Isotope Exchange Probes and Enzyme Mechanisms

PAUL D. BOYER

Molecular Biology Institute and Department of Chemistry, University of California, Los Angeles, California 90024

Received January 24, 1977

Through isotopes the physicist provided the chemist and biologist with a tool of enormous power. Among the myriad applications, one increasingly appreciated is use of isotope-exchange measurements to reveal detailed steps in enzyme-catalyzed reactions. My present purpose is to outline, with a historical perspective, some useful approaches based on isotope-exchange measurements. However, I will not discuss H exchange, an important area frequently considered in its own right. Also excluded from consideration are isotope effects on reaction rates.

Isotopes provide a convenient and sometimes the only way to measure the nature and rate of replacement of atoms or groups in one molecule by corresponding atoms or groups from another molecule. The occurrence of exchange must involve the formation and cleavage of covalent bonds at the catalytic site. By employment of isotopes, the occurrence and the rates of covalent steps, as well as of other catalytic steps affecting the covalent steps, can be probed.

Examples of catalytic events revealed by exchange measurements include the formation of intermediates in which substrates are covalently bound to enzymes, preferential orders of binding and release of substrates, and information about the relative rates of reactant release and chemical interconversion at catalytic sites. A current application of particular interest to me is oxygen exchange with carboxyl and phosphoryl groups. Such studies allow deductions to be made about the partitioning of catalytic intermediates between the forward and reverse catalytic pathways.

Before discussion of these and other applications, some background material is given on types of enzymic exchange reactions and their theoretical basis.

Types of Enzymic Exchange Reactions

Most isotopic exchanges catalyzed by enzymes can be grouped into two main types dependent upon whether or not all the substrates necessary for the occurrence of overall reactions (forward and/or reverse) leading to net chemical change[1] are present. Each of these main types can be further subdivided, as noted below.

Type 1: Exchange without All Substrates Present. In such instances, no change in reactant concentration can occur, and the reactions responsible for exchanges must be at equilibrium. Such exchange reactions are representative of what physical organic chemists call degenerate reacions—reactions in which the product is chemically identical with the reactant.

Three prominent subtypes warrant mention. In one, hydrolases catalyze incorporation of oxygen from water into one product of the hydrolytic cleavage in the absence of the other product. Oxygen exchange occurs with the product that gains an oxygen from water in net hydrolysis. An early example was the exchange of an amino acid carboxyl group with water, catalyzed by the proteolytic enzyme chrymotrypsin.[2] Another is exchange of inorganic phosphate oxygens catalyzed by alkaline phosphatase.[3] Such observations revealed a significant catalytic capacity of these hydrolases not previously recognized.

A second and frequently observed subtype of exchange reaction in this category occurs with enzymes that catalyze group-transfer reactions such as depicted in eq 1, where group A is transferred from B to C. A

$$\text{A-B} + \text{C} \rightleftharpoons \text{A-C} + \text{B} \qquad (1)$$

valuable distinction arises between those enzyme catalyzing a direct transfer of group A to C and those catalyzing indirect transfer through formation of a covalent E-A intermediate. If E-A is formed from E and A–B independent of the presence of C, addition of E, A–B, and B* will give a B* $\rightleftharpoons$ E–B exchange. (B* represents an isotopically labeled molecule.) Similarly, with A–C, C*, and enzyme, a C* $\rightleftharpoons$ A–C exchange would result. As an example, such exchanges are observed with phosphoryl transfers by nucleoside diphosphokinases, and involve the intermediate formation of a phosphoryl enzyme[4,5] as depicted in eq 2. In eq

$$\text{ATP} + \text{enzyme} \rightleftharpoons \text{enzyme-P} + \text{ADP} \qquad (2a)$$

$$\text{enzyme-P} + \text{GDP} \rightleftharpoons \text{enzyme} + \text{GTP} \qquad (2b)$$

$$\text{ATP} + \text{GDP} \rightleftharpoons \text{ADP} + \text{GTP} \qquad (2)$$

2a and 2b, enzyme-P represents a phosphoryl derivative of an enzyme; for this reaction it is a phosphorylated side chain of a histidine residue at the catalytic site. With an enzyme catalyzing the overall net reaction of eq 2, an ADP $\rightleftharpoons$ ATP exchange can occur in the absence of GTP and GDP by reversal of the first step, eq 2a. Similarly a GDP $\rightleftharpoons$ GTP exchange in the absence of ADP and ATP can occur by reversal of the second step, eq 2b.

A third subtype in this category often occurs when only two substrates are present for an enzyme that catalyzes a net reaction involving three substrates. Examples are found with some synthetases, such as the

Paul D. Boyer is Director of the Molecular Biology Institute and Professor of Chemistry, University of California, Los Angeles. He was born in Provo, Utah, and attended Brigham Young University for his undergraduate degree. After he received his Ph.D. from the University of Wisconsin in 1943, he spent 2 years at Stanford University as Instructor and Research Associate before moving to the University of Minnesota, where he remained until he joined the UCLA faculty in 1963. Professor Boyer is a member of the National Academy of Sciences. His research interests include enzyme mechanisms, oxidative phosphorylation, active transport, and other energy-linked membrane processes.

(1) By net chemical reaction is meant production of many molecules of product per mole of enzyme. A reaction stoichiometric with the enzyme, e.g., A–B + enzyme $\rightleftharpoons$ A–enzyme + B, is not considered to represent net chemical reaction.

(2) Stein, S. S., and Koshland, D. E. Jr., *Arch. Biochem. Biophys.* **39**, 229 (1952).

(3) Sprinson, D. B., and Rittenberg, D., *Nature (London)* **167**, 484 (1951).

(4) Mourad, N., and Parks, R. E., Jr., *J. Biol. Chem.* **241**, 3838 (1966).

(5) Uhr, M. L., Marcus, F., and Morrison, J. R., *J. Biol. Chem.* **241**, 5428 (1966).

0001-4842/78/0111-0218$01.00/0

pyrophosphate (PP_i) ⇌ ATP exchange catalyzed by amino acid activating enzymes in the presence of enzyme, ATP, and amino acid but in the absence of the acceptor RNA (tRNA).[6] Exchange occurs by the dynamic reversal of the reaction of eq 3. The amin-

$$\text{ATP} + \text{amino acid} \rightleftharpoons \text{aminoacyl adenylate} + PP_i \quad (3)$$

oacyl adenylate remains firmly bound to the enzyme, preventing net catalysis; in the presence of tRNA the aminoacyl-tRNA used in protein synthesis is formed.

Type 2: Exchanges with All Substrates Present. Two subtypes may be distinguished. The first includes exchanges that occur under equilibrium or quasi-equilibrium conditions as a result of dynamic reversal of the overall reaction. At equilibrium, although there is no net change in reactant concentrations, the forward and reverse reactions nevertheless occur at equal rates, and often rapidly. This is a very broad subtype in which fall many of the examples to be discussed later. All catalytic reactions at equilibrium must show exchange between reactants and products. The prominence of this subtype in experimental studies stems from theoretical and experimental advantages noted below.

An illustrative early example of such equilibrium exchange is the incorporation of glyceraldehyde 3-phosphate, in preference to dihydroxyacetone phosphate, into fructose 1,6-bisphosphate catalyzed by the glycolytic enzyme aldolase.[7] This occurs by reversal of the first step[4] of the sequence depicted by eq 4, prior

$$\text{fructose 1,6-bisphosphate} + H_2N\text{-enzyme} \rightleftharpoons RR'C{=}N\text{-enzyme} + \text{glyceraldehyde 3-phosphate} + HOH \quad (4a)$$

$$HOH + RR'C{=}N\text{-enzyme} \rightleftharpoons H_2N\text{-enzyme} + \text{dihydroxyacetone phosphate} \quad (4b)$$

$$\text{fructose 1,6-bisphosphate} \rightleftharpoons \text{glyceraldehyde 3-phosphate} + \text{dihydroxyacetone phosphate} \quad (4)$$

to occurrence of the second step (eq 4b); in eq 4, RR'C=N-enzyme represents a Schiff base formed with the enzyme and dihydroxyacetone phosphate. The relative rates of incorporation of the two isotopically labeled triose phosphates, added to the system at equilibrium, into fructose 1,6-bisphosphate are governed by the partitioning of the Schiff base between its two alternative reactions. If step 4b were very rapid compared to 4a, equal rates of incorporation of the labeled triose phosphates into fructose 1,6-bisphosphate would be observed.

The second prominent subtype includes exchanges accompanying net chemical change. Such exchanges are observable when some early step of the overall reaction is reversible, and when reversal of the step results in exchange. Exchange may also be observed when a sufficient amount of one or more products is present for some overall reversal to occur. The exchange may allow estimation of the partitioning of an intermediate between return to reactant and conversion to product. An example is the incorporation of oxygen but not hydrogen from water into malate, catalyzed by fumarase, during net fumarate formation from malate[8] (eq 5); the catalytic mechanism must account for the

$$\underset{\text{L-malate}}{{}^-OOCCHOH{-}CH_2COO^-} \rightarrow \underset{\text{fumarate}}{{}^-OOCCH{=}CHCOO^-} + HOH \quad (5)$$

replacement of the C-2 hydroxyl without replacement of a C-3 hydrogen.

Theory and Methodology

Most applications of isotope exchange measurements to enzyme reactions, and all approaches mentioned in this Account, are made under conditions such that measurements of isotope exchange give a precise or very nearly precise measure of the rate of chemical change or changes that occur in a time-independent equilibrium or steady state. Derivation of appropriate rate equations is simplified by assumptions like those made in most treatments of initial velocity enzyme kinetics, namely that total enzyme concentration is much less than the concentration of any substrate, and than any initial transients prior to establishment of a steady-state rate of chemical and isotope exchange can be ignored.

Many applications of isotope exchange allow important conclusions without any necessity to formulate the governing rate laws. The development of a more formal basis for isotope exchange measurements in enzyme catalysis and a better recognition of some of the power and limitations stem to a large extent from my 1959 paper, "Uses and Limitations of Measurements of Rates of Isotopic Exchange and Incorporation in Enzyme Catalyzed Reactions".[9] This paper developed rate equations governing exchange rates at equilibrium for several common types of enzyme reactions. It provided a theoretical base for the use of exchange measurements for determining whether substrates were bound in a random or ordered fashion as well as the relative rates of substrate binding and release steps compared to substrate interconversion steps.

The kinds of expressions derived are illustrated by that for the relatively simple case of reaction with two enzyme–substrate intermediates (eq 6). The expression

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_b}{\overset{k_a}{\rightleftharpoons}} EB \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} E + B \quad (6)$$

for R, the rate of conversion of A to B, or of B to A, at equilibrium is given by eq 7, where K_2 is k_{-2}/k_2, the

$$R = \frac{V_f\left(1 + \dfrac{k_{-2} + k_b}{k_a}\right)}{\left(1 + \dfrac{k_{-2}k_{-1} + k_{-2}k_a}{k_{-1}k_b}\right)\left(1 + \dfrac{k_b}{k_a} + \dfrac{K_2}{(B)}\right)} \quad (7)$$

dissociation constant for (EB), and V_f is the initial velocity of net reaction with excess A and no B present. Equation 7 simplifies to the form of eq 8, and the

$$R = \frac{\text{a constant}}{1 + \dfrac{k_b}{k_a} + \dfrac{K_{x(B)}}{(B)}} \quad (8)$$

slope/intercept of a plot of $1/R$ vs. $1/(B)$ will give the value of the exchange constant for B, $K_{X(B)}$, which is equal to $K_2/(1 + k_b/k_a)$, or, from a plot of $1/R$ vs. $1/(A)$, a value of $K_{x(A)}$ equal to $K_1/(1 + k_a/k_b)$.

The approach I used was amplified by Silverstein and Boyer.[10] More erudite and complete formulations were

(6) Hoagland, M. B., Keller, E. B., and Zamecnik, P. C., *J. Biol. Chem.* **218**, 345 (1956).
(7) Rose, I. W., *Proc. Natl. Acad. Sci. U.S.A.* **44**, 10 (1958).
(8) Hansen, J. N., Dinovo, E. C., and Boyer, P. D., *J. Biol. Chem.* **244**, 6270 (1969).
(9) Boyer, P. D., *Arch. Biochem. Biophys.* **82**, 387 (1959).
(10) Boyer, P. D., and Silverstein, E., *Acta Chem. Scand.* **17**, S195 (1963).

developed subsequently by others. Morales et al. presented a generalized equation for isotope distribution with time, including the initial transients prior to steady-state exchange,[11] but suggested that the earlier steady-state formulations[9] were not correct. However, as noted by Darvey,[12] with proper assumptions, the two approaches are in harmony. Other formulations for equilibrium exchange were presented by Alberty et al.[13] Subsequently, Cleland,[14] Cole and Schimmel,[15] Britton,[16] and Plowman[17] presented approaches for deriving isotope exchange catalysis when net reaction occurs. With appropriate conditions, such approaches simplify to equations for exchange at equilibrium. If equilibrium reaction rates are to be studied, starting with equilibrium conditions prior to equation development is often simpler.

Several suggestions have been made for aiding rate law derivations, including extensions of the King–Altman matrix approach,[18] a computerized equation development based on a connection matrix representation,[19] and equation development based on analogy to electrical circuits.[20] In my laboratory a simple approach based on partitioning of reactants among pathways was found useful.[8] Independently Yagil and Hoberman presented a more complete and generalized partition approach,[21] and I concur with Wong's evaluation[22] —"that (the method) devised by Yagil and Hoberman is particularly elegant in its simplicity". A recent useful extension of partition analysis has been made by Cleland,[23] but overlooks reference to the contribution of Yagil and Hoberman.

Some salient characteristics of equilibrium-exchange measurements merit mention at this stage. Concentration dependencies of exchange rates can lead to evaluation of exchange constants for substrates analogous to Michaelis constants for initial velocity studies. Such constants are closely related to (or in simple systems as first noted by Vaslow[24] equivalent to) enzyme–substrate dissociation constants.[7,10,25] Darvey[25] emphasizes that equilibrium isotope exchange measurements provide an alternate to direct binding measurements for determination of dissociation constants, and, further, that such determinations can be made with enzyme concentrations similar to those used in initial velocity measurements.

The maximum exchange rates obtainable at equilibrium (R_{max}) are expected to be less than the slower of the maximum velocity for the forward (V_f) or reverse reaction (V_r). This is readily evident because when all substrates are present a smaller fraction of the total enzyme will be participating in reaction with any one substrate. For a simple A ⇌ B reaction, the relationship is $R_{max} = V_f V_r/(V_f + V_r)$,[26] a relation that has been confirmed experimentally for adenylate kinase[27] and phosphorylase.[28]

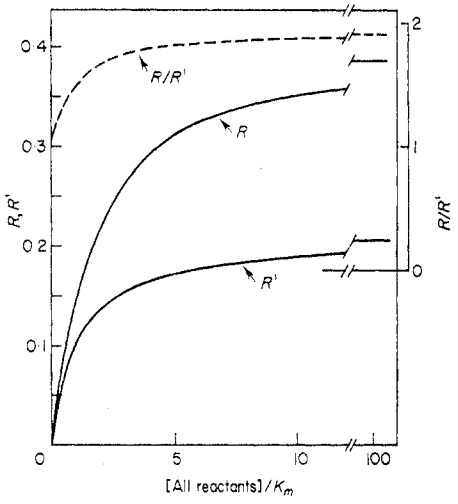
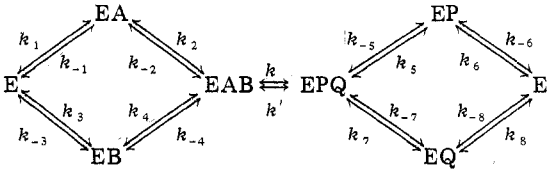


Figure 1. The effect of increasing reactant concentration at equilibrium on exchange rates for an enzyme reaction with a random substrate binding order.[29] The enzyme reaction catalyzed is A + B ⇌ P + Q. The patterns are for a sequential reaction proceeding through ternary complexes as follows:



Values assumed for rate constants are $k = 2k' = 10$; $k_1 = k_{-1} = k_4 = k_{-4} = k_6 = k_{-6} = k_7 = k_{-7} = 2$; $k_2 = k_{-2} = k_3 = k_{-3} = k_5 = k_{-5} = k_8 = k_{-8} = 1$. In a typical experiment, a trace of an isotopically labeled substrate, e.g. *A, would be added to an enzyme reaction already at equilibrium, and the rate of appearance of isotope in P measured. This would give the rate, R, of the A ⇌ P exchange. A similar measurement would give R' for the P ⇌ Q exchange. The figure gives calculated values for R and R' as the concentration of all substrates at equilibrium is increased. Both exchange rates rise smoothly to a maximum, but the maximum exchange rates differ because the ternary complex interconversion is not rate limiting, and off constants for reactants differ. Reprinted with permission from ref 29. Copyright 1973 Academic Press.

Equilibrium isotope exchange is a particularly powerful tool for assessing substrate binding orders and for determining whether substrate interconversion steps are rate limiting. For example, in random, sequential mechanisms for catalysis of A + B ⇌ P + Q, if substrate interconversion is definitely rate limiting all exchange rates between substrates and products must be equal. Contribution of a substrate dissociation step to rate limitation is readily revealed by a decreased rate for exchange involving this substrate. If compulsory binding orders exist, increase in concentrations of substrates that bind later will first increase, then decrease exchange rates for substrates that bind earlier. Examples of theoretical rate patterns[29] for random and ordered binding are indicated in Figures 1 and 2. Further examples are cited below.

(11) Morales, M. F., Horovitz, M., and Botts, J., *Arch. Biochem. Biophys.* **99** (1962).
(12) Darvey, I. G., *J. Theor. Biol.* **42**, 55 (1973).
(13) Alberty, R. A., Bloomfield, V., Peller, L., and King, E. L., *J. Am. Chem. Soc.* **84**, 4381 (1962).
(14) Cleland, W. W., *Annu. Rev. Biochem.* **36**, 77 (1967).
(15) Cole, F. X., and Schimmel, P. R., *Biochemistry* **9**, 480 (1970).
(16) Britton, H. G., *Arch. Biochem. Biophys.* **117**, 167 (1966).
(17) Plowman, K. M., "Enzyme Kinetics", McGraw-Hill, New York, N.Y., 1972, pp 76–91.
(18) Orsi, B. A., *Biochim. Biophys. Acta* **258**, 4 (1974).
(19) Schulz, A. R., and Fisher, D. D., *Can. J. Biochem.* **48**, 922 (1970).
(20) Flossdorf, J., and Kula, M.-R., *Eur. J. Biochem.* **30**, 325 (1972).
(21) Yagil, G., and Hoberman, H. D., *Biochemistry* **8**, 352 (1969).
(22) Wong, J. T., "Kinetics of Enzyme Mechanisms", Academic Press, New York, N.Y., 1975, p 174.
(23) Cleland, W. W., *Biochemistry* **14**, 3220 (1975).
(24) Vaslow, F., *C. R. Trav. Lab. Carlsberg, Ser. Chim.* **30**, 45 (1956).
(25) Darvey, I. G., *J. Theor. Biol.* **49**, 201 (1975).
(26) Fromm, H. J., Silverstein, E., and Boyer, P. D., *J. Biol. Chem.* **239**, 3645 (1964).
(27) Su, S., and Russell, P. J., Jr., *J. Biol. Chem.* **243**, 3826 (1968).
(28) Engers, H. D., Bridger, W. A., and Madsen, N. G., *J. Biol. Chem.* **244**, 5936 (1969).
(29) Wedler, F. C., and Boyer, P. D., *J. Theor. Biol.* **38**, 539 (1973).

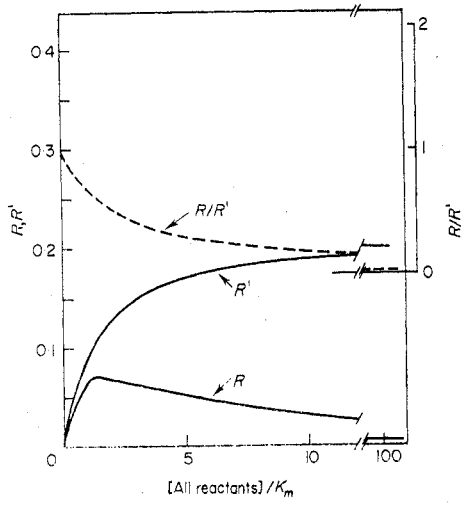


Figure 2. The effect of increasing reactant concentrations at equilibrium on exchange rates for an enzyme reaction with ordered substrate binding.[29] The reaction sequence is

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EAB \underset{k'}{\overset{k'}{\rightleftharpoons}} EPQ \underset{k_5}{\overset{k_{-5}}{\rightleftharpoons}} EP \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} E$$

with assumed values of rate constants as given with Figure 1. Both R and R′ initially rise, but as concentrations of B become sufficiently high the reaction EA + B ⇌ EAB predominates and the dissociation EA ⇌ E + A is presented. A similar effect occurs with Q preventing dissociation of EP. The A ⇌ P exchange is thus decreased but the B ⇌ Q exchange rises to a maximum as reactant concentrations are increased. Reprinted with permission from ref 29. Copyright 1973 Academic Press.

Applications of exchange measurements are often restricted by methodology—each data point requires a separation of reaction participants and determination of isotope concentration. Adequate and rapid separation methods are essential. For measurement of rates at equilibrium, there is the additional requirement that the equilibrium not be so far displaced as to make the concentration of any substrate too low for the requisite measurements. Adequate chemical and isotopic purity of labeled compounds is requisite. High specific activity is helpful, for it enables the isotopically labeled reactant to be added in tracer quantities so as not seriously to disturb the previously established equilibrium.

An interesting extension of isotope-exchange studies has been proposed for detection of enzyme isomerization as a possible step in a catalytic sequence. Such an isomerization may give a transient change in the distribution of an isotope during readjustment to a new equilibrium condition after a substrate is added to a system at equilibrium.[30] Thus if catalysis occurs by a reaction, $E_1 + S \rightleftharpoons EX \rightleftharpoons E_2 + S$, followed by an isomerization step, $E_2 \rightleftharpoons E_1$, some isotope redistribution will occur if the isomerization contributes to rate limitation of isotope exchange.

Isotope-exchange measurements show considerable promise as aids to the understanding of enzyme control mechanisms. Wedler and Boyer[29] have presented some theoretical background in this regard. Various types of action by positive or negative modifiers of enzyme activity have predictable consequences, some of them quite distinctive, on isotope exchange. Engers et al.[31] have pointed out the applicability of isotopic exchange studies to detection of possible allosteric cooperativity.[32] This area appears ripe for future development.

Detection and Characterization of Intermediate Steps

Detection of intermediate steps of an overall catalytic sequence, sometimes called partial reactions, is one of the most useful and common applications of isotopic exchange. In the classical experiment of Doudoroff et al., reported in 1947,[34] the ability of sucrose phosphorylase to catalyze the exchange of ^{32}P-labeled inorganic phosphate into glucose 1-phosphate was interpreted as showing formation of a glucosyl enzyme, as depicted by eq 9. An alternative explanation not

$$\text{glucose 1-phosphate} + \text{enzyme} \rightleftharpoons \text{glucosyl-enzyme} + \text{phosphate } (P_i) \qquad (9)$$

eliminated at that time was the possibility of formation of a noncovalently but firmly bound glucose residue. The original interpretation was recently shown to be correct by chemical characterization of the glucosyl enzyme and, further, by evidence of its stereochemical competency to participate as an intermediate.[35]

The formation of a covalent intermediate with an enzyme, accompanied by a product release as a catalytic step independent of the presence of other substrate(s), must always allow measurement of the predictable exchange. In early studies the lack of glucose 1-phosphate ⇌ phosphate exchange gave evidence against participation of a glucosyl-enzyme in α-glucan phosphorylase catalysis[36] and the lack of an ADP ⇌ ATP exchange gave evidence against participation of a phosphoryl-enzyme intermediate in pyruvate kinase catalysis.[37] Both inferences have been corroborated by later developments. The possibility must always be considered, however, that the other substrate(s), although not participating in chemical change, must bind to the enzyme in order for catalysis to occur.

With phosphotransacetylase, the lack of an exchange suggested the absence of an acetyl-enzyme intermediate. Henkin and Abeles have recently presented convincing evidence that an acetyl-enzyme is indeed not formed, based on the undetectability of [^{14}C]acetyl-enzyme under appropriate conditions, the failure of CoA analogues to induce exchange, and the demonstration that binary complexes with enzyme are catalytically active.[38]

Care must be exercised in the interpretation of weak exchange activities. For example, a detectable ADP ⇌ ATP exchange with 3-phosphoglycerate kinase led to suggestions of a phosphorylated enzyme intermediate.[39] However, the exchange activity appears to be due to contaminating adenylate kinase[40] or 3-phospho-

(30) Britton, H. G., and Clarke, J. B., *Biochem. J.* **110**, 161 (1968).
(31) Engers, H. D., Bridger, W. A., and Madsen, N. B., *Biochemistry*, **9**, 3281 (1970).
(32) An approach suggested by Darvey for assessing cooperativity[33] has subsequently been noted by him to be without value (I. G. Darvey, personal communication).
(33) Darvey, I. G., *Biochem. J.* **135**, 861 (1973).
(34) Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.* **168**, 725 (1947).
(35) Silverstein, R., Vogt, J., Reed, D., and Abeles, R. H., *J. Biol. Chem.* **242**, 1338 (1967).
(36) Cohn, M. and Cori, G. T., *J. Biol. Chem.* **175**, 89 (1948).
(37) Harrison, W. H., Boyer, P. D., and Falcone, A. B., *J. Biol. Chem.* **215**, 303 (1955).
(38) Henkin, J. and Abeles, R. H., *Biochemistry* **15**, 3427 (1976).
(39) Walsh, C. T., and Spector, L. B., *J. Biol. Chem.* **246**, 1255 (1971).
(40) Larsson-Raznikiewicz, M., and Schierbeck, B., *Biochem. Biophys. Res. Commun.* **57**, 627 (1974).

glycerate[41] in enzyme preparations, thus allowing reversal of the overall enzyme reactions. Further, a weak exchange might represent slow occurrence of a reaction not characteristic of the principal catalytic pathway. Such appears to be the case for the weak ADP ⇌ ATP exchange catalyzed by hexokinase.[42]

Substrate Binding Order and Relative Rates of Catalytic Steps

For sequential mechanisms (where all substrates must be bound to the enzyme before covalent change occurs) exchange measurements can frequently allow distinction between whether substrates bind independently of one another or whether the presence of one bound substrate influences the binding of another. Also, as mentioned earlier, inequalities of exchange rates at equilibrium may reflect compulsory binding orders for substrates, or random but kinetically preferred binding orders under the experimental conditions. For random or even for compulsory sequential mechanisms, prominent rate limitation by the chemical interconversion of bound substrates must result in equality of different exchanges. Adenylate kinase,[27,43] creatine kinase,[44] and phosphorylase[28] are among the enzymes for which substrates show equal rates of exchange; this indicates that interconversion of bound substrate at their catalytic sites is slow compared to flux between free and bound substrates. A lack of equality with random mechanisms is perhaps the most sensitive available criterion to show that the overall reaction rate is not limited by interconversion of bound substrates. Such inequality of exchanges is observed with many enzymes and is part of the growing evidence that substrate binding and release steps frequently form the principal barriers in the catalytic processes. Once the substrates are appropriately bound, chemical reaction is facile.

Applications in this area are numerous, and only a few can be mentioned here. In early studies with different alcohol dehydrogenases[45] and lactate dehydrogenases,[46] substrate concentration effects typical of compulsory, partially compulsory, or random binding orders were noted. For example, with lactate dehydrogenase (eq 10) at equilibrium, measurements can be made of NAD ⇌ NADH and lactate ⇌ pyruvate

$$\text{lactate} + \text{NAD}^+ \rightleftharpoons \text{pyruvate} + \text{NADH} + \text{H}^+ \qquad (10)$$

exchanges. Exchanges may be measured at different total concentrations of substrates while maintaining overall equilibrium, with increase in lactate and pyruvate concentration together, or of NAD^+ and NADH together, or of all substrates. Patterns akin to Figure 1 (random substrate binding) or Figure 2 (compulsory order of substrate binding) have been observed.

Wong and Hanes[47] have pointed out a potential limitation in detection of compusory binding. If an abortive ternary complex (such as E·NAD·ald for alcohol dehydrogenase) allows NAD dissociation but a productive complex (such as E·NAD·alc) does not, a compulsory binding might be overlooked. Although somewhat unlikely, such possibilities must be considered. As is frequently the case, conformity to a kinetic pattern can give evidence for or be consistent with a mechanism, but does not provide proof. Other tests may prove useful. Thus Rudolph and Fromm[48] note that, for the possibility suggested by Wong and Hanes, a hyperbolic increase in NAD ⇌ NADH exchange rate would not be expected with increase in alcohol and aldehyde concentrations at equilibrium.

A continued dispute about the kinetic behavior of yeast hexokinase appears to be largely resolved by demonstration by computer simulation that available data are consistent with an essentially random order of substrate binding—a finding clearly indicated by isotope exchange criteria.[48,49] Silverstein has continued his valuable services of exchange studies with demonstration of random substrate binding and substrate dissociation as rate limiting for oxidative alanine deamination by glutamate dehydrogenase.[50] A recent contribution in Cleland's fine series of kinetic studies indicates a similar behavior with isocitrate dehydrogenase.[51] With pepsin, ordered product release has been observed.[52]

For the enzymic synthesis of valyl-tRNA, Midelfort et al. used exchange studies to resolve a dispute in favor of an aminoacyl adenylate as an intermediate.[53] Moffet and Bridger with succinyl-CoA synthetase showed that a kinetically preferred initial binding of ATP does not represent an absolutely compulsory step.[54] Blostein,[55] by comparison of ADP ⇌ ATP exchange rates with net hydrolysis rates catalyzed by Na^+,K^+-ATPase, showed that the dissociation of ADP was likely a slow step at 0 °C but not at 37 °C.

Glutamine synthetase, as in other areas of enzyme catalysis, has been an excellent subject for exchange studies. Including oxygen exchanges, 10 different exchange reactions are possible with this enzyme.[56] Studies of some of them have given considerable information about relative rates of dissociation of various substrates.[57] An observation applicable to other studies was that the effect of increase of concentration of all substrates at equilibrium on exchange rates pointed to random substrate binding.[57] Rate inhibition noted when substrate pairs were increased, indicative of possible compulsory order, probably resulted from binding of one substrate at another substrate site. Other more recent studies have established that during net reaction of ADP, glutamine, and ammonia by the *E. coli* enzyme, the most rapid reaction catalyzed is a transfer of oxygen from P_i to glutamine.[56] This can occur only if the carboxylate of the bound glutamate, containing an oxygen derived from P_i, is bound so that either of the two oxygens can appear in P_i or in glut-

(41) Johnson, P. E., Abbott, S. J., Orr, G. A., Semeriva, M., and Knowles, J. R., *Biochem. Biophys. Res. Commun.* **62**, 382 (1975).
(42) Solomon, F., and Rose, I. A., *Arch. Biochem. Biophys.* **147**, 349 (1971).
(43) Rhoads, D. G., and Lowenstein, J. M., *J. Biol. Chem.* **243**, 3963 (1968).
(44) Morrison, J. F., and Cleland, W. W., *J. Biol. Chem.* **241**, 673 (1968).
(45) Silverstein, E., and Boyer, P. D., *J. Biol. Chem.* **239**, 3908 (1964).
(46) Silverstein, E., and Boyer, P. D., *J. Biol. Chem.*, **239**, 3901 (1964).
(47) Wong, J. T.-F., and Hanes, C. S., *Nature (London)* **203**, 492 (1964).
(48) Rudolph, F. B., and Fromm, H. J., *J. Biol. Chem.* **246**, 6611 (1971).
(49) Purich, D. J., Fromm, H. J., and Rudolph, F. B., *Adv. Enzymol.* **39**, 249 (1973).
(50) Silverstein, E., and Sulebele, G., *Biochemistry*, **13**, 1815 (1974).
(51) Uhr, M. L., Thompson, V. W., and Cleland, W. W., *J. Biol. Chem.* **249**, 2920 (1974).
(52) Ginodman, L. M., and Lutsenko, N. G., *Biokhimya* **37**, 101 (1972).
(53) Midelfort, C. F., Chakraburtty, K., Steinschneider, A., and Mehler, A. H., *J. Biol. Chem.* **250**, 3866 (1975).
(54) Moffet, F. J., and Bridger, W. A., *Can. J. Biochem.* **51**, 44 (1973).
(55) Blostein, R., *J. Biol. Chem.* **245**, 270 (1970).
(56) Stokes, B. O., and Boyer, P. D., *J. Biol. Chem.* **251**, 5558 (1976).
(57) Wedler, F. C., and Boyer, P. D., *J. Biol. Chem.* **247**, 984 (1972).

amine during the reversal of the interconversion step. Demonstration of such a fine point, although of no major import, is intellectually satisfying.

Of particular interest is the recent demonstration by Midelfort and Rose[58] that glutamine synthetase allows conversion of the β–γ-bridge oxygen of ATP to a nonbridge position in the presence of glutamate but the absence of ammonia. Such conversion gives strong evidence for formation of a bound γ-glutamyl intermediate and torsional symmetry of the β-phosphoryl group of the enzyme-bound ADP.[58] This approach should have applicability to some other ATP-using enzymes.

A related measurement that shows considerable promise is the exchange pattern for loss of ^{18}O from highly labeled P_i during exchange with water as catalyzed by phosphatase, pyrophosphatase, or transport ATPases.[59] Measurement of the mass of a suitable derivative reveals whether phosphate species present may contain one, two, three, or four ^{18}O atoms. The exchange of phosphate oxygens with water, catalyzed by a phosphatase or transport ATPase (E), involves steps as depicted in eq 11. In step 1 the substrate and

$$E + HOPO_3^{2-} \underset{1}{\longleftrightarrow} E \cdot HOPO_3^{2-} \overset{HOH}{\underset{2}{\longleftrightarrow}} E\text{-}PO_3^{2-} \qquad (11)$$

enzyme form a Michaelis complex; in step 2 water is eliminated and a phosphoryl enzyme is formed. Step 2 is the exchange step, and at least three and perhaps all four phosphate oxygens may be regarded as participating equally in exchange through reversal of step 2. If P_i with ^{18}O in all four positions undergoes exchange, the pattern of ^{18}O loss allows deduction of the relative rate of steps 1 and 2. If step 1 is fast compared to step 2, ^{18}O atoms will be lost from medium P_i one at a time in a random pattern. If step 2 is fast compared to step 1, each P_i that binds could exchange all four oxygens before returning to the medium; "all-or-none" loss of ^{18}O will result.

The inclusion of ^{18}O exchange measurements in addition to ^{14}C exchanges has allowed demonstration that the $O_{HCO_3^-} \rightleftharpoons O_{HOH}$ exchange catalyzed by intracellular carbonic anhydrase can be used to assess the rate of transfer of bicarbonate across the red blood cell membrane.[60]

Most of the applications described below present additional probes of the relative rates of catalytic steps, but they are more usefully presented in separate sections depending on the type of information yielded. Those interested in applications in this area will also want to read a recent publication by Cleland that gives a further analysis of patterns of exchange in ordered and sequential systems.[61]

Substrate Synergism

Substrate synergism occurs when one substrate at a catalytic site promotes the reactivity of another substrate although the two substrates do not react directly with each other.[62] Such promotion of one catalytic step at a site by another occupant at the site, not participating directly in the step, is probably frequent in enzyme catalysis and is a fruitful area for exchange studies. Substrate synergism offers an explanation for why exchange expected for a demonstrable intermediate step may be slow or not even observed. For example, with arginosuccinate synthetase (ATP + citrulline + aspartate → arginosuccinate + AMP + pyrophosphate) a pyrophosphate ⇌ ATP exchange in presence of citrulline is not observed even though there is convincing evidence for AMP–citrulline as an intermediate. Detectable release of the enzyme-bound pyrophosphate does not occur until aspartate adds.[63] With succinyl-CoA synthetase from *E. coli* (ATP + succcinate + CoASH → succinyl-S-CoA + ADP + P_i), the presence of succinyl CoA markedly accelerates an ADP ⇌ ATP exchange reaction expected from the step E + ATP ⇌ E–P + ADP.[62]

Pronounced substrate synergism might also account for the undetectability of exchanges with *E. coli* glutamine synthetase which would be expected if enzyme-bound γ-glutamyl phosphate were an intermediate.[58] The presence but not reaction of NH_3 might be necessary for phosphorylation of glutamate by ATP, even though substrates appear to bind randomly. The alternate possibility that the reaction is essentially concerted must, however, still be considered.[64]

As recognized by Bridger et al.[62] and pointed out more explicitly by Lueck and Fromm,[65] comparison of the V_{max} for initial velocity in one direction with R_{max} for a partial reaction for an enzyme with a "ping-pong" mechanism does not suffice as a test for substrate synergism. For a ping-pong mechanism applying to the reaction A + B ⇌ P + Q, the governing relation in absence of synergism is given by eq 12. All four kinetic parameters need to be evaluated to assess whether

$$\frac{1}{R_{max}(A \rightleftharpoons P)} + \frac{1}{R_{max}(B \rightleftharpoons Q)} = \frac{1}{V_f} + \frac{1}{V_r} \qquad (12)$$

substrate synergism is operative.

Substrate synergism that promotes binding of substrates rather than reaction of the bound substrate has been observed[66] and explored theoretically[67] by Wedler.

Control of Catalysis

Exchange techniques may reveal the mode of action of allosteric effectors. Such effectors may modulate substrate association, chemical conversions of bound substrates, or product release. Silverstein was first to report applications of exchange measurements to the study of control mechanisms. As an example of a series of studies from his laboratory, Sulebele and Silverstein assessed *p*-mercuribenzoate activation of malate dehydrogenase as a model for allosteric control.[68] In other studies, Kosow and Rose demonstrated that citrate activation of yeast hexokinase stimulated all measured exchanges equally, consistent with increase in the amount of active enzyme without change in kinetic characteristics.[69] Wedler and Boyer noted differing modes of action for modifers of glutamine synthetase activity,[57] including evidence that GDP acts primarily by decreasing ATP dissociation. Wedler and Gasser

(58) Midelfort, C. F., and Rose, I. A., *J. Biol. Chem.* **251**, 5881 (1976).
(59) Hackney, D., and Boyer, P. D., unpublished.
(60) Silverman, D. N., *Mol. Pharmacol.* **10**, 820 (1974).
(61) Cleland, W. W., *Adv. Enzymol.*, in press.
(62) Bridger, W. A., Millen, W. A., and Boyer, P. D., *Biochemistry* **7**, 3608 (1968).
(63) Rochovansky, O., and Ratner, S., *J. Biol. Chem.* **242**, 3839 (1967).
(64) Wedler, E. C., and Horn, B. R., *J. Biol. Chem.*, **251**, 7530 (1977).
(65) Lueck, J. D. and Fromm, H. J., *FEBS Lett.* **32**, 184 (1973).
(66) Wedler, F. C., *J. Biol. Chem.* **249**, 5080 (1974).
(67) Wedler, F. C., *Physiol. Chem. Phys.*, in press.
(68) Sulebele, G., and Silverstein, E., *Biochemistry*, **9**, 283 (1970).
(69) Kosow, D. P., and Rose, I. A., *J. Biol. Chem.* **246**, 2618 (1971).

clarified effects of ATP activation and CTP inhibition of aspartate transcarbamylase.[70] Also, as mentioned above, homotrophic behavior of allosteric enzymes leads to changes in patterns of substrate dependency of equilibrium exchange rates.[30,31]

Energy Transducing Mechanisms

Exchange studies are currently contributing prominently to the understanding of the energy transductions catalyzed by the actin–myosin system of muscle, the synthesis of ATP coupled to electron transport by mitochondria, chloroplasts, and related systems, and the osmotic work done by transport ATPase.

Discovery by Mildred Cohn[71] of the capacity of mitochondria to catalyze a rapid $P_i \rightleftharpoons HOH$ exchange (more properly but less conveniently designated as an $O_{P_i} \rightleftharpoons O_{HOH}$ exchange) led to the recognition of the $P_i \rightleftharpoons ATP$[72,73] and the $ATP \rightleftharpoons HOH$ exchanges.[74,75] The $P_i \rightleftharpoons ATP$ exchange capacity has served as a particularly useful probe in many subsequent studies. With myosin, Levy and Koshland[76] described catalysis of an "intermediate" exchange (incorporation of more than one oxygen from water into each P_i formed by hydrolysis) and Dempsey et al. noted[77] a "medium" exchange (a $P_i \rightleftharpoons HOH$ occurring with P_i added to the reaction medium). The value of these exchanges for aiding understanding of the energy-transducing mechanisms has increased markedly with the attaining of an adequate explanation of the molecular basis of the oxygen exchanges.

A prominent and probably the only source of the oxygen exchanges is dynamic reversal of the cleavage of enzyme-bound ATP to enzyme-bound ADP and either a transient phosphorylated derivative or P_i. If a phosphorylated intermediate forms, it must exchange P_i oxygens with water directly or indirectly. For example, a carboxyl group may exchange through reversible thioester formation. Measurements of the oxygen exchanges thus appear to offer a sensitive probe for the rate of interconversion of bound ATP to bound ADP and P_i at the catalytic site. With this information, the insensitivity of the intermediate $P_i \rightleftharpoons HOH$ exchange and the sensitivity of the very rapid medium $P_i \rightleftharpoons HOH$ exchange to uncouplers of oxidative phosphorylation allow identification of the catalytic steps modified by energy input. Important points are that transmembrane proton gradients are not used directly in the formation of ATP and that energy-linked conformational events may play a vital role.[78,79]

With muscle, the fine kinetic studies of Trentham and Bagshaw pointed to a reversal of bound ATP cleavage as a source of the oxygen exchange,[80] a mechanism also indicated by the independent demonstration of the incorporation of medium $^{32}P_i$ into myosin-bound ATP.[81] Subsequent studies have demonstrated incorporation of water oxygens into bound ATP at a rate consistent with an initial water oxygen incorporation into bound P_i and reversal of the ATP cleavage step.[82]

The $ADP \rightleftharpoons ATP$, $ATP \rightleftharpoons E\text{–}P$, $P_i \rightleftharpoons E\text{–}P$, and $P_i \rightleftharpoons HOH$ exchanges demonstrable with the transport ATPases are serving increasingly as recognized probes. In the $P_i \rightleftharpoons HOH$ exchange, the bridge oxygen of the C–O–P bond is furnished by the carboxyl group, and the displacement by a carboxylate group of an oxygen from P_i to form water serves as a reasonable explanation for the exchange.[80,83] Recent studies with the Ca^{2+},-Mg^{2+}-ATPase of sarcoplasmic reticulum demonstrate that such a reaction is the major and likely the only source of the oxygen exchange.[84] Thus, as with the mitochondrial systems, the oxygen exchange rate becomes a useful measure for the rate of reversal of a key intermediate step.

Concluding Remarks

This Account focuses on a technique for use in the understanding of biological systems, namely the nature and value of isotope exchange measurement for study of enzyme-catalyzed reactions. But the problem and not the techniques must remain paramount. This Account will be of value if it illustrates how better understanding of some problems was gained and if it aids in the recognition and solution of new problems.

Preparation of this Account was aided by Grant GM 11094 of the Institute of General Medical Sciences, U.S. Public Health Service, Contract E(04-3)-34, of the U.S. Energy Research and Development Administration, and Grant PCM 75-18884 of the National Science Foundation.

(70) Wedler, F. C., and Gasser, F. J., *Arch. Biochem. Biophys.* **163**, 69 (1974).
(71) Cohn, M., *J. Biol. Chem.* **201**, 735 (1953).
(72) Boyer, P. D., Falcone, A. S., and Harrison, W. H., *Nature (London)* **174**, 401 (1954).
(73) Swanson, M. A., *Biochim. Biophys. Acta* **20**, 85 (1956).
(74) Cohn, M., and Drysdale, G. R., *J. Biol. Chem.* **216**, 831 (1955).
(75) Boyer, P. D., Luchsinger, W. W., and Falcone, A. B., *J. Biol. Chem.* **223**, 405 (1956).
(76) Levy, H. M., and Koshland, D. E., Jr., *J. Biol. Chem.* **234**, 1102 (1959).
(77) Dempsey, M. E., Boyer, P. D., and Benson, E. S., *J. Biol. Chem.* **238**, 2708 (1963).
(78) Kayalar, C., Rosing, J., and Boyer, P. D., *J. Biol. Chem.* **252**, 2486 (1977).
(79) Smith, D. J., and Boyer, P. D., *Proc. Natl. Acad. Sci U.S.A.* **73**, 4314 (1976).
(80) Bagshaw, C. R., and Trentham, D. R., *Biochem. J.* **141**, 331 (1974).
(81) Wolcott, R. G., and Boyer, P. D., *Biochem. Biophys. Res. Commun.* **57**, 709 (1974).
(82) Bagshaw, C. R., Trentham, D. R., Wolcott, R. G., and Boyer, P. D., *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2492 (1975).
(83) Dahms, A. S., Kanazawa, T., and Boyer, P. D., *J. Biol. Chem.* **248**, 6592 (1973).
(84) Boyer, P. D., de Meis, L., Carvalho, M. G. C., and Hackney, D., *Biochemistry* **16**, 136 (1977).