Inhibition of Bound Enzymes. III. Diffusion Enhanced Regulatory Effect with Substrate Inhibition†

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ABSTRACT: The kinetic behavior of a bound enzyme that is inhibited by the substrate shows multiple steady states in a particular range of the macroenvironmental concentration of the substrate, provided its diffusion to the enzyme is sufficiently slow. This phenomenon may play a role in the regulation of metabolic processes in the highly compartmented cellular interior when the substrate has to cross a membrane in order to reach the enzyme, thus encountering significant diffusion resistance. The theoretical analysis and the resulting regulatory schemes show that in such systems the enzymic activity can be dramatically changed by small variations of any of the following variables: the substrate concentration, the membrane permeability, or the kinetic constants of the enzyme. Therefore the regulatory properties of enzymes known to be inhibited by the substrate could be more effective in the cell than suggested by experiments in free solution.

In the two preceding studies on enzyme inhibition in heterogeneous systems, the effect of diffusional limitations has been investigated on the inhibition by substances that do not participate in the reaction (Engasser and Horvath, 1974a) and by the product (Engasser and Horvath, 1974b).

Substrate inhibition is another type of enzyme inhibition which is assumed to play an important role in the regulation of intracellular metabolism. The following theoretical analysis shows that substrate inhibition in the presence of diffusion resistances for the substrate can yield multiple steady states, which in turn drastically alter the regulatory effect of substrate inhibition.

Theoretical Analysis

Mathematical Model. In the simplest case the enzyme is attached to the surface of a membrane and the substrate diffuses from the macroenvironment to the surface and reacts in the microenvironment of the bound enzyme. The rate of reaction with substrate inhibition is expressed by the following equation (Webb, 1963)

$$v = \frac{V_{max}}{1 + (K_1/[S_0]) + ([S_0]/K_2)} \quad (1)$$

where $[S_0]$ is the substrate concentration in the microenvironment, and V_{max}, K_1, and K_2 are the appropriate kinetic parameters.

In the absence of electrical effects the rate of substrate transport from the macroenvironment is given by the product of the transport coefficient, h_s, and the driving force, $[S] - [S_0]$, that is the difference between the macro- and microenvironmental substrate concentrations. The transport coefficient can be, for instance, the permeability coefficient for the diffusion of the substrate through the membrane. At steady state the rate of substrate transport is equal to the rate of substrate consumption, so that

$$h_s([S] - [S_0]) = \frac{V_{max}}{1 + (K_1/[S_0]) + ([S_0]/K_2)} \quad (2)$$

Introducing the dimensionless substrate concentration

$$\beta = [S]/K_1 \quad (3)$$

the dimensionless substrate modulus

$$\mu = V_{max}/h_s K_1 \quad (4)$$

and the dimensionless inhibition constant

$$\kappa = K_1/K_2 \quad (5)$$

we can write eq 2 in dimensionless form as

$$\beta - \beta_0 = \frac{\mu}{1 + (1/\beta_0) + \kappa\beta_0} \quad (6)$$

The rearrangement of eq 6 yields for β_0 the following third-order equation

$$\kappa\beta_0^3 + (1 - \kappa\beta)\beta_0^2 + (1 + \mu - \beta)\beta_0 - \beta = 0 \quad (7)$$

According to eq 7 the interplay between substrate inhibition and external transport at a given substrate concentration, β, is characterized by the two parameters, κ and μ. The inhibition constant κ expresses the degree of inhibition by the substrate as shown in Figure 1. On the other hand, the substrate modulus, μ, which has been defined as the ratio of a first-order kinetic constant to the transport coefficient for the heterogeneous system, reflects the importance of diffusion resistances. In the limits of low and high values of μ, the effective reaction rate is controlled either by the kinetics of the reaction at the surface or by the rate of diffusion of the substrate to the surface (Engasser and Horvath, 1974a).

Graphical Presentation of the Results. Equation 7 has been numerically solved and the influence of the different parameters on the activity of the bound enzyme is illustrated graphically. First β_0 is plotted against β with μ as the parameter in Figure 2 in order to show the effect of diffusion limitations on the microenvironmental substrate concentration. It is seen that in the acceptable domain of $0 < \beta_0 < \beta$ three different β_0's satisfy eq 7 at certain values of β when μ is sufficiently high. The plots also show that the substrate depletion, that is, the devia-

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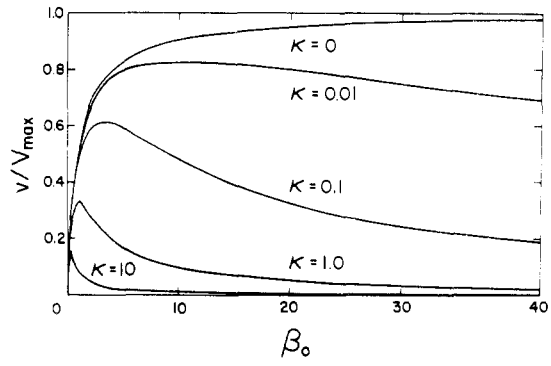

FIGURE 1: Graph illustrating the dependence of the normalized enzyme activity, v/V_{max}, on the dimensionless substrate concentration in the microenvironment, β_0, for different values of the inhibition constant, κ. The curves also represent the behavior of the enzyme in free solution when β_0 is the substrate concentration in the bulk.

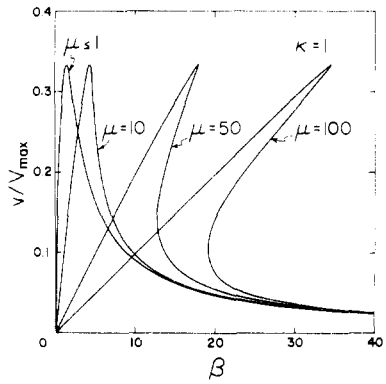

FIGURE 3: The effect of substrate inhibition in heterogeneous systems is illustrated by plots of the normalized activity, v/V_{max}, against the dimensionless substrate concentration in the macroenvironment, β. The curves represent different values of the substrate modulus, μ, which expresses the magnitude of the diffusion resistance for the substrate. At high values of μ, that is, with high diffusion resistances, three steady-state reaction rates are obtained at a given β in a particular concentration domain. The value of the inhibition constant, κ, is unity.

tion from the line representing the absence of diffusional resistances at $\mu \leq 1$, vanishes at high β's.

The values of β_0 shown in Figure 2 have been used to calculate the dimensionless enzyme activity, v/V_{max}, from eq 1, and the dependence of the activity on the substrate concentration in the macroenvironment is illustrated in Figure 3. When $\mu \leq 1$, the overall reaction is kinetically controlled and transport phenomena do not affect the enzyme behavior. At $\mu = 10$, substrate depletion occurs only at low β's and the substrate concentration that yields maximum enzyme activity is shifted toward higher values. With higher diffusion resistances, *e.g.*, when μ is equal to 50 and 100, the reaction rate is essentially diffusion and kinetically controlled at low and high substrate concentrations, respectively. The straight lines represent pseudo-first-order dependence which is characteristic for the diffusion controlled rate. At high β values all curves converge into that representing the kinetic control at $\mu \leq 1$. Figure 3 also shows that for a given system at high μ and intermediate β values there are three different steady-state rates.

In order to establish rigorously which of the possible rates can occur in practice a transient analysis of the problem is necessary (Aris, 1969). Simple steady-state considerations, however, can also be used to select the allowable rates as illustrated in Figure 4. Both the rate of the surface reaction at a given set of the kinetic parameters and the transport rate of the substrate at fixed h_s and β are plotted against the surface concentration of the substrate β_0. Since at steady state the two rates are equal the intersections of the two curves yield the appropriate concentrations in the microenvironment at the surface. Figure 4 shows the situation with three possible solutions: β_{0I}, β_{0II}, and β_{0III}.

The stability of the rate at β_{0I} is established by considering a small increase in the surface concentration at β_{0I}. The response of the system to this perturbation is readily inferred from Figure 4: the rate of reaction, that is, the rate of substrate consumption, increases, while the transport rate, that is, the rate of substrate supply to the surface, decreases. Since both effects tend to decrease β_0, the surface concentration returns to β_{0I}. Therefore the rate at β_{0I} is stable and can occur in practice. At β_{0II} the rate of both supply and consumption decreases when β_0 is slightly increased. The decrease in the reaction rate, however, is greater than that in the transport rate, as shown in Figure 4. Therefore, as a result of the perturbation, the surface concentration further increases and the system does not return to the rate at β_{0II}, which is consequently unstable. On the other hand, the rate at β_{0III} is found stable by similar considerations since the reaction rate decreases less than the transport rate upon perturbation. In view of this analysis only the highest and

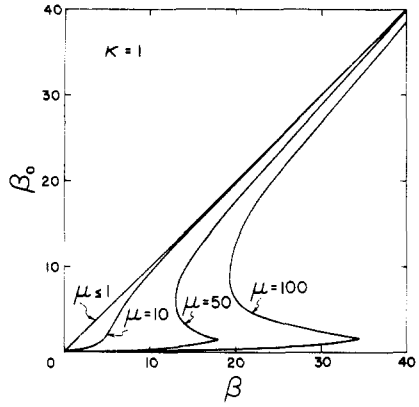

FIGURE 2: Plots of the microenvironmental substrate concentration, β_0, against the macroenvironmental concentration, β, with substrate inhibition in a heterogeneous system. The curves represent different values of the substrate modulus, μ, whose magnitude expresses the diffusion resistance for the substrate in the system. The value of the inhibition constant, κ, is unity.

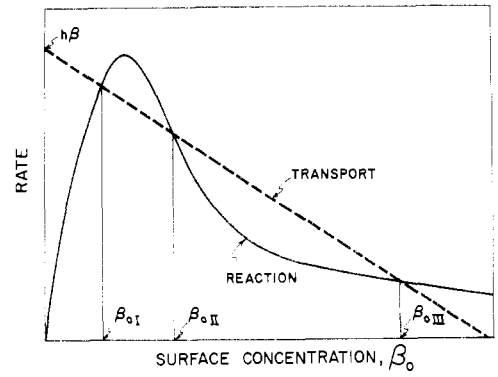

FIGURE 4: Schematic illustration of the rate of a substrate inhibited enzymic reaction and of substrate transport as a function of the microenvironmental substrate concentration at the surface, when three steady state rates of the overall reaction are possible at β_{0I}, β_{0II}, and β_{0III}. The graph can be used to show that only the rates obtained at β_{0I} and β_{0III} are stable, as described in the text.

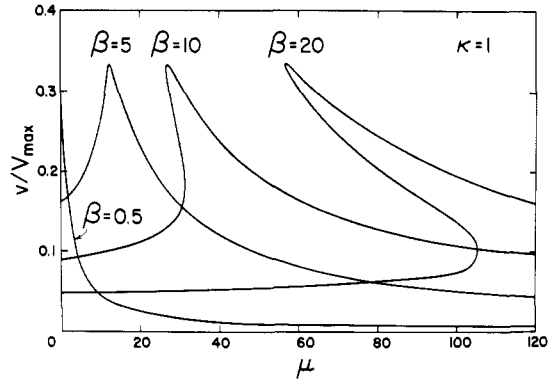

FIGURE 5: Graph illustrating the dependence of the normalized enzyme activity, v/V_{max}, with substrate inhibition in heterogeneous systems, on the substrate modulus, μ, which expresses the magnitude of diffusion resistances for the substrate. The different curves represent different values of the substrate concentration in the macroenvironment, β. The inhibition constant, κ, is unity. It is seen that at sufficiently high β's, for any given μ, three steady-state activities are possible in a particular range of the substrate modulus.

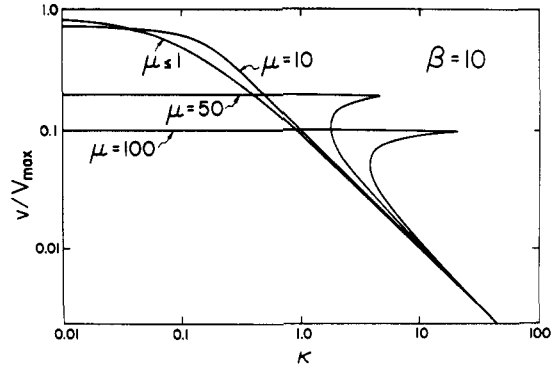

FIGURE 6: Plots of the normalized enzyme activity, v/V_{max}, in heterogeneous systems with substrate inhibition against the inhibition constant, κ. The dimensionless substrate concentration in the macroenvironment, β, is equal to 10. The different curves represent different values of the substrate modulus, μ, which expresses the magnitude of the diffusion resistance for the substrate. At sufficiently high values of μ and in a particular range of κ multiple steady-state activities of the enzyme are obtained at certain κ values.

lowest of the three steady-state rates shown in Figure 3 are stable. It is seen that the higher rate is obtained on the diffusion controlled branch of the curve whereas the lower rate is close to the kinetically controlled rate. Therefore the lower branch of the plots is termed the kinetically controlled branch.

Further insight is gained into the effect of diffusion resistances on substrate inhibition by plotting the normalized reaction rate against the substrate modulus, μ, as shown in Figure 5 for different values of β. As discussed before, the substrate concentration in the microenvironment decreases with respect to that in the macroenvironment when μ increases. In the absence of substrate inhibition, that is, when $\kappa = 0$, the depletion of the substrate always results in a decrease in the rate of reaction. When the substrate is an inhibitor, however, the enzyme activity increases with decreasing β_0, provided β_0 is higher than the substrate concentration at the maximum enzyme activity. As shown in Figure 1 this critical concentration is unity for $\kappa = 1$, the κ value used in Figure 5. In view of this dependence of the enzyme activity on the surface concentration the shape of the curves presented in Figure 5 for different macroenvironmental concentrations can easily be explained. When β is smaller than unity the enzymic activity always decreases with μ, as shown for $\beta = 0.5$. On the other hand, at β values greater than unity there is an increase of the activity with μ until β_0 reaches unity and the maximum activity is obtained. At sufficiently high macroenvironmental substrate concentrations, as seen for $\beta = 10$ and $\beta = 20$, three steady-state reaction rates are theoretically possible in a particular domain of the substrate modulus, but only two of them have practical relevance according to the analysis presented in Figure 4.

In order to assess the significance of the inhibition constant, κ, the normalized reaction rate has been plotted as a function of κ with $\beta = 10$ for different values of μ. As seen in Figure 6 at $\mu \leq 1$, *i.e.*, in the absence of diffusional resistances, the rate always decreases with increasing κ. At high values of the modulus the reaction rate is diffusion controlled, therefore, remains constant at small κ values, but reaches the kinetically controlled rate at high κ values. In an intermediate range of κ again two stable steady rates are obtained.

Discussion

The results of the numerical calculations, which have been presented graphically, enable us to postulate an enhanced regulatory function of substrate inhibition in the presence of diffusional limitations. Although this phenomenon may have wide ranging biological significance the existence of multiple steady states with substrate inhibition has not been observed experimentally as most studies were carried out in closed systems with the enzyme in free solution, that is, in the absence of diffusional effects. Since living cells are open systems close to steady state, the effect of substrate inhibition in the cellular milieu can be similar to the results of the theoretical study, provided there are significant diffusional resistances for the substrate. Therefore the role of the different variables in the possible control *via* substrate inhibition merits consideration.

The most plausible regulatory effect is expected from the variation of the substrate concentration in the macroenvironment. At sufficiently high diffusional resistances two different enzyme activities can be obtained for any given concentration in a particular range of substrate concentration, as has been shown in Figure 3. Under these conditions the direction of the change in the substrate concentration determines the actual rate of reaction as illustrated in Figure 7a. Therefore in a certain domain the dependence of the reaction rate on the substrate concentration shows hysteresis.

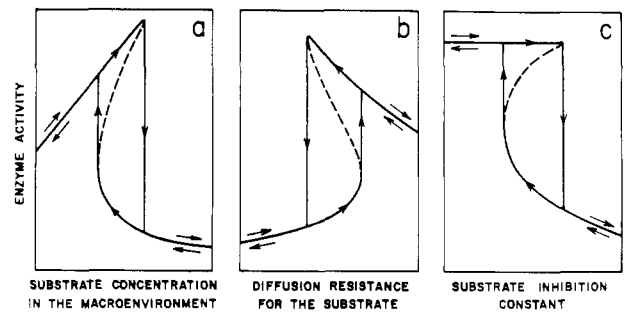

FIGURE 7: Possible regulatory schemes with substrate inhibition in heterogeneous enzyme systems. The variation of the enzyme activity is shown as a function of the substrate concentration in the macroenvironment (a), the diffusion resistance for the substrate (b), and the substrate inhibition constant (c). The solid lines represent the variation of the allowable enzymic activity with the corresponding variable. The arrows indicate the direction of change for the variable or the activity. It is seen that in each case small changes in the variable can bring about a dramatic decrease or increase in the enzymic activity as a result of sudden jumps from one allowed branch of the activity curve to the other. The broken lines indicate the theoretically possible but unstable, therefore, not allowable enzyme activities.

Beginning at low substrate concentrations the rate increases linearly with the concentration on the diffusion controlled branch, then drops suddenly and decreases further on the kinetically controlled branch. On the other hand, when an originally high substrate concentration decreases, the rate first increases slowly then jumps to a higher value on the diffusion controlled branch and subsequently further decreases. From the numerical calculations we obtain, for instance, that v/V_{max} decreases from 0.33 to 0.03 when β is slightly increased above its critical value of 34 at $\mu = 100$. Since at the discontinuous transitions from one branch of the curve to the other an infinitesimal concentration change causes a great increase or decrease in the reaction rate, the phenomenon may play a significant role in the regulation of the enzyme activity.

As illustrated in Figure 7b a similar effect can be observed with substrate inhibition when the magnitude of the diffusional resistance increases or decreases. It is recalled that changes in the kinetic parameters by some effectors or in the transport coefficient, *e.g.*, in the membrane permeability, alter the magnitude of diffusion limitations. Near the critical value of the diffusional resistance a small perturbation in any of these parameters can, therefore, dramatically affect the enzymic activity as shown in Figure 7b.

Another regulatory scheme is based on the variation of the inhibition constant by changes in the kinetic parameters. When substrate inhibition occurs at sufficiently high diffusion resistances the rate of reaction varies with the inhibition constant as depicted in Figure 7c. The abrupt transitions between the diffusionally and kinetically controlled branches of the enzyme activity are caused by slight changes in the inhibition constant. Therefore the enzyme activity can be drastically altered even by substances which only slightly affect the kinetic parameters.

Naturally, the relevance of the preceding theoretical analysis to biological systems depends on the existence of diffusional resistances for the substrate between the enzymic micro- and macroenvironment. The idea of multiple steady states with substrate inhibition has already been put forward (O'Neill *et al.*, 1971; Moo-Young and Kobayashi, 1972; Schuler *et al.*, 1973). Although the latter authors considered biological systems they rejected the possible existence of this phenomenon in living cells because the estimated diffusional resistances in cellular fluids were too low to affect the kinetics of the enzymic reaction. The short diffusion paths in the cell notwithstanding, significant transport resistances can be encountered by the substrate when it has to diffuse across a membrane to interact with the enzyme. In view of the highly compartmented structure of the cellular interior (Greville, 1969; Von Korff, 1972) and the very low membrane permeability coefficients even for small molecules (Davson and Danielli, 1952), the substrate modulus can be sufficiently high to make multiple steady states possible under such conditions (Engasser and Horvath, 1974a).

That the heterogeneity of the cellular milieu and diffusion resistances across membranes play a significant role in cellular events is also the fundamental concept of Mitchell's chemiosmotic theory of oxidative phosphorylation (Mitchell, 1961). Whereas orthodox theories take only chemical phenomena into account this hypothesis postulates that a transmembrane pH gradient is responsible for the enzymatic synthesis of adenosine 5′-triphosphate (ATP) in the mitochondria.

The theoretically found discontinuous transitions in enzyme activity due to small changes in the substrate concentration as well as in the magnitude of the kinetic parameters and membrane permeability, are not unlike certain "jumps" assumed to occur in living systems. Hahn *et al.* (1973) demonstrated the possibility of multiple steady states for enzymic reactions in the case when the membrane permeability for the substrate depends on the concentration of a metabolite. They suggested that the phenomenon can account for oscillations observed in cellular glycolysis. On the other hand, Blumenthal *et al.* (1970) have theoretically found that the membrane excitation following a small perturbation of the environment may be viewed as a "jump" between two stationary states of the membrane with two different diffusion resistances.

The results of the present investigation are generally applicable to the behavior of enzymes with restricted accessibility by the substrate. For instance, the enzyme may be located in the cytoplasm and the substrate is generated in an organelle so that it has to cross a membraneous barrier. The phosphorylation of fructose 6-phosphate to fructose 1,6-diphosphate by phosphofructokinase (PFK)[1] examplifies such a situation. PFK is located in the cytoplasm and ATP, the substrate which inhibits the enzyme at high concentrations, is mainly produced in the mitochondria. Thus, the substrate very likely encounters significant transport resistances in the heterogeneous cellular environment. On the basis of experiments in free solution, a major regulatory role in glucose metabolism has already been assigned to PFK due to its multiple allosteric properties (Passonneau and Lowry, 1964; Mansour, 1970). The results of this study, however, suggest that the regulation of the glycolytic pathway by PFK can be much more efficient and precise than inferred from experimental data. The physiological significance of possible regulatory schemes which take into account diffusional limitations and a discussion of the Pasteur effect will be presented in a forthcoming study.

Nevertheless the present results demonstrate that in heterogeneous systems substrate inhibition can produce dramatic changes in enzyme activity, when one of the pertinent parameters is slightly perturbed, thus, serving as an efficient regulatory mechanism. It is unlikely that nature would not use such a simple tool to control and trigger physiological processes.

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[1] Abbreviation used is: PFK, phosphofructokinase.

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Irreversible Inhibition of Aspartate Aminotransferase by 2-Amino-3-butenoic Acid†

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ABSTRACT: Pyridoxal-linked aspartate aminotransferase is irreversibly inactivated by 2-amino-3-butenoic acid. The mode of inhibition of this inhibitor requires that it be chemically converted into its active form by the target enzyme. The inhibitor has no effect on either the apoenzyme or the holoenzyme in the pyridoxamine form. A profound ultraviolet spectral change accompanies the inactivation process and ^{14}C inhibitor is covalently incorporated into the inactivated enzyme.

The microbial toxin *trans*-L-2-amino-4-methoxy-3-butenoic acid has been shown to be an irreversible inhibitor of soluble, pyridoxal linked, aspartate aminotransferase (Rando, 1974a). The inhibitor does not contain chemically reactive groups, as such, but is transformed into a reactive inhibitor by the target enzyme (Rando, 1974a). That is, aminomethoxybutenoic acid, a substrate for the enzyme, and one or several of the intermediates along the enzymatic pathway are sufficiently reactive to engage in a chemical reaction(s) with the enzyme. Thus, the enzyme is an agent of its own destruction.

The mechanism of action of this enzyme involves the initial sequence of steps shown in Scheme I (Hammes and Fasella, 1963). The transamination process is completed by the reversal of this sequence with α-ketoglutarate. The net reaction is: aspartate + α-ketoglutarate → oxaloacetate + glutamate. With 2-amino-4-methoxy-3-butenoic acid as the substrate, the following conversions can occur (Scheme II). Once the α C–H bond is enzymatically cleaved, the reactive intermediates can form. The highly reactive Michael acceptor (M) could be involved in the inactivation step by engaging in a reaction with an active-site Lewis base. However, the presence of the enol ether moiety renders the molecule potentially bifunctional in

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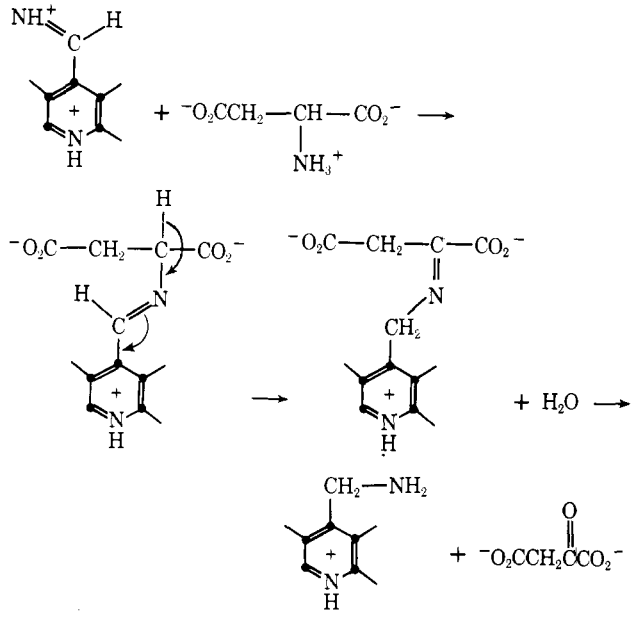

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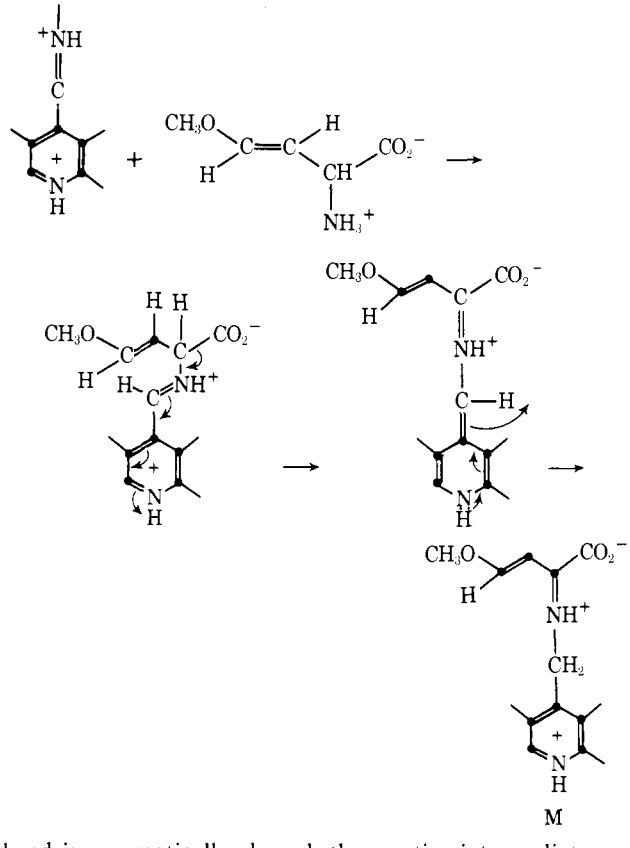

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