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Kinetic Studies of the Enzymatic Dopamine β-Hydroxylation Reaction*

Menek Goldstein,† Tong Hyub Joh, and Thomas Q. Garvey III

ABSTRACT: Acetate can replace fumarate in the stimulation of dopamine β-hydroxylase activity. Increased oxygen concentration enhances the enzymatic activity and can replace fumarate in the stimulation of the enzymatic β hydroxylation. Fumarate and oxygen stimulate the enzymatic activity at low substrate (RH) concentrations and both change the K_M of the substrate but not the V_{max}. Fumarate facilitates the interaction of the reduced enzyme intermediate with oxygen and most likely induces a conformational change of the enzyme. The initial velocity patterns are consistent with a mechanism in which the binding of the first substrate (ascorbate) to the enzyme is followed by the release of the product (dehydroascorbate) before a second substrate can react ("Ping-Pong" mechanism). The subsequent substrates (RH and O_2) add to the enzyme before either product is released. The data also suggest that regardless of which of these two substrates (RH or O_2) adds first, both steps are in a rapid equilibrium and the interconversion of the central ternary complexes most likely represents the rate-limiting step in the over-all β-hydroxylation reaction.

Dopamine β-hydroxylase is a mixed-function oxidase that catalyzes the conversion of 3,4-dihydroxyphenylethylamine into norepinephrine according to reaction I (Levin *et al.*, 1960).

$$\text{dopamine} + \text{ascorbate} + O_2 \xrightarrow{\text{fumarate}} \text{norepinephrine} + H_2O + \text{dehydroascorbate} \quad \text{(I)}$$

The enzyme is not specific for dopamine and catalyzes the β hydroxylation of many analogs of phenylethylamine (*e.g.*, tyramine) (Goldstein and Contrera, 1962; Levin and Kaufman, 1961; Creveling *et al.*, 1962). Fumarate and certain other dicarboxylic acids stimulate the hydroxylation reaction (Levin *et al.*, 1960) by a mechanism which is still obscure. The hydroxylating enzyme is a copper protein, and it was shown that part of the protein-bound copper undergoes cyclic reduction and oxidation during the over-all hydroxylation reaction (Goldstein *et al.*, 1965; Blumberg *et al.*, 1965; Friedman and Kaufman, 1965). The cupric copper of the enzyme is reduced by ascorbate and partially reoxidized when the reduced enzyme reacts with dopamine and oxygen. It was shown that the cupric copper of the enzyme can also be reduced by other reducing agents such as cysteine or reduced pteridine (Goldstein, 1966; Goldstein and Joh, 1967a). The reduced enzyme intermediate reacts with the substrate to give the hydroxylated product (Friedman and Kaufman, 1965). In the present study we have shown that the enzymatic activity is not only stimulated by fumarate but acetate also stimulates the β-hydroxylation reaction. We have also shown that increased oxygen concentration enhances the enzymatic activity and can replace fumarate in the stimulation of the enzymatic β hydroxylation. The results obtained with kinetic studies provide further information on the mechanism of the enzymatic β hydroxylation. Some of these results have been presented in preliminary reports (Goldstein and Joh, 1967b; Goldstein *et al.*, 1967).

Methods

Dopamine β-hydroxylase was prepared as previously described (Levin *et al.*, 1960). The enzyme was purified to the second DEAE column eluate stage as previously described in procedure A (Goldstein *et al.*, 1965). In some experiments an essentially homogeneous enzyme preparation was used. This enzyme preparation was prepared by the procedure of Friedman and Kaufman (1965). When dopamine was used as a substrate the enzyme activity was assayed fluorimetrically (Von

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† Research career development awardee of the U. S. Public Health Service (Grant No. 5-K3-MH-14,918-07).

TABLE I: The Effect of Fumarate and Acetate on the Enzymatic β Hydroxylation of Dopamine.[a]

Additions (μmoles)	Norepinephrine Formed (μmoles)	
	pH of the Incubation Mixture	
	5.5	6.4
Acetate (200)	1.30	1.20
Acetate (200) + fumarate (10)	1.40	1.30
Phosphate (100)	0.30	0.25
Phosphate (100) + fumarate (10)	1.40	1.25

[a] The incubation was carried out in a mixture that contained the following components: dopamine, 2 μmoles; ascorbic acid, 6 μmoles; catalase, 500 units (Sigma); and enzyme (0.1 mg of protein) in a total volume of 1 ml. Acetate, phosphate, and fumarate solutions were adjusted either to pH 5.5 or 6.4 and were added as indicated in the table. The reaction mixture was incubated for 5 min at 37°.

Euler *et al.*, 1955). When tyramine-^{3}H was used as a substrate the formation of the β-hydroxylated product octopamine was determined by the periodate method (Pisano *et al.*, 1960). In most studies the results with tyramine were similar to those obtained with dopamine. Since dopamine might reduce the cupric copper of the enzyme (Levin and Kaufman, 1961), tyramine was used in the studies where the effects of ascorbate on the initial velocities were investigated.

The reduced enzyme intermediate was formed by two procedures. In the first procedure the enzyme (an essentially homogeneous enzyme preparation, 0.1 mg of protein) was reduced aerobically with 0.02 μmole of ascorbate. One minute after the addition of ascorbate its excess was removed by addition of ascorbic acid oxidase (Friedman and Kaufman, 1965). In the second procedure the enzyme (0.1 mg of protein) was reduced anaerobically by adding 0.02 μmole of cysteine, and 1 min later PMB[1] (0.05 μmole) was added to react with the remaining sulfhydryl groups (Goldstein, 1966). In some experiments incubation under oxygen was carried out in Thunberg tubes and the delivery of gas was regulated by a reducing valve. In other experiments oxygen was delivered under atmospheric pressure.

For initial velocity studies the reaction mixtures were incubated for 1 min at 37°. In this time interval the velocity was linear with respect to time. Velocity, in all experiments to be reported here, is expressed in arbitrary units or as micromoles of product formed per minute.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: dopamine, 3,4-dihydroxyphenylethylamine; norepinephrine, 3,4-dihydroxyphenyl-2-aminoethanol; tyramine, 4-hydroxyphenylethylamine; octopamine, 4-hydroxyphenyl-2-aminoethanol; RH, dopamine or tyramine; PMB, *p*-mercuribenzoate.

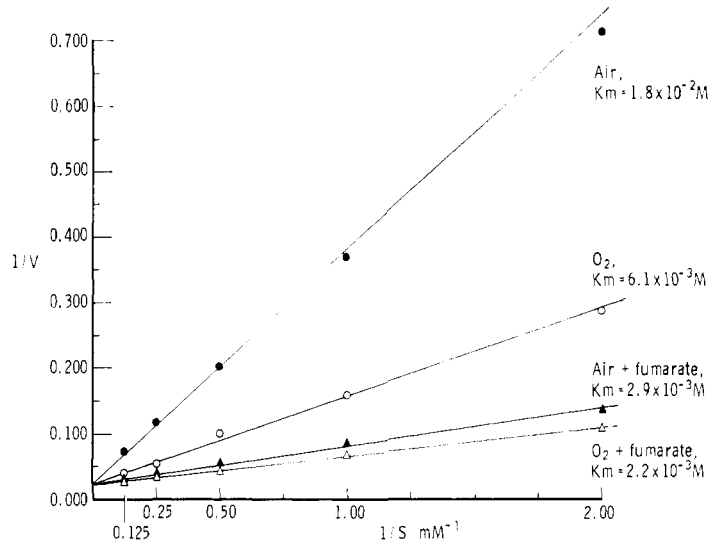

FIGURE 1: Double-reciprocal plots of velocity against dopamine concentration in the presence and absence of fumarate (10 μmoles). Experiments were performed under air or under hyperbaric oxygen in the presence of the following components: phosphate buffer (pH 6.4), 100 μmoles; ascorbic acid, 6 μmoles in 500 units of catalase (Sigma); and enzyme (0.1 mg of protein) in a total volume of 1 ml. The reaction mixture was incubated for 1 min at 37°. Velocity in arbitrary units, dopamine concentration millimolar.

Data Processing. Reciprocal velocities were first plotted graphically against the reciprocals of substrate concentration and the data were fitted to the rate question using the least-squares method (Wilkinson, 1961). All least-squares fits were performed by a digital computer using programs of Cleland (1963).

Results

The Effect of Acetate on the Enzymatic β Hydroxylation. The results presented in Table I show that in an incubation mixture at pH 5.5 or 6.4 in the presence of acetate buffer, fumarate only slightly stimulates the β-hydroxylation reaction. However, at pH 5.5 or 6.4 in the presence of phosphate buffer, the stimulation by fumarate is much more pronounced. It is evident that at both tested pH values fumarate can be replaced by acetate in stimulation of the enzymatic reaction. To obtain the same stimulation of dopamine β hydroxylation with acetate as with fumarate approximately a 20-fold higher concentration of the former is required.

The Effect of Increased Oxygen Tension on the Activity of the Reduced Enzyme Intermediate. To determine whether increased oxygen tension affects the enzymatic β hydroxylation by the reduced enzyme intermediate, the enzyme was exposed to 100% oxygen following its reduction by ascorbate or cysteine. The extent of the enzymatic β hydroxylation was determined under different experimental conditions. It is evident from the results presented in Table II that exposure to oxygen of the reduced enzyme intermediate in the absence of fumarate results in approximately a twofold increase in tyramine-^{3}H β hydroxylation. The data in Table II also show that the enzymatic activity of the reduced enzyme is

TABLE II: The Effect of Increased Oxygen Tension on the Activity of the Reduced Enzyme Intermediate.

Order of Additions			Extent of the Reaction[a]
Reduced enzyme	Air	Tyramine	1.20
Reduced enzyme	Oxygen	Tyramine	2.35
Reduced enzyme + fumarate	Air	Tyramine	3.00
Reduced enzyme + fumarate	Oxygen	Tyramine	3.10

[a] The extent of the reaction is expressed in millimicromoles of octopamine-^{3}H formed per milligram of protein. The enzyme was reduced with ascorbate as described under Methods. When the enzyme was reduced with cysteine the same results were obtained but the activities were 30–40% lower. The incubations were carried out in a mixture that contained the following components: reduced enzyme intermediate (0.1 mg of protein); potassium phosphate buffer (pH 6.4), 100 μmoles; catalase, 500 units (Sigma); and tyramine-^{3}H (uniformly labeled), 0.8 mμmole (500,000 cpm). The additions were made in the order shown in the table and the above time intervals were followed after each addition. After the addition of enzyme and phosphate buffer oxygen gas was delivered for 3 min prior to the addition of substrate and the incubations were carried out under oxygen for 5 min at room temperature. Fumarate (10 μmoles) was added to the reduced enzyme intermediate where indicated in the table prior to the oxygen exposure.

stimulated by fumarate to a greater extent than by the exposure to oxygen tension. In the presence of oxygen fumarate slightly stimulates the enzymatic activity, while in the presence of fumarate oxygen has no further effect on the enzymatic activity.

The Effect of Fumarate on Enzymatic β Hydroxylation at Various Concentrations of Substrate in Incubation Mixtures under 100% Oxygen or Air. A double-reciprocal plot of velocity against substrate concentrations in the presence of 10 μmoles of fumarate and in the absence of fumarate carried out in incubation mixtures under 100% oxygen or under air is shown in Figure 1. It is evident that fumarate and oxygen do not change the V_{max} but both lower the K_M. Fumarate as well as oxygen stimulate the enzymatic reaction at low substrate concentrations but have no effect at high substrate concentrations.

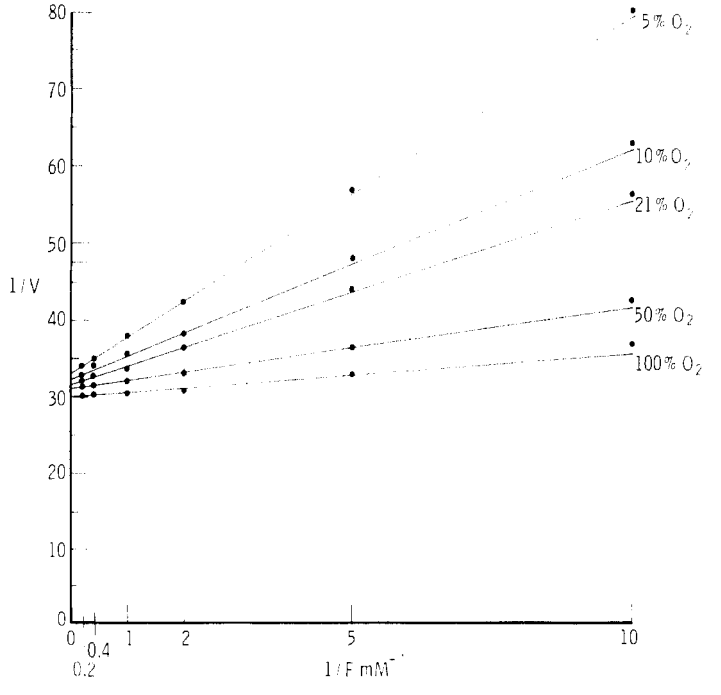

FIGURE 2: Double-reciprocal plots of velocity against fumarate concentration in the presence of different concentrations of oxygen. The experiments were performed in incubation mixtures with the same compositions as indicated in Figure 1, except tyramine (1 μmole) was used as a substrate. Velocity in micromoles per minute, fumarate concentration millimolar.

The Effect of Different Fumarate Concentrations on the Enzymatic β Hydroxylation at Different Concentrations of Oxygen. A double-reciprocal plot of velocity against fumarate concentration at a constant dopamine concentration in incubation mixtures exposed to different concentrations of oxygen is shown in Figure 2. It can be seen that at low fumarate concentrations increased oxygen concentration results in an increased rate of the β-hydroxylation reaction, while at high fumarate concentrations increased oxygen concentration has only a marginal effect.

Initial Velocity Analysis. THE EFFECTS OF ASCORBATE AT THE SATURATING CONCENTRATION OF FUMARATE. When tyramine was the variable substrate and ascorbate concentration was held constant at different concentrations from 2.5×10^{-4} to 2×10^{-3} M the double-reciprocal plots were linear and parallel to one another (Figure 3). A similar pattern was obtained with ascorbate as a variable substrate at different concentrations of tyramine (Figure 4). When the intercepts ($1/V$ at $1/S = 0$) from Figures 3 and 4 were replotted against the reciprocal concentration of ascorbate or tyramine (nonvaried substrate) the points formed a straight line (Figure 5). The slopes of Figures 3 and 4 do not vary significantly at different ascorbate or tyramine concentrations (Figure 5).

THE EFFECTS OF DIFFERENT CONCENTRATIONS OF OXYGEN ON THE ENZYMATIC β HYDROXYLATION IN THE ABSENCE OF FUMARATE. When dopamine was the variable substrate and oxygen concentration was held constant

at different concentrations (from 20 to 100%) the double-reciprocal plots were linear and intersected at one point on the ordinate (Figure 6). In Figure 7 the reciprocal of velocity is plotted against the concentration of oxygen for different concentrations of dopamine. The apparent K_M values of oxygen were calculated from Figure 7 and are listed in Table III. It can be seen that

TABLE III: The Apparent K_M Values of Oxygen at Different Dopamine Concentrations.

Dopamine Concn (M)	App K_M ($\times 10^{-3}$ M)
5×10^{-4}	1.14
1×10^{-3}	1.02
2×10^{-3}	0.75
3×10^{-3}	0.58
4×10^{-3}	0.55

the apparent K_M values of oxygen are lower at high dopamine concentrations. Thus at high dopamine concentrations lower oxygen concentrations are saturating the enzyme.

When the slopes from Figures 6 and 7 were replotted against the reciprocal concentration of oxygen or dopamine the points formed a straight line (Figure 8). It can also be seen from Figure 8 that the slope of the intercepts from Figure 6 is zero and that the intercept of the slopes from Figure 7 is also zero ($\phi_B = 0$).

Table IV lists the experimental values for the different kinetic coefficients and the relationships between the kinetic coefficients.

TABLE IV: Values of Various Kinetic Coefficients and Relationships between Kinetic Coefficients.[a]

ϕ_A	ϕ_B	ϕ_C	ϕ_{BC}
2.9 min	$\equiv 0$ min mM^{-1}	11.9 min	8.8 min mM
$\frac{k_2 + k_3}{k_1 k_3 E_0}$	$\frac{1}{k_4 E_0}$	$\frac{k_7 + k_8}{k_6 k_8 E_0}$	$\frac{k_5(k_7 + k_8)}{k_4 k_6 k_8 E_0}$

[a] ϕ_A value was calculated from Figure 5; ϕ_B, ϕ_C, and ϕ_{BC} were calculated from Figure 8 according to the method of Dalziel (1957). The data in the above table were obtained under the assumption that $S_B = O_2$ and S_C = RH (tyramine or dopamine). For S_B = RH and $S_C = O_2$, the following relationships were obtained:

$$\phi_B = \frac{k_7 + k_8}{k_6 k_8 E_0} \equiv 1 \text{ min mM}^{-1}$$

and

$$\phi_C = \frac{1}{k_4 E_0} = 11.9 \text{ min}$$

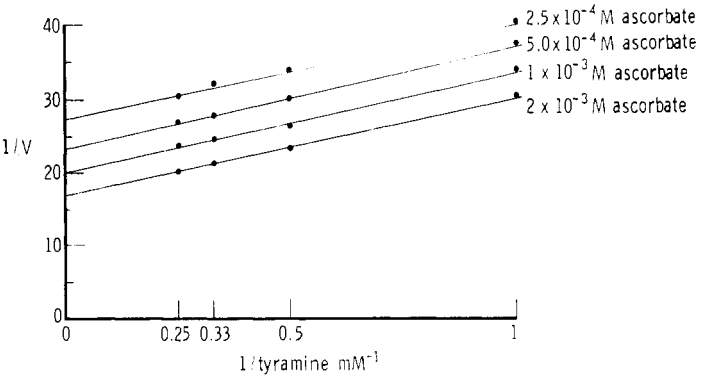

FIGURE 3: Initial velocity pattern with tyramine as the varied substrate at different concentrations of ascorbate in the presence of fumarate. Velocity in micromoles per minute, tyramine concentration millimolar.

Discussion

The finding that acetate can replace fumarate in the stimulation of dopamine β-hydroxylase activity shows that the stimulation is not specific for dicarboxylic acids. Fumarate and acetate can complex with Cu^{2+}, but the affinity of fumarate to Cu^{2+} is much greater than the affinity of acetate to Cu^{2+} (Martell, 1964). It is therefore noteworthy that a 20-fold higher concentration of acetate than of fumarate is required to obtain approximately the same stimulation of the enzymatic activity (See Table I). It is conceivable that the carboxylic acid combines with the copper of the hydroxylating enzyme and this might produce a conformational change which enhances the enzymatic activity. Furthermore, studies with the reduced enzyme intermediate suggest that fumarate facilitates the formation of the reduced enzyme–oxygen complex. This conclusion is also supported from the kinetic studies which show that fumarate stimulates the enzymatic activity only at low oxygen concentrations and that oxygen stimulates the enzymatic activity only at low fumarate concentrations.

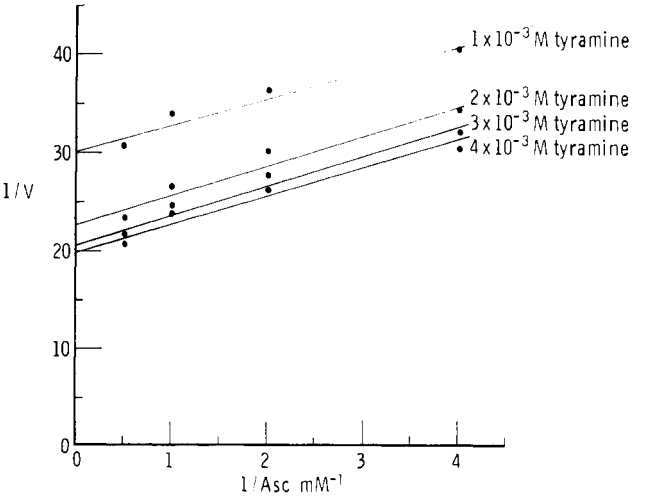

FIGURE 4: Initial velocity pattern with ascorbate as the varied substrate at different concentrations of tyramine in the presence of fumarate. Velocity in micromoles per minute, ascorbate concentration millimolar.

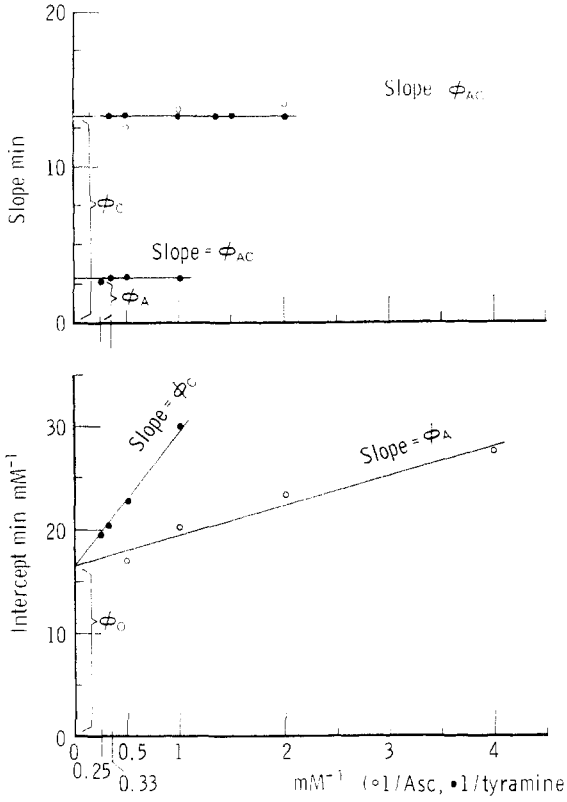

FIGURE 5: Secondary plots: variation of slopes and intercepts of Figure 3 (—○—) and of Figure 4 (—●—). The coefficients were calculated according to the method of Dalziel (1957) for a two-substrate system which fits

$$\frac{1}{V} = \phi_0 + \frac{\phi_A}{S_A} + \frac{\phi_C}{S_C} + \frac{\phi_{AC}}{S_A S_C}$$

where S_A = [ascorbate]; S_C = [tyramine].

From the electron paramagnetic resonance studies (Blumberg *et al.*, 1965; Goldstein, 1966), as well as from the chemical analysis of copper in purified dopamine β-hydroxylase (Friedman and Kaufman, 1965), it became obvious that copper is involved in the catalytic activity of the enzyme and that protein-bound copper undergoes cyclic reduction and oxidation during the over-all hydroxylation reaction. The present kinetic findings taken in conjunction with the previous investigations suggest Scheme I for the enzymatic β-hydroxylation reaction. In Scheme I E_1 is the oxidized form of the enzyme, E_2 is the reduced form of the enzyme, S_A is ascorbate, S_B is oxygen or RH (tyramine or dopamine), S_C is oxygen or RH (tyramine or dopamine), and ES_A, ES_B, and ES_BS_C are enzyme–substrate complexes.

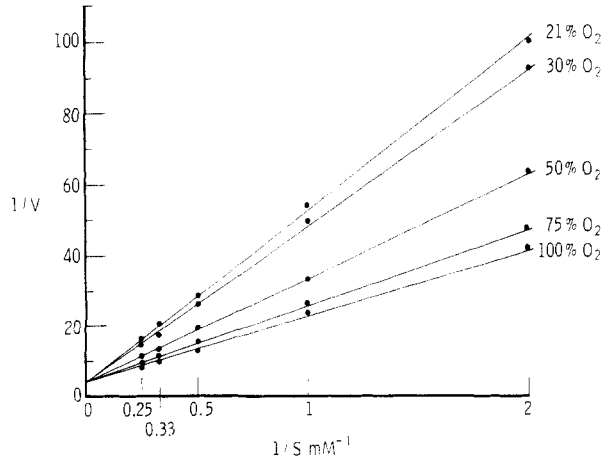

FIGURE 6: Initial velocity pattern with dopamine as the varied substrate at different concentrations of oxygen in the absence of fumarate. Velocity in micromoles per minute, dopamine concentration millimolar.

SCHEME I

$$E_1 + S_A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_A \xrightarrow{k_3} E_2$$

$$E_2 + S_B \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES_B$$

$$ES_B + S_C \underset{k_7}{\overset{k_6}{\rightleftharpoons}} ES_BS_C \xrightarrow{k_8} E_1 + \text{product}$$

The initial velocity data under the steady-state assumption for the mechanism presented in Scheme I is given by eq 1.

$$\frac{E_0}{V} = \frac{k_3 + k_8}{k_3 k_8} + \left(\frac{k_2 + k_3}{k_1 k_3} \times \frac{1}{S_A}\right) + \left(\frac{1}{k_4} \times \frac{1}{S_B}\right) + \left(\frac{k_7 + k_8}{k_6 k_8} \times \frac{1}{S_C}\right) + \left(\frac{k_5(k_7 + k_8)}{k_4 k_6 k_8} \times \frac{1}{S_B} \times \frac{1}{S_C}\right) \quad (1)$$

In Scheme I the over-all β-hydroxylation reaction can be considered in two parts. The first part involves the reaction of the enzyme with ascorbate while the second part involves the reaction of the reduced enzyme intermediate with oxygen and dopamine or tyramine. The present kinetic data can also be analyzed in two separate parts. In the first part we have analyzed the initial rates of the reaction at several concentrations of ascorbate and in the second part we have analyzed the initial rates of the reaction at different concentrations of oxygen. Oxygen can be omitted from the discussion of the first part since 20% oxygen is nearly saturating in the presence of high fumarate concentration. The parallel lines obtained in the double-reciprocal plots of initial rates (Figures 4 and 5) and the results obtained in Figure 5, $\phi_{AC} = 0$ (or $\phi_{AB} = 0$), imply that the binding of the first substrate (ascorbate) to the enzyme is followed by the release of a product (dehydroascorbate) from the enzyme before a second substrate reacts (Ping-Pong mechanism in the terminology of Cleland, 1963). This is also expressed in eq 1 by the absence of a term proportional to $1/S_AS_C$ or $1/S_AS_B$.

The kinetic studies in the presence of saturating concentrations of ascorbate and in the absence of fumarate

are presented in Figures 6 and 7. These data may shed some light on the second part of the reaction mechanism in which the substrates react with the reduced enzyme intermediate. The intersecting initial velocity patterns shown in Figure 6 suggest a sequential mechanism in which both substrates (dopamine or tyramine and oxygen) must add to the enzyme before a product is released.

The sequence of the addition of the two substrates oxygen and RH to the reduced enzyme intermediate could be analyzed under the following assumptions. If it is assumed that S_B is O_2 (second substrate) and S_C is RH (third substrate) then the rate constants (Table IV) could be defined as follows:

$$\phi_B = \frac{1}{E_0 k_4} = 0 \qquad (2)$$

$$\frac{\phi_{BC}}{\phi_C} = \frac{k_5}{k_4} = 0.7 \text{ mM} \qquad (3)$$

Equation 3 suggests that a substantial amount of the enzyme–O_2 complex is formed in the absence of RH and eq 2 shows that k_4 is large. Taken together, these two results indicate that the equilibrium of this step is rapid.

If one makes the alternative assumption that RH adds first (S_B = RH and S_C = O_2) then

$$\phi_B = \frac{k_7 + k_8}{E_0 k_6 k_8} = 0 \qquad (4)$$

which means that k_6 is large and O_2 would add rapidly to the enzyme–RH complex.

These data suggest that regardless of which of these two substrates (O_2 or RH) adds first, both steps are in a rapid equilibrium and the interconversion of the central ternary complexes most likely represents the rate-limiting step.

The present results have also shown that oxygen and fumarate decrease the K_M but do not affect the V_M. The V_M for high dopamine and ascorbate concentrations (assuming S_B = O_2 and S_C = RH) can be expressed by eq 5.

$$\frac{E_0}{V_M} = \frac{k_3 + k_8}{k_3 k_8} + \left(\frac{1}{k_4} \times \frac{1}{O_2}\right) \qquad (5)$$

Since k_4 is large the second term of eq 5 can be neglected. Thus it can be seen from eq 5 that fumarate and oxygen do not affect k_3 or k_8 since they do not change V_M (Figure 1).

The K_M for RH (at high ascorbate concentrations) can be expressed by eq 6.

$$K_M = \left(\frac{V_M}{E_0} \times \frac{k_7 + k_8}{k_6 k_8}\right)\left(1 + \frac{k_5}{k_4[O_2]}\right) \qquad (6)$$

Equation 6 suggests that the observed decrease of the K_M for RH by fumarate can be explained by a decrease of the k_5/k_4 ratio, an increase in k_6, or a decrease in k_7. Figure 2 shows that the V_{max} for fumarate does not change when [O_2] is varied. Since all experiments shown in Figure 2 were carried out at constant RH concentration, the effect of fumarate on the K_M for RH seems unlikely to be due to a change in k_6 or k_7, the rate constants which describe the interaction between RH and enzyme. Therefore our data imply that fumarate affects the K_M for RH by changing the ratio k_5/k_4, *i.e.*, fumarate shifts the O_2 binding equilibrium. Since fumarate is not involved in the formal binding reaction, the only way it can affect the equilibrium constant for binding is by altering the enzyme in some way such as a conformational change.

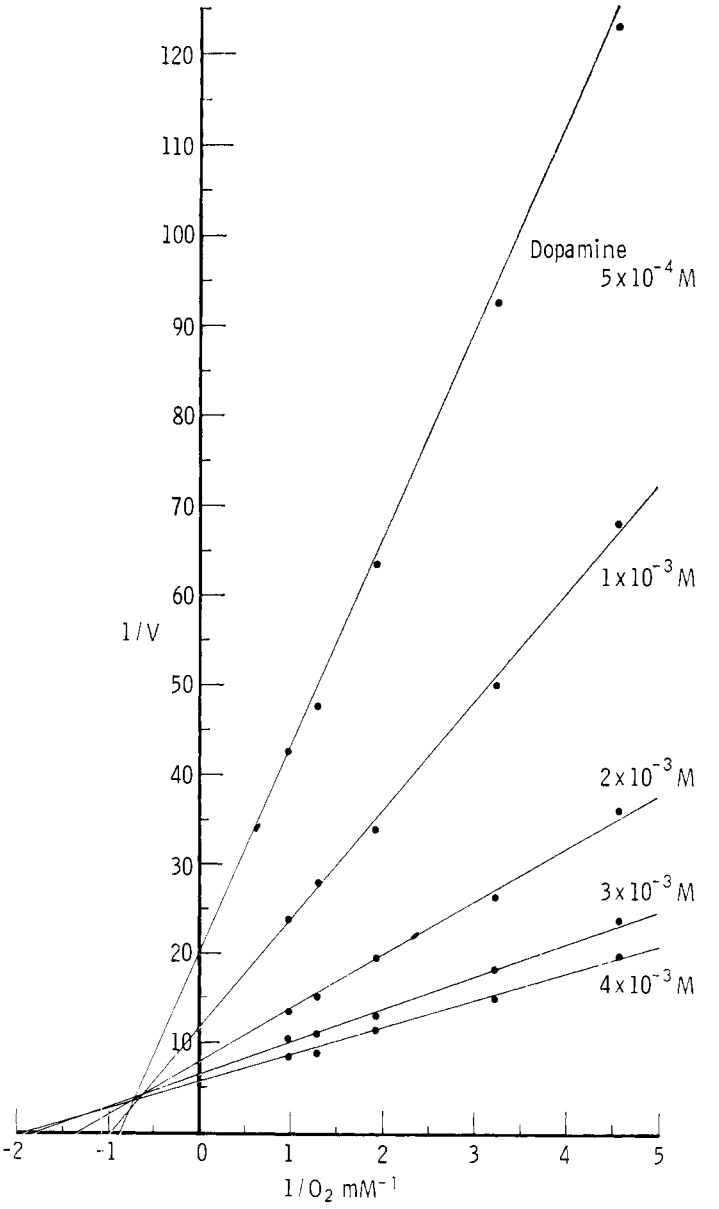

FIGURE 7: Initial velocity pattern with oxygen as the varied substrate at different concentrations of dopamine in the absence of fumarate. Velocity in micromoles per minute, oxygen concentration millimolar.

It has been suggested that fumarate favors the oxida-

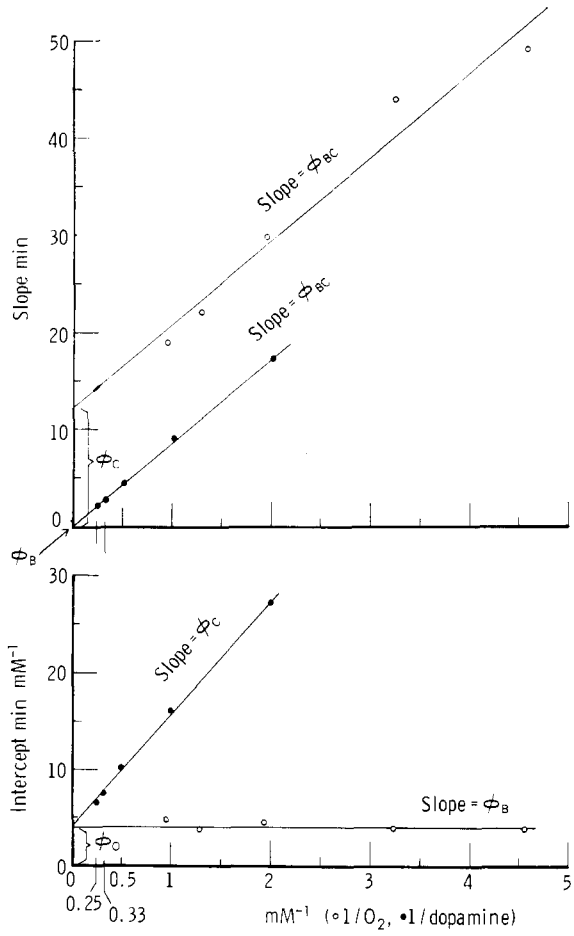

FIGURE 8: Secondary plots: variation of slopes and intercepts of Figure 6 (—○—) and of Figure 7 (—●—). The coefficients were calculated according to the method of Dalziel (1957) for a two-substrate system which fits

$$\frac{1}{V} = \phi_0 + \frac{\phi_B}{S_B} + \frac{\phi_C}{S_C} + \frac{\phi_{BC}}{S_B S_C}$$

where $S_B = [O_2]$; S_C = [tyramine].

tion of the enzyme Cu^+ to Cu^{2+} according to reaction b in Figure 9 (Friedman and Kaufman, 1966). Our findings are not incompatible with this possibility. It is also possible that fumarate, by displacing the equilibrium of reaction b (Figure 9), causes a shift in the equilibrium of reaction a to the right and therefore facilitates the formation of the reduced enzyme–oxygen complex.

The present data as well as the previous electron paramagnetic resonance studies (Blumberg *et al.*, 1965; Goldstein, 1966; Friedman and Kaufman, 1965) support a mechanism which is outlined in the scheme presented in Figure 9. In this scheme the first product dehydroascorbate leaves the enzyme before the addition of the subsequent substrates (Ping-Pong). The subsequent substrates (dopamine or tyramine and oxygen) add to the reduced enzyme intermediate before either product is released. The interconversion of the central ternary complexes seems to be the rate-limiting step in the over-all β-hydroxylation reaction.

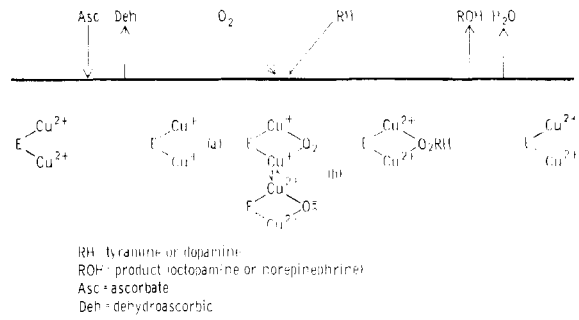

FIGURE 9: Schematic presentation of the enzymatic β hydroxylation.

Acknowledgment

The authors wish to thank Dr. W. Weber and Dr. S. Cohen from the Department of Pharmacology, New York University Medical Center, and Dr. Howard Devoe from the National Institute of Mental Health for their help in the evaluation of the kinetic data.

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