TABLE III

SUB-RESINOUS AMINOALKYLATION PRODUCTS USING SODIUM BISULFITE CATALYST WITH PARAFORMALDEHYDE

Molecular ratio of reactants				Analyses, %		Mol.
C_4H_4S	CH_2O	NH_4Cl	$NaHSO_3$	N	S	wt.[b]
2	6	1.5	0.5	6.64	22.0	706
2	6	2.0	.5	6.91	22.0	521
2	4	2.0	.5	8.00	26.5	416
2	6	2.0	1.0	8.67[a]	24.1[a]	...

[a] Resinous product, only partially soluble in hot benzene, obtained by adding the sodium bisulfite to the reaction mixture as a 40% solution while the reaction was proceeding at 75°. [b] Determined by boiling point method in benzene.

reacted in the presence of 25 ml. of acetic acid in the manner described in a prior reference.[3] When the temperature rose to 68° the addition of one liter of methanol was begun. The temperature immediately rose to 72° and external cooling was necessary to continue the addition. Rectification of the products yielded only 150 g. of 2-thenylamine and 90 g. of di-(2-thenyl)-amine. From a similar run in which methanol was excluded, 220 g. of 2-thenylamine and 390 g. of di-(2-thenyl)-amine were obtained.

The paraformaldehyde in the above experiment was replaced with 2000 g. of 30% formaldehyde (methanol-free) and the reaction was conducted to obtain a maximum yield of N-(2-thenyl)-formaldimine as described in reference 2. The reaction proceeded very rapidly and it was difficult to maintain the correct temperature of 65–68° by means of an ice-bath. Rectification of the products gave only 250 g. (35% based on thiophene reacted) of N-(2-thenyl)-formaldimine as the dimer, b. p. 110–145° (at 3 mm.), and larger amounts of resinous products. In a controlled experiment the 36% commercial formaldehyde containing 6% methanol reacted slower, permitting better temperature control. A 56% yield of the N-(2-thenyl)-formaldimine dimer was obtained. Thus, a small amount of alcohol is deemed necessary to slow down the reaction so that resinification and other side reaction are held at a minimum to assure formation of primary products.

Use of *n*-Butyl Alcohol as a Solvent in the Aminomethylation Reaction.—One mole each of thiophene, paraformaldehyde and ammonium chloride was mixed with 150 g. of *n*-butyl alcohol, vigorously stirred and warmed to 70–75° for three hours. The ammonium chloride, 54 g., was recovered by filtration. Distillation of the filtrate through a 6-plate column gave unreacted thiophene, water, unreacted butyl alcohol and 110 g. (92%) of di-(*n*-butyl)-formal, b. p. 180–181°.

Acknowledgment.—The authors are grateful to Dr. D. E. Badertscher for his advice and interest in this problem and to Emily Burns, Loretta Conley and Paul Gee for laboratory assistance.

Summary

1. A study of the action of sulfurous acid as a catalyst for the aminoalkylation reaction has led to the isolation of 2-thenylaminomethylsulfonic acid. Mechanism studies have been carried out which give further indication for the existence of $CH_2{=}NH$, formaldimine. This compound appears to be the reactive intermediate in the aminoalkylation reaction.

2. Alcohols have been found to have definite inhibitory action in the aminomethylation reaction due to formal formation.

3. The structures of resins formed from this reaction are discussed.

4. Generalizations concerning the mechanism for aminomethylation reactions with formaldehyde and ammonium halides, hydroxylamine salts and monoalkylamine hydrohalides are presented.

PAULSBORO, N. J. RECEIVED JANUARY 26, 1949

[CONTRIBUTION FROM THE MEDICAL NOBEL INSTITUTE, BIOCHEMICAL DEPARTMENT, STOCKHOLM]

The Properties of the Enzyme-Substrate Compounds of Lactoperoxidase[1]

BY BRITTON CHANCE[2]

Theorell[3] in addition to his work in crystallizing lactoperoxidase has obtained many important data upon which an explanation of the mode of action of this enzyme might be based. Among other things, two types of lactoperoxidase–hydrogen peroxide complexes have been found, one red and the other brown, probably corresponding to the horse-radish peroxidase-peroxide complexes II and III. In addition, a heme-linked hydroxyl group has been found as in the case of the horse-radish peroxidase.[4] However, the activity of lactoperoxidase and hydrogen peroxide toward pyrogallol was found[3] to be only P.Z. = 71.5 compared with P.Z. = 900 for the horse-radish peroxidase.[5] Such a great difference of activity could be attributed to the difference in the hemin or the protein components of these two peroxidases,[6] or to the fact that no primary lactoperoxidase–hydrogen peroxide complex was formed, in contrast with horse-radish peroxidase.

These experiments, however, show that lactoperoxidase not only forms a primary peroxide complex in the same way as horse-radish peroxidase but also oxidizes pyrogallol and ascorbic acid considerably faster than the horse-radish enzyme. Lactoperoxidase also forms highly active complexes with the alkyl hydrogen peroxides. There is a great similarity between the reaction

(1) This is paper 10 of a series on catalases and peroxidases.

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(3) H. Theorell and Å. Åkeson, *Ark. Kemi Mineral. Geol.*, **B17**, No. 7 (1943).

(4) H. Theorell and K. G. Paul, *Ark. Kemi. Mineral. Geol.*, **A18**, No. 12 (1944).

(5) H. Theorell, *Ark. Kemi Mineral. Geol.*, **A16**, No. 2 (1942).

(6) H. Theorell and K. O. Pedersen, "The Svedberg," Almquist & Wiksell, Uppsala, 1944.

kinetics of the milk and plant enzymes in spite of the differences between their prosthetic groups and protein moieties. The reactions studied are

$$\text{LPO·OH} + \text{HOOR} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{LPO(OOR)} - \text{I} + \text{H}_2\text{O} \quad (1)$$

$$\text{LPO(OOR)} - \text{I} \overset{k_7}{\rightleftarrows} \text{LPO(OOR)} - \text{II} \quad (2)$$

$$\text{LPO(OOR)} - \text{II} + \text{AH}_2 \xrightarrow{k_4 = k_3/(\text{AH}_2)} \text{LPO·OH} + \text{ROH} + \text{A} \quad (3)$$

LPO·OH represents the free enzyme in which the OH group is bound to the iron atom.

Experimental

Preparations.—Late in 1940, Dr. D. E. Green kindly supplied a sample of partially purified lactoperoxidase on which preliminary data on the reaction kinetics and activity toward acceptors were obtained. This project was set aside until 1948 when advantage could be taken of pure lactoperoxidase generously supplied by Prof. Hugo Theorell from his studies on the heme-linked groups of this enzyme. This preparation had a concentration of 86μM estimated spectrophotometrically (ϵ_{413} = 109 cm.$^{-1}$ × mM^{-1} Theorell and Petersen[6]) and was pale green in color.

Spectroscopic Data.—The spectrum of the secondary compound of lactoperoxidase and hydrogen peroxide is shown in Fig. 1. With rather dilute enzyme, it is possible to use an ordinary spectrophotometer and to cover the region from 380 to 450 mμ with a single addition of 9 μM hydrogen peroxide before the complex decomposes appreciably. These spectral data are checked by measurements in the rapid-flow apparatus which verify that this is the spectrum of complex II and not that of complex III. The spectrum is seen to be very similar to that of the horse-radish peroxidase-hydrogen peroxide complex II.[7]

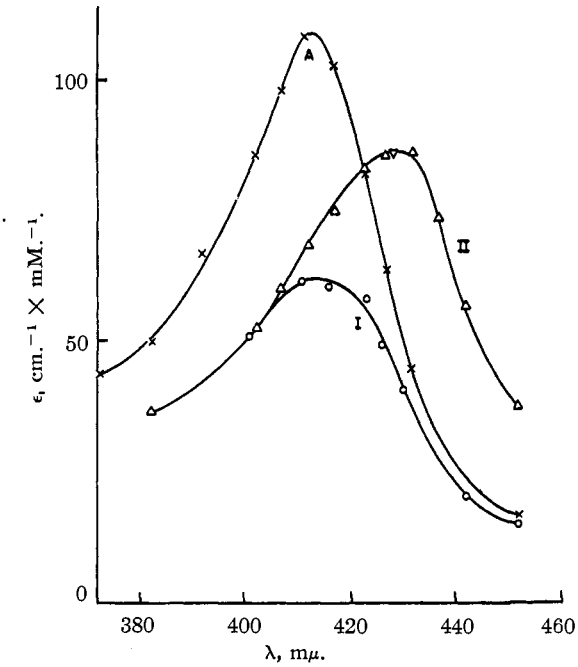

Fig. 1.—The Soret bands of the primary (I) and the secondary (II) lactoperoxidase–hydrogen peroxide complexes and the Soret band of lactoperoxidase (A); complex II spectrum: 1.3 μM. lactoperoxidase plus 9 μM. hydrogen peroxide in the Beckman spectrophotometer (expt. 287b). Point ▽ is obtained in the rapid-flow apparatus. Complex I spectrum: 0.86 μM. lactoperoxidase, 100 μM. hydrogen peroxide, rapid-flow apparatus (expt. 286c); *p*H, 7.0, 0.01 *M* phosphate buffer. The spectrum is obtained from changes of extinction coefficient with respect to both lactoperoxidase and complex II.

By using the rapid-flow apparatus,[8] the spectrum of the hitherto unknown primary lactoperoxidase-hydrogen peroxide complex is obtained by the method discussed elsewhere.[7] This spectrum is very similar to that of the primary compounds of horse-radish peroxidase[7] or catalase with peroxides.[9] The isosbestic point, usually found in the region 427–435 mμ lies beyond 450 mμ because the Soret band of lactoperoxidase lies at a longer wave length than that of horse-radish peroxidase.[6,10]

It is, therefore, not the lack of a primary lactoperoxidase-hydrogen peroxide complex that is responsible for the large difference in the activity of lacto- and horse-radish peroxidase.

The Combination of Lactoperoxidase with Hydrogen Peroxide.—The kinetic evidence for the separate existence of the primary and secondary lactoperoxidase hydrogen peroxide compounds is based upon studies in the rapid-flow apparatus and is given in Fig. 2. In the presence of

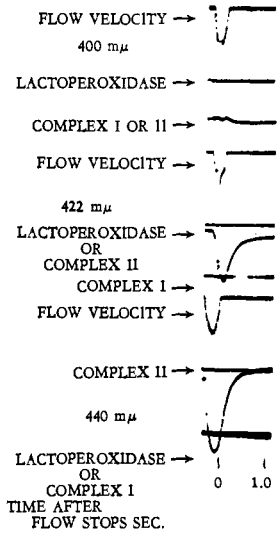

Fig. 2.—The transition from the complex I to II in lactoperoxidase. An upward deflection of the traces indicates an increase of optical density: rapid-flow apparatus, 0.86 μM. lactoperoxidase, 100 μM. hydrogen peroxide, *p*H 7.0, 0.01 *M* phosphate (expt. 286c).

an excess of hydrogen peroxide, the spectral shift at 400 mμ is complete at the earliest time measured owing to the very rapid formation of the primary complex. As the figure shows, the observation tube is initially filled with the enzyme-substrate complex, and a discharge of mixed reactants at the highest value of flow velocity causes no change of optical density; the reaction is complete at 5 ms. after mixing. Also, since the primary and secondary complexes are isosbestic at this wave length (see Fig. 1), no change of optical density is seen when the flow stops. At 422 mμ, the "cycle" of appearance and disappearance of the primary complex is shown. Before the flow starts, the optical density in the observation tube is equal to that of the free enzyme (but actually the observation tube is filled with the secondary complex remaining from a previous identical experiment). During the flow, the primary

(7) B. Chance, *Arch. Biochem.*, **21**, 416 (1949).
(8) B. Chance, *Rev. Sci. Instr.*, **18**, 601 (1947).
(9) B. Chance, *J. Biol. Chem.*, **179**, 1331 (1949).
(10) H. Theorell, *Enzymologia*, **10**, 250 (1941).

TABLE I

THE KINETICS OF FORMATION OF THE PRIMARY AND SECONDARY COMPLEXES OF LACTOPEROXIDASE AND HYDROGEN PEROXIDE: 0.43 μM LACTOPEROXIDASE, *p*H 7.0 (FLOW APPARATUS, EXPT. 286 A)

Formation of the primary complex at 410 mμ	Hydrogen peroxide concentration, μM	0.36	0.90	1.8	4.5	9.0
	Oscillograph deflection (D_1), mm.	30	32	32	(32)	(32)
	Oscillograph deflection (D_2), during flow, mm.	...	...	...	19	11
	Flow velocity indication (f), mm.	...	...	...	19	15
	$k = 27 f \log D_1/D_2$ (sec.$^{-1}$)	...	...	...	112	180
	$k_1 = k(H_2O_2)$ − M.$^{-1}$ sec.$^{-1}$ × 10^7	...	...	...	2.7	2.2
Formation of the secondary complex at 430 mμ	Oscillograph deflection, mm.	35	44	49	45	45
	Half time for formation of complex ($t_{1/2}$), − sec.	0.30	0.20	0.25	0.17	0.11
	$k_7 = 0.7/t_{1/2}$ − sec.$^{-1}$	2.3	3.5	2.8	4.1	6.2

complex is rapidly formed, as indicated by the decrease of optical density. As the flow stops, the primary complex disappears because of its conversion into the secondary complex. At 440 mμ, only the kinetics of formation of the secondary compound are seen; the observation tube is initially filled with the secondary complex, rapid flow of the mixed reactants gives an optical density nearly equal to that of the free enzyme, and as the flow stops, the secondary compound forms.

The kinetic data on these reactions are given in Table I.[11] With the rapid-flow method, the value of the velocity constant for the combination of lactoperoxidase and hydrogen peroxide is found to be $k_1 = 2.5 \times 10^7$ M.$^{-1}$ sec.$^{-1}$—somewhat larger than that found for horse-radish peroxidase. In consideration of the data given in Table III (see later), an average value of 2×10^7 M.$^{-1}$ sec.$^{-1}$ is taken for the velocity constant.

TABLE II

THE EFFECT OF SUCCESSIVE ADDITIONS OF HYDROGEN PEROXIDE TO LACTOPEROXIDASE UPON THE STABILITY OF LACTOPEROXIDASE·H_2O_2·II: 0.044 μM LACTOPEROXIDASE, *p*H 7.0 (CUVETTES, EXPT. 286 A), λ = 410 mμ.

Number of times peroxide had been added to solution previously (cycle number)	2	6	8
Concentration of hydrogen peroxide on each addition, μM	0.04	0.04	0.04
Time for half-decomposition of the complex, sec.	61	46	50
k_3, sec.$^{-1}$	0.026	0.035	0.032

ably caused by the removal of an acceptor is shown by repeated additions of peroxide to horse-radish peroxidase which greatly decelerated the primary to secondary shift.[12] But the data of Table II clearly show that repeated additions of peroxide to lactoperoxidase cause no decrease either in the rate at which the primary complex is transformed into the secondary complex or in the rate at which the secondary complex spontaneously decomposes into the free enzyme. Thus further purification of lactoperoxidase would probably not have enabled Theorell to see this primary–secondary transition. And the half-time for the transition is too short to permit its observation by ordinary visual spectroscopy.

The Kinetics and Equilibria of the Compounds of Lactoperoxidase and the Alkyl Hydrogen Peroxides.—These experiments were carried out in an open cuvette (1.33 cm.) in order to conserve material. The substrate was added with vigorous stirring as a drop on the end of a rod.

Since lactoperoxidase is apparently not inactivated by repeated additions of peroxide under these conditions, advantage is taken of this fact to conserve enzyme in the following experiments where an open cuvette is used. Repeated additions of substrate to the same enzyme solution at the end of each "cycle" are possible.

Since rapid kinetics were measured by this technique, the kinetics of formation of the primary complex of lactoperoxidase and hydrogen peroxide were repeated as shown in Table III. The values of k_1 are somewhat less than those obtained with the rapid-flow technique (2.5×10^7 M.$^{-1}$ sec.$^{-1}$). However, the values are consistent over a reasonable range of half-times, and it is felt that the mixing is rapid enough for the measurement of half-times of 1.0 sec. or greater. For half-times of 0.5 sec., it is necessary to compute the velocity constant using values obtained from 80–90% completion. The total interval between the insertion and withdrawal of the stirring rod is

TABLE III

The kinetics of formation of the primary compound of lactoperoxidase and hydrogen peroxide as measured in a 1.33-cm. depth open cuvette on adding the substrate as a drop on the end of a stirring rod: λ = 410 mμ, *p*H 7.0, 0.01 *M* phosphate (repeated additions of substrate are made to an enzyme solution of a given concentration).

Expt. no.	278b	278b	286a	286a	278d	278d	278d	278d	278
Lactoperoxidase concentration, μM	0.017	0.017	0.044	0.044	0.14	0.14	0.14	0.14	0.14
Hydrogen peroxide concentration, μM	0.08	0.16	0.04	0.04	0.02	0.04	0.08	0.12	0.20
Oscillograph deflection, mm.	.....	.....	.....	.....	5	14	34	38	48
Half-time for formation of complex, sec.	1.5	0.6	1.6	1.5	0.6	....	1.2	0.9	0.5
k_1 − M^{-1} × sec.$^{-1}$ × 10^{-7}	0.8	0.8	1.6	1.6	1.0	....	0.8	0.9	1.0

The formation of the secondary complex from the primary complex proceeds at a rate nearly independent of the hydrogen peroxide concentration, and the first order velocity constant (k_7) is about 4 sec.$^{-1}$.

With horse-radish peroxidase, the transition from the primary to the secondary complex was relatively slow in highly purified material and, under these conditions, Theorell first saw the green-red transition from primary to secondary complex.[10] That this slower transition is prob-

about one second which makes for some uncertainty in the time at which mixing is substantially complete. In all cases where doubt arises, the velocity constant calculated from the half-time should be checked against that calculated from longer times. A typical record of reaction kinetics measured by this technique is given in Fig. 3.

No evidence of the primary complex is obtained in these reaction kinetics as is clearly shown in Fig. 3 where no cycle is found at 421 mμ. This is another case where $k_1(H_2O_2) < k_7$.[12] From Table I, k_7 = 4 sec.$^{-1}$ while $k_1(H_2O_2)$

(11) The temperature of these and the following experiments is 25–30°.

(12) B. Chance, *Arch. Biochem.*, **22**, 224 (1949).

TABLE IV

The kinetics of formation and decomposition of the primary and secondary compounds of lactoperoxidase and alkyl hydrogen peroxides as measured in an open cuvette: λ = 410 mμ, *p*H 7.0, 0.01 *M* phosphate.

Expt. no.		278b	278d	278d	278d	278d	278d	278b	278d	278d	278d
Lactoperoxidase concentration, μM		0.034	0.14	0.14	0.14	0.14	0.14	0.034	0.14	0.14	0.14
Substrate		Ethyl hydrogen peroxide →						Methyl hydrogen peroxide			
Concentration, μM		0.08	0.04	0.08	0.1	0.20	0.4	0.10	0.10	0.16	0.21
Kinetics of formation of primary complex	Oscillograph deflection, mm.	(20)	9	24	30	46	52	.....	28	45	54
	Half-time for formation of complex, sec.	1.8	2.7	....	2.7	2.1	1.0	1.2	....	1.2	0.8
	k_1 — $M^{-1} \times sec.^{-1} \times 10^{-6}$	5	2	....	3	2	1.5	5	....	6	5
Kinetics of decomposition of secondary complex	Half-time for decomposition of complex, sec.		43	58	65	79	140	.....	29	46	86
	$k_3 = x_0/p_{max}t_{1/2}$ off. $sec.^{-1}$		0.04	0.02	0.02	0.02	0.02	.....	0.05	0.03	0.10
Average k_1, $M.^{-1}$ $sec.^{-1}$			2×10^6						6×10^6		
Average k_3, $sec.^{-1}$			0.02						0.03		
Calcd. dissociation constant, k_3/k_1, M.			1×10^{-8}						5×10^{-9}		

is only about one-tenth this value under the conditions of Fig. 3. Thus k_1 is accurately measured in terms of the kinetics of formation of complex II. Table IV summarizes kinetic data on the formation and decomposition of the primary and secondary complexes of lactoperoxidase and organic peroxides. The velocity constant for the formation of the complex decreases somewhat as the size of the substituent on the peroxide molecule increases but not nearly so much as is found in the reaction of catalase and peroxides. The velocity constants for the decomposition of the complexes are unaffected by the type of substituent on the peroxide molecule.

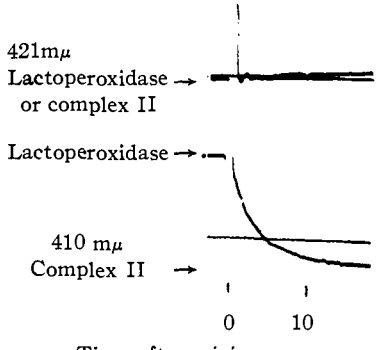

Fig. 3.—The kinetics of formation of complex II of lactoperoxidase and ethyl hydrogen peroxide: 1.33 cm. cuvettes, 0.14 μM. lactoperoxidase, 0.2 μM. ethyl hydrogen peroxide, *p*H 7.0, 0.01 *M* phosphate (expt. 278d).

From the kinetic data of Tables I, II and IV, the dissociation constants of the secondary complexes may be calculated from the ratio of k_3 to k_1, assuming that there is no reversible decomposition of the primary or secondary complexes into free enzyme and peroxide (see Eqn. 1). The actual dissociation constant is measured in Fig. 4, which shows a graph of the optical density of the lactoperoxidase-peroxide complex as a function of the initial peroxide concentration. These data so closely follow the straight line corresponding to a combination of all the peroxide with the enzyme that the data are unsuitable for an accurate calculation of the dissociation constant. However, at the 50% point, the free peroxide concentration is roughly 1×10^{-8} *M* hydrogen peroxide, 2×10^{-8} *M* methyl hydrogen peroxide, and 2×10^{-8} *M* ethyl hydrogen peroxide, while the kinetic values (k_3/k_1) are 3×10^{-9}, 5×10^{-9}, and 2×10^{-8} *M*, respectively. The discrepancies between the titration and kinetic data suggest that there is a reversible dissociation of the enzyme substrate complexes. However, dissociation constants calculated from the titration data involve the difference of two nearly equal quantities and are not suitable for a calculation of k_2.[12]

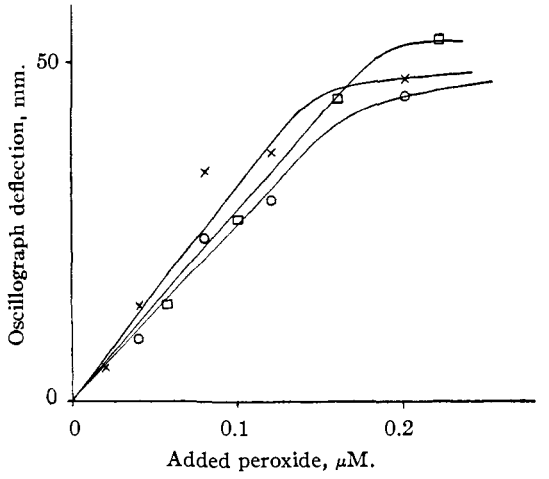

Fig. 4.—Titration of lactoperoxidase with hydrogen peroxide (×), methyl hydrogen peroxide (□) and ethyl hydrogen peroxide (○). The dissociation constants are roughly 1×10^{-8}, 2×10^{-8} and 2×10^{-8} *M*, respectively: 0.14 μM. lactoperoxidase, *p*H 7.0, 0.01 *M* phosphate, λ 410 mμ (expt. 278d).

Thus, in addition to spectral similarity, the kinetics and equilibria of the enzyme-substrate compounds of lacto- and horse-radish peroxidase are very similar in spite of the differences in the hemes and proteins.

The Reaction of Lactoperoxidase-Peroxides with Acceptors.—Again, in order to conserve enzyme, these reactions were carried out in an open cuvette. Whereas Theorell and Åkeson[3] found that this enzyme is quite unstable at high hydrogen peroxide concentrations, it is possible to make repeated activity determinations upon

TABLE V

The activity of lactoperoxidase and peroxides toward ascorbic acid·0.15 μM lactoperoxidase, 1.3 μM ascorbic acid, *p*H 7.0, λ = 410 mμ (Cuvettes, Expt. 278b).

Substrate	Ethyl hydrogen peroxide		Methyl hydrogen peroxide			Hydrogen peroxide		
Concentration, μM	4	8	2.6	5.2	10.0	2	4	8
Oscillograph deflection, mm. ($p = e$ = 31 mm.)	10	14	13	21	24	16	19	22
Half-time for decompn. of complex, sec.	36	45	11	18	26	4.2	6.6	11
k_3, sec.$^{-1}$	2.5	2.8	4.1	3.0	3.5	6.6	7.1	7.3
$k_4 = k_3/[\text{Asc}] - \text{M.}^{-1}\text{ sec.}^{-1} \times 10^{-3}$	1.9	2.2	3.1	2.3	2.7	5.1	5.5	5.6
Average k_4 M.$^{-1}$ sec.$^{-1}$	2100			2700			5400	

the same enzyme solution without loss of activity when using very dilute hydrogen peroxide.

Table V gives the results of a number of determinations of the activity of lactoperoxidase and peroxides with ascorbic acid. The method of measurement and the computation of the results is described in detail in references 12 and 13. Here the velocity constant increases somewhat on going from ethyl to methyl hydrogen peroxide and then increases considerably with hydrogen peroxide as a substrate. The actual values of the velocity constants are greater than those obtained with the horse-radish enzyme under identical conditions (see Table I in (14)).

Of great interest is the reaction of lactoperoxidase and hydrogen peroxide with pyrogallol. If this reaction is measured according to a modified procedure for P. Z. determination, Theorell and Åkeson found only one-fourteenth the activity of horse-radish peroxidase.[3] A truer picture of the relative activity of the milk and plant enzymes is given in Table VI where an extremely rapid reaction of pyrogallol and lactoperoxidase-hydrogen peroxide is indicated by the velocity constant $k_4 = 6.6 \times 10^6$ M.$^{-1}$ sec.$^{-1}$ more than ten times *greater* activity than that of horse-radish peroxidase-hydrogen peroxide and pyrogallol ($k_4 = 3 \times 10^5$ M.$^{-1}$ sec.$^{-1}$).[14]

TABLE VI

The activity of lactoperoxidase and hydrogen peroxide toward pyrogallol·0.14 μM lactoperoxidase, *p*H = 7.0, λ = 410 mμ (Cuvettes, Expt. 278 e).

Hydrogen peroxide concentration, μM	0.2	0.2	0.4
Pyrogallol concentration, μM	0	0.4	0.8
Oscillograph deflection, mm. ($p = e$ = 52 mm.)	52	17	17
Half-time for decomposition of complex, sec.	29	3.3	3.3
k_3, sec.$^{-1}$	0.05	1.3	2.7
$k_4 = k_3/[\text{pyrogallol}]$ M.$^{-1}$ × sec.$^{-1}$ × 10^{-6}	...	6.5	6.7
Average k_4 M^{-1} × sec.$^{-1}$		6.6×10^6	

In attempting to measure the velocity constant for the reaction of lactoperoxidase–hydrogen peroxide and hydroquinone, the saturation of the enzyme–substrate complex was too small for accurate measurements even when equal concentrations of substrate and acceptor were used. Thus the reaction velocity constant for the reaction of lactoperoxidase–hydrogen peroxide and hydroquinone is one of the same order of magnitude as that for the combination of lactoperoxidase and hydrogen peroxide (10^7 M.$^{-1}$ sec.$^{-1}$). An explanation of the discrepancy between these data and those of Theorell is given later.

The Effect of Acceptors upon the Transition from the Primary to the Secondary Lactoperoxidase–Peroxide Complexes.—The question of how the fairly slow transition from the primary to the secondary complex can be compatible with the high turnover number obtained during enzymatic activity is answered in Fig. 5 which shows the effect of acceptor upon the kinetics of the secondary complex as measured in the rapid-flow apparatus.[8] On the left, is shown the kinetics of formation of the secondary compound in the absence of acceptor. The observation tube is initially filled with the secondary complex. Rapid flow of the reactants gives the optical density of free peroxidase and, as the flow stops, the secondary complex forms with a velocity constant of 7 sec.$^{-1}$. In the right-hand record, ascorbic acid is present, and the observation tube is initially filled with free lactoperoxidase. On mixing lactoperoxidase with hydrogen peroxide and ascorbic acid, there is a very rapid reaction which is about half complete at the earliest time of measurement and, as

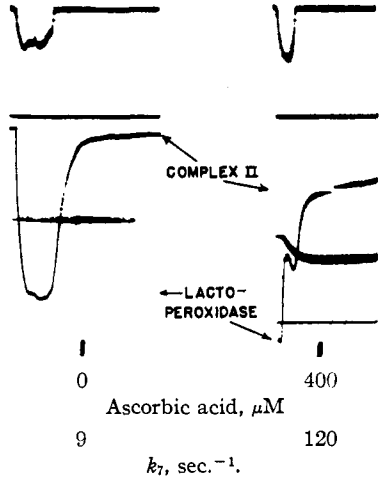

Fig. 5.—The effect of ascorbic acid upon the speed of transition from complex I to complex II in lactoperoxidase: 0.86 μM lactoperoxidase, 100 μM hydrogen peroxide, 430 mμ, *p*H 7.0, 0.01 *M* phosphate (expt. 286d).

TABLE VII

The effect of the acceptor upon the transition from the primary to the secondary complex of lactoperoxidase: 0.86 μM lactoperoxidase, *p*H = 7.0, λ = 430 mμ. (flow apparatus, Expt. 286 d).

Hydrogen peroxide concentration, μM	90	105	15 (from ascorbic acid)
Ascorbic acid concentration, μM	0	400	400
Oscillograph deflection (D_1), mm.	56	56	60
Oscillograph deflection (D_2) during flow, mm.	56	32	30
Flow velocity indication (f), mm.	..	18	15
Half-time for formation of complex ($t_{1/2}$), sec.	0.1	...	...
$k = 0.7/t_{1/2}$ or $k = 27 f \log D_1/D_2$ sec.$^{-1}$	7.0	120	120

(13) B. Chance, *J. Biol. Chem.*, **179**, 1341 (1949).

(14) B. Chance, *Arch Biochem.*, **24**, 410, (1949).

the flow stops, goes rapidly to completion. Here the velocity constant is 120 sec.$^{-1}$. Table VII clearly shows that this is a specific effect of the acceptor since the same velocity constant is obtained over a seven-fold range of the hydrogen peroxide concentration.

The Results

The spectrum of the hitherto unknown primary compound of lacto-peroxidase and hydrogen peroxide has been obtained in the region of the Soret band by means of the rapid-flow apparatus and bears a remarkable resemblance to the spectrum of the primary horse-radish peroxidase–peroxide complex. The change of extinction coefficient from lactoperoxidase to the primary complex at 413 mμ is 47 cm.$^{-1}$ × mM.$^{-1}$ and is about the same as the values found with horse-radish peroxidase and catalase on a hematin-iron basis.

The spectrum of the secondary lactoperoxidase–hydrogen peroxide complex can be obtained in the ordinary spectrophotometer and is very similar to that of the secondary horse-radish peroxidase–hydrogen peroxide complex and thereby strengthens the analogy between the complexes of lacto- and horse-radish peroxidase with peroxides.

As in the case of horse-radish peroxidase–peroxide complexes, there is only a remote analogy between the spectrum of the secondary lactoperoxidase peroxide complex and the secondary catalase–peroxide complex.

The primary and secondary compounds of lactoperoxidase with methyl or ethyl hydrogen peroxide have been studied kinetically, and the spectra of the secondary complexes are identical to that of the secondary hydrogen peroxide complex. The spectra of the primary complexes were not obtained owing to lack of material.

The formation of the three primary lactoperoxidase–peroxide complexes is very rapid, 2×10^7 M.$^{-1}$ × sec.$^{-1}$ for hydrogen peroxide, 6×10^6 M.$^{-1}$ sec.$^{-1}$ for methyl hydrogen peroxide, and 2×10^6 M.$^{-1}$ × sec.$^{-1}$ for ethyl hydrogen peroxide. Here there is a correlation between the size of substrate molecule and reaction velocity which was not found with horse-radish peroxidase. The magnitude of the effect here is much smaller than that found with catalase. However, it may be pointed out that the iron-porphyrin group of lactoperoxidase may not be exposed on the surface of the protein as may be the case with horse-radish peroxidase. The smaller accessibility of lactoperoxidase heme is also indicated by the fact that lactoperoxidase has not yet been successfully split (Theorell, personal communication) while horse-radish peroxidase is readily split.[15]

As suggested by Theorell as the cause of his failure to find the primary lactoperoxidase-hydrogen peroxide complex,[3] this complex is short-lived and rapidly changes into the secondary complex. In fact, in dilute lactoperoxidase solutions, this transition is half-complete in about 0.2 sec. (k = 4.0 sec.$^{-1}$). The transition is not, however, decelerated by repeated additions of peroxide as was found to be the case with horse-radish peroxidase. Thus it is unlikely that further purification or repeated additions of peroxide to lactoperoxidase will slow this transition and permit ordinary visual studies of the spectrum of the primary complex.

The three secondary lactoperoxidase–peroxide complexes decompose spontaneously into free peroxidase with a velocity constant of about 0.03 sec.$^{-1}$ as has been found true of horse-radish peroxidase and catalase peroxides.

The dissociation constants of the secondary lactoperoxidase hydrogen peroxide, and ethyl hydrogen peroxide complexes are found from titration data to be roughly 1×10^{-8} M, 2×10^{-8} M, 2×10^{-8} M and are calculated from kinetic data to be 3×10^{-9} M, 5×10^{-9} M, and 2×10^{-8} M, respectively. The values based upon the titration data must be regarded as maximum values and involve appreciable error; nevertheless, it is suggested that the primary or secondary complexes dissociate reversibly. There is an indication of a similar reversible dissociation in horse-radish peroxidase.[12]

The titration data show that these enzyme–substrate compounds consist of one molecule of peroxide per molecule of hematin.

Acceptors accelerate the transition of the primary to the secondary complexes of lactoperoxidase just as was found with horse-radish peroxidase. Thus both primary and secondary complexes are essential to the enzymatic activity. The rate-determining step in the enzymatic activity of lactoperoxidase is usually the reaction of the secondary complex with the acceptor.

The kinetics of the secondary lactoperoxidase–peroxide complex fulfill one of the criteria previously applied to the horse-radish peroxidase–peroxide complex[16] for a Michaelis enzyme–substrate complex: the velocity constant for the reaction with the acceptor can be accurately computed from the equation, $k_4 a_0 = x_0/p_{max.} t_{1/2}$,[12,13,16] based upon solutions of the theory of Michaelis and Menten.

The reaction velocity of the secondary lactoperoxidase–peroxide complexes with ascorbic acid is somewhat greater than that of the horse-radish peroxidase–peroxide complexes with hydrogen peroxide, methyl hydrogen peroxide, and ethyl hydrogen peroxide. Values of 5400, 2700, and 2100 M.$^{-1}$ and sec.$^{-1}$ are obtained with lactoperoxidase, and values of 2800, 2800, and 2200 M.$^{-1}$ × sec.$^{-1}$,[17] respectively, are obtained with horse-radish peroxidase in both cases at pH 7.0.

The oxidation of ferrocytochrome-c by lactoperoxidase–peroxides proceeds at about the same speed obtained with horse-radish peroxidase at pH = 7.0.[18]

(15) H. Theorell, *Arkiv. Kemi Mineral. Geol.*, **B14**, No. 20 (1940).

(16) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943).

(17) B. Chance, *Arch. Biochem.*, **24**, 389 (1949).

(18) B. Chance, *Science*, **109**, 204 (1949).

With pyrogallol, the reaction velocity constant for lactoperoxidase–hydrogen peroxide II is 7 $\times$ 10^6 $\times$ sec.$^{-1}$—17 times greater than the value obtained with horse-radish peroxidase–hydrogen peroxide at *p*H 7.0.[14] The velocity of combination of the enzyme-substrate compound with the acceptor (Eqn. 3) approaches the velocity of combination of enzyme with substrate (Eqn. 1)—a very interesting situation considering the differences in the size of the substrate and acceptor molecules and the quite different reactions that must occur in the two combinations.

The low P. Z. of lactoperoxidase[3] can now be explained. The velocity constant for the reaction of pyrogallol with lactoperoxidase–hydrogen peroxide is two-thirds that of the reaction of hydrogen peroxide with lactoperoxidase. Thus a ratio of 1.5:1 of acceptor to peroxide concentration means equal rates of substrate and acceptor reaction and will give half saturation of the enzyme-substrate complex—and half the maximum rate of over-all reaction (purpurogallin production or peroxide disappearance). But in the P. Z. test, 20 mM. pyrogallol and 0.74 mM. hydrogen peroxide are used. Under these conditions, the saturation of lactoperoxidase with hydrogen peroxide is only about 0.09 because its Michaelis constant is about 7 mM. ($7 \times 10^6 \times 0.02/2 \times 10^7$). Horse-radish peroxidase, on the other hand, is about 0.8 saturated under these conditions.

In order to compare the two enzymes at equal saturation with peroxide, the P. Z. value for lactoperoxidase should be increased by the ratio 0.8/0.09 to give $71.5 \times (0.8/0.09) = 640$.

Theorell has shown that lactoperoxidase is rapidly inactivated by large hydrogen peroxide concentrations and for this reason reduced the hydrogen peroxide concentration in his test to 0.176 mM.[3] That considerable inactivation of lactoperoxidase still occurs in this modified test is indicated by the fact that the corrected P. Z. of lactoperoxidase (640) is less than that of horse-radish peroxidase (900) whereas their k_4 values are 70×10^5 and 3×10^5 M.$^{-1}$ $\times$ sec.$^{-1}$, respectively.[14]

It is suggested that the conditions of the activity test for lactoperoxidase be revised to be more suitable to the nature of the enzyme. The amount of pyrogallol must be reduced to the neighborhood of the peroxide concentration which in turn should be as low as possible. A more suitable method is the spectrophotometric determination of ascorbic acid disappearance or tetraguaiacol production.[3,14]

In general, there are surprisingly small differences in the reaction-velocity constants that characterize the activity of horse-radish and lactoperoxidase when the great differences in their protein components (the molecular weights are 44,000[10] and 93,000[6], respectively), and their iron-porphyrin groups (the prosthetic groups are protohemin[10] and a green hemin of unknown constitution[3] respectively) are taken into consideration. The general theory that activity is determined by heme-linked groups[19] may serve to explain the similarity between these two enzymes. The iron atoms of these two enzymes do contain a heme-linked hydroxyl group.[4] Further similarities in the heme-linkages are not known and require investigation.

Summary

It is now clear that lactoperoxidase forms the same series of enzyme-substrate compounds as horse-radish peroxidase; a primary lactoperoxidase–hydrogen peroxide complex is here shown to be a precursor of the reddish secondary complex discovered by Theorell and Åkeson.[3] This primary complex has properties in common with those of catalase and horse-radish peroxidase: a Soret band which is lower than but very slightly shifted with respect to the Soret band of the free enzyme. In addition, kinetic evidence indicates that lactoperoxidase forms analogous primary and secondary compounds with methyl or ethyl hydrogen peroxide. The velocity constants for the formation of these primary complexes have been measured and are found to be larger than the corresponding values for horse-radish peroxidase. As in the case of horse-radish peroxidase, the transition from the primary to the secondary complex is greatly accelerated by the addition of an acceptor. And the transition to the secondary complex must occur before the free enzyme is obtained. Thus both the primary and secondary complexes are involved in the enzymatic activity. Under most conditions of enzymatic activity, the concentration of the primary complex is negligible and the secondary complex is the Michaelis enzyme-substrate compound in lactoperoxidase activity, in accord with studies of the primary and secondary enzyme-substrate compound of horse-radish peroxidase and peroxides. Thus the mechanism of action of lactoperoxidase appears to be identical to that of horse-radish peroxidase. However, the velocity constants for the combination of lactoperoxidase with peroxides and the reaction of the enzyme-substrate complexes with acceptors are significantly higher than the corresponding velocity constants for horse-radish peroxidase; lactoperoxidase is a more active enzyme than horse-radish peroxidase.

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(19) H. Theorell, in "Advances in Enzymology," **7**, 265 (1947).

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