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Oxygenation of Polyunsaturated Fatty Acids during Prostaglandin Biosynthesis by Sheep Vesicular Gland†

William L. Smith and William E. M. Lands*

ABSTRACT: We found that two types of fatty acid dioxygenase activity are present in acetone powder preparations of sheep vesicular gland. One activity (E_a) when stimulated by phenol may be suppressed by the functioning of glutathione peroxidase. Therefore, E_a seems to require hydroperoxide as an obligatory intermediate. The oxygenation of fatty acids catalyzed by freshly prepared homogenates of sheep vesicular gland was also inhibited by glutathione peroxidase in the presence of glutathione. The slower oxygenase activity (E_b) found in untreated vesicular gland acetone powder preparation was not affected by glutathione peroxidase and therefore did not seem to require hydroperoxide in its mechanism of action. E_b activity was inactivated in the presence of both substrates, fatty acid and oxygen, by a process which appeared to be first order with respect of enzyme concentration. E_a was inactivated by a kinetically similar process, and in addition also lost activity at a significant rate in the presence of hydroperoxide alone, apparently by a substrate-independent process. The kinetic formulation of E_a action resembles that for soybean lipoxygenase in being product activated and self-destructive.

The oxidative cyclization of certain polyunsaturated acids to form prostaglandins involves a series of reactions (Bergström, 1967; Samuelsson, 1967, 1969; Nugteren *et al.*, 1966). Two principal features indicated by the work of Samuelsson and coworkers are the initial attack by a dioxygenase (Samuelsson, 1965; Hamberg and Samuelsson, 1967a) and the existence of a cyclic endoperoxide intermediate (Samuel-

† From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104. *Received March 6, 1972.* This work was supported in part by a grant (GB-8243) from the National Science Foundation and a U. S. Public Health Service Health Science Predoctoral Traineeship (W. L. S.).

sson, 1967; Hamberg and Samuelsson, 1967a, Granström *et al.*, 1968). Some reports describe optimal conditions for producing either type E (Jouvenaz *et al.*, 1970; Yoshimoto *et al.*, 1970; Takeguchi *et al.*, 1971) or F (Samuelsson, 1969; Lee and Lands, 1971) or simply total prostaglandin derivatives (Lands *et al.*, 1971). The glutathione, dithiol, and antioxidant cofactors used were, in general, regarded to serve in converting the endoperoxide to one or the other products and not particularly in influencing the total amount of substrate acid oxidized by the initial dioxygenase activity. Stimulating the dioxygenase would however increase the overall production of hormonal intermediates without necessarily influencing the ratio of the hormones produced. Since the vesicular gland dioxygenase plays such an important role in initiating the process of prostaglandin formation, we examined its mechanism in more detail. When attempting to trap the presumed hydroperoxide product from the first oxidation step with the aid of glutathione peroxidase, we found that the vesicular gland dioxygenase activity was inhibited (Lands *et al.*, 1971). Further studies indicated a variety of unexpected properties for this oxidative activity. The present report describes the results which lead us to regard the major initial step in prostaglandin formation to be an activatable, product-dependent, self-destructive enzymic process.

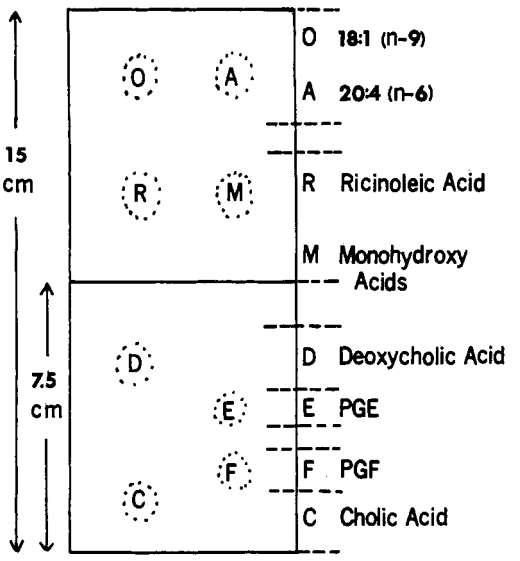

FIGURE 1: Thin-layer chromatographic system to separate products for the radioisotope assay of prostaglandin synthesis. Horizontal dotted lines indicate sections which were scraped into vials and counted.

Experimental Section

Materials

All solvents were reagent grade and were used without further purification. Silica gel G was obtained from Brinkmann Industries. [1-^{14}C]5,8,11,14-Eicosatetraenoic (52 Ci/mole) and [5,6,8,9,11,12,14,15-^{3}H]5,8,11,14-eicosatetraenoic (5 Ci/mmole) acids were purchased from Applied Science Laboratories and New England Nuclear Corp., respectively; unlabeled 5,8,11,14-eicosatetraenoic acid (high purity grade) was obtained from the Hormel Institute and Nuchek Preps; 8,11,14-eicosatrienoic acid was a gift from the Upjohn Co. The fatty acids were dissolved in benzene containing 0.03% (w/w) Santoquin (a gift of the Monsanto Co.) and stored at −20°.

Sheep vesicular glands, and an acetone powder preparation of vesicular gland, PGE_1 and $PGF_{1\alpha}$ were donated by the Upjohn Co. Reduced glutathione (GSH) was purchased from Sigma Chemical Co. All other chemicals were reagent grade obtained from common commercial sources.

Methods

Determination of Dioxygenase Activity by Radioisotope Assays. In the radioisotope procedure for measuring vesicular gland dioxygenase activity, reactions were initiated by addition of 0.05–0.2 ml of the enzyme preparations (1–5 mg of protein) to incubation mixtures containing [1-^{14}C]5,8,11,14-eicosatetraenoic acid in 0.1 M Tris·HCl (pH 7.4). The final volume of all incubation mixtures was 1.0 ml. Assays were performed at room temperature with occasional shaking. The incubations were stopped by the addition of 7.0 ml of chloroform–methanol (1:1). The suspension was centrifuged to remove the precipitated protein, and the supernatant was poured into tubes containing 3.0 ml of chloroform and 1.6 ml of 0.88% formic acid (v/v) and mixed. The resultant lower phase was removed and evaporated to dryness with the aid of a stream of nitrogen and the residue redissolved in a small volume of chloroform and then quantitatively transferred to a silica gel G thin-layer plate. Thin layer plates were developed to 15 cm in benzene–dioxane–acetic acid–formic acid (82:14:1:1), air-dried for 15 min, and then developed to 7.5 cm with acetone–methylene chloride (60:40). Oleic, ricinoleic (12-hydroxyoleic), cholic, and deoxycholic acids were routinely used as reference compounds. As shown in Figure 1, deoxycholic and cholic acids chromatographed immediately above PGE and below PGF, respectively. The standards were visualized with the aid of iodine vapor, and the plate was sectioned into regions corresponding to free fatty acid, hydroxy acids, PGE and PGF with appropriate unoccupied intermediate bands. After spontaneous loss of the adsorbed iodine, the sections were scraped into scintillation vials, suspended in 6 ml of toluene scintillation fluid (Arnold, 1963) containing 0.2 volume of isoamyl alcohol and assayed for radioactivity in a Packard series 2000 scintillation spectrometer. The amounts of radioactive material produced were estimated after subtracting the experimental results from the values obtained in control incubations without enzyme.

Preparation of Vesicular Gland Dioxygenase. Two types of preparation from sheep vesicular gland were used: A 3000*g*-min supernatant was prepared by homogenizing the vesicular gland in 1–3 volumes of 0.1 M Tris·HCl (pH 7.4) at 0–4°, in a Dounce ball-type homogenizer using a loose pestle. The pH was readjusted to 7.4 with a solution of 1% Tris. The homogenate was centrifuged at 600*g* for 5 min to yield the 3000*g*-min supernatant. An acetone powder was prepared as described elsewhere (Wallach and Daniels, 1971) and was routinely suspended by homogenization in 0.1 M Tris·HCl (pH 8.5) to give a concentration of 20–50 mg of powder/ml of buffer. Protein concentrations were estimated using a nomograph based on the extinction coefficients for enolase and nucleic acid given by Warburg and Christian (1942) using protein solubilized with 0.5% deoxycholate. The average of at least two spectrophotometric readings were used.

Determination of Vesicular Gland Dioxygenase Activity by Oxygen Absorption Measurements. All oxygen absorption measurements were performed on a Yellow Springs Instrument Co. Model 53 oxygen monitor at a constant temperature of 29.5 ± 0.5°. In most experiments, the total final volume of the reaction mixture in the sample chamber was 3.0 ml. The

TABLE I: Effect of GSH on the GSH Peroxidase Inhibition of the Oxygenation of 5,8,11,14-Eicosatetraenoic Acid by Vesicular Gland Homogenates.[a]

	Zero-Time Control	1	2	3	4	5	6
GSH peroxidase (units)		0	0	12	60	180	180
[GSH] (mM)		0.65	0	0.65	0.65	0.65	0
^{14}C counts per minute in free fatty acid fraction	7540	5704	5295	5585	5984	6753	5892
Per cent reaction	0	20.3	21.5	16.1	13.6	8.1	18.2

[a] Reactions of this experiment were initiated by the addition of 2.5 mg of protein from a sheep vesicular gland 3000*g*-min supernatant prepared as described in Methods in 0.1 M Tris·HCl (pH 7.4). The incubation mixtures contained [1-^{14}C]5,8,11,14-eicosatetraenoic acid and the indicated quantities of GSH and GSH peroxidase. After 6-min incubation time at room temperature, the reactions were stopped with $CHCl_3$–MeOH (1:1), lipids were extracted, and thin-layer chromatography was performed as described in Methods. The per cent reaction is based on the decrease in the percentage of total counts in the free fatty acid region for each experimental sample compared to that for the zero-time control.

maximum volume of all additions made through the side of the electrode holder during the assay was 0.25 ml (less than 10% of the total sample volume).

To determine if the oxygen uptake assayed on the monitor was a valid measure of fatty acid oxygenation an experiment was performed using [5,6,8,9,11,12,14,15-^3H]5,8,11,14-eicosatetraenoic acid as substrate. The substrate purity and O_2 electrode response were normalized by observing the maximum change attained when an equivalent amount of substrate was treated with excess soybean lipoxygenase. Some radioisotope (22%) still chromatographed in the substrate region after all lipoxygenase-catalyzed oxidation had ceased. Subsequent calculations of radioactive substrate converted were made with a correction for the nonmetabolized portion of the labeled substrate and were based on the fact that lipoxygenase consumes 1 mole of O_2/mole of fatty acid oxygenated. Figure 2 shows the correspondence between the radioisotope assay and oxygen electrode assay with two preparations of the acetone powder that had been treated with phenol and one of the untreated enzyme. The vesicular gland preparations consumed 2 moles of O_2/mole of product formed.

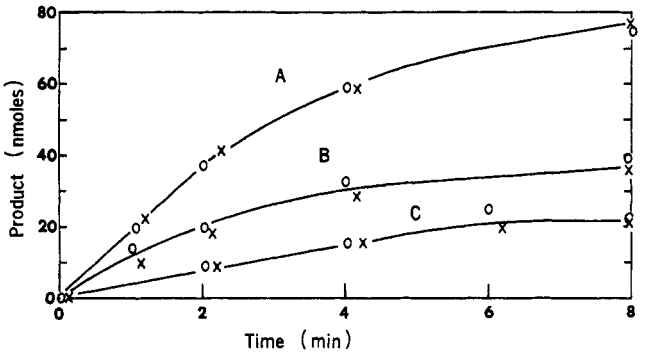

FIGURE 2: Comparison of the oxygen absorption and radioisotope assays for prostaglandin synthesis. All reactions were performed in 0.1 M Tris·HCl (pH 8.5) containing 3.3 μmoles of GSH in a final total volume of 3.5 ml. Reactions were initiated by the addition of 142 nmoles of [^3H]20:4 (n − 6) to the reaction mixture containing the acetone powder. At the indicated times the electrode was withdrawn from the sample and a 1.0-ml aliquot added to 7.0 ml of $CHCl_3$–MeOH (1:1). Product formation was measured directly by the radioisotope assay outlined in Methods. Per cent reaction for the O_2 electrode measurements was calculated as described in Methods. Per cent reactions for the radioisotope assays were obtained by calculating the per cent of counts in the free fatty acid region of the thin-layer chromatography plate in the zero-time control which subsequently chromatographed with lower R_F values after incubations were performed. The two calculated percentage values were multiplied by 142 nmoles of 20:4 n − 6 present initially to give the nmoles of product formed. Curve: (A) 4 mg of phenol-activated acetone powder, 2 μmoles of phenol; (B) 2 mg of phenol-activated acetone powder, 2 μmoles of phenol; and (C) 4 mg of untreated acetone powder, no phenol. Product formation calculated from fatty acid consumption (X) and one-half of the oxygen uptake (O).

Purification and Assay of GSH Peroxidase. GSH peroxidase was partially purified from rat liver by the procedure of O'Brien and Little (1969) and assayed by a modification of the method of Jocelyn (1962) as previously described (Smith and Lands, 1972).

Results

GSH Peroxidase Inhibition. Rat liver GSH peroxidase catalyzes the nucleophilic cleavage of a wide variety of hydroperoxides with a variety of mercaptans (Little and O'Brien, 1968). We previously demonstrated that GSH peroxidase in the presence of GSH inhibits the oxygenation of 5,8,11,14-eicosatetraenoate by sheep vesicular gland homogenates (Lands *et al.*, 1971). The results for tube 6 in Table I show that the inhibition does not occur without the concomitant presence of GSH in the reaction mixture. Addition of *N*-ethylmaleimide (and therefore removal of mercaptan from the GSH peroxidase inhibited reaction mixture; see Figure 3) reversed the partial inhibition by GSH peroxidase of the oxygenation of 5,8,11,14-eicosatetraenoic acid by the supernatant preparation. The control systems without added GSH peroxidase also produced somewhat higher amounts of prostaglandin after *N*-ethylmaleimide treatment. However, this effect was small and might be due to some endogenous peroxidase activity in the supernatant fraction of the vesicular gland homogenate.

In contrast to the results with fresh homogenates, no inhibition of the oxygenation of fatty acid catalyzed by the untreated acetone powder preparations was observed with several levels of added GSH peroxidase up to 25,000 units/ml. The experiments paralleled those described in Table II

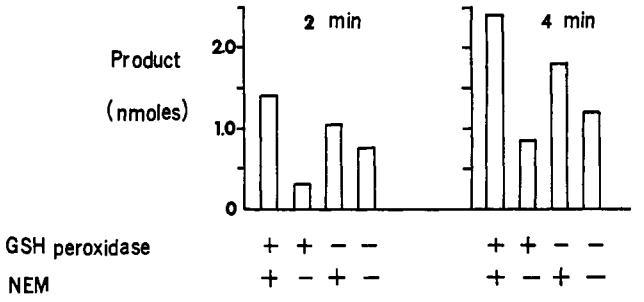

FIGURE 3: The effect of *N*-ethylmaleimide on the GSH peroxidase inhibition of the oxygenation of 5,8,11,14-eicosatetraenoic acid by homogenates. A 3000*g*-min supernatant (100 mg of protein) was allowed to stand for 3 min at room temperature with 1600 nmoles of GSH and 2500 units of GSH peroxidase in a final volume of 2.65 ml of 0.1 M Tris·HCl (pH 7.4). Then 1.0 ml was transferred to 0.25 ml of 10 mM *N*-ethylmaleimide and another 1.0-ml aliquot to 0.25 ml of 0.1 M Tris·HCl (pH 7.4) and both solutions were allowed to stand 3 min at room temperature. Two additional solutions were prepared in an identical manner except GSH peroxidase was omitted. After the preincubations, 0.4-ml aliquots of the protein solutions were added to 0.6 ml of the substrate solutions as indicated in the figure. Substrate solutions contained 650 nmoles of GSH, 10 nmoles of [^{14}C]20:4 ($n-6$) and either 0 or 1500 units of GSH peroxidase in a final volume of 0.4 ml of 0.1 M Tris·HCl (pH 7.4). After allowing each solution to stand 3 min at room temperature, 0.2 ml of 10 mM *N*-ethylmaleimide was added to the mixtures indicated and 0.2 ml of 0.1 M Tris·HCl (pH 7.4) was added to the remainder. The solutions were then allowed to stand 3 min at room temperature before the enzyme was added to initiate the oxygenation reactions.

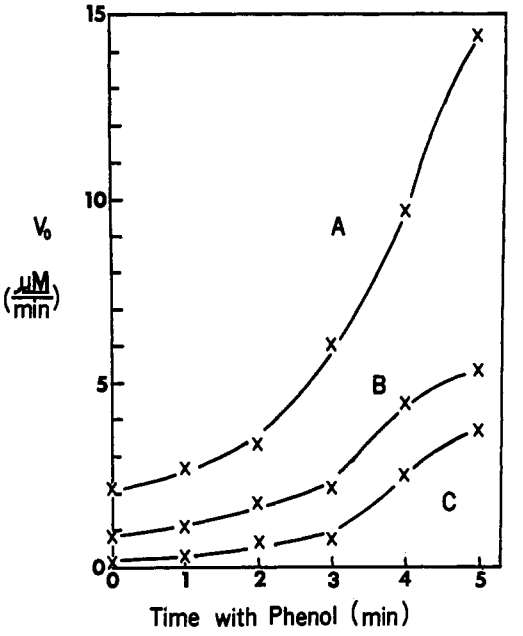

FIGURE 4: The effect of protein concentration on the rate of phenol activation. All rates were obtained by measurements of oxygen uptake as outlined in Methods. The acetone powder was incubated in 3.0 ml of 0.1 M Tris·HCl (pH 8.5) for a total of five min in the reaction chamber at 30° before addition of 5,8,11,14-eicosatetraenoic acid (190 nmoles) to initiate the reaction. Phenol (2 μmoles) addition preceded the substrate sample by the times indicated. The rates of oxygenation by controls at equivalent concentrations after 5-min preincubations without phenol are subtracted from all rates. Protein concentrations: (A) 1.33 mg/ml, (B) 0.66 mg/ml, and (C) 0.33 mg/ml.

but contained 5 mg of the acetone powder preparation and gave a velocity of 2.7 ● 0.3 μM per min for all levels of GSH peroxidase tested. Thus, the sensitivity to GSH peroxidase inhibition as well as much of the oxygenation activity appeared to be lost during preparation of the acetone powder from homogenates. We found, however, that the more active phenol-treated acetone powder (unlike the untreated powder) showed an inhibition by GSH peroxidase (Table II) and was thus more similar to the native 3000*g*-min supernatant from vesicular gland. The above results led us to study in more detail the nature of the phenol activation process and the kinetic properties of the phenol-treated enzyme preparation.

Phenol Activation. Figure 4 shows a sigmoidal increase in the observed oxygenation rate when acetone powder preparations were preincubated for different times with 0.66 mM phenol before addition of fatty acid substrate to the reaction chamber. The time required to reach half-maximal activity (3.5 min) was similar over the fourfold range of protein concentration examined. The activation by phenol appeared to be nearly complete after 5 min in this system. A phenol concentration of 0.66 mM was chosen for the activation experiments since we observed that the rate of oxygenation in the presence of phenol (without prior incubation of the powder with phenol) was maximal at this concentration (Figure 5). If the acetone powder was preincubated with phenol to achieve maximal activation and the phenol concentration then lowered by dilution, the activity of the enzyme rapidly fell to its original level (Figure 6). The deactivated enzyme could not then be completely reactivated by further treatment with phenol as indicated by the B′ curve in Figure 6.

Self-Catalyzed Destruction. When 5,8,11,14-eicosatetraenoic acid was combined with untreated acetone powder in the absence of phenol, the reaction proceeded, but the velocity decreased to zero before all of the fatty acid was oxidized. When more acetone powder was then added to the reac-

TABLE II: Effect of GSH Peroxidase on the Oxygenation of 5,8,11,14-Eicosatetraenoic Acid by Phenol-Treated Acetone Powder.[a]

Sample	GSH Peroxidase (Units)	Phenol-Treated Vesicular Gland Powder (mg)	Velocity (μM 20:4/min)	% Inhibn
1	0	0.5	2.9	0
2	6,300	0.5	1.45	50
3	0	1.0	4.5	0
4	6,300	1.0	2.6	42
5	0	2.0	7.6	0
6	6,300	2.0	5.2	32
7	0	2.0	7.1	0
8	12,500	2.0	4.4	38

[a] Oxygen uptake was measured as described in Methods. The acetone powder (20 mg) was homogenized in 1.0 ml of 0.1 M Tris·HCl (pH 8.5) containing 0.66 μmole of phenol. The homogenate was allowed to stand at room temperature for 30 min to allow complete activation and then placed in ice. Aliquots of the cooled homogenate were added to solutions containing 2 μmoles of phenol, 3.3 μmoles of GSH, 190 mmoles of 5,8,11,14-eicosatetraenoic acid, and the indicated units of GSH peroxidase to yield a total volume of 3.0 ml of 0.1 M Tris·HCl (pH 8.5). The GSH peroxidase preparation used in this experiment was also assayed at pH 8.5.

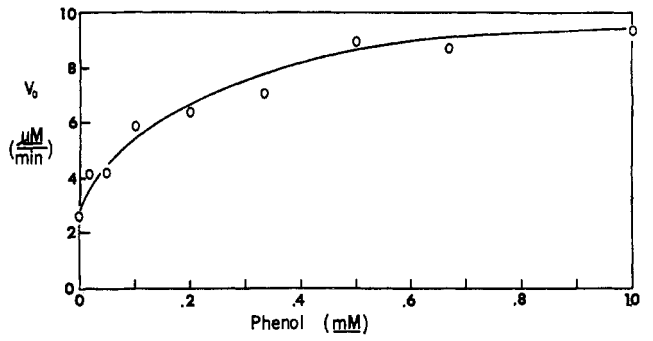

FIGURE 5: The effect of the phenol concentration on the rate of oxygenation. All rates were obtained by measurements of oxygen uptake as outlined in Methods. Suspensions of acetone powder (2.5 mg) in buffer were added to the reaction chamber containing 5,8,11,14-eicosatetraenoic acid (190 μmoles) and the indicated phenol concentration to initiate oxygen uptake.

tion mixture, the amount of product formed (at saturating substrate levels) was directly proportional to the amount of fresh enzyme added. When the instantaneous velocities for many time points were plotted against the concentration of substrate acid present at that time, the decrease in velocity with decreasing fatty acid substrate concentrations was almost linear except at velocities below 1 μM/min (Figure 7). The curvature at very low rates may be due to an inadequate correction for the slow basal drift in the O_2 sensor system. Experiments with varying initial substrate concentrations generated a series of parallel lines. The average slope of these parallel lines was 0.25 min^{-1} with 5,8,11,14-eicosatetraenoic acid as substrate. This enzyme preparation also gave similar values for the slopes when GSH and GSH peroxidase were included in reaction mixtures to remove newly formed hydroperoxides indicating that neither the initial velocities nor the rate of self-destruction was influenced by the added peroxidase system.

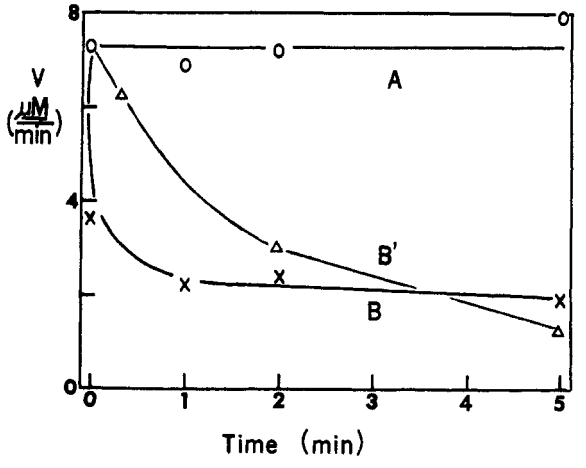

FIGURE 6: Deactivation of the phenol-activated preparation. All rates were obtained by measurements of oxygen absorption as outlined in Methods. The acetone powder was treated with 0.66 mM phenol for 30 min at room temperature and then kept at 0–4° before use. Aliquots of the activated enzyme were added to 3.0 ml of 0.1 M Tris·HCl (pH 8.5) containing (A) 0.66 mM phenol or (B) no additional phenol. *B'* values were obtained by readjusting the phenol concentration to 0.66 mM after the indicated time and allowing the system to stand an additional 5 min, and then initiating the reaction. Eicosatetraenoic acid (60 μM, final concentration) was added to initiate the reactions.

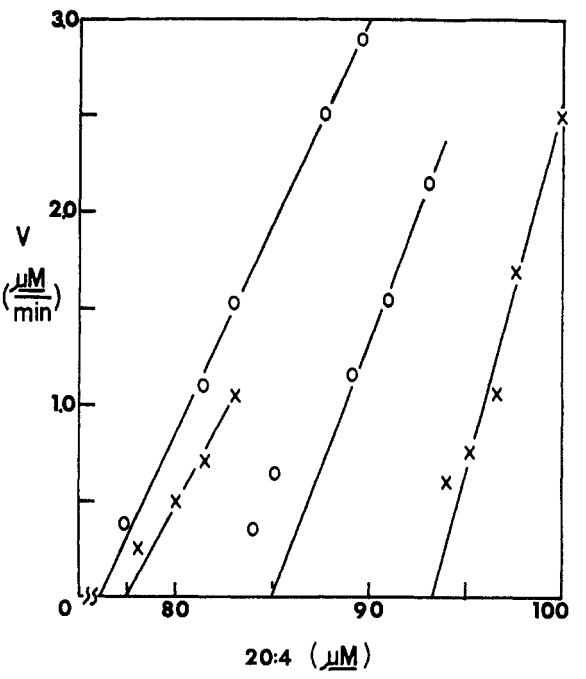

FIGURE 7: Velocity *vs.* substrate concentration for the oxygenation of 5,8,11,14-eicosatetraenoic acid. The instantaneous rate of substrate consumption by untreated acetone powder preparations at various concentrations of 5,8,11,14-eicosatetraenoic acid was determined from continuous oxygen-uptake tracings as described in Methods. Similar amounts of enzyme were used in each incubation but one preparation was less active. Final volume in all samples was 3.0 ml.

The phenol-treated powder was also inactivated during the oxygenation process by a kinetically similar process. This is shown with 8,11,14-eicosatrienoic acid (Figure 8, I) and with 5,8,11,14-eicosatetraenoic acid (Figure 8, II). The average of the slopes for the velocity versus substrate concentration plots for the phenol-activated acetone powder is 1.0 min^{-1} with the 20.3 acid as substrate and 0.55 min^{-1} with the 20:4 substrate. Added glutathione did not significantly alter the rate of loss of activity.

Phenol caused a stimulation in oxygenation rate when added during the first 3-min reaction in a mixture containing untreated acetone powder and 5,8,11,14-eicosatetraenoic acid (Figure 9A). However, no stimulation was observed upon phenol addition after 3 min even though the observed rate of oxygenation of fatty acid had not yet dropped to zero. The first-order rate constant ($k\Delta$) for the loss in phenol-stimulable activity was approximately 1.1 min^{-1} (Figure 9B). This was about four times greater than that for the loss of basal dioxygenase activity (0.25 min^{-1}). The results supported the concept that two types of fatty acid dioxygenase activity were present in the acetone powder preparation and that the latent activity (which is stimulable by phenol) was also rapidly inactivated during the oxygenation catalyzed by the untreated powder in the absence of phenol.

Two types of inactivating processes were recognized in our study of soybean lipoxygenase (Smith and Lands, 1971). One was due directly to lipid hydroperoxide (and occurred in the presence or absence of substrate) and the other to a self-catalyzed destruction (occurring only in the presence of substrate). We reasoned that if hydroperoxide could be continually removed during the oxygenation of fatty acid by untreated vesicular gland powder preparations, we might be

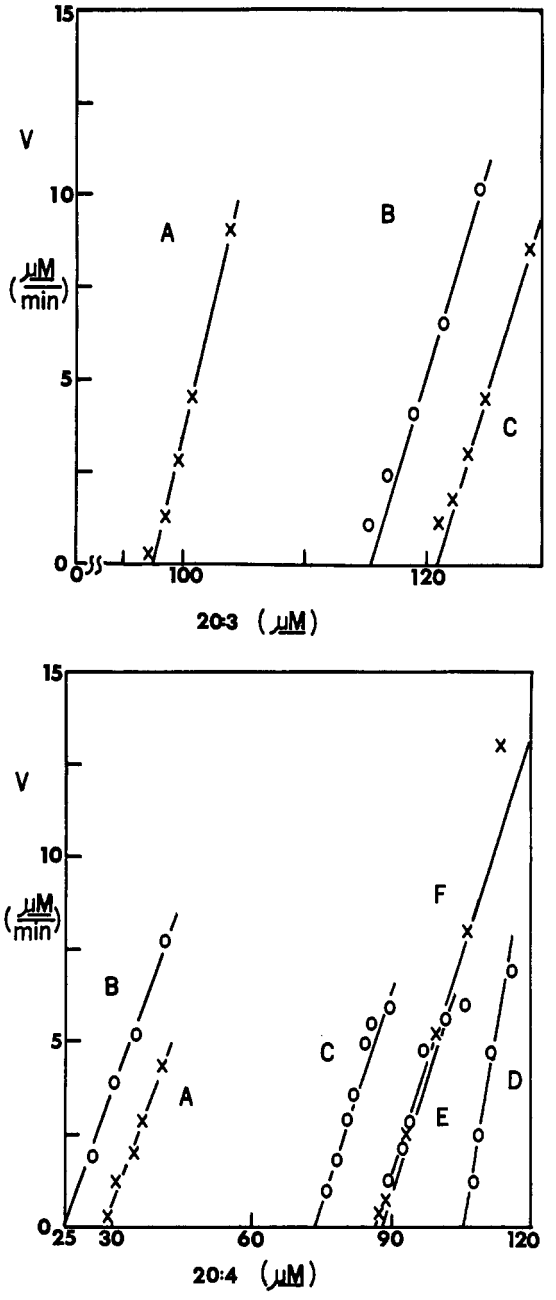

FIGURE 8: Velocity *vs.* substrate concentration for oxygenation by phenol-treated preparations of 8,11,14-eicosatrienoic acid (I). The instantaneous rate at various concentrations of 8,11,14-eicosatrienoic acid was determined from continuous oxygen uptake tracings. Reactions were initiated by the addition of acid to samples containing acetone powder which had been fully activated with 0.66 mM phenol. Initial protein concentrations were: (A) 0.66 mg/ml, (B) 1.33 mg/ml, and (C) 0.66 mg/ml. 5,8,11,14-Eicosatetraenoic acid (II) in the presence and absence of GSH. Initial concentrations of acetone powder and glutathione are: (A) 0.71 mg/ml, 0.93 mM GSH; (B) 1.4 mg/ml, 0.93 mM GSH; (C) 0.83 mg/ml; (D) 0.83 mg/ml; (E) 0.83 mg/ml; (F) 1.66 mg/ml. All reaction mixtures contained 0.66 mM phenol.

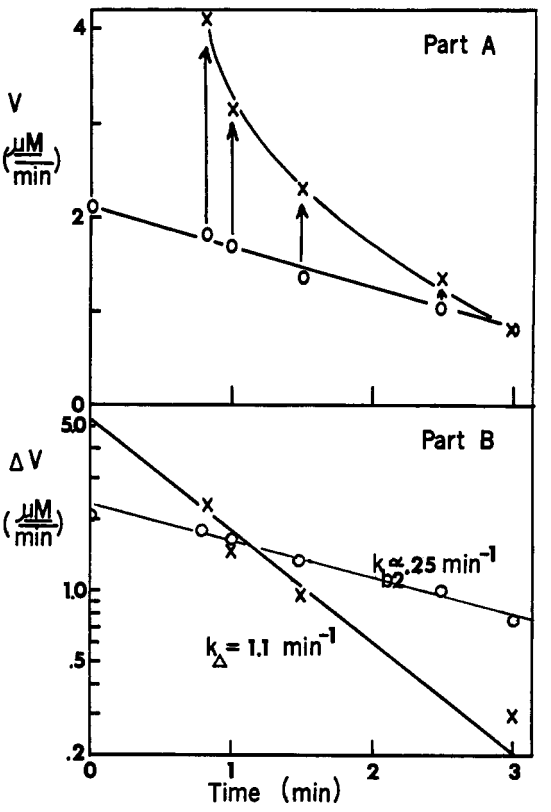

FIGURE 9: The loss of latent phenol-stimulatable activity during the oxygenation of 5,8,11,14-eicosatetraenoic acid by acetone powder preparations. Part A: oxygen absorption measurements were performed as described in Methods. The final volume of all solutions was 3.0 ml of 0.1 M Tris·HCl (pH 8.5). Acetone powder (80 μl, 4 mg) was added to the sample chamber and 2 min after addition of the untreated powder, 5,8,11,14-eicosatetraenoic acid was added to yield a final concentration of 60 μM. At 0.8, 1.0, 1.5, 2.5, and 3.0 min after this substrate addition, the solution was made 0.66 mM in phenol and the velocity was determined (upper curve, X). The lower curve (O) shows the instantaneous velocity of fatty acid substrate utilization just prior to the time of phenol addition. Part B: the log of the difference between the velocities from the two curves of part A (*i.e.*, the stimulatable activity) is plotted as a function of time (X). In addition, the observed velocities without added phenol (O) are replotted to estimate k_{b2}.

able to prevent the loss of the latent phenol-stimulable activity even though the basal dioxygenase activity was being inactivated by a self-destruction process (such as seen in Figure 7). Therefore the untreated vesicular gland powder was incubated with GSH, GSH peroxidase, and 5,8,11,14-eicosatetraenoic acid for various times (4–18 min), the reaction mixture was treated with phenol for 5 min and then *N*-ethylmaleimide was added to remove the GSH. Figure 10 shows the type of observation made in these experiments. The initial (basal, E_b) rate of reaction was not influenced by the peroxidase system and decreased in accord with the expected self-destruction constant (0.25 min^{-1}). After phenol was added, latent E_a was presumably converted to the activated form, E_a, which is not capable of sustained reaction in the presence of the GSH peroxidase system. When *N*-ethylmaleimide was then added to remove the glutathione, the oxygenation reaction was rapidly reestablished. Table III shows the rate of oxygenation initially and at the time of phenol addition as well as the rate observed after *N*-ethylmaleimide addition. After 18 min of reaction in the presence of either 1330 or 6750 units of GSH peroxidase, the rate of oxygenation of fatty acid by untreated acetone powder had dropped to zero; however, some phenol-stimulable activity still remained in the sample containing the higher level of peroxidase. The remaining phenol-stimulable activity shown in Table III confirms that, in the presence of GSH peroxidase, the latent phenol-stimulable activity was somewhat more stable than the basal activity which dropped so rapidly.

TABLE III: Protection of Latent Phenol-Sensitive Dioxygenase in the Presence of GSH Peroxidase.[a]

Expt	GSH Peroxidase (Units)	Velocity (μM 20:4/min)			% Remaining Activity	
		Initial	At Time of Phenol Addition	After NEM	Phenol Unactivatied	Phenol Sensitive
1	1,330	2.5	0 (18)	0	0	0
2	6,750	3.1	0 (18)	2.2	0	16
3	12,500	2.9	0.38 (8)	7.8	13	57
4	12,500	2.9	1.7 (4)	10	58	73

[a] All experiments were performed as described in the legend to Figure 11. The percentage of remaining phenol-insensitive activity was estimated from the ratio of the velocity at the time of phenol addition to the initial velocity. The percentage of remaining phenol-sensitive activity (latent E_a) was determined from the ratio of the velocity after *N*-ethylmaleimide (NEM) addition to the estimated initial (E_a) velocity of an uninhibited system. The latter value was estimated from the fact that corresponding control activated samples had 4.7 times the activity of untreated ones.

Oxygenation by the phenol-treated powder was only slightly inhibited by concentrations of 5,8,11,14-eicosatetraenoic acid above 500 μM. The degree of inhibition was similar in systems in which the preparation was preincubated with phenol before addition of substrate and in systems in which the enzyme was added to the sample containing both phenol and eicosatetraenoic acid. Such levels of substrate were far above the observed K_m value of 5.5 and suggest that the substrate inhibition is not as significant as found for the soybean lipoxygenase (Smith and Lands, 1972).

Discussion

Dependence of the Vesicular Gland Dioxygenases upon Hydroperoxide. We observed that added GSH peroxidase caused a 60% inhibition in the oxygenation of 5,8,11,14-eicosatetraenoic acid by vesicular gland homogenates. This inhibition required the presence of GSH and was reversed by adding *N*-ethylmaleimide. The results are similar to that seen for soybean lipoxygenase (Smith and Lands, 1972) and suggest that the fresh homogenate contains a dioxygenase which requires its product hydroperoxide for sustained activity.

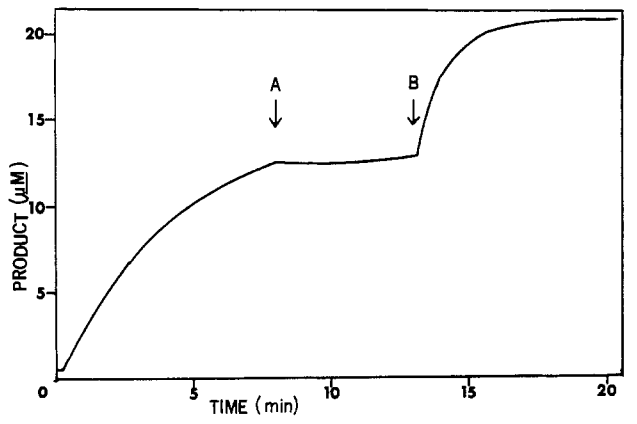

FIGURE 10: Product formation *vs.* time for the nonactivated and phenol-activated vesicular gland dioxygenase activities in the presence of GSH peroxidase. The reaction was initiated by the addition of a suspension of the acetone powder (5 mg) at zero minutes to a sample containing 190 nmoles of 5,8,11,14-eicosatetraenoic acid, 3.3 μmoles of GSH, and 12,500 units of GSH peroxidase. The volume of the sample after addition of powder was 3.0 ml of 0.1 M Tris·HCl (pH 8.5). Further additions to the sample chamber: (A) 2.0 μmoles of phenol (0.20 ml) and (B) 0.9 mmole of *N*-ethylmaleimide (0.10 ml) 5 min after the phenol addition. Product formation was calculated from oxygen consumption as described in the Methods.

In contrast to the results with homogenates, no evidence could be found for a product-dependent dioxygenase activity in untreated acetone powder preparations of vesicular glands. GSH peroxidase, at a level sufficient to catalyze the destruction of 47 mM hydroperoxide/min at saturating concentrations of hydroperoxide, did not inhibit the low rate of oxygenation catalyzed by the untreated acetone powder. This result indicates that the basal type of dioxygenase activity (E_b) did not depend on hydroperoxide (or if it did, it required only extremely low concentrations to maintain full activity).

Treatment of the acetone powder with phenol increased the oxygenase activity almost fivefold and restored the sensitivity of the preparation to GSH peroxidase. Oxygenation catalyzed by the phenol-treated preparations was inhibited 50% with the levels of GSH peroxidase tested. Thus, with respect to a presumed need for hydroperoxide, the phenol-treated acetone powder is more similar than the untreated powder to the enzyme system found in freshly prepared vesicular gland homogenates. The half-life for the phenol-stimulated activation process (approximately 3.5 min at 30°) was similar at several levels of protein concentration. If the sigmoidal shape of the activation curve were due to an autocatalytic production of an activator (such as seen in the product activation of lipoxygenase; Smith and Lands, 1972) or to the removal of an inhibitor, the half-life for maximal activation might be expected to decrease with increasing concentrations of acetone powder.

We found it necessary to have phenol continuously present in our assay mixture because the enzyme, once activated, was rapidly deactivated by the removal of phenol. Although this result suggested that phenol may be continually consumed both during the activation process and during the oxygenation by the fully activated enzyme, we detected no measurable oxygen consumption during the activation process.

No rigorous estimate of dioxygenase rate can be made for the homogenates which were assayed by the radioisotope technique because endogenous substrates in the vesicular gland homogenate cause an unknown dilution of the isotopically labeled fatty acid. On the other hand, a comparison of the radioisotope assay to the oxygen absorption assay with acetone powder preparations showed that both the un-

treated (E_b activity) and phenol-treated preparations (E_b plus E_a activity) consumed 2 moles of oxygen/mole of fatty acid. These results indicate that E_b and E_a could both function as a first enzyme in the formation of prostaglandins from free fatty acid substrate. The E_b activity could provide a "basal" level of prostaglandins whereas the other, more rapid dioxygenase (E_a) may serve in a regulatable or activatable manner (Smith and Lands, 1971).

Additional evidence for the two separate types of oxygenase activity came from experiments in which the untreated acetone powder became completely inactivated during oxygenation of fatty acid in the presence of GSH peroxidase (see Table III). Although no increased activity was observed after treatment of the apparently inactivated acetone powder with phenol, additional dioxygenase activity (E_a) did appear following the removal of the GSH with *N*-ethylmaleimide. This newly observed E_a activity must have been completely suppressed by the functioning of the GSH peroxidase system and therefore may resemble soybean lipoxygenase in requiring its product hydroperoxide, P, as an obligatory intermediate (Smith and Lands, 1971a)

$$E_a + P \rightleftharpoons [E_aP] \overset{S+O_2}{\rightleftharpoons} [E_aPO_2S] \xrightarrow{k_{a3}} E_aP + P$$

The fact that we observed only partial inhibition of both the phenol-treated powder and the crude homogenate may therefore have been due to the activity of the GSH peroxidase insensitive dioxygenase (E_b). E_b not only catalyzes fatty acid oxygenation in the presence of GSH peroxidase but could also continually and rapidly supply hydroperoxide for E_a so that a partial E_a activity may also occur.

Self-Catalyzed Destruction of the Vesicular Gland Dioxygenases. The loss in dioxygenase E_b activity during the oxygenation reaction was a linear function of substrate consumption and is therefore kinetically analogous to the self-catalyzed destruction of lipoxygenase (Smith and Lands, 1970; Lands *et al.*, 1971; Smith and Lands, 1972). At relatively high substrate concentrations, the untreated acetone powder was inactivated at similar rates in either the presence or absence of GSH peroxidase. Since the level of GSH peroxidase added was sufficient to destroy 8 mM hydroperoxide per minute under maximum conditions, it seems reasonable that free lipid hydroperoxide was not accumulating appreciably during the oxygenation reaction to cause the inactivation of E_b. Therefore, this inactivation seems more likely to have been due to a destructive reaction of some active intermediate such as was observed with the soybean lipoxygenase (Smith and Lands, 1972). Nevertheless, we cannot rule out again the possibility that a very low level of product (which also might still be saturating a hydroperoxide activation process) might also be capable of saturating the destruction process. In such an instance, the process would not respond to usually observed changes in the hydroperoxide concentrations.

The capacity to stimulate E_a with phenol appeared to be lost more rapidly (1.1 min^{-1}) than E_b activity (0.25 min^{-1}) during oxygenation in the absence of phenol, GSH, and GSH peroxidase. However, in the absence of phenol, but in the presence of GSH and GSH peroxidase, the latent activatable E_a activity was lost more slowly than the E_b activity. Since the latent E_a was lost at different rates in the presence and absence of GSH and GSH peroxidase, whereas E_b was inactivated at the same rate in both systems, the latent E_a seems to be more susceptible than either E_b or active E_a to direct attack by hydroperoxides.

The E_a dioxygenase activity was also inactivated while catalyzing the oxygenation of fatty acid. This loss of enzyme, like that observed with E_b, occurred with a linear loss in velocity with substrate consumption although at different rates with different acids. The rate constant observed for inactivation of E_a during oxygenation of arachidonate (0.55 min^{-1}, see Figure 8, II is less than the rate constant for loss of phenol-stimulable activity (*i.e.*, latent E_a) in the presence of hydroperoxide (1.1 min^{-1}, see Figure 9B). This suggests that the presence of substrate fatty acid protects the activated E_a activity against inactivation by product. We noted a similar conclusion for soybean lipoxygenase (Smith and Lands, 1972). An alternate interpretation is that phenol provides a more stable form of the enzyme. If inactivation of E_a were caused only by product hydroperoxide, independent of substrate, the loss of E_a activity might be expected to occur more rapidly during the oxygenation reaction as the ratio of product to substrate concentration increased. Since the rate of inactivation remains relatively constant during reaction, loss of E_a by a k_{a4} process seems less likely than by a self-destruction (k_{a2}) process under these conditions

$$E_a + P \rightleftharpoons [E_aP] \overset{S+O_2}{\rightleftharpoons} [E_aPO_2S] \begin{cases} \xrightarrow{k_{a2}} E_a^* \\ \xrightarrow{k_{a3}} E_aP + P \end{cases}$$
$$[E_aP] \xrightarrow{k_{a4}} E^*$$

Pace-Asciak and Wolfe (1968) reported that 9,12-octadecadienoic acid and 6,9,12-octadecatrienoic irreversibly inhibited the oxygenation of 5,8,11,14-eicosatetraenoic acid by a hydroquinone-treated acetone powder of rat stomach. Since fatty acids containing two or more double bonds may serve as substrate for the vesicular gland dioxygenase (Samuelsson, 1967; Hamberg and Samuelsson, 1967b) this irreversible inhibition might have been due to inactivation of the dioxygenase(s) by either a self-destruction process (k_2) or a substrate-independent process (k_4) described here (or both).

Overall Kinetic Mechanisms for Vesicular Gland Dioxygenases. E_b appears to catalyze the oxygenation of fatty acid substrate in a product-independent reaction according to the following formulation

$$E_b + S + O_2 \rightleftharpoons [E_bO_2S] \begin{cases} \nearrow E^{**} \\ \searrow E_b + P \end{cases}$$

This formulation is described by a steady-state rate equation

$$V_b = \frac{k_{b3}[E_b^0] - k_{b2}([S_0] - [S])}{\frac{K_{bM}}{[S]} + 1} \qquad (1)$$

We determined from a double-reciprocal plot that for the untreated acetone powder (E_b), K_{bM} is 5.5 μM and k_{b3} ($V_{max}/[E_b^0]$) is 3.4 min^{-1} (nmoles of 20:4/mg of protein). A k_{b2} value of 0.25 min^{-1} was determined from the slopes of plots of instantaneous velocity *vs.* substrate concentration (Figure 7).

The fully activated form of E_a appears to catalyze the oxygenation of polyunsaturated fatty acids by a product-depend-

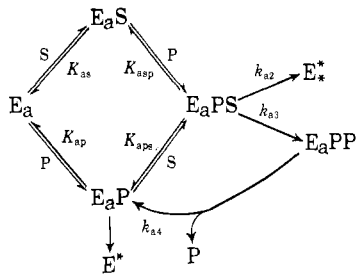

FIGURE 11: A proposed kinetic formulation for the phenol-activated vesicular gland dioxygenase activity.

ent mechanism (see Figure 11) similar to that observed for soybean lipoxygenase (Smith and Lands, 1972).

Equation 2 is the equilibrium rate expression for this kinetic mechanism

$$V = \frac{k_{a3}([E_a{}^0] - [E_a{}^*]) - k_{a2}([S_0] - [S])}{\frac{K_{aps}}{[S]}\left(1 + \frac{K_{ap}}{[P]}\right) + \frac{K_{asp}}{[P]} + 1} \quad (2)$$

Constants for eq 2 were estimated in the following manner. The K_{aps} value was estimated from the K_M of the phenol-treated enzyme preparation since with high concentrations of product, $K_M \simeq K_{aps}$. For 5,8,11,14-eicosatetraenoic acid the K_M is on the order of 5 μM. The value for k_{a2} (0.55 min^{-1}) was determined from the slopes of plots of instantaneous velocities *vs.* remaining substrate concentration (Figure 8, II). Plots of reciprocal velocities and substrate concentrations were used to estimate k_{a3} (12.5 min^{-1} (nmoles of 20:4/mg of protein)) since for the phenol-treated powder $V_{max} \simeq k_{b3}[E_b{}^0] + k_{a3}[E_a{}^0]$ whereas for the untreated powder $V_{max} \simeq k_{b3}[E_b{}^0]$.

Since, at a measurable product concentration (0.3 μM), we observed no product-dependent lag in initial velocity for the oxygenation of 5,8,11,14-eicosatetraenoic acid by phenol-treated acetone powder preparations, we reasoned that the dioxygenase E_a must be operating at maximum velocity with respect to product at that product concentration. Therefore, we estimated that the K_p value was no greater than 0.1 μM. Our initial velocity determinations were obtained from sections of the kinetic curves where the apparent product concentrations were no larger than 0.3 μM. This upper limit of 1 μM for [P] (above which no increase in velocity occurred with increasing [P]) was then used in our subsequent calculations.

In phenol-treated acetone powder systems, both E_b and E_a were assumed to be active, and therefore the total rate of oxygenation is represented by the sum of eq 1 and 2

$$V_{total} = V_b + V_a = \frac{k_{b3}[E_b{}^0] - k_{b2}([S_0] - [S])}{\frac{K_{bM}}{[S]} + 1} + \frac{k_{a3}([E_a{}^0] - [E_a{}^*]) - k_{a2}([S_0] - [S])}{\frac{K_{aps}}{[S]}\left(1 + \frac{K_{ap}}{[P]}\right) + 1 + \frac{K_{asp}}{[P]}} \quad (3)$$

At a product concentration ([P]) of 1 μM, enzyme concentrations $[E_b{}^0] = [E_a{}^0]$ of 0.84 mg/ml and using $K_{ap} = 0.1$ μM; $k_{a2} = 0.55$ min^{-1}; $k_{b2} = 0.25$ min^{-1}; $k_{a3} = 12.5$ min^{-1} (nmoles of 20:4/mg); $k_{b3} = 3.4$ min^{-1} (nmoles of 20.4/mg);

TABLE IV: Calculation of the Velocities for the Oxygenation of 5,8,11,14-Eicosatetraenoic Acid by Phenol-Activated Vesicular Gland Acetone Power from the Overall Kinetic Equation for Dioxygenases E_b and E_a.

Expt[a]	$[E_0]$ (mg/ml)	[P] (μM)	[S] (μM)	V_{obsd} (μM 20:4 (n − 6)/min)	V_{calcd} (μM 20:4 (n − 6)/min)
1	0.84	1	3.6	5.2	5.2
2	0.84	1	6.0	6.4	6.9
3	0.84	1	12.0	9.4	9.0
4	0.84	1	24.0	11.0	10.6

[a] Values of $[E_0]$, [S], and V_{obsd} are from Figure 12. [P] is the estimated product concentration at which V_{obsd} is determined. V_{calcd} is calculated from eq 3 and the estimated values of the necessary constants indicated in the text.

and $K_{bM} = 5.5$ μM, we calculated values of K_{aps} and K_{asp} which, when substituted into eq 3 with the above constants, would predict the observed velocity for the oxygenation of different concentrations of 5,8,11,14-eicosatetraenoic by phenol-treated acetone powder. The values of K_{aps} and K_{asp} which allowed a reasonable prediction of the observed velocities under these conditions were 5 and 0.03 μM, respectively. The value of K_s (which equals $K_p \cdot K_{ps}/K_{sp}$) was then calculated to be 17 μM. Table IV indicates the correspondence between the observed and predicted velocities for several incubations using eq 3 and the constants noted above.

We have recently proposed a mechanism to account for the product-dependent action of lipoxygenase (Smith and Lands, 1972). In this hypothesis the lipid hydroperoxide may react with oxygen to form a tetroxide transition state. Then, interaction with an enzyme-bound substrate acid could conceivably supply orientational energy leading to the production of a planar tetroxide and then a perepoxide metastable intermediate without free singlet state oxygen actually being required.

Both enzymes, lipoxygenase or dioxygenase E_a, would presumably function in this mechanism by positioning the appropriate double bond of the substrate in such a way that the driving force for formation of a planar tetroxide intermediate would be available. With lipoxygenase, this double bond would be at the n − 6 position whereas with the vesicular gland dioxygenase E_a the double bond would be at the n − 9 position. Perepoxide formation across either double bond could result in the eventual abstraction of the L-(n − 8) hydrogen atom of the substrate. The enzymes may make cleavage of this particular carbon–hydrogen bond more favorable.

Using hydroquinone-treated lyophilized particle preparations, Nugteren (1970) determined a K_i value of 0.12 μM for the apparent competitive inhibition of the oxygenation of 8,11,14-eicosatrienoic acid by 8-*cis*,12-*trans*,14-*cis*-eicosatrienoic acid. This K_i value was more than 300 times smaller than the apparent K_M (40 μM) for the 8-*cis*,11-*cis*,14-*cis*-eicosatrienoic acid substrate. We found in our studies that the estimated K_p value (0.1 μM) was 50 times less than the apparent K_M (K_{aps}) for 5,8,11,14-eicosatetraenoic acid. The trans inhibitor is structurally similar to 11-hydroperoxy-8-*cis*,12-*trans*,14-*cis*-eicosatrienoic acid, one of the hydroperoxides believed

to be produced in the oxygenation catalyzed by sheep vesicular gland (Hamberg and Samuelsson, 1967b). The similarity in binding constants leads us to believe that the inhibitors studied by Nugteren may not have competed with substrate alone, but might have also been competing with product for the product binding site. We also would expect these trans isomers to have less effect on the rate of oxygenation catalyzed by untreated acetone powders since a product-dependent site has not been recognized for the E_b dioxygenase.

In addition to finding that 8-*cis*,12-*trans*,14-*cis*-eicostatrienoic acid inhibited the vesicular gland dioxygenase, Nugteren also noted that this acid did not inhibit soybean lipoxygenase. However, if the above formulation if correct, we would anticipate that another isomer, 8-*cis*,11-*cis*,13-*trans*-eicosatrienoic acid, would be a more effective competitive inhibitor of lipoxygenase. This latter acid has a carbon structure similar to the lipoxygenase product, 15-hydroperoxy-8-*cis*,11-*cis*,-13-*trans*-eicosatrienoic acid, which has been shown to activate the soybean enzyme presumably by attachment to a product-selective site (Smith and Lands, 1972). Lipoxygenase catalyzes oxygen insertion at the $n - 6$ carbon atom of the substrate whereas the vesicular gland dioxygenase E_a catalyzes oxygen insertion at the $n - 10$ position. The position of this insertion may be effected by the corresponding products of the two enzymes: 15-hydroperoxy-8-*cis*,11-*cis*,13-*trans*-eicosatrienoic acid for lipoxygenase and 11-hydroperoxy-8-*cis*,-12-*trans*,14-*cis*-eicosatrienoic acid for the dioxygenase E_a.

A striking feature of these product-activated oxygenases is the wide range of the control that is theoretically feasible in regulating the rates of reaction. An uninterrupted product activation would provide an explosive increase to maximal velocity whereas action of tissue peroxidases might lower E_a-type activity to near zero. The rate of removal of hydroperoxide could also be a function of the [RSH]:K_m(RSH) ratio for the peroxidase. Another unusual feature complementing the positive feedback in initiating the dioxygenase action is the automatic negative feedback due to the k_2 process. The latter process would tend to ensure that once the enzyme begins to function, it will produce no more than a fixed, finite amount of hydroperoxide to serve as precursor of prostaglandin hormones.

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