymes which, on the basis of both spectroscopic and magnetic susceptibility data, form definite chemical compounds with their substrate, hydrogen peroxide. These enzyme-substrate compounds cause the very rapid oxidation of oxidizable substances (acceptors) such as ascorbic acid, pyrogallol, etc. One type of peroxidase is widely distributed in plants and is usually prepared from horse-radish root (horse-radish peroxidase). Another type is found in milk and is called lactoperoxidase. The pioneer work of Keilin and Mann (*10*) on horse-radish peroxidase and Theorell's (*16*) purification and extensive studies of both horse-radish and lactoperoxidase now make it possible to study in detail the properties of the several compounds which these enzymes form with hydrogen peroxide and the mechanism by which these compounds oxidize acceptors.

[1] The horse-radish peroxidase, lactoperoxidase, and cytochrome C preparations used in these studies were generously supplied by Hugo Theorell and K. G. Paul. Many thanks are due to H. Theorell, D. Keilin, and E. F. Hartree for their criticism and advice in these researches. A special acknowledgment is made to the memory of Glenn Millikan, who greatly stimulated this development of the rapid-flow method for studies of enzyme-substrate compounds.

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Theories (*12*) dating from 1913 have postulated that enzyme reactions involve enzyme-substrate compounds, and now these studies (*4*) give direct records of their reactions. Direct evidence of these chemical compounds of enzyme and substrate clearly shows that short-range, not long-range, forces are required for the action of these enzymes. The possibility that the long-range forces postulated by Rothen (*14*) play any part in these enzymatic reactions is very remote.

When the iron atom of horse-radish peroxidase combines with hydrogen peroxide, three distinct colored compounds are formed: green (I) (*18*), pale red (II) (*10*), and bright red (III) (*10*). All of them rapidly disappear when an oxidizable substance like ascorbic acid is added, with the result that the free enzyme and oxidized acceptor are obtained. Here studies have been made to show which of these three enzyme-substrate compounds are involved in the enzymatic activity of peroxidases and how the active complex reacts with the acceptor molecule.

These color changes are so rapid that a special method is needed, not only to measure the rates of formation and disappearance of the compounds, but also to obtain their absorption spectra. Various improvements (*5*) of the flow method of Hartridge and Roughton (*9*) and Millikan (*15*) now permit studies of the reaction kinetics and the absorption spectrum of an unstable enzyme-substrate complex that has a half-life of only several milliseconds and a molar concentration as small as 1×10^{-6}.

The spectra of the primary and secondary enzyme-substrate complexes. There are two striking features of the combination of these enzymes with their substrates; first, the brown enzyme solution becomes green, and secondly, this change appears to occur instantaneously. Theorell (*18*) first saw this reaction occur upon the addition of hydrogen peroxide to horse-radish peroxidase, and in these researches a similar lactoperoxidase-hydrogen peroxide complex has been found. In addition, a green primary hydrogen peroxide complex of the related enzyme, catalase, has been found (*6*). These three enzymes can also combine with substituted peroxides (methyl [CH_3OOH] or ethyl [C_2H_5OOH] hydrogen peroxide), and here again green primary complexes are found. In all nine cases studied, enzyme and substrate were found to combine in very rapid reactions.

By using the rapid flow apparatus, quantitative data on the absorption spectra of these green primary compounds have been obtained. The addition of peroxide to these enzymes causes a very large decrease in the intensity of their major absorption band as shown by the shift from curve A to curve I of Fig. 1. The spectra of the nine primary compounds studied are very similar, and it is concluded that they all involve the same type of iron-peroxide bond. From Theorell's studies of the magnetic susceptibility of related peroxidase compounds (peroxidase fluoride) (*17*), it is probable that the iron atoms of these primary enzyme-substrate compounds are bound by essentially ionic bonds in the primary complexes.

In peroxidases, these primary peroxide complexes rapidly shift into a red secondary form in about 0.1 sec under these conditions. These red forms were first seen and their visible absorption bands were measured by Keilin and Mann (*10*) using horse-radish peroxidase and by Theorell and Åkeson (*19*) using lactoperoxidase. Curve II of Fig. 1 shows that the absorption band of the secondary complex differs considerably from that of the primary complex. Curve II closely resembles the absorption band of horse-radish peroxidase-cyanide which has been found by Theorell to have covalent bonding. Thus it is probable that these secondary peroxidase-peroxide complexes also have covalent bonding. These data show that the substrate can combine with the enzyme so as to alter profoundly the absorption spectrum and the nature of the chemical bonds in the enzyme.

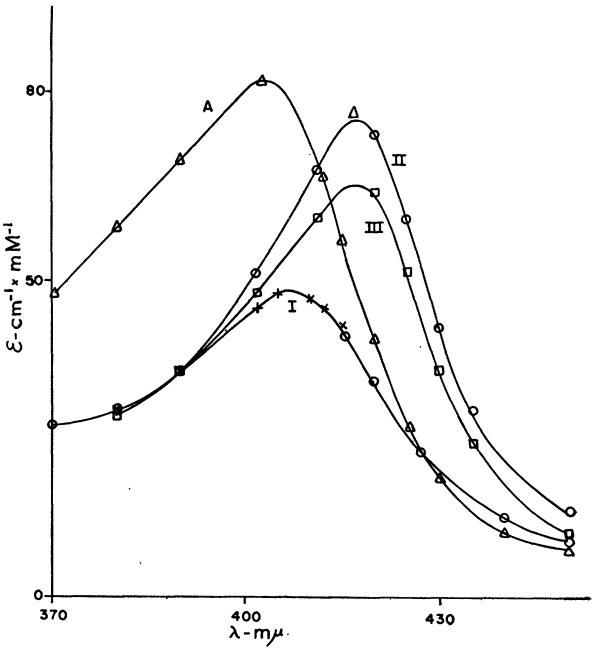

FIG. 1. The Soret bands of free peroxidase (A) and peroxidase combined with hydrogen peroxide to form the primary (I), secondary (II), and tertiary compounds (III). Complex I is very unstable and the points on this spectrum were measured by means of the rapid-flow apparatus. Under proper conditions, Complex II is relatively stable and both the rapid-flow apparatus and the Beckman spectrophotometer were used to measure its spectrum. Complex III forms in the presence of a large excess (10- to 100-fold) of hydrogen peroxide; its spectrum was measured in the Beckman spectrophotometer.

The reaction kinetics of the primary and secondary complexes. By timing the rate of formation of the primary complexes in the rapid-flow apparatus, the reaction-velocity constants for the union of peroxidase with peroxide molecules have been studied. In this very rapid reaction, the hydroxyl group bound by ionic bonds to the iron atom of peroxidase (*16*) is replaced by the peroxide group:

$$\underset{\text{brown}}{\text{Per} \cdot \text{OH}} + \text{HOOR} \underset{k_2}{\overset{k_1}{\longleftrightarrow}} \underset{\text{green}}{\text{Per} \cdot \text{OOR(I)}} + H_2O \qquad (1)$$

R may be H– (*18*), CH_3–, or C_2H_5–, and the values of k_1

for these substrates are given in Table 1. These enzymes combine with their substrates at about the same speed as muscle hemoglobin combines with oxygen (*13*).

ticular experiment. The quantity $t_{\frac{1}{2}off}$ is the time from $t = 0$ until the concentration of complex II has fallen to half its maximum value. Thereby the enzyme-substrate

TABLE 1

REACTION VELOCITY CONSTANTS FOR EQUATIONS 1, 2, AND 3

Enzyme	Substrate	k_1 ($M^{-1} \times sec^{-1}$)	Km_2(M) k_7 (sec^{-1}) k_3 (sec^{-1}) for zero acceptor concentration			k_4($M^{-1} \times sec^{-1}$) for ascorbic acid (pH 7.0)	k_4($M^{-1} \times sec^{-1}$) for pyrogallol (pH 7.0)
Horse-radish peroxidase	hydrogen peroxide	1×10^7	1.5×10^{-8}	4.0	0.05	2,800	2.1×10^5
"	methyl-hydrogen peroxide	1.5×10^6	3×10^{-7}	4.0	0.03	2,800	2.1×10^5
"	ethyl-hydrogen peroxide	3.6×10^6	....	4.0	0.02	2,200	1.8×10^5
Lacto-peroxidase	hydrogen peroxide	2×10^7	1×10^{-8}	4.0	0.03	5,400	7×10^6
"	methyl-hydrogen peroxide	6×10^6	2×10^{-8}	4.0	0.03	2,700	....
"	ethyl-hydrogen peroxide	2×10^6	2×10^{-8}	4.0	0.02	2,100	....

Complex I is spontaneously converted into complex II in a first-order reaction:

$$\underset{\text{green}}{\text{Per} \cdot \text{OOR (I)}} \overset{k_7}{\longleftrightarrow} \underset{\text{red}}{\text{Per} \cdot \text{OOR (II)}} \qquad (2)$$

The oxidation reaction occurs on combination of the acceptor molecule (AH_2) with the secondary complex and results in the liberation of the free enzyme:

$$\underset{\text{red}}{\text{Per} \cdot \text{OOR (II)}} + AH_2 \overset{k_4 = \frac{k_3'}{[AH_2]}}{\longleftrightarrow} \underset{\text{brown}}{\text{Per} \cdot \text{OH}} + A + \text{ROH} \qquad (3)$$

The velocity constant (k_4) for the reaction of complex II with acceptor is here taken as a measure of the enzyme activity (see Table 1). The values of k_7 given in the table increase greatly when an acceptor is added. Therefore, the transition from complex I to complex II is not a rate-determining step in peroxidase kinetics. Only the combination of enzyme and substrate and their reactions with the acceptor are rate-determining steps in accordance with previous data (*4*). When $[AH_2] = 0$, complex II nevertheless spontaneously decomposes in a first-order reaction of rate k_3 (see Table 1).

In these studies, the utilization of peroxide by the enzyme system can be readily measured from the graph of the concentration of complex II as a function of time as shown in Fig. 2. Complete solutions of the equations for peroxidase kinetics (*4*) have shown that a simple relation exists between k_4 and values readily measured from the kinetics of complex II:

$$k_4 = \frac{[\text{HOOR}]}{[\text{Per} \cdot \text{OOH} - \text{II}]\,[AH_2]\,t_{\frac{1}{2}off}}\ M^{-1} \times sec^{-1} \qquad (4)$$

where [HOOR] and $[AH_2]$ are initial concentrations ($[AH_2] > [\text{HOOR}]$) and [Per · OOH (II)] is the maximum concentration to which complex II rises in the par-

complex is used as a spectrophotometric indicator of its activity. This method permits studies of enzyme activity in the presence of substrate concentrations which are much less than those measurable by ordinary techniques. Thus enzyme inactivation caused by excessive substrate concentrations is avoided.

The values of k_4 given in Table 1 show that, usually, the reaction of enzyme and substrate is considerably more rapidly than the reaction of complex II with the acceptor. This is not a general rule; the reaction of complex II of lactoperoxidase and hydrogen peroxide with hydroquinone is nearly as rapid as the combination with hydrogen peroxide.

The values of k_4 show that lactoperoxidase is a more active enzyme with these two acceptors than is the plant enzyme. In addition, the milk enzyme combines more rapidly with hydrogen peroxide. These differences may be attributed to differences between the protein parts of these two enzymes.

In general, the peroxidase complexes formed from the substituted peroxides react just as rapidly with acceptors as do the complexes formed from hydrogen peroxide. The same result was obtained in the reactions of the catalase-peroxide complexes with alcohols and other substances (*6*). The rate of formation of the primary complexes, however, decreases somewhat with the substituted peroxides, possibly owing to a steric effect. This effect is much less with peroxidases than with catalases (*6*).

Fig. 2 illustrates the effect of using three different substrates upon the kinetics of complex II. With hydrogen peroxide, k_1 is largest, and the concentration of complex II rises to very nearly its saturation value. Because nearly all the enzyme is in the form of this reactive complex, the oxidation of ascorbic acid is rapid, and consequently the hydrogen peroxide is consumed in about two

seconds. Since methyl hydrogen peroxide combines more slowly with peroxidase, the concentration of complex II rises to a smaller value in this test, and the consumption of the peroxide requires a longer time. A similar effect is observed with ethyl hydrogen peroxide. But in all three cases, the values of k_4 calculated from equation 4 are nearly the same (2,800, 2,800, and 2,200 $M^{-1} \times sec^{-1}$;

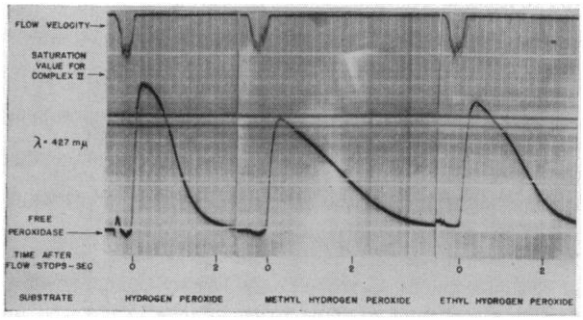

FIG. 2. Illustrating the use of the rapid-flow apparatus in the measurement of the rapid formation (fast rise of traces) and disappearance (slower fall of traces) of the enzyme-substrate complexes of horse-radish peroxidase (2.9×10^{-6} M) caused by reactions with hydrogen peroxide (3.6×10^{-6} M), methyl hydrogen peroxide (4.2×10^{-6} M), and ethyl hydrogen peroxide (3.2×10^{-6} M) in the presence of ascorbic acid (40×10^{-6} M). These three enzyme-substrate complexes are here used as spectrophotometric indicators of the peroxide concentration; the velocity of their reaction with ascorbic acid is calculated by equation 5. The results are given in Table 1.

see Table 1). The earlier conclusion of Wieland and Sutter (*20*) that the activity of horse-radish peroxidase with ethyl hydrogen peroxide is ½ that with hydrogen peroxide is apparently incorrect.

In the absence of added acceptor, the very high affinity of the enzyme for its substrate can be demonstrated; Table 1 lists as Km_2 the concentrations of peroxide which give the half-saturation value of complex II. These concentrations are much less than those required to half-saturate muscle hemoglobin with oxygen and about as small as the concentration of oxygen estimated to give half-saturation of the respiratory enzyme (*13*).

With horse-radish peroxidase, but not with lactoperoxidase, the values of k_7 and k_8 can be reduced more than tenfold by repeated additions of an equivalent of peroxide. But k_3 has never been reduced to zero; the enzyme-substrate compounds of both peroxidase and catalases slowly undergo "spontaneous" decomposition (whose mechanism is not understood) into the free enzyme.

The reversible decomposition of the enzyme-substrate complexes (k_2 of equation 1) does not appear to play an important role in the reaction kinetics; in fact, final proof of the existence of k_2 is lacking. Hence, the rapidly reversible combination of enzyme and substrate pictured in the theory of Michaelis and Menten (*12*) is not characteristic of these enzymes.

The relation between the enzyme-substrate bond and the activity. In peroxidase, the covalent secondary complex must be formed before oxidation of the acceptor occurs and the enzyme is liberated from the complex. But in catalases, the primary complex with presumably ionic bonds appears to react directly with the acceptor; the enzyme is liberated from the primary complex without the formation of a measurable amount of a secondary complex of the type that peroxidase forms. This fundamental difference in the mechanism of catalase and peroxidase reactions may ultimately be resolved by further search of an undiscovered covalent complex in catalase reactions. Nevertheless, based on the nine similar primary complexes of catalase, horse-radish peroxidase, and lactoperoxidase, which surely have the same bond type (probably ionic), there is support for the generalization that this type of compound of enzyme and substrate forms first and more rapidly than the covalent compound.

None of these oxidations caused by these hematin-peroxide complexes can be inhibited by carbon monoxide (*6, 7, 8, 10*), and therefore, the iron atom of these peroxidases is concluded to be trivalent and to remain trivalent in reactions with the substrates and acceptors.

The relation between heme-linked groups and activity. By a variation of the pH, linkages between the iron atom of peroxidases and the protein molecule can be altered, and their effect upon peroxidase activity may be determined. In alkaline solution above pH 9, peroxidase acquires a covalently bound hydroxyl group (*17*). Under these conditions the enzyme-substrate complexes do not form, and the enzyme is inactive, the effect being very similar to that observed when cyanide is bound to the iron atom of peroxidase by covalent bonds. Apparently the exchange reaction of equation 1 between the hydroxyl group bound by ionic bonds and the peroxide group is inhibited when the hydroxyl group is bound to the iron atom of peroxidase by covalent bonds.

The dissociation constant (Km_2) and reaction kinetics of the primary and secondary complexes of horse-radish peroxidase are found to be practically unchanged from pH 8.8 to 3.6. This is in accordance with studies of this particular peroxidase preparation which shows no changes of heme linkage in this pH region. Below pH 3.6, a change of heme linkage of pK 3.5 is found by direct spectrophotometric studies and by the effect of pH upon the dissociation constant of the horse-radish peroxidase fluoride and cyanide compounds.

In accordance with these data, the values of k_4 for the reaction of complex II with a number of acceptors show no systematic variation in the region pH 3.6 to 6.7. In general, nearly constant activity is found in this region, for example, in the case of hydroquinone, guaiacol, and pyrogallol. Where changes of k_4 are observed, changes in the course of the oxidation are also observed (leuco-malachite green).

As a consequence of these experiments, the previous concept of sharp "pH optima" for peroxidases and catalases may be discarded. When the actual reaction velocity constants involved in the enzymatic activity are measured directly from the kinetics of the enzyme-substrate complex, errors caused by enzyme inactivation, by partial saturation of the enzyme with substrate, or by

a variable lag between the disappearance of substrate and the appearance of a colored reaction product are eliminated. The activity is practically constant in the broad region where no changes of heme-linkages occur.

Acceptor specificity. Balls and Hale (*3*) generally describe peroxidase as a dehydrogenase removing two hydrogen atoms from different carbon atoms. Ascorbic acid, dihydroxymaleic acid, and *o*-dihydroxybenzenes are examples of the enediol group, $-\overset{OH}{\underset{|}{C}} = \overset{OH}{\underset{|}{C}}-$, which on reaction with peroxidase-peroxide complexes is dehydrogenated to the diketo or o-quinone group, $-\overset{O}{\underset{||}{C}}-\overset{O}{\underset{||}{C}}-$. Such a reaction is related to the action of catalase-peroxides on the $-\overset{H\cdot}{\underset{|}{C}}-OH$ group of a lower alcohol, methylene glycol, or formic acid, where two hydrogen atoms attached to the same carbon atom are removed (*6*) to give the group $-\overset{|}{C}=O$. While catalase has been found to be highly specific for small molecules of related structure, peroxidase-peroxides oxidize a wide range of rather different substances, for example, iodide or leucomalachite green. It is clear, however, that the broader acceptor specificity of peroxidase includes reactions analogous to those of catalase except that the two hydrogen atoms are attached to different carbon atoms. Since catalase hematin is accessible only to small molecules while peroxidase hematin is accessible to rather large molecules, further analogies between catalase and peroxidase specificity are difficult to demonstrate.

The oxidation of a number of substances previously considered to be inhibitors of peroxidase activity (*3*), e.g., phenol, aniline, resorcinol, and phloroglucinol, can be demonstrated by these more sensitive methods.

A particularly interesting reaction is the oxidation of ferrocytochrome C by the peroxidase-peroxide complexes. Contrary to the conclusions of Altschul, Abrams, and Hogness (*1*), horse-radish peroxidase has considerable activity in this reaction as does lactoperoxidase. At pH 4.6, the turnover numbers of these enzymes are $k_s = 3.1$ and 2.5 sec^{-1}, respectively, as calculated from the rate of oxidation of ferro- to ferricytochrome C. In view of the acceptor specificity of peroxidase, it is more likely that the peroxidase-peroxide complex initially oxidizes the imidazole group of ferrocytochrome C and not the iron atom (see Theorell, *16*).

The ternary complex of enzyme substrate and acceptor. The kinetics of oxidation of ferrocytochrome C oxidation by the peroxidase-peroxide complexes, instead of being first order as equation 3 indicates, approximate the zero order until the ferrocytochrome C concentration has fallen to a small value. These reaction kinetics suggest that the rate-limiting step is the decomposition of a ternary complex of enzyme-substrate and acceptor:

$$\begin{array}{l} \text{Per}\cdot\text{OOR (II)} + AH_2 \overset{k_4}{\longrightarrow} \\ \qquad\qquad \swarrow AH_2 \qquad k_s \\ \text{Per}\cdot\text{OOR (II)} \longrightarrow \text{Per}\cdot\text{OH} + A + \text{ROH} \end{array} \qquad (5)$$

Usually k_s is fairly large—with pyrogallol it may be greater than 2,000 sec^{-1}. However, with ascorbic acid, k_s is roughly 20 sec^{-1}. Values for k_s in the oxidation of methanol by catalase peroxides are about 10 sec^{-1}. The unusually small value of k_s (~ 3 sec^{-1}) in the ferrocytochrome C reaction is possibly caused by the complexity of the cytochrome C molecule and the stoichiometry of the reaction. There is ample kinetic evidence for equation 5 but, as yet, no spectroscopic evidence for such a ternary complex has been obtained (see also LuValle and Goddard, *11*).

Activity and oxidation-reduction potential. The velocity constant (k_4) for the reaction of complex II with acceptor molecules of different oxidation-reduction potential has been studied. First, the variation of pH from 3.6 to 6.7 causes no systematic decrease in the values of k_4 for hydroquinone, guaiacol, and pyrogallol, yet their oxidation-reduction potential decreases by 0.186 v because of this pH change (*2*). If hydroquinone is replaced by quinhydrone at constant pH, the value of k_4 calculated on the basis of the hydroquinone molarity is nearly constant. Thus other factors are much more important than oxidation-reduction potential in determining the value of k_4. For example, hydroquinone and pyrogallol have about the same oxidation-reduction potential but hydroquinone reacts about 10 times more rapidly ($k_4 = 25 \times 10^5$ compared to 2.1×10^5 $M^{-1} \times sec^{-1}$).

It is concluded that the rates of these biological oxidations are remotely related to the oxidation-reduction potentials of the acceptors.

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