Ionic Intermediates in Enzyme-Catalyzed Carbon–Carbon Bond Formation: Patterns, Prototypes, Probes, and Proposals

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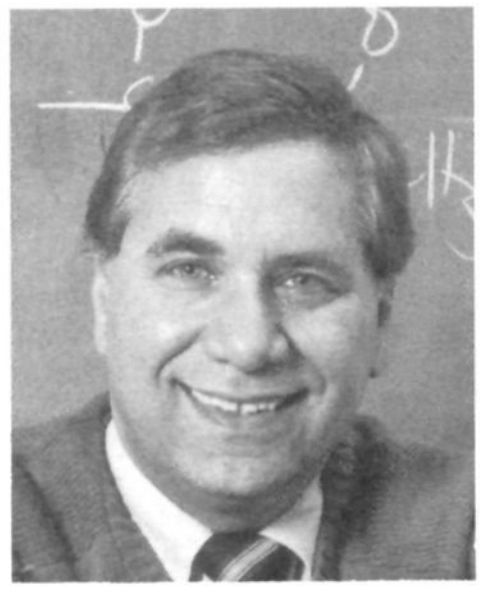

Ronald Kluger graduated from Columbia (A.B. 1965, chemistry) where he worked in Gilbert Stork's laboratory for 3 years. His graduate research was done at Harvard with Frank Westheimer (phosphate reaction mechanisms). After receiving his Ph.D. in 1969, he was an NIH postdoctoral fellow with Robert Abeles at Brandeis, working on enzyme mechanisms. He was at the University of Chicago from 1970 to 1974 where he began research in bioorganic chemistry and was awarded a Sloan fellowship. In 1974, he moved to the University of Toronto where he is currently a Professor of chemistry. He has received national awards in Canada for organic and biochemical research: the Merck Sharp and Dohme Award of the Chemical Institute of Canada in 1983 and the John Labatt Award of the Canadian Society for Chemistry in 1990. His research interests are in bioorganic chemistry, especially mechanisms related to coenzymes, biochemical phosphates and anhydrides, site-directed reagents for protein modification, and mechanistically designed enzyme inhibitors. Other reviews that he has written recently have been on the physical organic basis of enzyme catalysis, thiamin mechanisms, biotin carboxylation, phosphate substitution mechanisms, and the enzymology of carbon–carbon bond formation and cleavage.

I. Introduction

The organic chemist views chemical structures associated with biochemical phenomena from a perspective developed from experience acquired in dealing with similar matters. We admire their complexity but seek patterns which simplify the structural relationships. One way of finding these patterns requires the discovery of intermediates in biosynthetic pathways. This field of research has appealed to chemists for over a century and has led to an extensive body of knowledge in the area which continues to expand technically and conceptually while essential principles are elucidated. In these processes the formation of carbon–carbon bonds is the most obvious source of structural regularity. Thus, enzymes that catalyze formation of carbon–carbon bonds are an important target for investigation. While there are obviously diverse sets of substrates and highly developed specificity, there are mechanistic themes that lead to the conclusion that evolution has selected efficient pathways. The organization of this review is based upon the general nature of the intermediates that are likely to occur in the step of the enzymic reaction which produces a carbon–carbon bond.[1]

II. Enzymic Carbon–Carbon Bond Formation with Carbocations

Carbocationic intermediates are common to many organic reactions that involve reactions at tertiary, allylic, or benzylic carbon centers. Since enzymes usually function in an aqueous, polar environment and a polar medium can encourage the conversion of a neutral species to a more reactive ion pair, it is not surprising that ionic mechanisms are common in enzymic catalysis. Early studies suggested that the driving force for the cyclization of squalene to cholesterol is driven by the formation of an electron deficient species. Later it was shown that the intermediate in the reaction is an epoxide and it is not clear whether or not an intermediate cation is actually involved. However, later work on the

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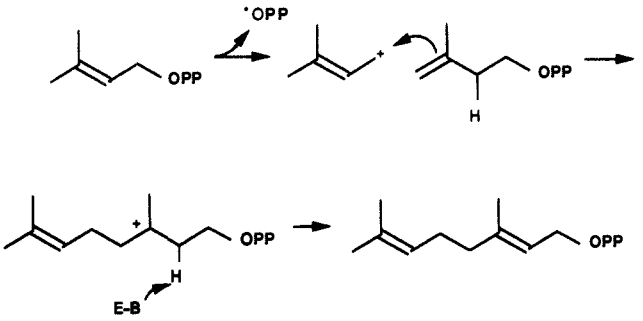
SCHEME 1. Generalized Prenyl Transfer



biosynthesis of terpenes has given clearer evidence in support of the existence of carbocations as intermediates. Convincing studies, particularly by Poulter and his co-workers makes it reasonable to assume that carbocations form during the course of enzymic catalysis.

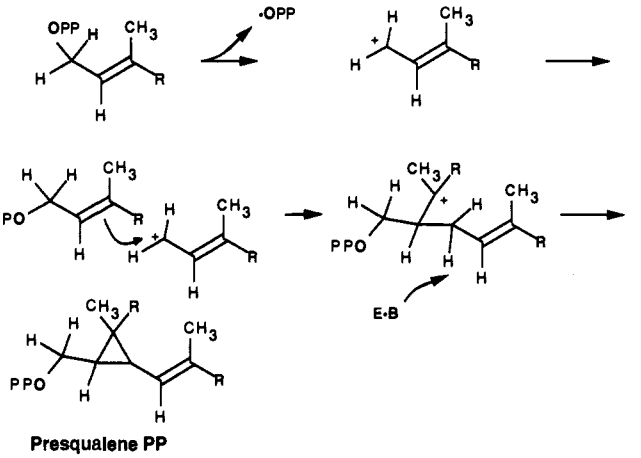
A. Reactions with Cationic Intermediates

Electron-deficient ionic intermediates or transition states are most common in pathways in which the carbon–carbon bond formation process occurs through substitution at an allylic center. The formation of terpenes and other isoprenoid lipids involves the condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate,[2] a process promoted by prenyl transferase. The enzyme is widely distributed and promotes similar transfers from oligomeric substrates. The leaving group is the pyrophosphate moiety of the allylic pyrophosphate, dimethylallyl pyrophosphate or a homologue. The reaction can be rationalized in terms of the formation of a substituted allylic carbonium ion (and inorganic pyrophosphate which forms as an ion pair with the carbonium ion). The electron-rich agent which adds to the electron-deficient center to form the carbon–carbon bond is the π electron density of the isopentenyl pyrophosphate. The stereochemical course of the biosynthesis of complex natural products has been determined and is consistent with such a mechanism.[3] A generalized picture of this process is shown in Scheme 1.

The stereochemical course of the reaction in a nonenzymic system would normally be a probe of the nature of the carbonium ion intermediate. The formation of a symmetrically solvated intermediate would give complete stereochemical scrambling of the reaction center. Ion pairing between the carbonium ion and the leaving group would lead to a degree of inversion while solvent participation would lead to retention of relative configuration. This analysis does not apply in an enzyme-catalyzed reaction because the chirotopic environment (terminology of Mislow and Siegel[4]) of the enzyme site controls the stereochemical outcome, whatever the nature of the intermediate. Isotope scrambling experiments[5] could also give similar information, but these have not been observed in enzymes catalyzing this class of reactions.

The assumption that carbocationic intermediates are involved in these reactions has received strong support from studies which demonstrate that cationic analogues serve as powerful enzyme inhibitors. In terms of transition-state theory, enzymes promote reactions by the selective stabilization of transition-state structures.

SCHEME 2. Formation of Presqualene PP from Farnesyl PP



Presqualene PP

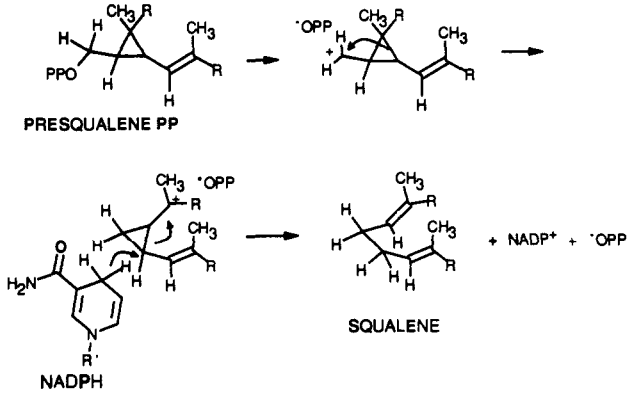
This is equivalent to having selectively tighter binding of transition states as compared to reactants or products. Thus, materials whose structure resembles that of a transition state should bind tightly to an enzyme. Information about intermediates in prenyl transfer comes from studies in which ammonium analogues of the carbonium ion are used as inhibitors. If one of the enzyme's main functions is to stabilize cationic intermediates, then other cations with structures similar to those expected in the reaction should bind tightly. Since the cation is generated with the loss of an anionic leaving group, the best analogue of the transition state should be a pair of oppositely charged ions.

Poulter and co-workers[6,7] have shown that, in enzymes which promote prenyl transfer, an alkylammonium compound which is a structural analogue of the proposed intermediate is only a very weak inhibitor in the absence of pyrophosphate ion. In the presence of pyrophosphate ion, binding becomes dramatically stronger. In a molecule in which the pyrophosphate is covalently attached to form an internal ion pair, an even more powerful inhibitor is generated since the ion pair is held in place.

1. Squalene Synthetase and Related Processes

Dramatic proof of the involvement of carbocationic intermediates comes from the observation of a spectacular rearrangement related to the remarkable properties of the cyclopropylcarbinyl system. The head-to-head union of two molecules of farnesyl pyrophosphate to produce squalene is promoted by squalene synthase.[8] The enzyme utilizes NADPH as a cofactor. If the cofactor is omitted, another species, presqualene pyrophosphate accumulates.[9] Upon addition of NADPH, this is converted to squalene. The structure of presqualene pyrophosphate was elucidated by Epstein and Rilling who found that the material contains a highly substituted cyclopropane ring as a central feature.[10] Two groups[11,12] independently proposed the route which now is accepted as that which leads to presqualene pyrophosphate and to squalene. The head-to-head carbon–carbon bond forming reaction, which produces presqualene pyrophosphate, formally involves the combination of the elements of two farnesyl moieties, one as the pyrophosphate and the other as the ion from which pyrophosphate has been cleaved (Scheme 2). In contrast, the more common head-to-tail

SCHEME 3. Formation of Squalene from Presqualene PP



carbon–carbon bond forming process involves two different initial species as reactants.

The conversion of presqualene pyrophosphate to squalene is an example of the rearrangement of a substituted cyclopropylcarbinyl carbonium ion (Scheme 3).[13] This ion has been studied as an example of a σ-delocalized system,[14] and the formation of squalene is a logical possibility of one of the established rearrangements of the ion. However, the enzyme is clearly involved in directing the rearrangement since Poulter has shown that the spontaneously formed products that would result are derived from structural isomers of the squalene skeleton.[15]

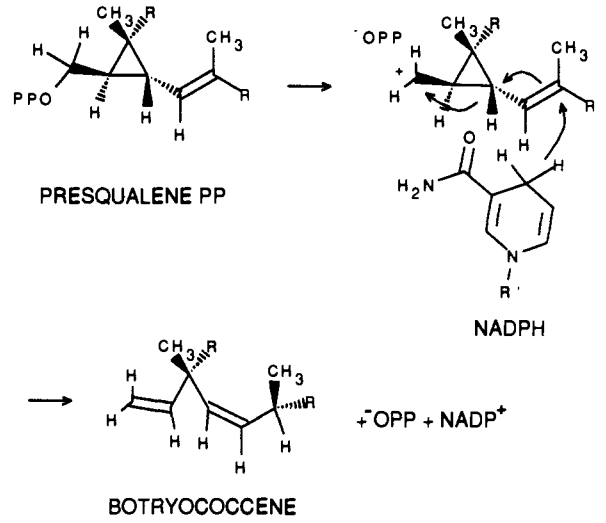
While the head-to-head linkage of farnesyl units produces squalene, there are many other terpene-related materials that appear to result from linkages other than common 1′,4 (head-to-tail) condensation between an allylic pyrophosphate and a homoallylic pyrophosphate. Huang and Poulter have provided a mechanistically detailed picture of the mechanism of 1′,3 bond formation in the biosynthesis of the triterpene botryococcene.[16] This C_{30} compound is formed from two equivalents of farnesyl pyrophosphate. Presqualene pyrophosphate, the precursor to squalene, is also a precursor to botryococcene. As noted in the previous section, cyclopropylcarbinyl model systems for presqualene pyrophosphate do not produce the structure characteristic of squalene. In fact, they react almost exclusively to produce the 1,3′ linkage characteristic of botryococcene.

Using stereospecifically labeled precursors, Huang and Poulter have provided convincing evidence that squalene and botryococcene arise from a common cyclopropylcarbinyl intermediate, presqualene pyrophosphate.[16] Huang and Poulter propose that the relative binding position of NADPH and the cyclopropane intermediate will control the outcome with respect to the final carbon–carbon bond which is produced (Scheme 4). This supports the existence of carbonium ion intermediates and implicates such intermediates in analogous reactions of other substrates in prenyl transfer systems.

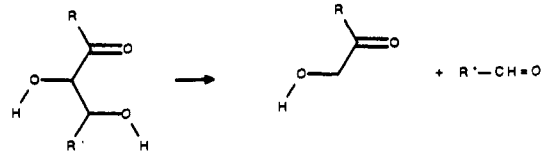
2. Generalization

Enzymes catalyze the formation of carbon–carbon bonds between allylic and homoallylic pyrophosphate species by mechanisms which involve carbocations, stabilized as ion pairs and generated from allylic pyrophosphates. Reaction patterns are consistent with model systems and the mechanisms are based on

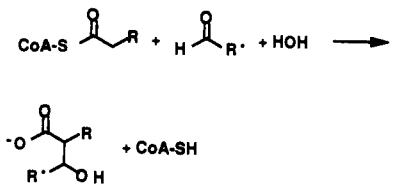
SCHEME 4. Formation of Botryococcene from Presqualene PP



SCHEME 5. Aldolase Reaction



SCHEME 6. Synthase Reaction



analogies with the models, stereochemical information (which is subject to interpretation) and the structural requirements for inhibitors.

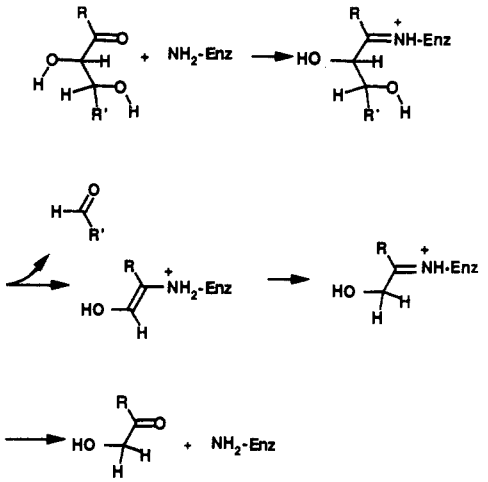
B. Carbanionic Transition States

The formation of carbon–carbon bonds by the addition of a nucleophilic carbon center to a carbonyl or other unsaturated electrophilic carbon center is a common enzymatic reaction pattern. The mechanisms can be classified by the participants in the carbon–carbon bond forming process:

(1) Carbanions adjacent to imines, better described as enamines, add to carbonyl centers. These are found in the aldolase enzymes catalyzing carbon–carbon bond formation and cleavage (Scheme 5).[16] Bond formation is thermodynamically favored but coupling to a metabolic process can make the overall reaction efficient in either direction.

(2) Enolates and enols typically are derived from a ketone or thiolester. These add to the carbonyl group of the ketone in an α-ketoacid or other carbonyl compound.[17] The loss of a proton from the α-position of a carboxylic acid has a very high thermodynamic barrier due to the resulting loss of resonance interactions within the carboxylic acid.[18] Therefore, carboxylic acids do not normally react directly but are converted first to thiolester derivatives of coenzyme A (CoA) (Scheme 6). Since a thiolester is a more acidic carbon acid at its α-position due to the increased availability of the car-

SCHEME 7. Aldolase Mechanism



bonyl group for electronic delocalization,[18] carbanion formation is catalyzed by the process which specifies the substrate as a derivative of CoA.[19]

It has been proposed that some aldolases function by a carbanion rather than enamine mechanism in which case a metal ion serves a catalytic role.[17] In these cases metal ion complexes of sugars may act as electrophiles which in turn react with enolate carbanions derived from the other reaction partner. Alternatively, the metal ion may promote the formation of the enolate by coordination of the base and substrate as has been demonstrated by studies of metal ion catalyzed enolization reactions.[20] In those studies, it was shown that pyridine bases catalyze the enolization of 3-ketophosphonates and the reaction is subject to steric effects by substituents on the pyridine ring. In the reaction catalyzed by a combination of magnesium ion or manganese ion and a substituted pyridine base there is a much reduced steric sensitivity. This is consistent with a mechanism in which water coordinated to the metal ion which is coordinated to the substrates reacts with the pyridine base. Since the reaction no longer involves a direct proton transfer from the ketone to the base, steric interactions are reduced.

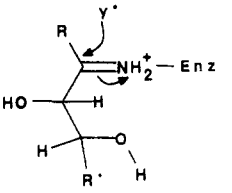
1. Aldolases Utilizing Enamine Intermediates

If a ketone or aldehyde reacts with an amine to form a protonated imine in neutral solution, the corresponding equivalent of a carbanion generated by deprotonation of the α-position is an enamine. The ability of enamines to serve as carbanion equivalents was noted in studies of the catalysis of aldol reactions by amines.[21,22] Enamines are now widely used as carbanion equivalents in synthetic organic chemistry.

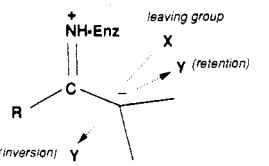
Adolases produce an imine as an initial intermediate from the reaction of a carbonyl group of the substrate with the ϵ-amino group of a lysine residue of the enzyme. The initially formed imine can be deprotonated by a relatively weak base to give a carbanion equivalent. This in turn is involved in the condensation reaction with a carbonyl compound (Scheme 7).

The existence of imines as intermediates has been implied from the results of chemical trapping experiments. The addition of sodium borohydride to a solution of the enzyme alone, followed by dialysis to remove borohydride, produces active enzyme. However, in the presence of the ketonic substrate, the enzyme is inactivated.[23] Analysis of the inactivated enzyme reveals that the substrate has been trapped by reduction of the imine which results from addition of the ϵ-amino group of a lysine residue to the carbonyl group of the substrate (Scheme 8).[23] Further confirmation comes from the use of hydrogen cyanide to intercept the imine. The Strecker synthesis of amino acids involves the addition of hydrogen cyanide to an imine to form a cyanoamine, followed by hydrolysis of the nitrile. The cyanoamine is stable and thus the imine does not re-form nor can it be converted to the corresponding enamine. Similarly, trapping of the enamine from an enzyme can be accomplished by the same addition process.[24]

SCHEME 8. Trapping of Enamines



SCHEME 9. Stereochemical Considerations



Kinetic evidence for the existence of imine–enamine intermediates comes from the observation that aldolases catalyze preequilibrium proton exchange at the nucleophilic carbon center. The rate is consistent with the intermediate involvement of the conjugate base in the condensation reaction.[25]

The aldolase reaction is formally an electrophilic substitution of a carbonyl carbon for a proton at the α-carbon atom of the enamine. Stereochemical studies have shown that the proton and carbonyl group bind to the same face of the enamine carbon (carbanionic center).[26] A substitution reaction in which the leaving group and incoming group are on the same face of a carbanion is indicative of a reaction in which the carbanion is stabilized by solvent or by a neighboring electrophile (Scheme 9).

2. Direct Carbon–Carbon Bond Formation from Acetyl Coenzyme A

A group of carbon–carbon lyases promote carbon–carbon bond formation between the α-carbon of a thiolester (typically acetyl-CoA) and the carbonyl carbon of a ketone or aldehyde. These enzymes catalyze a reversible Claisen-type condensation reaction, analogous to an aldol condensation, which is made irreversible by the hydrolysis of the thiolester. Unlike the aldolases, these enzymes do not catalyze preequilibrium proton exchange at the potentially carbanionic center[27] and the reactions proceed with backside displacement as indicated by the observation of net inversion of relative configuration.[28] The lack of exchange does not reveal whether an intermediate carbanion forms since there is no way to tell if such a carbanion would be accessible to the medium. The finding that the electrophiles depart and enter from opposite faces is consistent with an intermediate carbanion stabilized as an

ion pair with the entering and leaving groups.

Enzymes which catalyze carbonyl condensation reactions involving one component which is not an aldehyde or ketone have been called "Claisen enzymes".[29] Although these reactions are not formally Claisen condensations they are distinguished by their mechanisms from the other adolases.

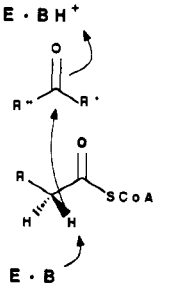
a. Carbanions in Claisen Enzymes. A carbanion (enolate) is a good carbon-centered nucleophile and it will add readily to a carbonyl electrophilic partner. Since enolates derived from ketones or esters are very strong bases,[18] proton removal by a base within an enzyme should be a highly endergonic process resulting necessarily in a large kinetic barrier unless the environment specifically stabilizes the transition state for the transfer. One mechanistic alternative to the formation of the enolate might be a concerted process in which the electrophilic carbonyl compound is attacked as the proton is removed. A third possibility is a concerted transformation of the ketone to the enol with general base catalyzed attack of the enol upon the carbonyl compound. Although it is clear that a Claisen enzyme must be able to promote the removal of an α-proton, the intermediate which forms need not be in equilibrium with the solvent under the conditions of the catalytic reaction. Both malate synthetase and citrate synthase catalyze the addition of the equivalent of the conjugate base of the carbon acid of acetyl-CoA to the keto group of a 2-keto acid but neither catalyzes exchange reactions of the α-protons.[27] If proton transfer and carbon–carbon bond formation occur in a single step, then no proton exchange would be observed. Alternatively, lack of exchange might indicate that the intermediate is simply inaccessible to the bulk medium.

b. Trapping of Carbanions as Evidence of Their Existence in a Mechanism. Oxidizing reagents which react with carbanions have been utilized to provide indirect evidence of the involvement of carbanions in catalytic pathways. Tetranitromethane serves as a source of the equivalent of the powerful electrophile, NO_2^+.[30] In aldolase-catalyzed reactions and other reactions where independent evidence exists for reversible carbanion formation, tetranitromethane intercepts the carbanion and generates nitroformate. However, this reagent does not affect Claisen enzymes.[29] Again, this can be interpreted as indicating that the carbanion is inaccessible, locally stabilized by a counterion, is converted to the enol, or does not form.

c. Stereochemical Patterns of Claisen Enzymes. Stereochemical studies reveal patterns for the substitution process in Claisen enzymes. In these reactions, carbon–carbon bond formation occurs on the face of the nucleophile opposite to that from which the proton is abstracted. Srere summarized the stereochemical patterns in enzymes responsible for the formation of citrate and its breakdown.[27] Citrate is formed by citrate synthase from the reaction of acetyl-CoA and oxaloacetate and is cleaved by citrate lyase to acetate and oxaloacetate. Citrate cleavage enzyme converts citrate, coenzyme A, and ATP to acetyl-CoA, oxaloacetate, and ADP.

The condensation of the acetyl methyl carbon of acetyl-CoA with the carbonyl group of oxaloacetate is common to the surmised transition state of all these enzymes. The acetyl methyl carbon becomes deprotonated during the course of the condensation process and the 2-maleyl group, from oxaloacetate, replaces the proton. Normally one would expect that removal of the proton would be a slow process, given the high thermodynamic barrier to formation of such a carbanion. One way to avoid the carbanion is to have the proton removal be part of a process concerted with carbon–carbon bond formation, but is such a mechanism reasonable?

SCHEME 10. Reaction Diagram for Claisen Enzymes



Stereochemical studies have followed the course of the reaction at the α-position through the use of the "chiral methyl" group. The reaction takes place with inversion of relative configuration.[31,32] While most enzyme are specific for the *si* face of the carbonyl group of oxaloacetate, some bacterial citrate synthases promote reaction at the *re* face. The facial selectivity therefore does not have any mechanistic significance since it is inconsistent, but the substitution pattern at carbon may indicate an evolved feature based on mechanistic advantage.[34]

The implications of mechanisms from such data can be derived from the pioneering work of Cram on the stereochemical consequences of carbanionic mechanisms. The stereochemical outcome is the result of several competing factors. Carbanionic reactions in general are not concerted and special factors would have to be present to change the mechanism.[35] Formation of an intimate ion pair between the conjugate base of the substrate and the conjugate acid of a catalytic base in a poor solvent, forces attack of the incoming electrophile to the opposite face of the carbanion.[36] Preassociation of the incoming electrophile could serve to trap the high energy species so that the reaction would proceed efficiently. The intermediate would form but would not be not equilibrated with the solvent leading to no observable exchange. Alternatively, retention and racemization result from the influence of the counter ion and the solvent.

d. Reaction Coordinate Diagram for Claisen Enzymes. The issues involved in consideration of whether it is possible for processes to be concerted are readily seen through the use of a three-coordinate reaction diagram.[37-40] For a condensation reaction, on one axis we plot the progress of carbon–carbon bond formation and on the other axis we plot the progress of C–H bond cleavage. Free energy is the third coordinate of the diagram and the path with the lowest energy transition state for its rate determining step will be favored. C–H bond cleavage involves transfer of the proton to a basic group on the enzyme and therefore proton transfer to a "B–E" species is synchronous with C–H bond cleavage (Scheme 10).

The next step is addition of the carbanion (enolate) to the carbonyl carbon of the other reactant and this

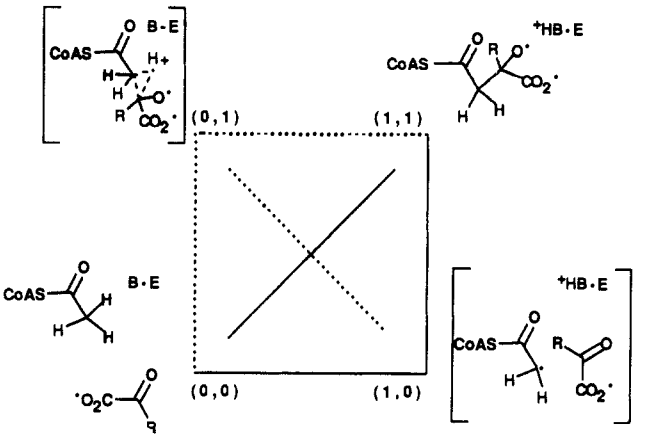
follows the second coordinate. The path of a concerted reaction avoids the corner corresponding to the intermediate carbanion. Conceptualizing the structure of any transition state for a concerted reaction requires analysis as to the meaning of departure from the edges of the diagram. Grunwald's procedure enhances this approach.[39] The corner of the diagram corresponding to the intermediate carbanion is set opposite to a corner representing another intermediate which corresponds to a path in which events take place in the opposite order, even if this is improbable. A line connecting reactants and products is termed the *main reaction* and the perpendicular diagonal, between intermediates, is called the *disparity reaction*. If there is a well in the energy surface of the disparity reaction, then a concerted reaction will be able to occur. Since energy surfaces are continuous, finding such a well anywhere is sufficient to assure that a concerted reaction is possible. Alternatively, if the stepwise path involves an intermediate with no possibility of a lifetime of more than one vibration (ca. 10^{-13} s), the concerted path is a necessary alternative. An example of such a diagram is in the following section.

e. Malate Synthase Intermediates. As an illustration, for the malate synthase reaction, we plot reaction progress in the general direction of C–H bond cleavage on the *X* axis and we plot the formation of a new carbon–carbon bond on the *Y* axis. The coordinates are labeled (0,0) for the start of the reaction, (1,1) for the products, and (0,1) and (1,0) for the intermediates. Energy increases in the direction above the *X*–*Y* plane and decreases below it. Carbon–carbon bond formation is exergonic so that (1,1) is downhill from (0,0). Breaking the C–H bond (by transfer of the proton to a base on the enzyme, B–E) without formation of a carbon–carbon bond leads to the carbanion (enolate) intermediate which characterizes the stepwise mechanism. This is above the reactants by the free energy corresponding to the formation of the enolate by the base on the enzyme (B–E becomes $^{+}$HB–E). Since the formation of a carbon–carbon bond without breaking the C–H bond leads to unconventional bonding, this axis label is not directly carbon–carbon bond formation but indicates that whatever bonding that can occur without departure of the proton is complete at the point marked (0,1).

f. Use of a Grunwald Diagram for Malate Synthase. Recognition of the disparity reaction is a useful way to devise a basis from which to interpolate the structure of the transition state in a concerted reaction and to consider the existence of such a path (Scheme 11).

The line for the main reaction mode then can be visualized as sliding like a string along the "solid" energy coordinate of the disparity reaction. The disparity coordinate can be analyzed for the possibility of a concave section through which the main reaction can take a lower energy route than permitted by the stepwise processes. The intermediates and products are shown as coenzyme A derivatives (although hydrolysis occurs during the course of the reaction) and proton transfer to oxygen has not been included. The intermediate in the disparity reaction results from the improbable situation of initial carbon–carbon bond formation without proton transfer. Since this necessarily involves an expansion of valence at carbon, the intermediate contains two 5-coordinate carbon atoms analogous to edge protonated cyclopropane species where the proton is delocalized across the σ-bonds and there are two electrons distributed among three nuclear centers. This should be a considerably higher free energy point than the enolate and its partners at the opposite corner.

SCHEME 11. Grunwald Disparity Diagram for Malate Synthase



To the extent that the concerted path for the main reaction involves a transition state which does not have a full carbanionic center, it must bear some resemblance to the intermediate of the disparity reaction. It is unlikely that the intermediate with 5-coordinate carbon atoms would exist in a stepwise process under normal circumstances. The energetic problem with the concerted reaction, besides its higher entropic demands, is that it must bear some component of this high energy structure. There is no apparent reason for there to be a concave section on the axis of the disparity reaction in this case: partial transfer of the proton from the substrate to a base of the enzyme does not stabilize the situation until the proton is fully transferred. Therefore, the carbanion–enolate in the stepwise main reaction should always lie on a lower energy path. The only alternative reason for a concerted reaction would be if the carbanion were too unstable to exist.[38] In that case, the lifetime of the species is less than a vibration and by the definitions of transition-state theory, this is not distinguishable from a transition state.

g. Double Isotopic Fractionation and Carbanion Intermediates. Proof of the existence of a carbanion–enolate intermediate has been attempted by a number of approaches. The most direct evidence is the observation of isotopic exchange of the α-hydrogen for deuterium or tritium (in deuterated or tritiated water) at a kinetically competent rate. If the carbanion is accessible to the solvent, instead of reacting with the carbonyl carbon of the second substrate, the carbanion should be able to react with the solvent to produce the isotope-exchanged reactant. Dissociation of the reactant from the enzyme leads to the observed exchange. However, in all enzyme reactions where this method has been attempted, exchange is only observed for materials which are not substrates.[29] This does not rule out carbanions as intermediates in these cases but rather suggests that the enzyme operates most effectively by protecting the carbanion or its equivalent from reaction with solvent so that it can react more efficiently with the second substrate. Therefore, other methods have

been developed to test for the involvement of an intermediate.

The interdependence of the magnitude of the kinetic consequences of two different isotopic substitutions can establish whether two bond-making or bond-breaking events occur in one or more steps.[41,42] The method involves the observation of the magnitude of the *change* in the kinetic isotope effect on the second process of the sequence due to isotopic substitution affecting the first process. If the reaction can occur in two steps, isotopes are substituted at sites associated with each step, first separately then in tandem. If the intrinsic effect on the second step (that which is observed when there is no other isotopic substitution) is greater than the observed effect (when the second isotope is present) the first step must be partially rate-limiting.

In a single-step process which involves both isotopically sensitive processes (as primary isotope effects; the bond to the isotope is broken), the isotope effects should be independent, according to the fundamental assumption of transition-state theory since the bond to the atom (the isotopic atom in this case) in the transition state has no residual vibrational component.[1] Therefore, the isotope effect is due primarily to differences in ground-state vibrational levels. In a single step in which both isotopic bonds are involved for the two processes of interest, it is reasonable to expect that the isotope effects should be expressed independently. In a sequential system, the first isotope effect perturbs the proportioning between isotopes in the second.

h. Isotopic Fractionation in the Malate Synthetase Reaction. Malate synthase catalyzes the production of malate by the condensation of acetyl-CoA and glyoxylate. This Claisen enzyme has also been studied by the double isotope fractionation method.[43] Eggerer and Klette[44] found that malate synthase does not catalyze exchange of the methyl protons of acetyl-CoA with solvent in the absence of glyoxylate. The stereochemical course of the reaction leads to inversion at the acetyl carbon, a result which is consistent with either a stepwise or concerted process.[43,44] Therefore, these results do not establish whether the reaction involves an intermediate.

The most reasonable stepwise chemical mechanisms have C–H bond cleavage precede carbon–carbon bond formation. The alternative stepwise mechanism, in which carbon–carbon bond formation precedes proton removal, necessarily involves a 5-coordinate carbon center, a species which is certain to be very high in energy. Concerted reactions will have characteristics of that structure in a transition state and therefore in general will tend to be avoided in favor of stepwise process with initial C–H bond fission if this is possible. The test of such predictions was done by Knowles and co-workers who specifically tested whether cleavage of the carbon–hydrogen bond and formation of the carbon–carbon bond occur in the same step by using double isotopic fractionation.[43] In a double isotopic fractionation study, it first must be determined that there is a hydrogen isotope effect on V_{max}/K_m. Then the isotope effect for V_{max}/K_m, with ^{12}C replaced with ^{13}C at a position involved in carbon–carbon bond formation, is determined for substrates with hydrogen and deuterium in the bond that is cleaved. Northrop's shorthand nomenclature is convenient to use: $V_{max}/K_m = V/K$. The ratio of V/K for substrates with ^{12}C to those with ^{13}C is written as $^{13}(V/K)$. The ratio for substrates containing hydrogen in the position where carbon–hydrogen bond cleavage occurs is $^{13}(V/K)_H$, and the ratio for substrates in which a C–D bond is broken is $^{13}(V/K)_D$.

The carbon isotope effect on V/K at C-1 of glyoxylate was determined by using natural abundance carbon isotopic mixtures of acetyl-CoA and trideuterio-acetyl-CoA as the nucleophilic reactant. It is known for this enzyme that the H/D isotope effect on V/K and V is significant.[46] The observed carbon isotope effect is based upon analysis of product distributions and not on measurement of rates. Therefore, the ratio gives a direct reflection of the competition in the carbon–carbon bond forming step between the isotopomers. If proton removal occurs in a step which precedes that in which the carbon isotope is manifested, and both steps are partially rate-determining, the observed carbon isotope effect will be reduced. If the reaction is concerted, then deuteration will have no effect on the observed $^{13}(V/K)$ since in the transition state both bonds are broken and the H/D effect will not affect the proportioning of the intermediate and the observed $^{13}(V/K)$ isotope effect. The analysis of the product's isotopic composition compared to that of the reactant must be done at early stages of the reaction. The need for accurate measurements requires that the carbon source measured for isotopic distribution be done by isotope ratio mass spectrometry of carbon dioxide.

Knowles and co-workers observed $^{13}(V/K)$ at C-1 of glyoxylate by analyzing the isotopic composition of the malate produced in the reaction at early and late stages.[43] The value of $^{13}(V/K)_H$ for the condensation of acetyl-CoA with glyoxylate catalyzed by malate synthase was determined to be 1.0037. When trideuterio-acetyl-CoA was used as a substrate in place of acetyl-CoA, $^{13}(V/K)_D$ was also 1.0037. The deuterium isotope effect itself $^D(V/K)$ was determined to be 1.3 and ^DK was 1.0, in agreement with earlier reports of Eggerer's group.[44-46] Since there is a $^{13}(V/K)$ effect, one would conclude that a step involving formation of a bond to C-1 of glyoxylate is kinetically significant. Since there also is a $^D(V/K)$ effect, one would also conclude that carbon–hydrogen bond cleavage is also kinetically significant. Does this mean that the processes are concerted?

If $^{13}(V/K)_D$ and $^{13}(V/K)_H$ for malate synthase are identical, one would expect that both centers to be involved in rate-determining processes. In a simplified energy diagram, the presence of a deuterium isotope effect ahead of a carbon isotope effect causes the (apparent) observed carbon isotope effect to become larger. In a steady-state model, the observed rate constant, k_{obs}, depends on $k_2/(k_{-1} + k_2)$. If isotopic substitution decreases k_1 and k_{-1}, then the observed rate constant becomes less affected by the magnitude of k_2. Therefore, in this case the apparent $^{13}C/^{12}C$ isotope effect will decrease if deuteration reduces the size of k_1 and k_{-1}. If the isotope effect is constant, then the expression for k_{obs} does not apply. If the two processes occur in the only kinetically significant step, then proton removal will not affect the observed $^{13}C/^{12}C$ isotope effect. In the absence of other information, one might conclude that these data indicate that C–H bond cleavage and

C–C bond formation occur in the same step. However, other kinetic information (discussed in the next section), the stereochemical observation of inversion in these systems, and the Grunwald diagram analysis, show that there is a significant disadvantage for a concerted mechanism versus a stepwise mechanism.

The results do not *require* that the reaction occurs by a concerted mechanism involving simultaneous C–H cleavage and C–C formation, they simply rule out a mechanism in which the two steps are each partially rate-determining. If the processes occur in different steps and if one or both steps occur after the rate-determining step, then the isotope effects are independent. Two further pieces of information clarify the picture and establish that (1) a kinetically significant step which is independent of isotope effects precedes both C–H bond breakage and carbon–carbon bond formation, (2) the observed carbon isotope effect is due to the effects of ^{13}C on an uncatalyzed preequilibrium process, and (3) the carbon–carbon bond forming step is kinetically insignificant.[43]

A comparison of the primary hydrogen isotope effect measured in two different ways for the malate synthase reaction provides the clue that permits interpretation of the strange result in the double isotope fractionation study. It leads to the conclusion that a kinetically significant step which is isotope-insensitive precedes C–H bond cleavage. Therefore the observed hydrogen isotope effect is not a measure of the properties of the rate-determining step.

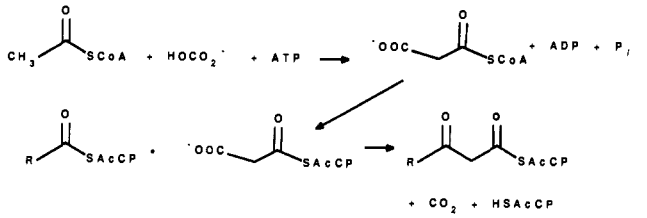
In an earlier study, Lenz and Eggerer found that the *intramolecular* ^{D}k effect is 3.8.[45] An intramolecular isotope effect measures the results of competition between breaking a C–H bond versus a C–D bond in the methyl group of partially deuterated acetyl-CoA. The usual kinetic isotope effect is *intermolecular*, where samples of different isotopic composition react under parallel conditions. If the step in which C–H bond breaking occurs is after the rate-determining step, there should be no observable intermolecular isotope effect. In the case of an intramolecular isotope effect, the measured quantity is the isotopic distribution in the products. Every C–D bond cleavage process competes internally with C–H bond cleavage in the same reacting molecule and the deuterated species is necessarily carried forward by C–H cleavage. Therefore, even if the bond-breaking step occurs after the rate-determining step, the product distribution will reflect a preferential cleavage of the C–H bond. In the case of an *intermolecular* measurement, the C–D bond is necessarily cleaved in the deuterated substrate and therefore an isotope effect is noted only if C–D cleavage slows the net rate and this must precede the rate-determining step. Thus, the *intramolecular* effect is not a measure of the relative rates of reaction of deuterated and undeuterated substrates but only of the branching in the step which determines whether the product results from the loss of deuterium or hydrogen, a distinction that must occur whether the step affects the rate of the reaction or not.

Under conditions where an intermolecular isotope is measured, if the rate law is a mixture of expressions some of which are dependent and others which are independent of isotope effects, the observed rate constants for deuterated and undeuterated substrates will have a smaller ratio than the product distribution measured in the intramolecular case. For malate synthase, the measured intermolecular isotope effect is only 1.36 while the intramolecular effect is 3.8.[43,45] If proton abstraction occurred in the first irreversible step, there would be no difference between the measured intramolecular and intermolecular and isotope effects. The fact that the two isotope effects are different indicates that the C–H bond cleavage step is preceded by an isotopically insensitive but kinetically significant step. Therefore, if the C–H bond cleavage step is concerted with carbon–carbon bond formation and that step is partially rate-limiting (which it must be since an isotope effect is observed), that concerted step becomes more rate-limiting upon deuteration and the magnitude observed for $^{13}(V/K)$ increases.

Since there is an isotopically insensitive but kinetically significant step preceding the C–H bond cleavage step, the observation of an invariant $^{13}(V/K)$ effect upon deuteration is obviously not the result of a concerted mechanism, since that would require the two events to be in one step and it was shown that the two processes are on either side of the rate-determining process in the reaction sequence. Chemical reasonableness requires that carbon–carbon bond formation cannot precede carbon–hydrogen bond cleavage yet there is an invariant $^{13}(V/K)$ effect which is greater than unity.

Is any reasonable mechanism consistent with the data? The answer lies in an observation of a probable isotope effect in a coupled non-enzymic phenomenon. The double isotope fractionation method does not enter into the analysis. The keto group of glyoxylate is actually present as a covalent hydrate to the extent of about 99% of the total glyoxylate concentration.[47] However, the ketone will react in the enzymic process and the concentration of ketone determines the rate of reaction and binding to the enzyme. The equilibration of ketone and hydrate is not catalyzed by the enzyme and as a result the isotope effect on this equilibrium will appear in the measured kinetic isotope effects. The extent of this equilibrium will not be affected by deuteration of the methyl group of acetyl-CoA. Therefore, the observed $^{13}(V/K)$ is not an indication of kinetically significant carbon–carbon bond formation but of a preequilibrium hydration, a process that is independent of the enzyme. The value for $^{13}(V/K)$ of 1.0037 is consistent with measured equilibrium isotope effects in related molecules.[43] Therefore the deuteration of acetyl CoA has no effect on the observed kinetic $^{13}(V/K)$ since that value in fact is due to a preequilibrium and not the rate-determining step. Since proton removal is kinetically significant, if this were concerted with carbon–carbon bond formation the observed $^{13}(V/K)$ would have necessarily have increased because it is in a step after a kinetically significant, isotopically insensitive step. It is concluded that on the basis of the magnitude of the ratio of the intramolecular and intermolecular isotope effects a concerted reaction would have seen $^{13}(V/K)$ increase to 1.011. (The intramolecular effect is about three times the intermolecular effect and the heavy atom effect is predicted to change by the same ratio if the two processes are concerted). What is the kinetically significant but isotopically insensitive step which precedes C–H bond breaking and carbon–

SCHEME 12. Fatty Acid Synthase Reaction



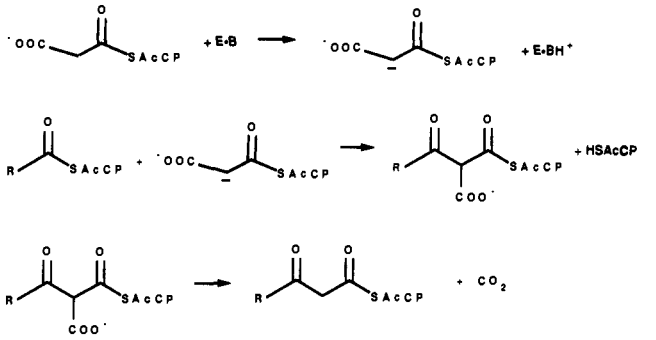
carbon bond formation? The magnitude of V/K (which equates to a second order rate constant) is small enough to show that the reaction is not diffusion-limited. It is suggested that the enzyme undergoes a conformational change after binding the two substrates.[43] Therefore, the data are consistent with the expected stepwise mechanism of carbon–carbon bond formation catalyzed by this enzyme.

i. Concertedness in Condensation Reactions. In non-enzymic reactions, competition between concerted and stepwise reactions is not known for condensation processes. In all cases of intermolecular and intramolecular condensation reactions, there is initial formation of an enolate or enol followed by the formation of the carbon–carbon bond. However, in the case of elimination and substitution reactions there is a competition between stepwise and concerted processes. The concerted processes (E2 and S_N2) compete with stepwise processes (E1 and E1cB, S_N1). In the case of the E2 reaction, a proton is removed and a carbon-based leaving group bond is broken in a single step while the stepwise mechanisms do these steps in sequence. Depending on the extent of proton transfer and leaving group departure, the E2 reaction transition state can have E1 or E1cB character. The synthase reactions do not have a good analogy to this since no leaving group is involved. Formally there are three chemical processes involved in the condensation reaction: (1) removal of the proton from the α-carbon, (2) addition of the carbanion to the carbonyl group of the reaction partner, and (3) protonation of the alkoxide product. However, in the consideration of stepwise versus concerted reaction in Knowles' study, only the synchronization of the first two processes is of significance since protonation on oxygen was not evaluated.[42]

j. Fatty Acid Synthase. The chain elongation reaction in the formation of straight chain fatty acids occurs by a process which is formally related to the reactions of Claisen enzymes and the issue of the involvement of carbanions has been specifically investigated. A two carbon fragment is introduced from malonyl-CoA which undergoes transesterification to form a thiol derivative with the acyl carrier protein (malonyl-AcP). The malonyl group is derived from acetyl-CoA by the reaction catalyzed by acetyl-CoA carboxylase. The malonyl-AcP derivative is decarboxylated and functions as a nucleophile toward the carbonyl of the thiol ester of the acyl carrier protein of the growing fatty acid (Scheme 12).

The reaction involves decarboxylation of the malonyl derivative and carbon–carbon bond formation. The enzyme does not catalyze partial exchange or acetyl proton exchange. Metzler proposes that the conjugate base of the carbon acid derived from malonate condenses with the other acyl component (Scheme 13).[48] The resulting keto acid then undergoes decarboxylation.

SCHEME 13. Metzler's Mechanism for Fatty Acid Synthase



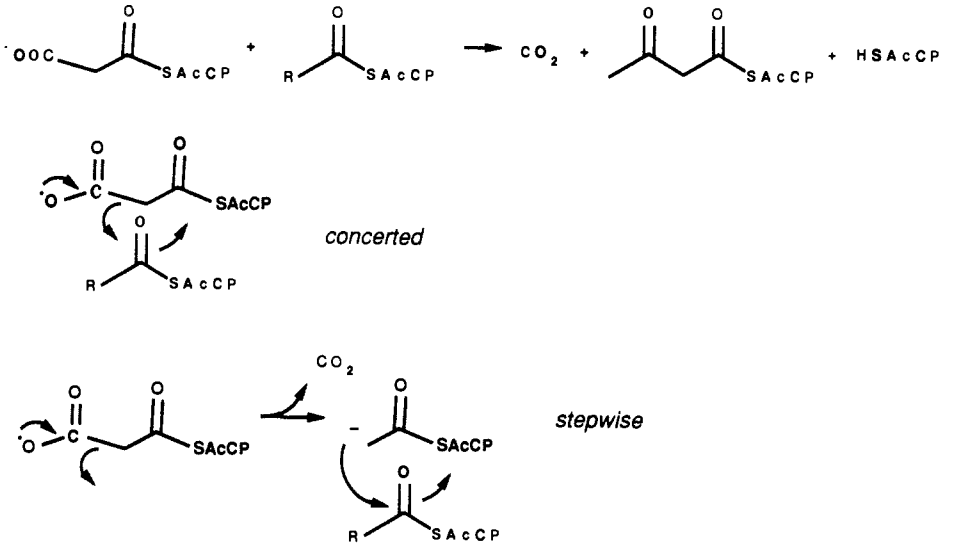
The mechanism is attractive in that the carboxyl group which is added in the acetyl-CoA carboxylase reaction serves to acidify the carbon acid (malonyl-AcP) and also provides a basis for driving the reaction in the forward direction after condensation, through the decarboxylation process. Vagelos and co-workers have shown that the enzyme does not promote rapid decarboxylation of malonyl-AcP[48] and Metzler's mechanism is consistent with this observation. Presumably, the enzyme can use conformational control to prevent decarboxylation.

Walsh proposes that the reaction involves initial decarboxylation of malonyl-AcP and that this either generates the enethiolate ester as an intermediate or there is concerted decarboxylation and condensation (Scheme 14).[29] This poses the same question as in the citrate synthase and malate synthase reaction mechanisms.

Dewar and Dieter addressed variations of Walsh's mechanism using MNDO and AM1 theoretical orbital calculations.[49] They propose that the decarboxylation is the initial reaction and that there either is general acid catalysis by a lysine, leading to the enol of acetyl-AcP as an intermediate or there is a direct decarboxylation to the enolate. They find that the enol is not a sufficiently basic nucleophile to participate in the condensation reaction and that deprotonation involves a large energy barrier. The mechanism favored by the interpreted calculations involves generation of the enethiolate which is stabilized by electrostatic interactions with a neighboring protonated amino group of a lysine residue. The enethiolate then undergoes condensation to the product.

k. Dithio Esters and Carbanions. Recently, a new method for implicating carbanions in enzymatic carbon–carbon bond formation for systems which utilize coenzyme A derivatives as substrates has been developed by Anderson and co-workers.[50–52] They use thioacetyl-CoA as an analogue of acetyl-CoA. The thioacetyl species contains a thiocarbonyl moiety in place of the acetyl carbonyl group and is a much stronger carbon acid than the corresponding acetyl species (pK_a = 12.5) and consequently will form a carbanion much more readily. The resulting enethiolate from thioacetyl-CoA will be a much weaker base and thus a poorer nucleophile than the corresponding enolate derived from acetyl-CoA, but its concentration will be higher. In the case of citrate synthase, the thio analogue is a very slowly reacting substrate (k_{cat} and V/K are lower than those of acetyl-CoA by a factor of 10^7). The thio substrate also undergoes enzyme-catalyzed proton exchange in the absence of oxaloacetate.[51] Spectro-

SCHEME 14. Stepwise and Concerted Mechanisms for Fatty Acid Synthase



scopic studies of the presteady state system indicate that the enethiolate forms reversibly at pH 6–8. Since the pK_a is 12.5, the enzyme must be stabilizing the ionized form, providing evidence by analogy that the enolate would also be stabilized and exist as a discrete intermediate.[50]

Anderson's group has recently studied the reaction of thioacetyl-CoA as a substrate for thiolase, which catalyzes the Claisen-like condensation of two CoA esters to give a β-keto CoA ester and CoA.[52] While a thioacetyl analogue functions competently as the nucleophile in the reaction through formation of the enethiolate, it does not function as the electrophile. Since the enzyme functions by the intermediate formation of a thiolacyl enzyme derived from a cysteine residue, the inability of the cysteine residue to react with the thioacetyl derivative prevents the complete reaction. The enzyme catalyzes the exchange of the methyl protons of the thioacetyl substrate in the absence of the overall reaction at a rate that is kinetically competent for generation of the intermediate anion as the nucleophile toward the normal substrate. Again, this is consistent with the enzyme promoting the formation of the carbanion, in place of a concerted condensation process. The enzyme also catalyzes an acetyl exchange reaction in the reverse of the normal reaction. Thus, when the cysteine of the enzyme is acetylated, it functions as the electrophilic reaction partner in forming the acetoacetyl derivative. When equal quantities of acetyl-CoA and thioacetyl-CoA are combined with acetylated enzyme, equal quantities of acetoacetyl-CoA and acetothioacetyl-CoA are produced. This requires that the two substrates react at equal rates (V/K).[52] Anderson cites this as evidence that acetyl-CoA and thioacetyl-CoA are kinetically equivalent nucleophiles, which is true if the rate determining step is related to the addition process.

It is interesting to compare this reaction with the fatty acid synthase reaction where decarboxylation accompanies carbon–carbon bond formation. Clearly, decarboxylation is not a requirement for the condensation which produces a similar product. In the case of fatty acid synthase, ATP is consumed in the carboxylation process promoted by acetyl CoA carboxylase and the carbon dioxide is lost in the condensation process.

l. Rationale for Stereochemical Observations. The observed stereochemistry of inversion in the enolate reactions is consistent with a mechanism in which a base on the enzyme removes the α-proton and forms a stabilized ion pair. The addition reaction then proceeds from the opposite face of the carbanion.

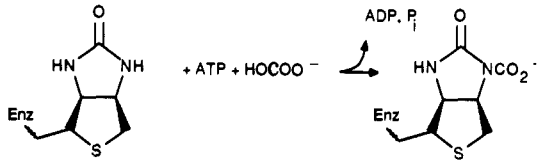
3. Generalization

Enzyme-catalyzed reactions in which bonds are formed between a carbon atom adjacent to a carbonyl group in one molecule and the carbonyl carbon of another molecule have been shown to be stepwise processes by convincing studies on several enzymes as well as by theoretical modeling. However, further studies are needed to permit this to be a confident generalization. Since there are many reasons why exchange reactions might not be observed, their absence cannot be used to rule out an intermediate. Jencks has proposed that in non-enzymic reactions in the case where an intermediate would be too unstable to exist we expect to find a concerted process[38] and none has yet been demonstrated for Claisen enzymes. Since intermediates associated with enzymes can be expected to have properties that provide kinetic competence that would not be predicted from their properties in isolation, it is reasonable to expect that apparently strongly basic enolates can be intermediates that need not be avoided.

III. Coenzymes in Carbon–Carbon Bond Formation via Carbanionic Intermediates

The general reaction patterns in the preceding parts of this review dealt with the nature of the intermediates involved in the carbon–carbon bond formation process. These were divided into categories based on whether carbon–carbon bond formation involved cationic or carbanionic intermediates. Enzymes which utilize coenzymes to accomplish carbon–carbon bond formation in general utilize pathways which involve carbanionic intermediates (cases involving radicals also exist but will not be covered in this review). Coenzymes can provide temporary functional groups for substrates which lack properly positioned functional groups to permit generation of carbanions. Reaction patterns of this type are typical of the coenzymes thiamin diphosphate and

SCHEME 15. Formation of *N*-Carboxybiotin



pyridoxal phosphate. Another common function of coenzymes is the activation of a species which serves as an electrophile in the coupling process to a carbanion. Biotin and flavin mononucleotide normally function in this mode in activating species used for the addition of single carbon units.

A. Biotin and Carboxylation Reactions

The formation of a bond between the carboxylate group derived from bicarbonate and a carbon atom adjacent to a carbonyl group is indicative of a reaction catalyzed by an enzyme that utilizes biotin as a cofactor. Most biotin-dependent enzymes promote a two step process in which *N*-carboxybiotin serves as an intermediate in a process involving the exchange of the carboxylate group derived from bicarbonate for a proton at the α-carbon of the carbonyl compound.

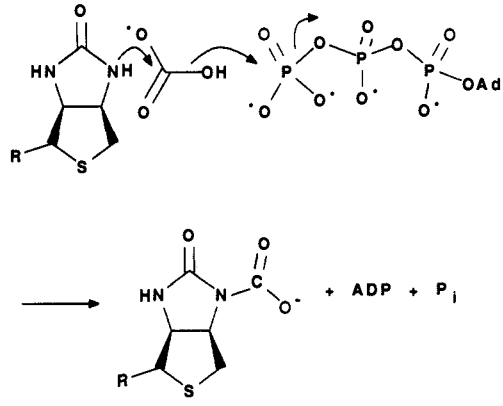
The hydrolysis of ATP is coupled to formation of the carbon–carbon bond[53] but is not explicitly involved in the apparent stoichiometry of the biosynthetic process. The excess oxygen from bicarbonate which is not transferred as the carboxyl group has been shown by isotopic labeling of the oxygen atoms to be transferred to the terminal phosphoanhydride group of ATP, appearing in the inorganic phosphate product (Scheme 15).[54]

1. *Mechanism of ATP-Dependent Carboxylations*

Biotin-dependent enzymes do not catalyze partial exchange reactions which would provide insight into the possibility of intermediates in the reaction.[55] The incubation of labeled ADP and unlabeled ATP in the absence of other substrates does not lead to interconversion. Such an exchange would implicate a phosphorylated intermediate: its absence does not provide evidence for or against such an intermediate.

Climent and Rubio reported that biotin carboxylase promotes the hydrolysis of ATP in the presence of bicarbonate and absence of biotin.[56] They cite this as evidence of a partial reaction between ATP and bicarbonate occurring in the normal catalytic cycle and propose that this supports the existence of carboxy phosphate as an intermediate. The rate of this reaction is only 0.005 times that of the reaction in the presence of biotin (where ATP hydrolysis is coupled to carboxylation). Alternatively, the site that normally contains biotin might be occupied by water or bicarbonate. Since the enzyme is likely to provide a Brönsted base to remove a proton from biotin,[57] it will also enhance the nucleophilicity of other bound species. If biotin normally attacks either ATP or bicarbonate (mechanisms which do not involve carboxy phosphate) then one would predict that when water or bicarbonate bind in the biotin site, they would promote the cleavage of ATP but at a slow rate. Alternatively, the slow rate of the bicarbonate dependent ATPase reaction can be considered as a kinetically incompetent reaction whose interpretation is ambiguous.

SCHEME 16. Concerted Reaction: ATP + Bicarbonate + Enzyme



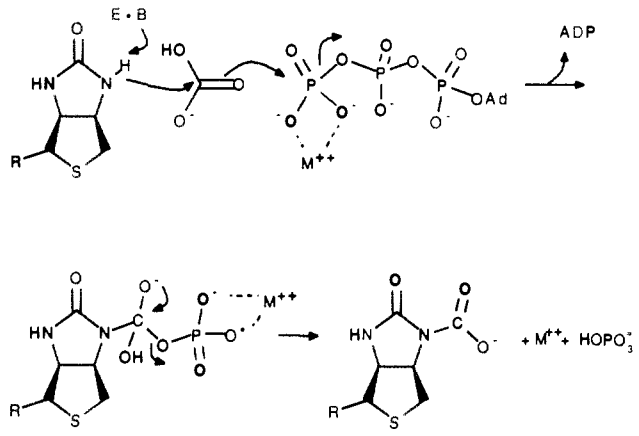
A more subtle exchange, which is indicative of internal return in the formation of an intermediate, can be detected in some enzymes through the use of positional isotope exchange.[5,58] In such a procedure, the terminal bridging oxygen atom of ATP is unlabeled while the nonbridging oxygen atoms are labeled. If the reisolated reactant incorporates label into the bridging position more rapidly than the overall reaction occurs, this is evidence that the terminal phosphate has been cleaved and readded, as would be the case if a loosely held ion pair were to form without the phosphate separating from the enzyme prior to completion of carbon–carbon bond formation. Since efforts to detect such an exchange in the ATP-dependent carboxylation of biotin have given negative results, any intermediate must be very tightly held. In summary, the exchange results show that all the reaction components, biotin, ATP, bicarbonate, and substrate, are necessary in order for an enzyme to produce *N*-carboxybiotin.

The early oxygen-labeling studies mentioned previously showed that oxygen from [^{18}O]bicarbonate is incorporated into the inorganic phosphate derived from ATP.[54] This logically implicates a direct interaction of bicarbonate and the terminal phosphorus of ATP. The lack of exchange and the direct interaction can be most readily accommodated by a mechanism involving a rate-determining transition state in the formation of *N*-carboxybiotin, consisting of the enzyme, bicarbonate, and ATP. In this transition state, an oxygen ligand on bicarbonate attacks ATP, and biotin attacks bicarbonate (Scheme 16).

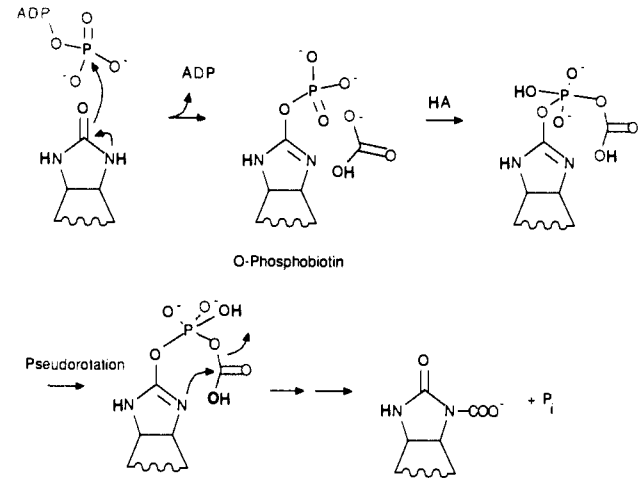
While satisfying the observed criteria, this mechanism is not in accord with expectations from reasonable chemical analogies. A direct displacement on a carboxyl center with hydroxide as a leaving group is unreasonable. Furthermore, there is then no mechanistic function for the cleavage of ATP in promoting the reaction. Knowles has observed that the function of promoting the departure of hydroxide could just as well be done by a proton derived from any Brönsted acid.[55] The choice of this mechanism is made less compelling by the well-known kinetic complications that are possible in enzymic reactions. The lack of exchange does not exclude stepwise processes if the enzyme utilizes a complex mechanism that is the result of favorable evolution.

The mechanism involving a single transition state is based on the further assumption that no further step is necessary to give all the products. Unfortunately, this

SCHEME 17. Termolecular Transition State Leading to the Formation of a Phosphorylated Tetrahedral Intermediate



SCHEME 18. Carboxylation



leads to what is a chemically unreasonable assumption of an S_N2 reaction at bicarbonate in which phosphate from ATP is simultaneously displaced. Such a reaction, in which hydroxide is a leaving group and a nucleophile at once is extremely unlikely. Less demanding mechanisms require ad hoc assumptions to explain the lack of exchange.

In fact, the data do not require that all the bond-making and bond-breaking processes of the reaction be complete in only one step.[53] Instead of envisioning the expulsion of hydroxide from carbonate, biotin can add to bicarbonate while one of the oxygen ligands attacks ATP without departing. In this case, ATP acts as a Lewis acid catalyst and the oxygen ligand is converted into a better leaving group.

A general approach to the question of possible mechanisms ignores the lack of observable exchange processes, since this can be rationalized as originating from the demands of ordered binding. The three reactants which are bound to the enzyme (biotin, ATP, bicarbonate) produce three enzyme-bound products (*N*-carboxybiotin, ADP, and inorganic phosphate) (Scheme 17). The first mechanism, which has all products resulting in a single step, appears to be mechanistically unreasonable. The chemically more reasonable mechanism (see scheme) involves formation of a phosphorylated tetrahedral intermediate in a single step from ATP, bicarbonate, and biotin. This intermediate would decompose to *N*-carboxybiotin. The mechanism involves trapping of the addition product between bicarbonate and the conjugate base of biotin by the terminal group of ATP. This mechanism might give positional isotope exchange if the groups were bound loosely.

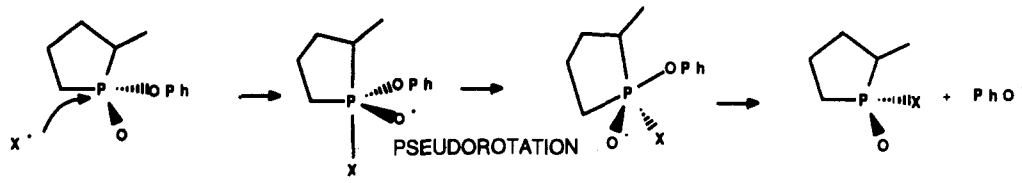
If the lack of exchange is disregarded, we can consider mechanisms in which two reactants can produce an intermediate which reacts with then with the third component. The first possible combination we consider involves the initial reaction of bicarbonate and ATP followed by the reaction of the intermediate with biotin (see scheme). The transfer of oxygen from bicarbonate to phosphate occurs in the first step with the formation of ADP and carboxy phosphate. The latter species might either react directly with biotin or initially decompose to inorganic phosphate and carbon dioxide which in turn reacts with biotin. The barrier to addition of the conjugate base of biotin to carboxy phosphate should not be significantly lower than the addition to bicarbonate itself. The advantage of phosphorylation is in the enhancement of the leaving group, not in the electrophilicity of the carbonate since either a proton or a phosphate is electron-withdrawing.

Since carboxy phosphate is expected to have a very short lifetime,[59,60] what advantages are possessed by a route that involves this intermediate? The leaving group (phosphate) is already bound, while addition to bicarbonate requires subsequent transfer of the equivalent of PO_3^{2-} from ATP to provide the intermediate with a good leaving group. Since the tetrahedral intermediate derived from addition of biotin to bicarbonate should be a better nucleophile toward ATP than bicarbonate itself, the route via carboxy phosphate is at a disadvantage in this respect. If carboxy phosphate decomposes to carbon dioxide and inorganic phosphate prior to reaction with biotin, the carbon dioxide concentration in the area adjacent to biotin will be high, providing an entropic advantage for reaction compared to a situation in which the enzyme would have to bind the species from solution.[59]

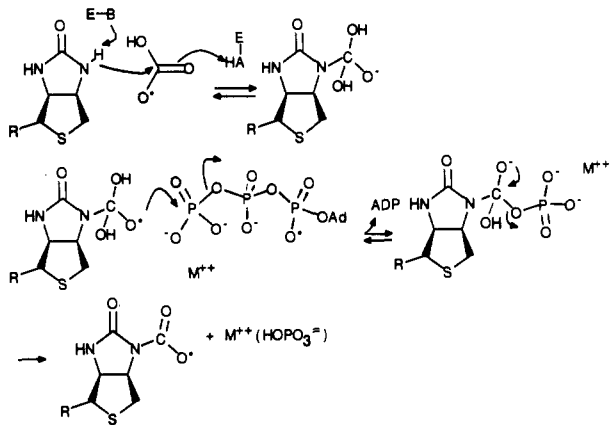
Another mechanism involves the initial reaction of biotin with ATP, forming ADP and a phosphorylated biotin species (Scheme 18). It has been proposed, based on model studies, that such a species would be *O*-phosphobiotin.[61,62] This reacts with bicarbonate to produce *N*-carboxybiotin and inorganic phosphate. The transfer of oxygen from bicarbonate occurs in the second step in this case. Models for the O-phosphorylation of biotin demonstrate that such a process can occur readily.[61,62]

The results of stereochemical studies by Hansen and Knowles have placed restrictions on the possible steps of such a mechanism.[63] However, a reasonable interpretation of this mechanism is consistent with the stereochemical results. The stereochemical studies show that the net effect at phosphorus is overall inversion. That is, the oxygen from bicarbonate is added to the face opposite to that from which ADP leaves at the terminal phosphate of ATP. The attack of biotin upon ATP is a direct displacement process which should lead to inversion at phosphorus.[64-66] Therefore, the reaction of *O*-phosphobiotin with bicarbonate must be a separate process and occur with retention of relative configuration about phosphorus.[62] Westheimer proposed how substitution at phosphorus can occur

SCHEME 19. Retention Mechanisms for Substitution at Phosphorus



SCHEME 20. Reaction of Biotin with Bicarbonate Followed by Reaction with ATP To Form a Phosphorylated Tetrahedral Intermediate



with retention or inversion at phosphorus.[67] The process involving retention of relative configuration involves a step in which the pentacovalent adduct formed from the addition of a nucleophile to a phosphate undergoes an isomerization belonging to the class of molecular rearrangements known as pseudorotations (Scheme 19). Addition, pseudorotation, and decomposition[67,68] lead to net retention of configuration about phosphorus competitive with inversion. If carbon dioxide is to be generated it must be near the N-1′ position of biotin with which it must react. In the retention mechanism, the carbon atom is considerably closer to the nitrogen than in the inversion mechanism. While pseudorotation mechanisms have not been observed for any enzymic reaction studied thus far, molecular mechanics calculations in our laboratory indicate that in the retention mechanism, the carboxyl carbon is 3.1 Å from the nitrogen while in the inversion mechanism the carboxyl carbon is separated by 4.5 Å.

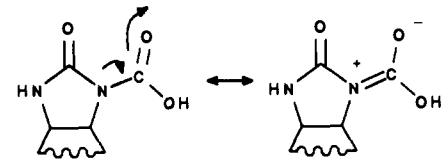
A third member of the "bimolecular then unimolecular" reaction class is a variant of the previous mechanism. In this case, the conjugate base of biotin reacts with bicarbonate to produce an addition intermediate which then reacts with ATP (Scheme 20).

It is likely that the γ-phosphorus atom of ATP would preassociate with an oxygen atom of bicarbonate. In particular, if the anionic center of bicarbonate associates with a cation, the π-electron density of bicarbonate would align with the phosphorus of the terminal phosphate of ATP. The addition of the conjugate base of a urea to a carboxylate is an appropriate model for this mechanism.[69] The intermediate should be very reactive toward ATP on the basis of the observation that the conjugate base of a carbonyl hydrate reacts rapidly with an internal phosphate ester.[70]

2. Relating Structure and Function

The unique structure of biotin has been the subject of speculation with regard to its relationship to mechanism. The bicyclic system has no immediately obvious reason for existence. Yet, it is unlikely that the structure is not optimal for its purpose.[71,72] The imidazolidinone ring of biotin is the reaction site and it has been shown to be appropriate for the reactions in which it is involved. Perrin and Dwyer found that the exchange of the protons attached to the nitrogen atoms of the urea group occurs sufficiently rapidly that the conjugate base is a reasonable intermediate for any reaction in which substitution for the proton occurs, as in carboxylation.[57] The need for acid catalysis that had been proposed is not consistent with this observation nor is there a necessity for an enol urea intermediate.

SCHEME 21. Resonance and Rotation in *N*-Carboxybiotin

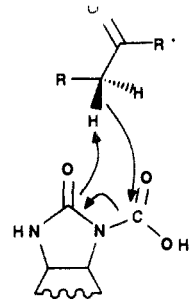


Another interesting feature in which structure and function may be related concerns the conformation of the carboxyl group in *N*-carboxybiotin. Biotin has a dual function: it preserves a carboxyl group after the ATP-dependent carboxylation has occurred and it readily transfers the carboxyl group to an acceptor. Wallace and co-workers showed that the reactivity is "triggered" by binding of *N*-carboxybiotin to the transfer site when substrate or an analogue of the substrate is present.[73] That is, *N*-carboxybiotin must be inherently unreactive in order to preserve the carboxyl group but it must become reactive in the triggering situation.

The change in reactivity might be accomplished simply by rotation about the bond between the nitrogen of biotin and the carboxyl group.[74] If the carboxyl group is in the plane of the urea moiety, resonance overlap stabilizes the carbon oxygen bond. Rotation of the carboxyl group out of the plane destroys this stabilization and enhances the reactivity of the carboxyl toward nucleophilic attack (Scheme 21).

Such a change in reactivity can best be accomplished if the ureido group is held in a planar conformation. If the urea function is forced out of the plane, then resonance interactions are disrupted. Examination of reported structures of biotin and analogues of biotin suggests that the fused ring containing sulfur is of the precisely correct geometry to hold the imidazolidinone ring planar. X-ray studies of analogues in which sulfur is replaced by oxygen or carbon result in structures in which the imidazolidinone ring is distorted.[75] It is likely that the long carbon-sulfur bond serves to produce the correct bridge length while shorter bonds cause distortion. The sulfur atom is also an electron-withdrawing substituent and this can also affect the reactivity of the urea group, and in particular should stabilize the conjugate base.

SCHEME 22. Carboxyl Transfer by a Concerted Mechanism



N-Carboxybiotin

3. Carboxyl Transfer from Biotin

Transfer of the carboxyl from *N*-carboxybiotin to an anionic acceptor is analogous to the reactions described for Claisen enzymes earlier in this review. The reaction is an electrophilic substitution at the substrate carbon of the carboxyl for a proton. Rētey and Lynen had shown that this reaction occurs with retention of relative configuration.[76] In response to this observation, they wrote what is a concerted mechanism (Scheme 22).

Kuo and Rose showed that the proton that is removed from one substrate molecule is retained by the enzyme and transferred to another substrate.[77] Stubbe and Abeles tested 3-fluoropropionyl-CoA as alternative substrate for propionyl-CoA carboxylase and transcarboxylase.[78] They found that elimination of hydrogen fluoride from the alternative substrate to produce acryloyl-CoA competes with the substitution process involved in carboxylation to the extent that no carboxylation product results.[78,79] Both processes involve initial removal of the proton from the a position of the substrate, but only carboxylation necessarily involves decarboxylation of *N*-carboxybiotin. The rate of production of acryloyl-CoA is equal to the rate of hydrolysis of ATP. Since ATP hydrolysis is necessary for the formation of *N*-carboxybiotin from biotin and bicarbonate (and no other process promoted by the enzyme promotes ATP hydrolysis), the result suggests that decarboxylation of *N*-carboxybiotin stoichiometrically accompanies elimination of HF from fluoropropionyl-CoA. If the enzyme were capable of removing a proton from the substrate without decarboxylation of *N*-carboxybiotin, the concerted mechanism of carboxyl transfer would have been ruled out. This result suggests that the substrate is carboxylated and the resulting material loses carbon dioxide and fluoride. The net stoichiometry is the same as direct elimination and therefore the experiment does not rule out any of the possible mechanisms.

The use of double isotopic fractionation was applied to the question of whether the transfer of carbon dioxide from *N*-carboxybiotin is concerted with transfer of the proton from the substrate.[80,81] The carboxyl of a transcarboxylase substrate was labeled with ^{13}C and the proton to be transferred was replaced with deuterium. The results are consistent with a mechanism in which proton removal from the substrate to generate the carbanion and transfer of the carboxyl occur in distinct steps since the substitution of deuterium for hydrogen reduces the observed magnitude of the ^{13}C kinetic isotope effect. The assumption for this conclusion is that if the two transfers occurred in the same step, the isotopic substitutions could not affect each other's magnitude. For this to be rigorously correct, the intrinsic isotope effect must not change although the position of the transition state might change. A test of the assumption is not possible due to conditions which follow from the principle of microscopic reversibility: the isotope effects for both instances would have to be measured for calibration and would have to be shown to be different. Then the case in question would be measured. In effect, there would have to be a changeover from a stepwise mechanism to a concerted mechanism with a clearly distinct change in isotope fractionation patterns demonstrated. Since mechanisms are not a matter of choice but a consequence of the lowest energy rate-determining transition state (or a combination of comparably energetic transition states), such a calibration must be the result of estimation rather than measurement.

4. Carboxylation and Related Reactions

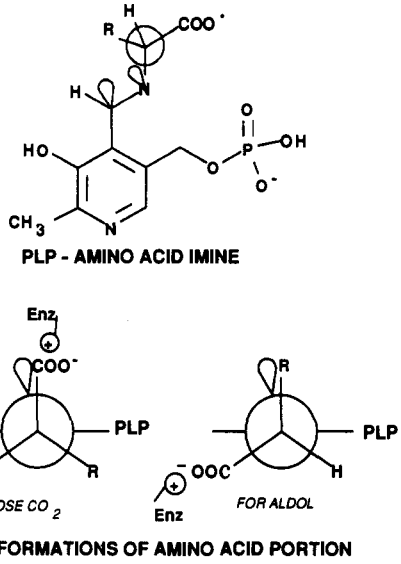
N-Carboxybiotin has the dual function of stabilizing the carboxyl group in the absence of an acceptor and then facilitating the transfer in the presence of an acceptor.[55,74] Resonance structures and molecular orbital evaluation of structural possibilities provide an insight into the function significance of the particular groups which are involved in the reaction. The planar imidazolidinone ring on biotin is structurally suited for controlling the reactivity of the carboxyl moiety.[70]

While *N*-carboxybiotin is responsible for transfer of the carboxyl group, biotin derivatives are not involved in the transfer of more reduced one carbon units. The next lowest oxidation state at carbon involves the transfer of an aldehyde carbonyl. This is equivalent to substitution for the hydroxyl group of formic acid. The leaving group in this case is a derivative of tetrahydrofolate which contains the carbon as a formamide derivative.[29] Amide resonance is a powerful factor in maintaining the C–N bond. Rotation of the carboxyl out of the plane of the amine will weaken the bond by disruption of resonance.

B. Carbon-Carbon Bond Formation and Cleavage with Pyridoxal Phosphate and Tetrahydrofolate Derivatives

Many enzymes catalyze reactions in which the amino group of an amino acid is condensed with the aldehyde moiety of enzyme-bound pyridoxal phosphate, producing an enzyme-bound imine. This unsaturated functional group can stabilize an adjacent carbanion derived from the amino acid by a combination of resonance and inductive effects.[82] The carbanion can be generated by any process that removes a ligand heterolytically so that the carbanion remains. The three ligands are the carboxyl group (which leaves as carbon dioxide), a hydrogen atom (which is transferred as a proton to a base), and the side chain (which is transferred as the equivalent of the carbocation). Processes which thus are promoted include decarboxylation, racemization of the amino acid derivative, and aldol reactions.[83] The mechanisms of enzymes utilizing pyridoxal phosphate have been reviewed extensively and the function of pyridoxal phosphate is well-understood. Specificity is likely to be controlled enzymically through

SCHEME 23. Conformations of Pyridoxal Phosphate Adducts



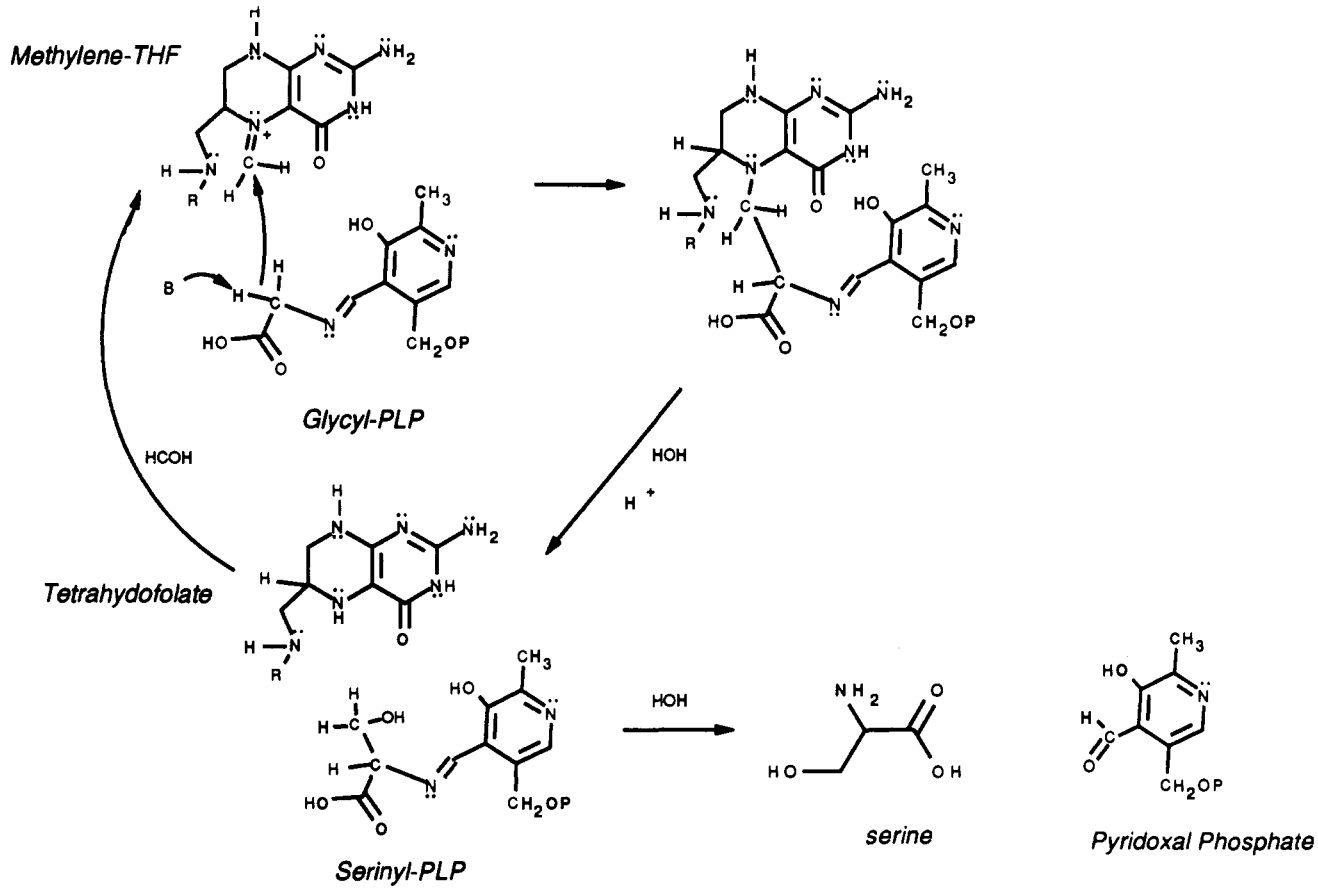
the torsional angle about the C–N bond originally in the amino acid.[83] The π-electron system of the imine must overlap the σ-bond to the ligand which departs so that the transition state leading to the carbanion can be stabilized by π-interactions. The enzyme controls the conformation which determines the reaction pattern. Although the mode through which conformational specificity is achieved is not known, one possibility is electrostatic interactions between the carboxyl group derived from the amino acid and a cationic site of the enzyme (Scheme 23).

If this is the case, decarboxylases will be oriented correctly regardless of the identity of the other ligands (However, the interaction of the carboxylate with a cation would probably retard decarboxylation). That is, a decarboxylase in principle should stabilize a carbanion from either enantiomer of an amino acid if both are capable of binding to the enzyme.[84] If other bonds are activated and binding is specified by the interaction of the carboxylate with the enzyme, enantiomers will react differently and enantiotopic ligands will be readily distinguished.

The most common process involving carbon–carbon formation and pyridoxal phosphate is exemplified by the reaction catalyzed by serine hydroxymethylase, a reaction which utilizes a derivative of tetrahydrofolate as a cofactor.[85] In this system, the adduct of glycine and pyridoxal phosphate is formed and the carbanion generated as in the previous example. Formally, the production of serine from glycine requires the condensation of the carbanion derived from glycine with formaldehyde. According to the Dunathan hypothesis, the enzyme should distinguish the two enantiotopic hydrogens at the α position. Akhtar and Jordan observed that such is the case and used this to produce pure enantiomers of α-monodeuterioglycine.[85]

The addition of formaldehyde to a carbanion does not involve the free formaldehyde molecule. Instead, N^5,N^{10}-methylenetetrahydrofolate, an adduct of tetrahydrofolate which can provide the chemical equivalent of formaldehyde, is utilized (Scheme 24).[29] The carbon center with two nitrogen substituents is highly polarized by these strongly electron-withdrawing groups and can react with water to produce the carbinolamine deriva-

SCHEME 24. Reaction of Methylene Tetrahydrofolate



tive of formaldehyde. (Attack of the carbanion equivalent derived from glycine and pyridoxal phosphate would not lead to the product but rather to an amino alkane derivative).

The resulting intermediate could then react with the carbanion equivalent by a number of mechanistic possibilities which lead to the addition of the hydroxymethyl group to the carbanionic carbon center.

The transfer of a more highly oxidized one carbon unit, as in carboxylation reactions, is not accomplished via an analogous derivative of tetrahydrofolate but instead involves *N*-carboxybiotin. However, transfer of less oxidized one carbon units involves tetrahydrofolate as a cofactor. These provide for the addition of a hydroxymethyl group or methyl groups. The latter can also be transferred from *S*-adenosylmethionine.

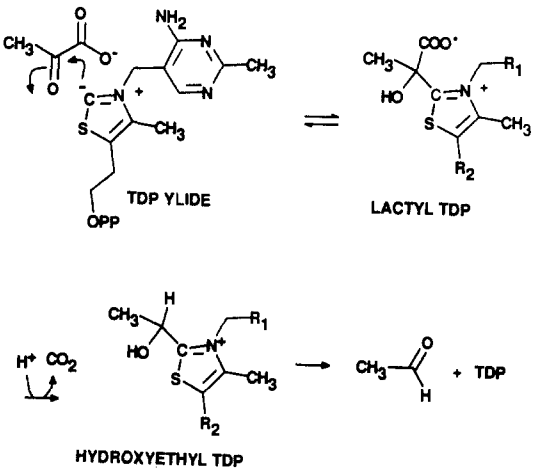
The basis for the diversity of cofactors for transfer of one carbon units in biosynthetic pathways is unknown. However, the source of the equivalent of carbon dioxide for carboxylation of biotin is bicarbonate in an ATP-dependent reaction. The reactions involving tetrahydrofolate do not require ATP and utilize a direct reaction. The adducts of formaldehyde and reduced equivalents are more stable than carboxylated derivatives. Therefore biotin appears to be necessary for controlling the reactivity of the carboxylate group. In addition, tetrahydrofolate derivatives involve double attachments which might cause a carboxyl group derivative to be too stable. Considerable effort on the mechanism of interconversion of folate derivatives and their role in cell development in recent years has led to a detailed understanding of these processes.[86,87]

C. Thiamin Diphosphate Derivatives in Carbon–Carbon Bond Formation

Thiamin diphosphate functions as a cofactor to overcome a chemically difficult problem in carbon–carbon formation and cleavage.[83] The reaction pattern is exemplified by the decarboxylation of pyruvate to give acetaldehyde (or a more highly oxidized species) and carbon dioxide. In this reaction, the bond that is broken is not inherently activated toward the reaction. The bond that is to be cleaved is between two carbonyl carbons. Since these groups are similarly polarized, heterolytic cleavage is not a likely process. Non-enzymically, the direct cleavage of such a bond involves a homolytic process.

Thiamin diphosphate permits the enzyme to promote substitution in a way that is of considerable mechanistic and synthetic interest. The enzyme promotes formation of a readily formed carbon–carbon bond between the conjugate base of thiamin diphosphate and the carbon adjacent to the carboxyl group of the substrate by a nucleophilic addition process. Since the carbonyl group of the substrate is adjacent to a carboxyl group, the inductive and polar interactions of the groups make the carbonyl carbon very electrophilic and the carbanion derived from thiamin diphosphate can add readily. The resulting adduct has β-unsaturation which promotes decarboxylation of the substrate adduct. The resulting product can be derived by elimination of thiamin diphosphate which in turn serves as a catalyst for another turnover. The mechanism was proposed by Breslow based on studies of the acidities of carbon acids in thiazolium compounds which are models for thiamin

SCHEME 25. Mechanism of Thiamin-Catalyzed Decarboxylation of Pyruvate



diphosphate.[89,90] The mechanism has been confirmed with the intermediates themselves[90–102] and is summarized below and in (Scheme 25).

1. The Ionization of Thiamin Diphosphate

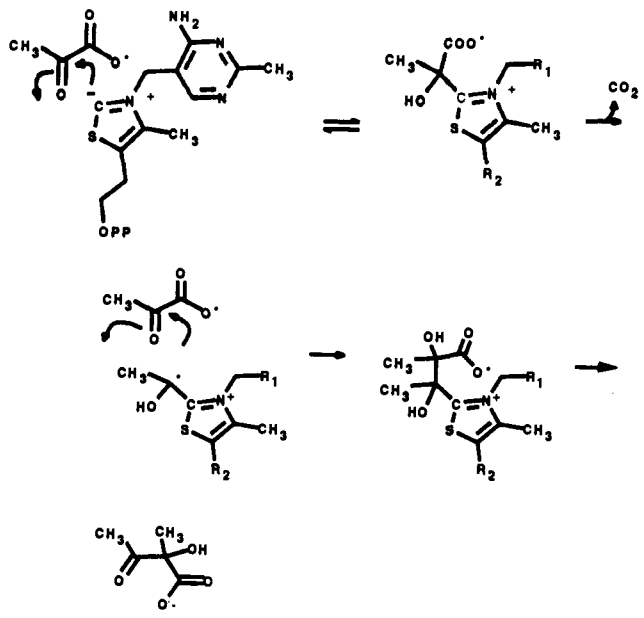
The ionization of thiamin diphosphate is the key step in understanding the mechanism. Thiamin diphosphate is a Brönsted acid and its conjugate base, which is an ylide, functions as a nucleophile toward the electrophilic center at the carbon atom of the keto group of pyruvate. The pK_a of the carbon acid is approximately 18 and the ionization is general base catalyzed.[103–105] The adduct, lactylthiamin diphosphate,[95,96] has a carbon–carbon bond which is polarized by the thiazolium ring toward decarboxylation. The thiazolium ring derived from thiamin diphosphate serves to delocalize excess electron density generated by the loss of carbon dioxide (which is a Lewis acid). The reaction amounts to an electrophilic substitution reaction (of a proton for carbon dioxide) at a carbanionic center. The reaction cycle is completed by the cleavage of the carbon–carbon bond between thiamin diphosphate and the precursor of the aldehyde.

While the pK_a of thiamin is high, the rate of proton removal is sufficiently rapid to be competent for an enzyme-catalyzed reaction.[103] The equilibrium constant for the formation of the adduct is about 1 M[94] which means that immobilized materials on the enzyme would form the adduct with a considerable amount of energy released due to the reduced entropic barrier. We have shown that the enzyme utilizes the energy of formation of lactylthiamin diphosphate on the enzyme (from pyruvate and thiamin diphosphate) to promote the decarboxylation step.[95] Warshel has noted that since pyruvate is preferentially solved in a polar medium, passive catalysis by extraction into a nonpolar active site would not promote reaction and therefore energy must be made available or the site must be polar.[106]

2. Transketolase and Transaldolase

The reactions catalyzed by transketolase and transaldolase also utilize thiamin diphosphate as a cofactor and the mechanism parallels that of pyruvate decarboxylases in the formation of an initial adduct between the ylide derived from thiamin diphosphate and

SCHEME 26. Mechanism of the Transketolase Reaction



the carbonyl group of the substrate (Scheme 26).[107] The enzyme interchanges the hydroxyl alkyl function with an aldehyde. The reaction actually is two processes: α-cleavage and α-condensation via a thiamin diphosphate adduct. These involve generation of a second carbanion after the ylide has added to the carbonyl of the substrate.

The carbanion which is expected to be generated in the reactions is of some interest. Sable and co-workers demonstrated that such a carbanion can form by showing that 2-(1-hydroxyethyl)thiamin will undergo base-catalyzed exchange of hydrogen for deuterium (in deuterium oxide) at the α-carbon (Scheme 27). The ion is also generated by decarboxylation of lactylthiamin as demonstrated by the fact that reaction occurs with racemization.[98] Jordan, Bordwell, and co-workers have prepared ethers which are models for the conjugate base and have studied the ionization of these species in nonaqueous media. They have concluded that the carbon acid has a pK_a of 14 in water,[110] which is considerably lower than the value of about 18 which has been estimated from extrapolation by linear free energy relationships.[111] Recent work by Washabaugh suggests that the higher value for the aqueous ionization may be correct.[112]

3. Acetolactate Synthase

The reaction catalyzed by acetolactate synthase combines the mechanisms of pyruvate decarboxylases and transketolases. The product, α-acetolactate, is formally derived from the reaction of the acyl carbanion generated by the decarboxylation of pyruvate with a second molecule of pyruvate (Scheme 28).

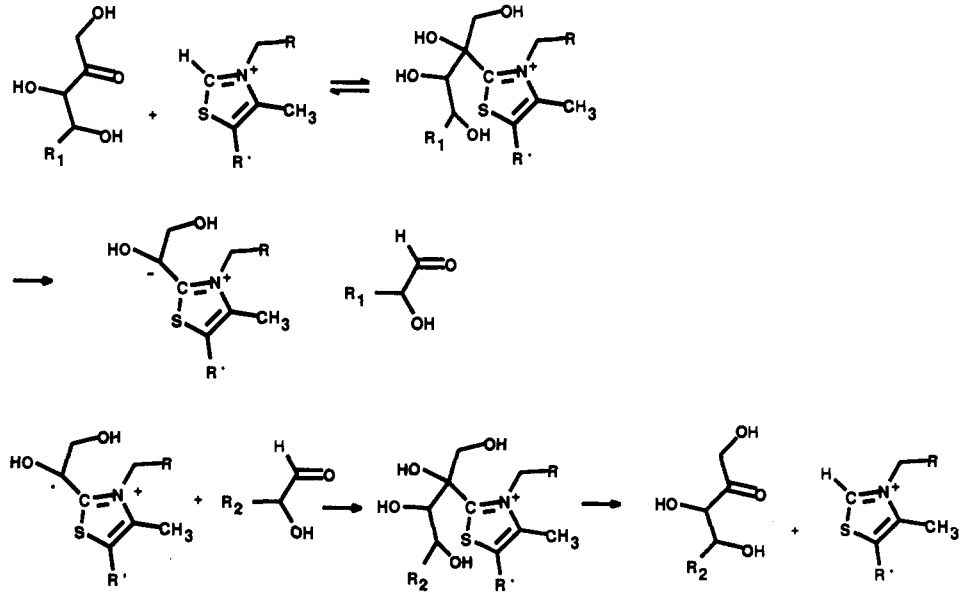
The formation of lactylthiamin diphosphate on the enzyme generates initially the unprotonated adduct which contains the enamine equivalent of a carbanion. This can add to the carbonyl group of a second molecule of pyruvate to form acetolactate.

The carbanion should be a very strong base and therefore would be expected to add rapidly to any electrophile, including any Brönsted acid. If the carbanion is trapped by the carbonyl group of a bound pyruvate molecule, the alternative quench is avoided as is the thermodynamic problem of deprotonation. The enzyme contains a flavin cofactor in addition to thiamin diphosphate although there appears to be no function for the flavin in the reaction mechanism.[113] While there is no direct evidence as yet for a catalytic function for the flavin, we can speculate that it might serve to protect the carbanion as a reducing equivalent. The carbanion is generated under conditions where it is readily lost if a second molecule of pyruvate is not bound. The alternative is that the carbanion reversibly reduces the bound flavin and is in turn re-reduced in the presence of the second molecule of pyruvate. In this case the carbanion may be stabilized as an adduct of the flavin.

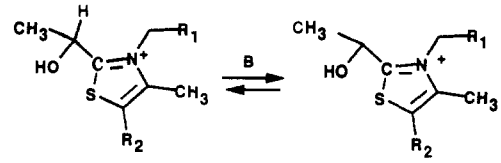
IV. Additional Scope

This review has not been intended to be exhaustive and many important enzymes and mechanisms have been omitted. Reactions involving radical intermediates

SCHEME 27. Formation of a Stabilized Carbanion from Hydroxyethylthiamin



SCHEME 28. Acetolactate Synthase Reaction



are of considerable importance and have been reviewed recently.[114,115] Carboxylation reactions involving enzymes that utilize vitamin K are in an early stage of mechanistic explication but appear to follow the patterns of the carbanion systems.[116] Other reactions which appear to be enzyme-catalyzed thermal rearrangements, such as that promoted by chorismate mutase, may involve electronic reorganization according to the rules of conservation of orbital symmetry.[117]

In summary, patterns of carbon–carbon bond formation are clearly emerging with the development of modern mechanistic methodology and its application to enzymes. The formation of delocalized carbocations and carbanions is widespread and concerted pathways which avoid these have yet to be identified. It appears that the Jencks' generalization that the major reason intermediates will be bypassed is if their lifetime is less than a vibration applies to enzyme catalyzed processes.[38] The energy barriers to the formation of the ionic species at carbon in active sites of enzymes are reduced compared to corresponding barriers for forming similar ions in model reactions in solution. The charge type of the intermediate is directly related to the properties of its precursor. Allylic systems from carbocations, carbonyl systems form carbanions. Theoretical studies have led to the proposal that enzymes can promote reactions by substituting electrostatic stabilization for solvation.[106] If this is the case, then, in general, reactions involving discrete, charged intermediates will be preferred over concerted processes. Recent activity in the design of enzyme-like catalysts by generation of antibodies[118,119] and by synthesis of biomimetic catalysts[120,121] appears to have been successful when transition states resembling specifically charged intermediates can be targeted.

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