In the preceding paper[2] we alluded to the possibility of obtaining kinetic evidence for the order of combination of species by examination of the steady state rate law under certain conditions, *e.g.*, both reactants but only *one* product present initially. Underlying the foregoing analyses has been the assumption that this sequence is known, *i.e.*, appropriate species could be identified as A, B, etc. A variety of indirect studies has suggested that the pyridine nucleotide coenzyme combines first with the enzyme. We have made this assumption in calculating the quantities in Table II.

However, we are now in a position to examine the feasibility of the procedure outlined in paper II for determining the order of addition of substrates. We can refer to eq. 21b of that manuscript[2] for the steady state velocity for the reduction of DPN^+ by alcohol with the addition of the first product, Q, to dissociate from the enzyme. By choosing (A) $= K_{AB}/K_B$, (B) $= K_{AB}/K_A$ and (Q) $= K_Q$ and selecting representative values of these kinetic parameters from Table II, it can be seen that all terms in the expression for V_{AB}/v_f are of the order of unity with the exception of the last term. This last term, which equals $K_{AB}K_Q/K_{ABQ}$, is of the order of 10^{-2} to 10^{-1}. The above method of assessing the importance of terms in the steady state rate law is an obvious extension of the "rule of thumb" statement that for simple enzymatic reactions one must measure steady state velocities at concentrations somewhat higher than the Michaelis constant to evaluate this parameter. The estimate presented above does not encourage too sanguine a view concerning the possibility of a direct experimental determination of the parameter K_{ABQ}.

A substantially similar situation confronts us if we consider the corresponding term for the velocity of the reverse reaction, *i.e.*, the oxidation of DPNH by an aldehyde with B added initially. By an identical argument, it is the magnitude of the term $K_{QR}K_B/K_{BQR}$ compared to unity which is significant. Again values in the range 10^{-2}–10^{-1} are encountered.

The data are of course quite sparse, and any generalization is perhaps hasty. In passing, it should be noted that for ribitol dehydrogenase values of the order of unity are found for $K_{AB}K_Q/K_{ABQ}$ and $K_{QR}K_B/K_{BQR}$. However, even here a precision of better than 15% in the steady state velocity data would be necessary. It remains possible that particularly propitious experimental conditions for a given dehydrogenase system can be found where such studies will be reasonably practicable. More experimental data from conventional steady state studies must be available before any really unequivocal judgments can be formed.

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Multiple Intermediates in Steady-state Enzyme Kinetics. IV. The Steady State Kinetics of Isotopic Exchange in Enzyme-catalyzed Reactions

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Steady state isotopic exchange kinetics is examined for three different types of enzymatic reactions. The concentration dependence of the rate of interchange of label between various species is derived for mechanisms with an arbitrary number of intermediates. In some instances kinetic parameters not present in the steady state rate law for the reaction appear in these expressions. The relative magnitudes of the exchange rates provides a means of establishing the sequence of combination of substrates with the enzyme.

Introduction

Isotopic exchange experiments have been utilized to elucidate a number of interesting features of enzyme catalyzed reactions. The location of the position of bond scission in substrates for hydrolytic and transferase enzymes[2] provides an illustration of one type of study where isotopic labeling has been a useful tool. A somewhat different sort of study is the demonstration of exchange processes without any over-all enzymatic reaction as exemplified by the exchange between orthophosphate and glucose-1-phosphate catalyzed by sucrose phosphorylase in the absence of fructose.[3] Results of this nature on many systems have been widely interpreted as providing evidence for covalently bonded enzyme substrate complexes.[4]

In this paper we will be concerned with the kinetics of the exchange processes themselves. Recently Boyer[5] has analyzed the kinetics of exchange of label between reactant and product under the conditions that: (1) the enzyme reaction is readily reversible, (2) the unlabeled substrates are present at their equilibrium concentrations, (3) exchange takes place under steady state conditions for the labeled species, (4) exchange occurs *via* the *same* path as the over-all enzymatic reaction, and (5) the effect of isotopic substitution on the kinetic parameters is negligible. Boyer has evaluated the exchange rates for several special mechanisms with a specified number of intermediates. Application of these results has been made

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(2) M. Cohn, *J. Biol. Chem.*, **180**, 771 (1949).

(3) M. Doudoroff, H. A. Barker and W. Z. Hassid, *ibid.*, **168**, 725 (1947).

(4) D. E. Koshland, *Disc. Faraday Soc.*, **20**, 143 (1955).

(5) P. D. Boyer, *Arch. Biochem. Biophys.*, **82**, 387 (1959).

to data for some enzyme systems, notably glutamine synthetase and acetate thiokinase.[6a,b]

It should be remarked that an analysis of the transient state kinetics of isotopic exchange is also practicable in view of the fact that the differential equations governing these reactions become linear when unlabeled species are in large excess over the labeled species. Morales[7] has presented a preliminary report of an analysis of these kinetics for a particular reaction scheme under the same conditions as Boyer's study with the exception of the relaxation of condition 3 stated above. More recently Morales and co-workers[8] have considered in some detail the transient state for isotopic exchange for a reaction scheme representation of the class of transferase enzymes. Steady-state kinetics on the exchange of label between glutamate and α-ketoglutarate catalyzed by glutamic–aspartic transaminase, an enzyme of this type, have recently been reported.[9]

It is the purpose of this paper to present the results of a general steady-state analysis of exchange of isotopic label for reversible enzymatic reactions involving two reactants and two products. The conditions assumed are identical to Boyer's,[5] but the mechanisms used involve an arbitrary number of kinetically significant intermediates.

Dehydrogenase Reactions

Mechanisms with Ternary Complexes.—We consider a reaction scheme like eq. 2 of paper II.[10] Four types of exchange can be distinguished

$$A^* \rightleftarrows R^*,\ A^* \rightleftarrows Q^*,\ B^* \rightleftarrows R^*, \text{ and } B^* \rightleftarrows Q^*.$$

A* ⇄ R* Exchange.—In this case the label appears in *all* the intermediates. Hence, invoking the steady state assumption for the labeled intermediates, we have

$$-d(A^*)/dt = k_1(E)(A^*) - k_{-1}(X_1^*) = v^* \quad (1a)$$

$$-d(X_i^*)/dt = 0 \qquad (i = \alpha, \beta, \gamma) \quad (1b)$$

$$d(R^*)/dt = k_{(n+1)}(X_n^*) - k_{-(n+1)}(E)(R^*) \quad (1c)$$

where the starred quantities bear the isotopic label: v^* is the steady state velocity of exchange. Since it is assumed that all unlabeled material is at equilibrium, the following relations must apply for the unlabeled intermediates and free enzyme

$$(X_\alpha) = K_\alpha^0 (E)(A) \qquad (\alpha = 1 \ldots f-1) \quad (2a)$$

$$(X_\beta) = K_\beta^0 (E)(A)(B) \qquad (\beta = f \ldots g-1) \quad (2b)$$

$$(X_\gamma) = k^{n+1} (E)(R) \qquad (\gamma = g \ldots n) \quad (2c)$$

$$(E) = \frac{(E)_0}{1 + (A)\sum_{\alpha=1}^{f-1} K_\alpha^0 + (A)(B)\sum_{\beta=f}^{g-1} K_\beta^0 + (R)\sum_{\gamma=g}^{n} K_\gamma^{n+1}} \quad (2d)$$

where the definition of the K's has been given in reference 10. By use of the expression for equilibrium between the unlabeled reactants and products

$$(A)(B)/(Q)(R) = K_0^{n+1} = 1/K_{eq} = V_{QR}K_{AB}/V_{AB}K_{QR}$$

several alternative ways of writing eq. 2a–2d are available.

With the above assumptions v^*, the steady state velocity of exchange, must be given by eq. 3 of reference 10 where (A) is replaced by (A*) in the numerator and (R) is replaced by (R*). Invoking the conservation of isotopic label, $(A^*)_0 = (A^*) + (R^*)$, we get

$$v^* = \frac{\frac{V_{AB}}{K_{AB}}(A)(B)\left\{\left[\frac{1}{(A)} + \frac{1}{(R)}\right](A^*) - \left[\frac{(A^*)_0}{(R)}\right]\right\}}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(Q)}{K_Q} + \frac{(R)}{K_R} + \frac{(A)(B)}{K_{AB}} + \frac{(Q)(R)}{K_{QR}} + \frac{(A)(Q)}{K_{AQ}} + \frac{(B)(R)}{K_{BR}} + \frac{(A)(B)(Q)}{K_{ABQ}} + \frac{(B)(Q)(R)}{K_{BQR}}} \quad (3a)$$

In terms of the extent of attainment of isotopic equilibrium, F, where

$$F = ((A^*)_0 - (A^*))/((A^*)_0 - (A^*)_{eq})$$

eq. 3a becomes

$$\frac{dF}{dt} = \frac{(V_{AB}/K_{AB})(A)(B)}{D}\left[\frac{(A) + (R)}{(A)(R)}\right](1 - F) \quad (3b)$$

where D represents the denominator of eq. 3a. This integrates to give the familiar result[11]

$$\ln(1 - F) = -R_{AR}\left[\frac{(A) + (R)}{(A)(R)}\right]t \quad (4)$$

where the exchange rate R_{AR} is given by

$$R_{AR} = \frac{(V_{AB}/K_{AB})(A)(B)}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(Q)}{K_Q} + \frac{(R)}{K_R} + \frac{(A)(B)}{K_{AB}} + \frac{(Q)(R)}{K_{QR}} + \frac{(A)(Q)}{K_{AQ}} + \frac{(B)(R)}{K_{BR}} + \frac{(A)(B)(Q)}{K_{ABQ}} + \frac{(B)(Q)(R)}{K_{BQR}}} \quad (5)$$

Equation 4 is the general expression for the attainment of isotopic equilibrium, and its form is independent of the mechanism of equilibration.[12,13]

Our interest, however, centers on the actual *form* of the exchange rate whose dependence, *inter alia*, on the concentrations of unlabeled reactant species will be related to the mechanism of the reaction. It should be noted that for the special case in which the reaction scheme involves only two binary complexes and one ternary complex, *i.e.*, $f + 1 = g = n = 2$, eq. 5 reduces to an expression previously derived by Boyer.[14]

A* ⇄ Q* Exchange.—Turning to the case with the label being equilibrated between A* and Q*, eq. 2a applies but eq. 2b holds only for $(i = \alpha,\beta)$. There are no binary complexes designated by the subscript γ which are labeled. The concentrations of X are given by eq. 2c. For this case

$$d(Q^*)/dt = k_g(X_{g-1}^*) - k_{-g}(X_g)(Q^*) \quad (6)$$

(6) (a) P. D. Boyer, R. C. Mills and H. J. Fromm, *Arch. Biochem. Biophys.*, **81**, 249 (1959); (b) P. D. Boyer, Vth International Congress of Biochemistry, Moscow, August, 1961.

(7) M. F. Morales, 21st International Congress of Physiological Sciences, Buenos Aires, 1959.

(8) M. F. Morales, M. Horovitz and J. D. Botts, *Arch. Biochem. Biophys.*, in press.

(9) W. T. Jenkins and I. W. Sizer, *J. Biol. Chem.*, **234**, 1179 (1959).

(10) V. Bloomfield, L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **84**, 4367 (1962).

(11) See, for example, A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1961, p. 192.

(12) H. A. C. McKay, *Nature*, **142**, 997 (1938).

(13) G. M. Harris, *Trans. Faraday Soc.*, **37**, 716 (1951).

(14) The form of eq. 5 is to be preferred over that of eq. 56 of reference 5 which involves a negative sign and a denominator which is written as the product of two factors which could not be identified separately from experimental studies.

Utilizing eq. 2d and 2c for the unlabeled free enzyme and binary complexes and solving eq. 1a, 1b and 6 recursively yields a differential equation of the same form as eq. 3b but with a new exchange rate R_{AQ} given by

$$R_{AQ} = \frac{(V_{AB}/K_{AB})(A)(B)}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(R)}{K_R} + \frac{(B)(R)}{K_{BR}} + \frac{(B)(Q)(R)}{K_{BQR}} + \frac{(A)(B)}{K_{AQ}}} \quad (7)$$

All the kinetic parameters in eq. 7 are identical to those appearing in eq. 3 of reference 10 save for K_{AQ} which is characteristic of the $A^* \rightleftarrows Q^*$ *exchange.* In terms of the elementary rate and equilibrium constants, K_{AQ} can be expressed as

$$K_{AQ} = \frac{\sum_{s=f-1}^{g-1} \frac{K_0^s}{k_{(s+1)}}}{\sum_{\beta=f}^{g-1} \sum_{s=f-1}^{g-1} \frac{K_\beta^s}{k_{(s+1)}} + \sum_{\alpha=1}^{f-1} \sum_{s=0}^{f-2} \frac{K_\alpha^s}{k_{(s+1)}}} \quad (8)$$

B* ⇄ R* Exchange.—This is symmetrically related to the exchange between A and Q. The expression for R_{BR} follows from that of R_{AQ}, *mutatis mutandis.*

B* ⇄ Q* Exchange.—With the label interchanged between B and Q, the only labeled intermediates are ternary complexes, *i.e.*, X_β^*. In this situation, eq. 1b applies only for $i = \beta$ and also

$$-d(B^*)/dt = k_f(B^*)(X_{f-1}) - k_{-f}(X_f^*) \quad (9a)$$

$$d(Q^*)/dt = k_g(X_{g-1}^*) - k_{-g}(Q^*)(X_g) \quad (9b)$$

Solving eq. 1b, 9a and 9b recursively and employing eq. 2a and 2c for the unlabeled intermediates again yields a differential equation of the form of 3b. The new exchange rate R_{BQ} is given by

$$R_{BQ} = \frac{(V_{AB}/K_{AB})(A)(B)}{1 + \frac{(A)}{K_A} + \frac{(R)}{K_R} + \frac{(A)(B)}{K_{BQ}}} \quad (10)$$

where the new parameter K_{BQ} can be expressed by

$$K_{BQ} = \frac{1}{\sum_{\beta=f}^{g-1} K_\beta^0} \quad (11)$$

The expression for R_{BQ} has also been previously derived by Boyer[5] for the case in which there is just one of each kind of intermediate. However, there is a serious typographical error in Boyer's equation 57.

Two points should be noted about the exchange rates given in eq. 5, 7 and 10. Firstly R_{AQ} (and R_{BR}) and R_{BQ} involve new kinetic parameters not present in the over-all steady-state rate law. Hence, it is only the exchange rate R_{AR} which can be predicted in advance from steady state kinetics studies.

Secondly, examination of the denominators of eq. 5, 7 and 10 shows that

$$R_{BQ} > R_{AQ}, R_{BR} > R_{AR} \quad (12)$$

for a given set of equilibrium concentrations of the unlabeled species. This order is *not* dependent on any assumptions concerning the details of the isotopic exchange mechanisms. In principle then such studies should lead to the determination of the order of combination of substrates with the enzyme. Inequality 12 finds a ready rationalization in the expectation that isotopic exchange should be fastest between the "interior" species, *e.g.*, B and Q, as label is passed through a smaller number of intermediates.

Mechanisms Involving only Binary Complexes.—We refer to the reaction scheme given by eq. 22 of reference 10. The equilibrium concentrations of the unlabeled intermediates and free enzyme are given by

$$(X_\alpha) = K_\alpha^0 (E)(A) \quad \alpha = 1 \ldots f-1 \quad (13a)$$

$$(X_\beta) = K_{\beta^{n+1}} (E)(R) \quad \beta = f \ldots n \quad (13b)$$

$$(E) = \frac{(E)_0}{1 + (A)\sum_{\alpha=1}^{f-1} K_\alpha^0 + (R)\sum_{\beta=f}^{n} K_{\beta^{n+1}}} \quad (13c)$$

There are again four distinguishable exchange processes.

A* ⇄ R* Exchange.—Consideration of equations analogous to 1a–1c and employing eq. 13c for the concentration of free enzyme shows that the A*⇄R* rate of exchange is again governed by the over-all steady-state rate law. Hence, the exchange rate R_{AR} is expressable by

$$R_{AR} = \frac{(V_{AB}/K_{AB})(A)(B)}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(R)}{K_R} + \frac{(Q)}{K_Q} + \frac{(A)(B)}{K_{AB}} + \frac{(Q)(R)}{K_{QR}} + \frac{(A)(Q)}{K_{AQ}} + \frac{(B)(R)}{K_{BR}}} \quad (14)$$

A* ⇄ Q* Exchange.—For this reaction the only labeled intermediates are those designated by α, *i.e.*, X_α^*. Applying the steady state condition to the interconversion of these intermediates and the equilibrium conditions eq. 13b and 13c to X_β and E yields the customary differential equation with the exchange rate R_{AQ} expressed by

$$R_{AQ} = \frac{(V_{AB}/K_{AB})(A)(B)}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(R)}{K_R} + \frac{(B)(R)}{K_{BR}} + \frac{(A)(B)}{K_{AQ}}} \quad (15)$$

with

$$K_{AQ} = \frac{K_0^{f-1}/k_f}{\sum_{\alpha=1}^{f-1} \sum_{s=0}^{f-2} \frac{K^s{}_\alpha}{k_{(s+1)}}} \quad (16)$$

The exchange rate R_{BR} is symmetrically related to R_{AQ}.

B* ⇄ Q* Exchange.—As this exchange occurs in one step in the absence of ternary complexes, no labeled intermediates appear. The appropriate exchange rate R_{BQ} is given by

$$R_{BQ} = \frac{(V_{AB}/K_{AB})(A)(B)}{1 + \frac{(A)}{K_A} + \frac{(R)}{K_R}} \quad (17)$$

The relative order of magnitudes of the exchange rates is again given by relation 12.

Transaminase Reactions

For transaminase reactions with a reaction scheme described by eq. 28 of reference 10, only two types of exchange processes need be considered, A*⇄R* and A*⇄Q*. The B*⇄R* exchange is symmetrically related to the latter. The unlabeled

intermediates and free enzyme are at equilibrium concentrations given by the expressions

$$(X_\alpha) = K_\alpha^0 (E)(A) \quad \alpha = 1 \ldots f-1 \quad (18a)$$

$$(X_\beta) = K_\beta^0 \frac{(E)(A)}{(Q)} \quad \beta = f \ldots g-1 \quad (18b)$$

$$(X_\gamma) = K_\gamma^{n+1} (E)(R) \quad \gamma = g \ldots n \quad (18c)$$

$$(E) = \frac{(E)_0}{1 + (A)\sum_{\alpha=1}^{f-1} K_\alpha^0 + \frac{(A)}{(Q)}\sum_{B=f}^{g-1} K_\beta^0 + (R)\sum_{\gamma=g}^{n} K_\gamma^{n+1}} \quad (18d)$$

A* ⇄ R* Exchange.—Analogously to the previously discussed mechanisms, the A* ⇄ R* exchange rate is obtainable from the over-all steady-state rate law, *i.e.*

$$R_{AR} = \frac{(V_{AB}/K_{AB})(A)(B)}{\frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(Q)}{K_Q} + \frac{(R)}{K_R} + \frac{(A)(B)}{K_{AB}} + \frac{(Q)(R)}{K_{QR}} + \frac{(A)(Q)}{K_{AQ}} + \frac{(B)(R)}{K_{BR}} + \frac{(B)(Q)}{K_{BQ}} + \frac{(A)(B)(Q)}{K_{ABQ}} + \frac{(B)(Q)(R)}{K_{BQR}}} \quad (19)$$

A* ⇄ Q* Exchange.—When the isotope is exchanged between A* and Q*, the only labeled intermediates are designated by the subscript α, *i.e.*, X^*_α. Applying the steady state condition to the rate of change of concentration of these intermediates and the equilibrium relations 18b–18d to the other intermediates and free enzyme yields a differential equation for the approach to isotopic equilibrium with an exchange rate R_{AQ} defined where

$$R_{AQ} = \frac{(V_{AB}/K_{AB})(A)(B)}{\frac{(B)}{K_B} + \frac{(R)}{K_R} + \frac{(B)(R)}{K_{BR}} + \frac{(A)(B)}{K_{AQ}}} \quad (20)$$

$$K_{AQ} = \frac{1}{\sum_{\alpha=1}^{f-1}\sum_{s=0}^{f-1} \frac{K^s_\alpha}{k_{(s+1)}}} \quad (21)$$

Again it can be seen that

$$R_{AQ}, R_{BR} > R_{AR} \quad (22)$$

As a consequence of the application of the steady state condition in the above treatment, the information derivable from the exchange rates is in the main of the same type as that accessible from ordinary steady state kinetic studies. The appearance of certain new kinetic parameters in the expressions for some of the exchange rates does provide some ancillary information, however. Studies of exchange kinetics *alone* are less fruitful than ordinary steady-state kinetic studies in view of the fact that the latitude provided by the variation of the concentrations of the unlabeled species is restricted by the condition of over-all thermodynamic equilibrium.

The chief recommendation of such studies seems to lie in the opportunity to establish the sequence of combination of substrates with the enzyme through the anticipated inequalities in the exchange rates. This is particularly apparent for the dehydrogenase systems without ternary complexes where it has been pointed out previously that steady-state kinetic studies are insufficient to establish this order.[10]

The inequalities in exchange rates plainly derive only from the sequence of reaction of the various substrates and are in no way contingent on the number and steady-state concentrations of intermediates. Consequently, the contention that these inequalities provide a basis for *any* inferences about "rate-limiting steps"[5,6a,b] is seen to be without foundation.

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Measurement of the Stability of Metachromatic Compounds[1]

BY MEDINI KANTA PAL[2] AND MAXWELL SCHUBERT

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Two methods, ultracentrifuging and adsorption to $CaHPO_4$, which had previously been shown to remove from solution the metachromatic compound of chondroitin sulfate and methylene blue, have been extended to study metachromatic compounds of a wider variety of dyes and polyanions. Though in most cases the two methods are equivalent, the centrifugal method was found more generally effective than the adsorption method. An extension of this study to metachromatic solutions in mixtures of ethanol and water led to the development of a new method that makes possible a quantitative estimate of the relative stabilities of different metachromatic compounds in terms of the concentration of ethanol or urea that brings about their destruction. Results with ethanol and urea are nearly equivalent in twelve combinations of dye and polyanion and show it is possible to set up a single scale of stabilities of metachromatic compounds containing different dyes and polyanions. On such a scale it becomes clear that not only do polyanions differ markedly among themselves in the stabilities of the compounds they form with a single dye, but dyes also differ correspondingly. With any particular polyanion, methylene blue and crystal violet form much less stable metachromatic compounds than do toluidine blue and acridine orange.

In earlier work two independent methods were developed which showed that the metachromatic color produced in a solution containing chondroitin sulfate and methylene blue was mainly due to the formation of a single compound consisting of equiva-

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