

EVIDENCE FOR AN ACTIVATING FACTOR FORMED DURING PROSTAGLANDIN BIOSYNTHESIS

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SUMMARY: The oxygenation of 5,8,11,14-eicosatetraenoic acid by acetone powder preparations of sheep vesicular glands proceeded with a lag or accelerative phase when inhibitory concentrations of NaCN were present. This accelerative feature of the reaction suggested that an activating material might be produced as the oxygenation reaction proceeds. When a second addition of fresh enzyme was made to a reaction mixture, the lag phase was as short as in uninhibited controls. This indicated that an activating factor was required for optimal activity of this dioxygenase, and that it accumulated during the oxygenation reaction in the presence of NaCN. The factor was extracted from an aqueous incubation medium with cold diethyl ether. There was a positive relationship between the amount of activating factor added and the resultant increase in the initial velocity of the oxygenation system.

Oxygenation of 5,8,11,14-eicosatetraenoic acid by enzyme preparations from sheep vesicular glands was suppressed in the presence of functioning glutathione peroxidase activity (1) and, therefore, it was suggested that this phenol-activated enzyme-catalyzed reaction may require a hydroperoxide as an essential intermediate in a kinetic mechanism similar to one proposed for soybean lipoxygenase (2). The soybean lipoxygenase exhibits a lag phase that can be extended by inhibitory concentrations of substrate or glutathione peroxidase and decreased by addition of the product hydroperoxide. The acceleration in velocity can be explained by an activation of the enzyme by a product of the reaction binding to a specific site on the enzyme (2). We have now observed a similar lag phenomenon with the fatty acid dioxygenase of vesicular gland providing evidence that an activating factor needed for the oxygenation step in prostaglandin biosynthesis is produced during the reaction.

Analysis of the changes in reaction velocities was facilitated in our experiments by the continuous recording of the change in oxygen content with time (dO_2/dt) with an electronic differentiator attached to the biological oxygen monitor. Examples of the changes in the rate of oxygen consumption during oxy-

generation of 5,8,11,14-eicosatetraenoic acid with oxygenase preparations from vesicular gland are shown in Fig. 1. In an uninhibited system (A), the reaction velocity reached an optimal value at 12 sec after addition of the final reactant and then decreased. At 9 sec (the earliest time that an accurate measurement of the reaction velocity could be obtained) the velocity was generally 60-75% of the optimal velocity.

Like several other agents that complex metal ions, sodium cyanide inhibits the oxygenation of arachidonic acid by phenol-activated acetone powder preparations of vesicular gland (3). The optimal rate of oxygen consumption, indicated by the peak of the differentiator tracings (Fig. 1), was reduced considerably in the presence of cyanide. As the concentration of sodium cyanide in the reaction mixtures was increased, the lag period until the optimal velocity was reached became longer (Fig. 1A-E). For example, with 0.5 mM NaCN in the re-

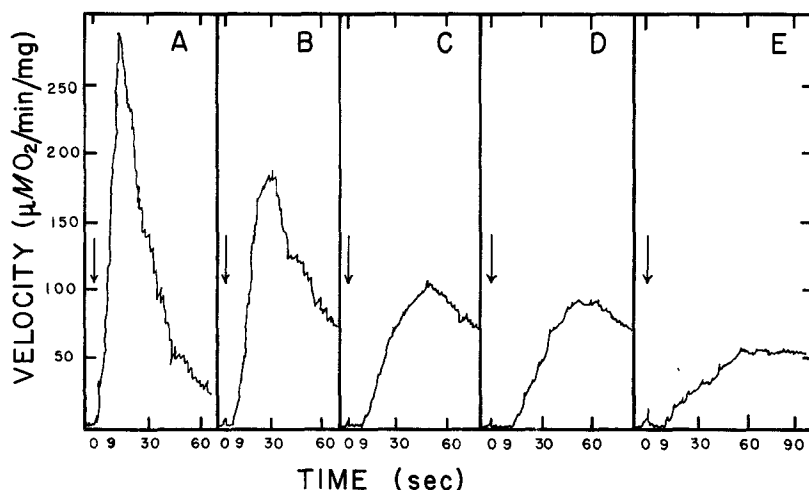


Figure 1. Velocity of oxygen consumption by acetone powder preparation of vesicular gland in the absence and presence of NaCN. The oxygenation of 210 nmoles of 5,8,11,14-eicosatetraenoic in 3.0 ml of 0.1 M Tris-HCl (pH 8.5) containing 0.67 mM phenol was determined with an oxygen electrode. The signal was monitored by an electronic differentiator adjusted so that 1 mV on the recorder was equivalent to a rate of 1.74 $\mu\text{M O}_2/\text{min}$. The concentration of NaCN was A - 0; B - 0.5 mM; C - 1.0 mM; D - 2.0 mM; E - 2.5 mM. At zero time, indicated by the arrows, 250 μg of phenol activated acetone powder was used.

action mixture, the velocity at 9 sec was decreased to 58 from 180 $\mu\text{M}/\text{min}/\text{mg}$ and the time to optimal velocity increased to 26 sec compared to 12 sec with no NaCN present. There was no detectable velocity at 9 sec with concentrations of NaCN 1 mM or greater, and the time to optimal velocity extended to more than 60 sec.

After no further oxygen consumption could be detected, another addition of enzyme was made to the reaction mixture to test its suitability for reaction since the original 5,8,11,14-eicosatetraenoic acid concentration had been reduced by less than 20%. This time the optimal velocity was 30-50% greater (Fig. 2) than that obtained with the first addition of the same amount of enzyme. The lag period following the second addition was brief with all concentrations of NaCN tested, and the velocity at 9 sec was about 65% of the optimal velocity.

The time until the optimal velocity is reached is a useful index of the severity of the lag phase during which the reaction is accelerating. Often, as seen in Fig. 1 for levels of NaCN that give greater than 50% inhibition, this value is an estimation, since it is difficult to determine precisely the point

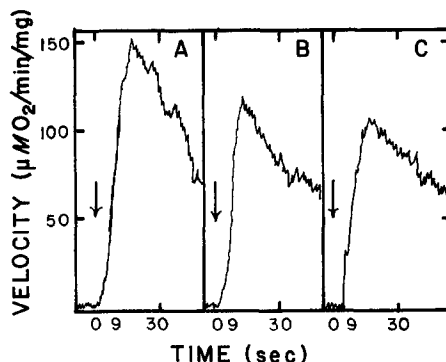


Figure 2. Effect of second addition of enzyme on the reaction velocity in the presence of NaCN. An additional 250 μg of phenol-activated acetone powder was added at 10 min (arrows) to previously reacted mixtures that contained NaCN and 55-60 μM unreacted fatty acid substrate. Reaction mixtures for A, B and C correspond to those in C, D and E of Fig. 1, respectively.

on the differentiator tracing where the velocity begins to decline. Nevertheless, differences are often great enough to make the values of time to optimal velocity a convenient indication of the lag phenomenon. The velocity at 9 sec, which potentially is useful in quantitating the amount of activator initially present in the reaction mixture (4), can be determined accurately from the differentiator tracings. Observed times to optimal velocity and velocities at 9 sec determined for first addition of enzyme in the presence of 0-2.5 mM NaCN and for second addition of enzyme 10 min after the first are summarized in Fig. 3. Although no velocity at 9 sec could be detected with the first addition of enzyme in the presence of 1.0 mM NaCN or greater, the progressively greater times to optimal velocity indicate progressively greater inhibition of the oxygenase system. However, with a second addition of enzyme to the reaction mixture, the velocity at 9 sec was consistently about 60% of the optimal velocity obtained (data not shown), and the time to optimal velocity was brief and unchanged by the cyanide content in all cases. Addition of more NaCN at the time

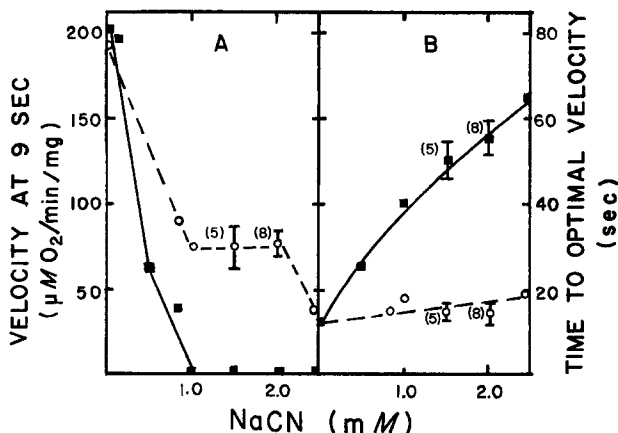


Figure 3. Effect of NaCN on the velocity at 9 sec and the time to optimal velocity. 250 μ g of acetone powder preparation of vesicular gland enzyme were added to 3.0 ml of 0.1 M Tris-HCl (pH 8.5) containing 0.67 mM and 210 nmoles of 20:4 (■—■). After 10 min another 250 μ g of enzyme was added (○--○). Velocity at 9 sec (A) and time to optimal velocity (B) were determined from differentiator tracings as described in the text. Bars represent SEM for the number of experiments indicated in brackets.

of second addition of enzyme to increase the total amount to 2-4 μM NaCN generally resulted in only a slightly lower (5-10%) optimal velocity and a 10-30% lower velocity at 9 sec compared to values with no additional NaCN. Addition of more fatty acid substrate at that time did not alter either the optimal velocity or velocity at 9 sec. A TLC analysis of the products formed in the presence of 2 mM NaCN indicated that greater than 60% of the total reaction products corresponded to prostaglandin E_2 and $F_{2\alpha}$ standards.

From these results, it appeared that the vesicular gland oxygenase formed an activator that was required for optimal velocity, and that NaCN slowed the formation of this activator as well as the overall reaction rate of the dioxygenase. Apparently, the activating factor accumulated in the reaction mixture so that the second addition of enzyme could operate at near optimal velocity without the need of generating the activator. Thus, the lag was abolished and the velocity at 9 sec was increased greatly. Because a self-catalyzed destruction of enzyme (1) inevitably accompanies activator production during the accelerative phase, the presence of activator allowed a somewhat greater optimal velocity after the second addition than was seen with the first addition of enzyme.

Since the activating factor that accumulated in reaction mixtures containing NaCN was apparently stable for at least 10 min in the aqueous medium, we tested the effect of adding aliquots of a previous reaction mixture containing 2 mM NaCN to fresh assay systems prior to enzyme addition (Fig. 4). The velocity at 9 sec after adding enzyme was proportional to the aliquot of previous reaction mixture in the assay (Fig. 4A). This type of relationship between the amount of activator and the velocity at 9 sec was similar to that found for the activation of soybean lipoyxygenase (4). In addition, the optimal velocity attained by a given amount of enzyme was little altered except that a 3.0 ml aliquot gave an increase of about 20% even though the final concentration of NaCN in the assay was 4.0 mM . In accord with the increased initial velocities, the time required to reach optimal velocity became shorter as the amount of added reaction mixture was increased (Fig. 4B).

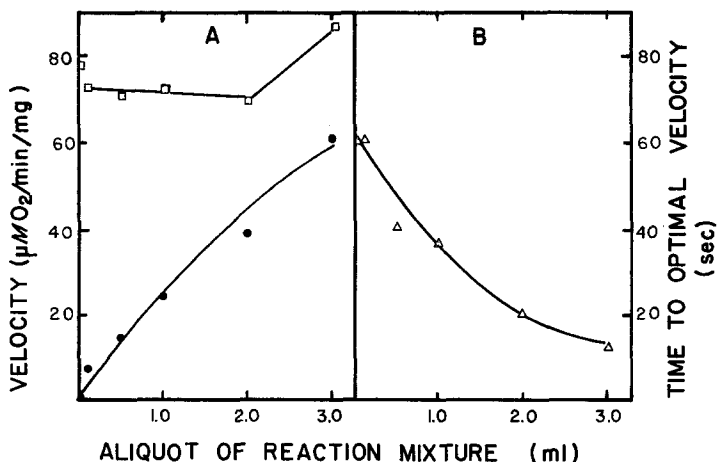


Figure 4. Effect of aliquots of a previous reaction mixture on reaction velocity. Initially 250 μg of a phenol-activated acetone powder preparation was incubated in 3.0 ml of 0.1 M Tris-HCl (pH 8.5) containing 0.67 mM phenol, 2 mM NaCN and 70 μM 5,8,11,14-eicosatetraenoic acid. Aliquots of this reaction mixture were added to 210 nmoles of 5,8,11,14-eicosatetraenoic acid with 2 mM NaCN in 3.0 ml final volume. Reaction was initiated by addition of 250 μg fresh enzyme. A. \square — \square optimal velocity, \bullet — \bullet velocity at 9 sec; B. \triangle — \triangle time to optimal velocity.

Reaction mixtures similar to those above were incubated and treated with diethyl ether. Aliquots of this organic extract were evaporated and assayed for their ability to alter the reaction velocity of enzyme in the presence of 2 mM NaCN. The effectiveness of these aliquots was compared to that for aliquots of an extract of an incubation of enzyme and substrate with no NaCN present or of aliquots of an extract of a mixture of 5,8,11,14-eicosatetraenoic acid and 2 mM NaCN in the absence of enzyme (Fig. 5). Values for the latter material indicated the background level for the system and only slight increases in the velocity at 9 sec occurred with aliquots of the extract from this mixture; the maximum effect was to allow a velocity at 9 sec that was 15-20% of the optimal velocity and in all cases the time to optimal velocity was 55-60 sec. With increased aliquots of the ether extracts from reaction mixtures that contained no NaCN, there was a 1 to 6 fold greater increase in the velocity at 9 sec and a greater decrease in the time until the optimal velocity was reached. A more

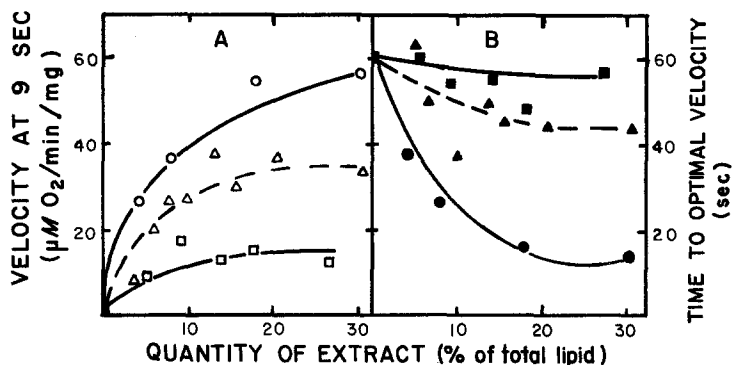


Figure 5. Assay of diethyl ether extracts of reaction mixtures for ability to alter the reaction velocity. Diethyl ether extracts were obtained from pre-incubations of 1.0 μ mole of [1- 14 C]5,8,11,14-eicosatetraenoic acid in 10 ml of 0.1 M Tris-HCl (pH 8.5) containing 0.67 mM phenol that were carried out as follows: A. 7.5 mg of phenol-activated acetone powder preparation for 2 min. B. 7.5 mg of phenol-activated acetone powder preparation and 2 mM NaCN for 2 min. C. 20 μ moles NaCN followed by 7.5 mg enzyme after the mixture was quenched. All reactions were terminated by addition of 30 mM citric acid. The aqueous mixtures were extracted twice with 20 ml diethyl ether at 0° and dried over Na₂SO₄. After filtering, the solvent was removed under vacuum. Aliquots of the lipid extract were suspended in buffer in reaction chambers. Assay mixtures contained 3.0 ml of 0.1 M Tris-HCl (pH 8.5) with 0.67 mM phenol, 2 mM NaCN and 50 μ M 5,8,11,14-eicosatetraenoic acid. Reaction was initiated by addition of 250 μ g enzyme. Quantity of extract assayed was determined as a percentage of total in the preincubation mixtures: A. Δ — Δ , velocity at 9 sec; \blacktriangle — \blacktriangle , time to optimal velocity; B. \circ — \circ , velocity at 9 sec; \bullet — \bullet , time to optimal velocity; C. \square — \square , velocity at 9 sec; \blacksquare — \blacksquare , time to optimal velocity.

dramatic effect was observed with the extract from the incubation mixture containing 2 mM NaCN. There was an apparent hyperbolic relationship between the quantity of extract assayed and the velocity observed at 9 sec and, when 18% of the total extract was assayed, the velocity at 9 sec was 68% of the optimal velocity reached by 16 sec after initiation of reaction. These latter values for velocity at 9 sec and time to the optimal velocity closely corresponded to those obtained with the second addition of enzyme to a reaction mixture containing 2 mM NaCN (see Fig. 3). Thus, the activating factor was organic in nature and was stable enough to permit extraction from an aqueous reaction mixture with diethyl ether at 0°. Clearly, the stimulatory effect of this material on the reaction velocity of an acetone powder preparation was proportional to the concentration of factor.

It seems that an activating factor essential to oxygenation may be produced during all fatty acid dioxygenase reactions. With 5,8,11,14-eicosatetraenoic acid and oxygenase preparations from vesicular glands, the production apparently occurs very rapidly in the absence of sodium cyanide, or else lower amounts are needed for optimal enzyme activity. On the other hand, the activator seems also to be dissipated relatively quickly in these preparations in the absence of cyanide so that its activation properties are not retained. Clearly, further study of the character and specificity of the activating factor produced in reaction mixtures in the presence of NaCN, and possibly other inhibitors, provides a means for a better understanding of the mechanism of the reaction and insight to the possible regulation of prostaglandin biosynthesis in vesicular gland and other tissues.

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