BIOORGANIC CHEMISTRY 2, 311–321 (1973)

Covalent Enzyme-Substrate Intermediates in Transferase Reactions

LEONARD B. SPECTOR

The Rockefeller University, New York, New York 10021

Received January 25, 1973

Enzymatic transfer reactions are often depicted as occurring on the surface of the enzyme by direct transfer of a chemical group from a donor substrate molecule to an acceptor, in a single-displacement reaction without covalent participation of the enzyme. This picture of enzyme action is purely speculative, because no positive evidence in support of it exists. The kinetic argument, which formerly afforded the sole support for the single-displacement mechanism, is now known to be in error. Contrasting sharply with this dearth of proof is the substantial and growing body of evidence which upholds the double-displacement mechanism of enzymatic transfer. In this mechanism, a catalytic group (or atom) within the active site of the enzyme links covalently with one of the substrates, or some fragment of it, at some stage of the reaction. Through use of the covalent enzyme–substrate intermediate it may be that the enzyme is enabled to overcome certain entropic difficulties, thus accounting in part for the well-known speed of enzymatic reactions. Covalent participation by the enzyme also brings enzymatic catalysis into closer accord with homogenous and heterogeneous catalysis, in which the key reaction is the transient formation of a covalent bond between substrate and catalyst.

There are grounds for believing that enzymatic transfer reactions proceed via an intermediate which is composed of enzyme and substrate (or fragment thereof) joined together in covalent linkage. Reaction (1), accordingly,

$$A-B+C \overset{E}{\rightleftharpoons} A+B-C \tag{1}$$

is more truly represented by the two partial Reactions (2) and (3).

$$A-B+E \rightleftharpoons A+E-B \tag{2}$$

$$E-B+C \rightleftharpoons E+B-C \tag{3}$$

in which the enzyme (E) transfers the fragment B reversibly between A and C by way of E – B, the covalent enzyme–substrate intermediate. This has been called the double-displacement mechanism. It is clearly marked off from the single-displacement mechanism, in which a group is transferred directly between substrate molecules without covalent intervention by the enzyme (*1*). It is this latter mechanism which first captured the imagination of biochemists, and to this day it enjoys the greatest influence. Yet

abundant testimony exists to show that transferases probably use only the double-displacement mechanism. This conclusion follows from a survey of the known transferase reactions (see below). The double-displacement mechanism conforms, moreover, with the fundamental mechanism of homogenous and heterogeneous catalysis. In order to see enzymatic catalysis in its proper relation to these other forms of chemical catalysis, it may be well to recall a few familiar facts about homogenous and heterogeneous catalysis.

Homogenous and Heterogeneous Catalysis

Chemical reactions in homogenous solution are often catalyzed by electrophiles or nucleophiles or both (*2*, *3*). Acid–base catalysis is a special case of electrophilic–nucleophilic catalysis. Many kinds of molecules and ions can effect such catalyses, all of which entail the covalent bonding of the catalyst to the reacting molecule or some detachable fragment of it. Homogenous catalysis by transition-metal complexes also requires the covalent (coordinate) bonding of the substrate to the transition-metal atom (*4*). To the extent that experimental data permit firm conclusions to be drawn, catalysis in homogenous solution seems always to demand the transient existence of a covalent link between substrate and catalyst.

Catalysis by a solid surface—whether from the gaseous or liquid state—requires that the reactant molecules first be adsorbed to that surface (*5*, *6*). Adsorption resulting in chemical reaction proceeds in two stages. First there is physisorption, in which the reactant binds to the catalytic surface by van der Waal's forces while retaining its molecular integrity. Physisorption is succeeded by chemisorption, in which the adsorbed molecule forms a covalent bond with one or more atoms in the surface (*7*). Chemisorption often takes the form of a dissociative binding in which the reactant breaks into fragments, each of which is chemisorbed to the surface. Molecular hydrogen, for instance, dissociates easily on a transition-metal surface to give a metallic hydride, which can react with other susceptible, adsorbed molecules such as olefins, carbonyl compounds, and aromatics. Desorption of the new molecules from the surface completes the catalysis. In the chemisorbed state the substrate molecule (or fragment) binds to a surface atom through mutual overlap with lobes of bonding orbitals (dangling orbitals) which project from the surface of the catalyst (*8*).

Enzymatic Catalysis

An aqueous solution of a protein is, as is well-known, not truly homogenous. Such solutions are colloidal. The "soluble" protein (enzyme) affords a surface upon which adsorption and chemical reaction can take place, much as they do on the solid surface of a heterogeneous catalyst. Catalysis by soluble enzymes lies thus between the extremes of homogenous and heterogeneous catalysis. Catalysis by insoluble (membrane-bound) enzymes has, of course, a greater inherent likeness to heterogeneous catalysis. The physisorbed and chemisorbed states in heterogeneous catalysis are strikingly analogous, respectively, to the Michaelis–Menten complex and the covalent enzyme–substrate intermediate of enzymatic catalysis. Since homogenous and heterogeneous catalysis require that a covalent bond to the catalyst share intimately in the process, it seems only natural and fitting that the same covalent principle should govern the action of enzymes. This reasonable expectation is on the way to fulfillment, as attested

by the sizable and growing number of enzymes for which positive evidence exists that they act through a covalent enzyme-substrate intermediate.

The Transferases

In 1961 the Enzyme Commission, which was created by the International Union of Biochemistry, arranged the known enzymes into six major classes: the oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (*9*). Of these, the second class of enzymes—the transferases—forms the special subject of the present article. Each of the six major classes of enzymes was divided into subclasses, and these were further divided into subsubclasses. The class of transferases was broken down by the Commission into a total of 23 categories (i.e., subsubclasses), each of which was assigned an Enzyme Commission (EC) number (*9*). These category numbers are listed in the first column of Table 1. Beside each number is placed the name of an enzyme from the indicated category which is believed to act through a covalent enzyme–substrate intermediate. It is seen that, of 23 categories of transferases, 21 are represented in the table by an enzyme (*10*).

The group which links covalently to each transferase in Table 1 is, without exception, something other than a proton. Yet, during the formation and further reaction of the covalent enzyme–substrate intermediate, it is possible—and in some cases even probable—that a proton transfers from the substrate to the enzyme, as a consequence of concurrent general base catalysis by the enzyme. The protonated enzyme, thus formed, qualifies too as a covalent enzyme–substrate intermediate, and may be the sole such intermediate in some isomerase reactions. But the expectation is that few, if any, transferases use the protonated enzyme as the sole intermediate during reaction.

A Reasonable Assumption

The Enzyme Commission's classification system is built upon the internal chemical resemblance among the reactions within each of its classes. The enzymes in any one of the six major classes catalyze reactions which are, at the very least, grossly similar in their overall chemistry. As the hierarchy of classification is descended, the resemblance among the reactions within a subordinate class becomes closer and more sharply defined. Thus, the category of mutases (EC 2.7.5), for instance, includes the enzymes phosphoglucomutase, phosphoglyceromutase, diphosphoglyceromutase, and phosphoacetylglucosamine mutase. They all catalyze the transfer of a phosphoryl from one hydroxyl group of the substrate to another. The general sameness of these chemical reactions prompts the reasonable assumption of a sameness in their respective chemical mechanisms. More specifically, it is assumed here that the proved participation of a covalent enzyme–substrate intermediate in any reaction of a category means that a like intermediate shares in the action of the other enzymes in that category.

The foregoing assumption is not without experimental basis. In the category of mutases cited above, the first two of the four named enzymes are known to act through phosphoenzymes (*11*, *12*). In the category of carboxylate kinases (EC 2.7.2) the enzymes catalyze the transfer of a phosphoryl group from ATP to the carboxylate group of an acid to yield an acyl phosphate. A detailed study has now been made of four carboxylate kinases: acetate kinase (*13*), 3-*P*-glycerate kinase (*14*), ATP citrate lyase (*15*, *16*), and succinyl-CoA synthetase (*15*, *17*). The constant finding is that each reaction is mediated

TABLE 1

SOME TRANSFERASES WHICH ACT THROUGH A COVALENT ENZYME–SUBSTRATE INTERMEDIATE[a]

EC No.	Trivial name of enzyme[b]	Criteria[c]	Reference
2.1.1	Methionine synthetase (cobalamine)[d]	I,[e] P	(*36*)
2.1.2	Serine transhydroxymethylase (pyridoxal-P)	I[f]	(*37*)
2.1.3	Transcarboxylase (biotin)	I	(*38*)
2.2.1	Transaldolase	I, E	(*39*)
2.3.1	Arylamine acetylase	I, E, P, V_{max}	(*18*)
2.3.2	Transglutaminase[d]	I,[g] E, P	(*40*)
2.4.1	Sucrose phosphorylase	I, E, P	(*41*)
2.4.2	AMP pyrophosphorylase	I[e]	(*42*)
2.5.1	Thiaminase I	V_{max}	(*23*)
2.6.1	Aspartate aminotransferase (pyridoxal-P)	I, P	(*43*)
2.6.2	Glycine amidinotransferase	I	(*44*)
2.6.3			
2.7.1	Phosphofructokinase	E, P	(*45*)
2.7.2	Acetate kinase	I, E, P	(*13*)
2.7.3	Arginine kinase	E	(*46*)
2.7.4	Nucleosidediphosphate kinase	I, E, P	(*47*)
2.7.5	Phosphoglucomutase	I,[e] P	(*11*)
2.7.6	Ribosephosphate pyrophosphokinase	E	(*48*)
2.7.7	Polynucleotide ligase[d,h]	I, E	(*49*)
2.7.8			
2.8.1	Rhodanese	I[e]	(*50*)
2.8.2	Adenylyl sulfate reductase (FAD)[d,i]	I[f]	(*51*)
2.8.3	3-Ketoacid CoA-transferase	I, E, P	(*52*)

[a] With two exceptions an example is given for each of the Enzyme Commission categories.

[b] Coenzymes are named in parentheses. Abbreviations: AMP, adenosine monophosphate; FAD, flavin adenine dinucleotide; CoA, coenzyme A.

[c] The criteria symbols mean the following: I, the covalent enzyme–substrate intermediate has been isolated from a reaction mixture in chemically competent condition; E, the enzyme catalyzes exchanges which conform with the exchange-specificity rule; P, the enzyme reaction follows authentic ping-pong kinetics; V_{max}, the enzyme acts on diverse substrates in conformity with the maximum velocity principle.

[d] Although this enzyme was not listed by the Enzyme Commission in 1964 it clearly falls into this category.

[e] This enzyme is actually isolated from tissue as the covalent enzyme–substrate intermediate; that is, the transferring portion of the substrate is already fixed to the enzyme.

[f] Isolated and identified spectroscopically.

[g] Transglutaminase does not form a stable intermediate with its natural substrate. But, like chymotrypsin, transglutaminase reacts with unnatural substrates, such as *p*-nitrophenyl acetate and *p*-nitrophenyl trimethylacetate, to form, respectively, an acetyl- and a trimethyl-acetyl enzyme. The latter is quite stable to hydrolysis. The trimethyl-acetyl group is fixed in thiolester linkage to an enzymatic cysteine, the same cysteine which reacts with iodoacetamide during inactivation of the enzyme.

[h] This enzyme is a ligase, but it is also a nucleotidyl transferase.

[i] This enzyme is an oxidoreductase, but it is also a sulphotransferase.

by its phosphorylated enzyme. All four of the intermediary phosphoenzymes have been isolated from reaction mixtures and shown to be chemically competent. This uniform enzyme action, exerted upon a diversity of acid structures, upholds the view that all carboxylate kinases use the phosphoenzyme pathway (*13*, *14*).

Categories of transferase activity other than the phosphotransferases also furnish grounds for the aforestated assumption. Among the acyl transferases (EC 2.3.1) several acyl-enzymes have been isolated after specific reaction of the enzymes with the appropriate acyl-coenzyme A's. Such acyl-enzymes, upon reaction with their respective acceptors, transfer the acyl group, as the case may be, to an amino function [arylamine acetylase (*18*)], a carbon atom [fatty acid synthetase (*19*)], or a sulfhydryl group [thiolase (*20*)].

But other categories of transferases (about half of them) have supplied till now only one example each of an enzyme which acts through a covalent enzyme–substrate intermediate. Our premise predicts that for such categories, too, a covalent enzyme–substrate compound mediates all reactions.

Criteria

Table 1 also gives the criteria by means of which it is judged that an enzyme makes use of the double-displacement mechanism (*21*). The most direct proof is the actual physical isolation of the covalent enzyme–substrate intermediate in the chemically competent state (indicated in the table by the letter I). Of the 21 representative enzymes listed in the table, 17 meet this criterion. Much to be desired is proof that the isolated intermediate is also kinetically competent, but this has so far been achieved with very few enzymes.

A less direct proof for the double-displacement mechanism depends upon the V_{max} (maximal velocity) criterion (*22*). Thiaminase I illustrates the use of this criterion. This enzyme catalyzes the transfer, to aniline, of the pyrimidinylmethyl portion of thiamine and of thiamine analogs at a maximal rate which is the same for all of the donor molecules. It is inferred from this that the reaction proceeds in two stages. The first stage is the formation of a covalent intermediate composed of the enzyme and the transferring portion of the donor molecule. In the second, rate-determining, stage, aniline reacts with the covalent intermediate to complete the transfer (*23*). Of the enzymes in Table 1, only thiaminase I depends solely upon this criterion for inclusion in the table. Arylamine acetylase also meets the V_{max} criterion, but it meets all of the others as well (*18*).

An enzyme which follows ping-pong kinetics (indicated in the table by the letter P) is thought to act via a two-stage chemical mechanism in which a covalent enzyme–substrate intermediate participates as a connecting link in each half-reaction. To conform to this criterion an enzyme must exhibit the predicted inhibition patterns as well as the parallel line kinetics (*24*). Of the different kinds of enzyme kinetics only the ping-pong variety is explicable directly in terms of chemical mechanism, since the kinetic mechanism makes it clear that the product of the first half-reaction leaves the enzyme before the substrate of the second half-reaction adsorbs. Such kinetic behavior infers the existence of a covalent enzyme–substrate intermediate.

Another indirect proof for the double-displacement mechanism makes use of the exchange-specificity criterion (indicated in the table by the letter E). The principle underlying this criterion is well-known (*25*). It is clear that if Reaction (1) truly proceeds in discrete chemical stages [i.e., through Reactions (2) and (3)], then the enzyme ought to catalyze an exchange of A into A – B in the absence of the substrate components of Reaction (3). In like manner, the exchange of C into B – C ought to be possible in the absence of the substrate components of Reaction (2). The exchanges implicit in these

partial reactions are often much slower than the exchanges in the overall reaction. This relative slowness may be ascribed, among other things, to the "substrate synergism" phenomenon (*26*). About half of the enzymes of Table 1 conform to the exchange-specificity criterion. Only two rely for inclusion solely upon it.

Other criteria have on occasion been used to detect covalent enzyme–substrate intermediates in enzyme reactions (*21*), but only the four described above were used in compiling Table 1.

The Two Gaps in Table 1

The list of transferases in Table 1 falls short of completion owing to the omission of two categories: EC 2.6.3 and EC 2.7.8. The first of these includes the oximinotransferases, of which few are known (*27*). These enzymes have been little studied from the mechanistic standpoint. They catalyze the transfer of the oximino group between the ketonic functions of substrate molecules. They all have pyridoxal-*P* as prosthetic group. General experience with pyridoxal-*P* as co-enzyme makes it a virtual certainty that the oximino group binds covalently to pyridoxal-*P* during oximinotransferase action. But since there are no experimental data bearing directly on this point the space for EC 2.6.3 is held open.

The second vacancy in Table 1 (EC 2.7.8) is reserved for the category of enzymes that catalyze the transfer of a substituted phosphoryl group other than the pyrophosphoryl and nucleotidyl groups. Of these enzymes, few are known, and they, too, want mechanistic study. It happens that phospholipase D, which is a hydrolase, also possesses a strong phosphatidyl transferase activity. Among other reactions, this enzyme catalyzes a choline—phosphatidylcholine exchange, implying the existence of an intermediate phosphatidyl-enzyme (*28*). But the experimental conditions described for this exchange are less exacting than is desired for inclusion of the enzyme in Table 1, so EC 2.7.8 is left unrepresented. Yet, significantly, this category is the last of eight listed categories of phosphotransferases (EC 2.7.1–2.7.8, inclusive), of which the first seven are represented in the table. The reasonable expectation is that EC 2.7.8, too, will in time be represented there.

Other Classes of Enzymes

It is plain from Table 1 that nearly every one of the categories of enzymes designated by the Enzyme Commission as transferases includes at least one enzyme which acts through a covalent enzyme–substrate intermediate. And the prospects are that the enzymes in the two unrepresented categories use the same chemical mechanism as the other 21. If, moreover, all of the enzymes in a category use the double-displacement mechanism if any one of them does, then it follows that the transferases, as a class of enzymes, operate uniformly on the covalent principle.

If such is true for the transferases, one wonders then what chemical mechanism is used by the five other classes of enzymes. In this connection it is possible, broadly speaking, to conceive of all enzymes as transferases—irrespective of their denomination as oxidoreductases, hydrolases, lyases, isomerases, or ligases. These names were devised solely for the convenience of classification. Closer analysis reveals that, despite class names, the enzymes of all classes have this in common—that they catalyze reactions in which a chemical group transfers from one substrate molecule to another or between

different sites within the same molecule. On these grounds all enzymes qualify as transferases. It follows that all enzymes ought to act like transferases. They ought to catalyze their reactions in discrete chemical stages, with the intervention of a covalent enzyme–substrate intermediate at each stage.

The Single-displacement Versus the Double-displacement Mechanism in Enzyme Action

The notion that reactions take place by single displacement on the surface of an enzyme comes down to us as a time-honoured heritage, which still enjoys pride of place among some students of enzyme action. Yet the plain truth is that this notion now has little foundation in experimental fact, and is therefore manifestly speculative. Until 1970 it seemed that the single-displacement mechanism had an experimental base in the fact that many enzymes do not follow ping-pong kinetics. It was reasoned that if ping-pong kinetics connotes a double-displacement mechanism, then non-ping-pong kinetics must connote a single-displacement mechanism. But such reasoning became untenable when it was discovered in 1970 that succinyl-CoA synthetase of *E. coli*, acting through a kinetically competent phosphoenzyme intermediate (*26*), exhibits a kinetic pattern which is clearly non-ping-pong (*29*). A similar observation was made with ATP citrate lyase (*30*), whose complex reaction is mediated by no less than two covalent enzyme–substrate intermediates (*31*). The patent fact is that if an enzyme follows authentic ping-pong kinetics, the covalent principle almost certainly applies, but that any other kind of kinetics is silent on the question of chemical mechanism (*29*). Thus, the kinetic argument—hitherto the only real argument—for the single-displacement mechanism has become a dead letter.

With the demise of the kinetic argument, any "proof" for the single-displacement mechanism relies now on the failure to find positive evidence for the double-displacement mechanism. But to rely thus on negative evidence is to stand on slippery ground. A case in point is the enzyme acetate kinase. In 1954 this enzyme was pronounced, on negative evidence, to proceed by the single-displacement mechanism (*32*). Yet 16 years later, with newer methods, phosphorylated acetate kinase could easily be isolated—proof positive for the double-displacement mechanism (*13*).

While no hard evidence can be found for the single-displacement mechanism, covalent participation of the enzyme has been proved for at least 140 of the 1100 known enzymes, and the list is growing (*33*). It comprises thus about 10% of all the known enzymes, of which the vast majority (about 80%) have not yet been studied mechanistically. The 140 enzymes include representatives of all six major Enzyme Commission classes. It is clear that a strong trend has set in, and that it favors only the double-displacement mechanism. Adding point to the argument is the natural kinship of enzymatic catalysis to homogenous and heterogeneous catalysis. Of the three great domains of chemical catalysis, the two latter have undergone by far the longest and most searching scrutiny. The evidence is overwhelming that both kinds of nonenzymatic catalysis are governed by the covalent principle. And the same conclusion seems so far to hold good for enzymatic catalysis.

Why Do Enzymes Use the Covalent Enzyme–Substrate Intermediate?

It may be that enzymes make use of the covalent principle because it eases the entropy problem. Maybe catalysis is expedited if the enzyme is not obliged to arrange a simul-

taneous, precisional alignment of all substrates within the active site. The expression "precisional alignment" is used here to convey a particular notion. One senses that the positioning of a substrate molecule for imminent reaction—and hence for maximal orbital overlap with its reacting partner—is a more delicately precise process than the positioning of a molecule which is not destined for immediate reaction. In the nature of things a precise alignment entails a greater loss of entropy than a less precise alignment.

The multiple, precisional alignment of substrates within the active site can be circumvented if the overall reaction is conducted in discrete chemical stages. In the first stage, the first substrate is precision aligned in order that it may easily react with the catalytic group in the active site of the enzyme and form the covalent enzyme–substrate intermediate. The latter can be regarded as a new holoenzyme. The second substrate will, in its turn, undergo precision alignment in order to maximize orbital overlap of its own reacting group with the catalytic group of the new holoenzyme. By proceeding in this stepwise fashion, the enzyme is enabled always to deal with its substrates on a one-at-a-time basis.

Although the new holoenzyme (that is, the covalent enzyme–substrate intermediate) has but one substrate to manage, it must nonetheless precision align this substrate with a second functionality, which is now, of course, a part of the enzyme. Why, it will be asked, should the enzyme prefer this mode of action to the simultaneous precision alignment of the two substrates, both of which, according to prevailing conceptions, are external to the enzyme on its surface? The answer is that the enzyme ought to have a finer degree of control over a reacting group which is a part of itself than it has over one which is external to itself. Arranging a favorable orbital overlap of two reacting groups ought to be easier if one of the groups is a part of the enzyme, much as the alignment of one's own left hand for insertion into its glove is easier than aligning someone's left hand for insertion into the same glove.

For multisubstrate enzymes the advantage of a stepwise procedure is especially clear. Consider the ATP citrate lyase reaction

$$\text{ATP} + \text{citrate} + \text{CoA} \rightleftharpoons \text{oxalacetate} + \text{acetyl-CoA} + \text{ADP} + \text{P}_\text{i}$$

Upon reversal, four substrate molecules must interact. To obviate the mutual precision alignment of four substrates within the active site, ATP citrate lyase conducts its reaction in discrete stages, with a phosphoenzyme and a citryl enzyme as isolable intermediates (*31*). The existence of yet a third intermediate—an acetyl enzyme—is strongly intimated (*34*). Such easement of the entropy burden may well account for some of an enzyme's capacity for speed in catalysis. This entropy effect can be thought of as a part of the "entropy trap" function which is subserved by all enzymes (*35*). It will be understood, moreover, that the covalent principle advocated here operates in close conjunction with the other factors which are thought to contribute to enzymatic catalysis, such as the effects of proximity, orientation, van der Waal's and electrostatic attraction, strain, general acid–base catalysis, and so on.

ACKNOWLEDGMENTS

For their careful reading of this paper my thanks are due to Drs. F. Lipmann, D. Mauzerall, E. B. Keller, C. T. Walsh, J. G. Hildebrand, and O. W. Griffith. I thank the National Science Foundation and the American Cancer Society for support.

REFERENCES

1. Reactions (2) and (3), as written, correspond to the so-called ping-pong kinetic mechanism, in which product A leaves the enzyme before substrate C adsorbs. But, as will appear in the sequel, E – B can also participate in a non-ping-pong kinetic mechanism, in which C adsorbs to the enzyme before A leaves.
2. M. L. BENDER, "Mechanisms of Homogenous Catalysis from Protons to Proteins," pp. 19–397, Wiley–Interscience, New York, 1971; T. C. BRUICE AND S. J. BENKOVIC, "Bioorganic Mechanisms," vol. 1. Benjamin, New York, 1966.
3. W. P. JENCKS, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, 1969.
4. L. E. ORGEL, "An Introduction to Transition Metal Chemistry," 2nd. ed., pp. 117–135. Methuen, London, 1966.
5. R. P. H. GASSER, *Quart. Rev.* **25**, 223 (1971).
6. J. R. ANDERSON (Ed.), "Chemisorption and Reactions on Metallic Films" vols. 1 and 2. Academic Press, New York, 1971.
7. J. R. ANDERSON AND B. G. BAKER, in Ref. 6, vol. 2, Chap. 8.
8. E. M. MÜLLER, *Quart. Rev.* **23**, 171 (1969); G. C. BOND, *Discuss. Faraday Soc.* **41**, 200 (1966); D. O. HAYWARD, in Ref. 6, vol. 1, pp. 255–258.
9. "Report of the Commission on Enzymes of the International Union of Biochemistry." Pergamon Press, London, 1961; "Enzyme Nomenclature: Recommendations of the International Union of Biochemistry on Nomenclature and Classification of Enzymes." Elsevier, Amsterdam, 1965.
10. Some of the enzymes in Table 1 have an attached coenzyme, which is indicated in parentheses. In every case the holoenzyme forms a covalent bond to substrate through its coenzyme, which may or may not be covalently fixed to the apoenzyme. This last point is regarded as immaterial, since only the holoenzyme is enzymatically active.
11. V. A. NAJJAR AND M. E. PULLMAN, *Science* **119**, 631 (1954); W. J. RAY, JR., AND G. A. ROSCELLI, *J. Biol. Chem.* **239**, 1228 (1964).
12. Z. B. ROSE, *Arch. Biochem. Biophys.* **140**, 508 (1970).
13. R. S. ANTHONY AND L. B. SPECTOR, *J. Biol. Chem.* **245**, 6739 (1970); R. S. ANTHONY AND L. B. SPECTOR, *J. Biol. Chem.* **246**, 6129 (1971); R. S. ANTHONY AND L. B. SPECTOR, *J. Biol. Chem.* **247**, 2120 (1972); D. L. PURICH AND H. J. FROMM, *Arch. Biochem. Biophys.* **149**, 307 (1972).
14. C. T. WALSH AND L. B. SPECTOR, *J. Biol. Chem.* **246**, 1255 (1971).
15. The Enzyme Commission did not classify this enzyme as a transferase. But the Commission recognized that some enzymes, such as this one, might be classified in more than one way.
16. H. INOUE, F. SUZUKI, H. TANIOKA, AND Y. TAKEDA, *Biochem. Biophys. Res. Commun.* **26**, 602 (1967). ATP citrate lyase (EC 4.1.3.8) catalyzes a multisubstrate reaction whose overall effect is that of a lyase. But early in its sequence of partial reactions the enzyme catalyzes the phosphorylation of an outer carboxyl group of citrate to form enzyme-bound citryl phosphate. Therefore, the enzyme also qualifies as a carboxylate kinase [C. T. WALSH AND L. B. SPECTOR, *J. Biol. Chem.* **244**, 4366 (1969)].
17. G. KREIL AND P. D. BOYER, *Biochem. Biophys. Res. Commun.* **16**, 551 (1964). Succinyl-CoA synthetase (EC 6.2.1.5) is a ligase. Yet among the partial reactions which it catalyzes is the phosphorylation of a carboxyl group of succinate to yield enzyme-bound succinyl phosphate. Therefore, the enzyme is also a carboxylate kinase [J. S. NISHIMURA AND A. MEISTER, *Biochemistry* **4**, 1457 (1965); J. G. HILDEBRAND AND L. B. SPECTOR, *J. Biol. Chem.* **244**, 2606 (1969)].
18. S. P. BESSMAN AND F. LIPMANN, *Arch. Biochem. Biophys.* **46**, 252 (1953); J. W. JENNE AND P. D. BOYER, *Biochim. Biophys. Acta* **65**, 121 (1962); M. S. STEINBERG, S. N. COHEN, AND W. W. WEBER, *Biochim. Biophys. Acta* **235**, 89 (1971); W. W. WEBER AND S. N. COHEN, *Mol. Pharmacol.* **3**, 266 (1967); B. RIDDLE AND W. P. JENCKS, *J. Biol. Chem.* **246**, 3250 (1971).
19. F. LYNEN, D. OESTERHELT, E. SCHWEIZER, AND K. WILLECKE, in "Cellular Compartmentalization and Control of Fatty Acid Metabolism" (F. C. Gran, Ed.), p. 1. Universitetsforlaget, Oslo, 1968; V. C. JOSHI, C. A. PLATE, AND S. J. WAKIL, *J. Biol. Chem.* **245**, 2857 (1970).
20. U. GEHRING, C. RIEPERTINGER, AND F. LYNEN, *Eur. J. Biochem.* **6**, 264 (1968).
21. Ref. 3, pp. 44–67.

22. Ref. 3, pp. 50–53.
23. G. E. LIENHARD, *Biochemistry* **9**, 3011 (1970).
24. R. A. ALBERTY, *Advan. Enzymol.* **17**, 1 (1956); W. W. CLELAND, *Biochim. Biophys. Acta* **67**, 104, 173, 188 (1963); Ref. 3, pp. 56–60.
25. D. E. KOSHLAND, JR., *Biol. Rev. (Cambridge)* **28**, 416 (1953); D. E. KOSHLAND, JR., *Discuss. Faraday Soc.* **20**, 142 (1956); R. M. BELL AND D. E. KOSHLAND, JR., *Biochem. Biophys. Res. Commun.* **38**, 539 (1970); Ref. 3, pp. 60–64.
26. W. A. BRIDGER, W. A. MILLEN, AND P. D. BOYER, *Biochemistry* **7**, 3608 (1968).
27. K. YAMAFUJI, H. OMURA, F. YOSHIHARA, M. YOSHITAKE, AND N. OYAMA, *Enzymologia* **19**, 180 (1958).
28. S. F. YANG, S. FREER, AND A. A. BENSON, *J. Biol. Chem.* **242**, 477 (1967).
29. F. J. MOFFET AND W. A. BRIDGER, *J. Biol. Chem.* **245**, 2758 (1970).
30. K. M. PLOWMAN AND W. W. CLELAND, *J. Biol. Chem.* **242**, 4239 (1967).
31. H. INOUE, F. SUZUKI, H. TANIOKA, AND Y. TAKEDA, *J. Biochem. (Tokyo)* **63**, 89 (1968).
32. I. A. ROSE, M. GRUNBERG-MANAGO, S. R. KOREY, AND S. OCHOA, *J. Biol. Chem.* **211**, 737 (1954),
33. A partial list of 60 enzymes which act through covalent-enzyme–substrate intermediates has been published [R. M. BELL AND D. E. KOSHLAND, JR., *Science* **172**, 1253 (1971)]. The count of known enzymes was 1100 in November, 1970 [A. L. LEHNINGER, *Fed. Proc.* **30**, 1397 (1971)]. The number is certain to be somewhat larger now (January, 1973).
34. W. BUCKEL, V. BUSCHMEIER, AND H. EGGERER, *Z. Physiol. Chem.* **352**, 1195 (1971); H. INOUE, T. TSUNEMI, F. SUZUKI, AND Y. YAKEDA, *J. Biochem. (Tokyo)* **65**, 889 (1969).
35. F. H. WESTHEIMER, *Advan. Enzymol.* **24**, 455 (1962).
36. J. STAVRIANOPOULOS AND L. JAENICKE, *Eur. J. Biochem.* **3**, 95 (1967); H. RÜDIGER AND L. JAENICKE, *Eur. J. Biochem.* **10**, 557 (1967); H. RÜDIGER AND L. JAENICKE, *Eur. J. Biochem.* **16**, 92 (1970); R. T. TAYLOR AND H. WEISSBACH, *Arch. Biochem. Biophys.* **123**, 109 (1968); R. T. TAYLOR AND H. WEISSBACH, *Arch. Biochem. Biophys.* **129**, 745 (1969); G. T. BURKE, J. H. MANGUM, AND J. D. BRODIE, *Biochemistry* **9**, 4297 (1970); G. T. BURKE, J. H. MANGUM, AND J. D. BRODIE, *Biochemistry* **10**, 3079 (1971).
37. L. SCHIRCH AND W. T. JENKINS, *J. Biol. Chem.* **239**, 3801 (1964).
38. H. G. WOOD, H. LOCHMÜLLER, C. RIEPERTINGER, AND F. LYNEN, *Biochem. Z.* **337**, 247 (1963).
39. A. G. DATTA AND E. RACKER, *Fed. Proc.* **19**, 82 (1960); B. L. HORECKER, S. PONTREMOLI, C. RICCI, AND T. CHENG, *Proc. Nat. Acad. Sci. USA* **47**, 1949 (1961); L. LJUNGDAHL, H. G. WOOD, E. RACKER, AND D. COURI, *J. Biol. Chem.* **236**, 1622 (1961).
40. J. E. FOLK, *J. Biol. Chem.* **244**, 3705 (1969); J. E. FOLK, P. W. COLE, AND J. P. MULLOOLY, *J. Biol. Chem.* **242**, 4239 (1967).
41. M. DOUDOROFF, H. A. BARKER, AND W. Z. HASSID, *J. Biol. Chem.* **168**, 725 (1947); J. VOET AND R. H. ABELES, *J. Biol. Chem.* **241**, 2731 (1966); R. SILVERSTEIN, J. VOET, D. REED, AND R. H. ABELES, *J. Biol. Chem.* **242**, 1338 (1967); J. VOET AND R. H. ABELES, *J. Biol. Chem.* **245**, 1020 (1970).
42. D. P. GROTH AND L. G. YOUNG, *Biochem. Biophys. Res. Commun.* **43**, 82 (1971).
43. H. WADA AND E. E. SNELL, *J. Biol. Chem.* **237**, 127 (1962); S. J. VELICK AND J. VAVRA, *J. Biol. Chem.* **237**, 2109 (1962); W. T. JENKINS AND L. D'ARI, *J. Biol. Chem.* **241**, 2845 (1966); C. P. HENSON AND W. W. CLELAND, *Biochemistry* **3**, 338 (1964).
44. E. GRAZI, F. CONCONI, AND V. VIGI, *J. Biol. Chem.* **240**, 2465 (1965).
45. K. UYEDA, *J. Biol. Chem.* **245**, 2268 (1970).
46. M. L. UHR, F. MARCUS, AND J. F. MORRISON, *J. Biol. Chem.* **241**, 5428 (1966); E. SMITH AND J. F. MORRISON, *J. Biol. Chem.* **244**, 4224 (1969); C. ROUSTAN, L.-A. PRADEL, R. KASSAB, AND N. V. THOAI, *Biochim. Biophys. Acta* **250**, 109 (1971).
47. N. MOURAD AND R. E. PARKS, JR., *Biochem. Biophys. Res. Commun.* **19**, 312 (1965); N. MOURAD AND R. E. PARKS, JR., *J. Biol. Chem.* **241**, 271, 3838 (1966); M. G. COLOMB, J. G. LATURAZE, AND P. V. VIGNAIS, *Biochem. Biophys. Res. Commun.* **24**, 909 (1966). E. GARCES AND W. W. CLELAND, *Biochemistry* **8**, 633 (1969).
48. R. L. SWITZER, *J. Biol. Chem.* **245**, 483 (1970); R. L. SWITZER, *J. Biol. Chem.* **246**, 2447 (1971).
49. B. WEISS AND C. C. RICHARDSON, *J. Biol. Chem.* **242**, 4270 (1967); B. WEISS, A. THOMPSON, AND C. C. RICHARDSON, *J. Biol. Chem.* **243**, 4556 (1968); C. L. HARVEY, T. F. GABRIEL, E. M. WILT, AND C. C. RICHARDSON, *J. Biol. Chem.* **246**, 4523 (1971); J. W. LITTLE, S. B. ZIMMERMAN, C. K.

OSHINSKY, AND M. GELLERT, *Proc. Nat. Acad. Sci. USA* **58**, 2004 (1967); B. M. OLIVERA, Z. W. HALL, Y. ANRAKU, J. R. CHIEN, AND I. R. LEHMAN, *Cold Spring Harbor Symp. Quant. Biol.* **33**, 27 (1968).

50. J. R. GREEN AND J. WESTLEY, *J. Biol. Chem.* **236**, 3047 (1961); J. WESTLEY AND T. NAKAMOTO, *J. Biol. Chem.* **237**, 547 (1962).
51. G. B. MICHAELS, J. T. DAVIDSON, AND H. D. PECK, JR., *Biochem. Biophys. Res. Commun.* **39**, 321 (1970).
52. L. B. HERSH AND W. P. JENCKS, *J. Biol. Chem.* **242**, 3468, 3481 (1967).