Tetrahedron. Vol. 34, pp. 813 to 832. Pergamon Press 1978. Printed in Great Britain

TETRAHEDRON REPORT NUMBER 43

SOME BIOLOGICAL TRANSFORMATIONS INVOLVING UNSATURATED LINKAGES: THE IMPORTANCE OF CHARGE SEPARATION AND CHARGE NEUTRALIZATION IN ENZYME CATALYSIS

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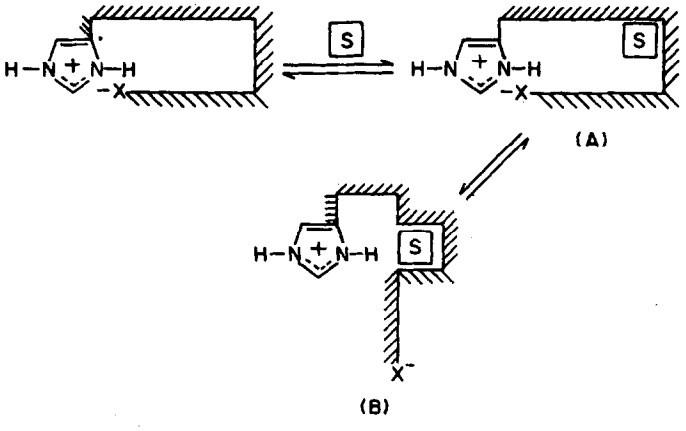
(*Received in the UK for publication* 13 *July* 1976)

Abstract—Current status of knowledge on the biological reduction of C=C and C=O is briefly reviewed. It is argued that the crucial event in the reduction of C=C is the addition of a proton to the more electron-rich terminal of the double bond to produce an electron-deficient species which is then neutralized through hydride transfer from NADPH. The activation for the reduction of a C=O group may also be achieved by a related process in which the carbonyl oxygen is polarized by H-bonding to an acidic group on the enzyme, prior to hydride transfer from NAD(P)H. Thus with both these systems an early event in catalysis is the protonation of the substrate for which, normally, strong acids will be required. Since the groups available at the active-sites of enzymes are weak acids, a mechanism through which powerful proton donating species could be transiently generated from them is proposed. The salient features of this mechanism may be enunciated as follows: Let us consider the enzyme-substrate complex (A) in which an imidazolium group is about to play a role as a proton donating species. It is argued that rearrangement of the initial complex (A) to the catalytic-complex (B), in which the negatively charged counter ion is removed away from the imidazolium cation, would transiently convert the latter group into a powerful proton donating species. The rearrangement (A)→(B) could occur through a protein conformational change or via a charge-relay system or a combination of both processes.



The activation of both the redox forms of pyridine nucleotides may also be achieved through the extension of the hypothesis. Thus the pyridinium ring of NAD(P) deprived of its counter ion would be a good hydride accepting species while hydrogen transfer from the dihydropyridine ring of NAD(P)H would be facilitated in the presence of an approaching counter anion (Scheme 13). The generality of the charge-separation hypothesis is further exemplified by considering the S-adenosylmethionine dependent biological alkylation reactions. The main feature of the proposal once again is the creation, within the micro-environment of the catalytic complex, of a sulphonium ion deprived of its counter anion which activates the S-adenosylmethionine for the crucial methyl transfer step. Broadly speaking, the hypothesis discussed in the review extends the theory of Jencks and assumes that the transient creation of a destabilized environment within the active-site region is the key to enzyme catalysis.

In the last 10 years we at Southampton have been engaged in the elucidation of the mechanisms of enzymic reactions involved in the biosynthesis of several groups of natural products. Our concern in this review is, however, not with a systematic discussion of the pathways but an examination of the molecular details of those steps which illustrate or highlight a new mechanistic feature, and to explore whether the principles thus emerging could be applied to other areas of enzymology. The pathway for the biological conversion of lanosterol into cholesterol[1] includes three stages in which olefinic linkages are reduced (Scheme 1). In the first section we consider the molecular nature of the processes which may be involved in the reduction of these double bonds.

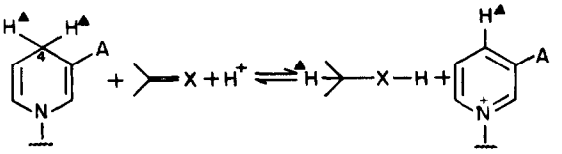
SECTION 1. ENZYMIC REDUCTION OF OLEFINIC DOUBLE BONDS

Reduction of the 7,8-double bond of 7-dehydrocholesterol and the emergence of a mechanism involving an electrophilic addition

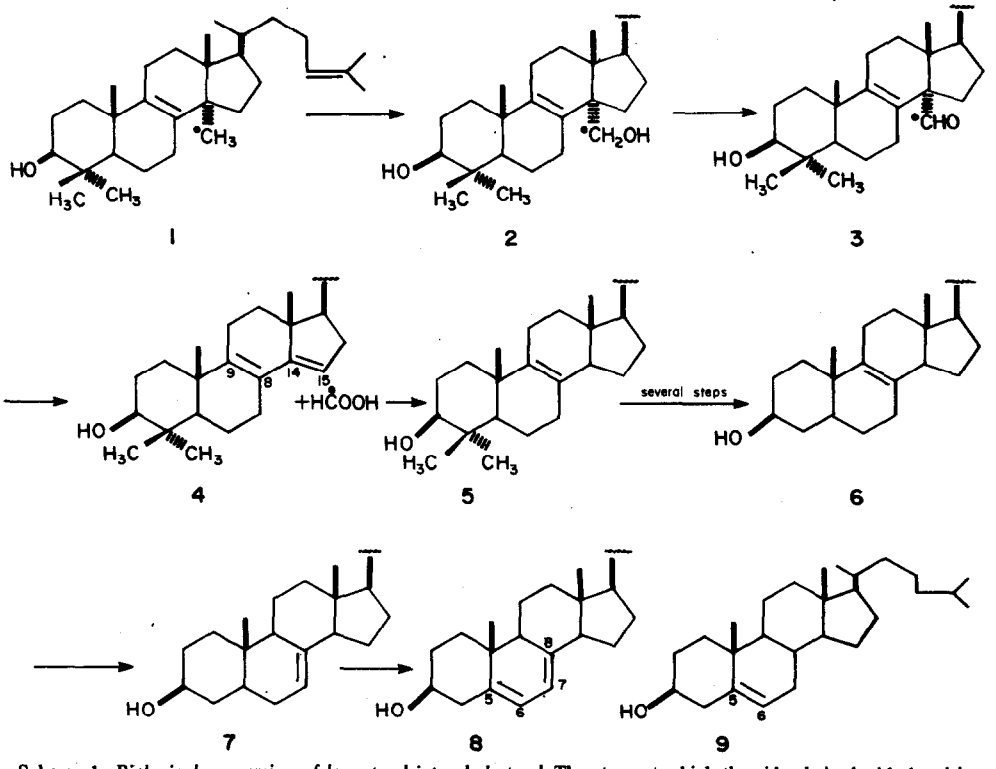
During the late 1950s cumulative work from several laboratories pointed to the fact the 5,6-double bond of cholesterol (**9**, Scheme 1) may originate from the Δ^8-precursor zymosterol (**6**) through an indirect process involving three discrete steps,[1b,2] the first reaction being the isomerization of the 8,9-double bond to the 7,8-position (**6**→**7**), followed by a desaturation reaction giving a 5,7-diene-system (**7**→**8**), and finally the reduction of the 7,8-double bond of the latter to furnish cholesterol.

At the time no satisfactory analogy or mechanistic framework was available from the realm of preparative organic chemistry to enable an understanding in molecular terms of the processes involved in the biological saturation of olefinic double bonds; especially when the bonds were not activated by electron withdrawing groups, as is the case with conversion of 7-dehydrocholesterol into cholesterol.

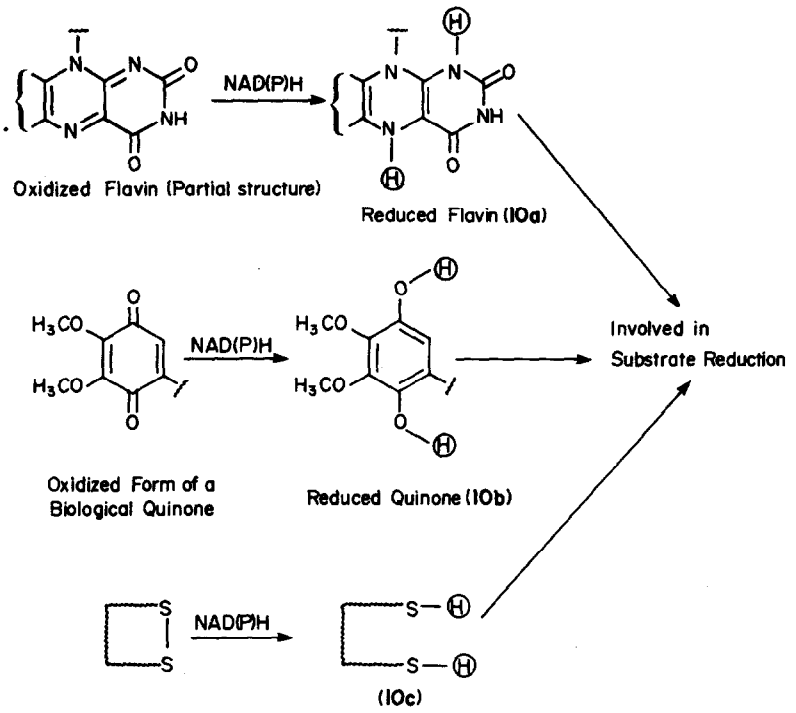
The examination of biochemical literature suggested that reduced pyridine nucleotides, which are the primary reducing agents in most enzymic reduction reactions, catalyze hydride transfer by one of two broad mechanisms. First, a direct hydride transfer from the 4 position of NAD(P)H to the substrate as:



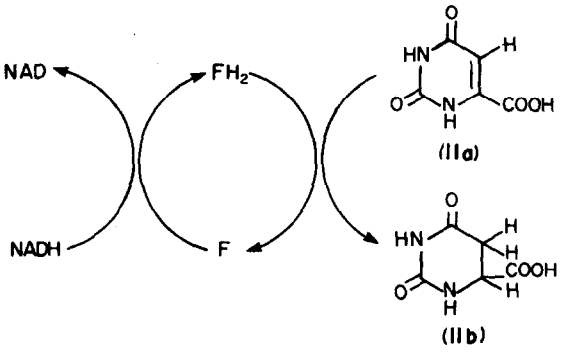
This type of mechanism operates in the reduction of carbonyl and imino compounds, as well as in the conversion of α, β-unsaturated ketones to the corresponding saturated ketones. The second mechanism is that in which the hydride transfer from the reduced pyridine nucleotides occurs indirectly through the involvement of intermediary electron carriers; which may be a flavin, a quinone or a disulphide moiety (Scheme 2a). Such a mechanism is involved in the NADH dependent conversion of orotic acid into dihydroorotic acid catalyzed by dihydroorotic dehydrogenase.[3] It was found that in the enzymic reaction tritium from [4-$^3H_2$]NADH was not incorporated into dihydroorotic acid due to its loss to the medium through an exchange process at the stage of the reduced flavin (Scheme 2b). If the enzymic reduction of olefins occurred through the second type of mechanism then both the hydrogen atoms in the product



Scheme 1. *Biological conversion of lanosterol into cholesterol.* The stage at which the side-chain double bond is reduced is not shown.



Scheme 2(a). *Hydride transfer from pyridine nucleotides through the involvement of intermediary electron carriers,* Ⓗ = *exchangeable hydrogens.*



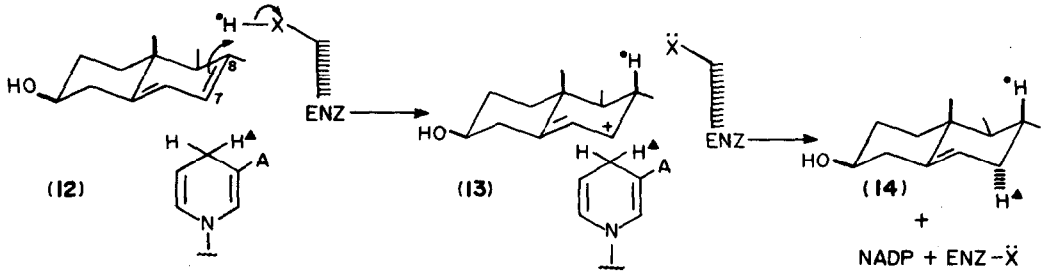
Scheme 2(b). *The reaction catalyzed by dihydroorotic dehydrogenase.* F and FH_2 represent respectively oxidized and reduced flavin prosthetic group.

would originate from the protons of the medium. This is because the groups of the intermediate (**10a–10c**) which participate in the terminal hydrogen transfer step are ionizable, hence in rapid exchange with the medium.

With this background 7-dehydrocholesterol was incubated[4] in tritiated water with rat liver microsomes, which contain the enzyme responsible for the reaction (**8→9**; Scheme 1), in the presence of NADPH. The cholesterol obtained from the incubation was shown to contain only one tritium exclusively located at the 8β-position. Thus a mechanistic hypothesis such as that shown in Scheme 2a was made unlikely, since according to this mechanism hydrogen atoms added to C-7 as well as C-8 should contain tritium.

In the next series of experiments 7-dehydrocholesterol was incubated in a non-radioactive medium with the microsomes in the presence of NADPH which was labelled either in the Pro-4R or Pro-4S position with tritium.[4b] The biosynthetic cholesterol from these experiments was subjected to specific degradations and the results showed that in the reduction of 7,8-double bond the pro-4-S-hydrogen atom of NADPH was transferred to the steroid nucleus where it occupied the 7α-position (Scheme 3). These results, showing that the reduction of the double bond involved a *trans*-addition,[2b] excluded several possible mechanisms not discussed in this review.

A quantitative study aimed at the determination of



Scheme 3. *Electrophilic addition mechanism for the saturation of the 7,8 double bond.* The scheme merely shows the order in which the two C–H bonds are formed and does not make any prediction regarding the extent of the positive charge on the intermediate (13) or its life time.

isotope effects revealed[5] that whereas the tritium product isotope effect in the formation of the medium derived 8α-hydrogen was about 7.5, the 7α-hydrogen atom of cholesterol originating from the 4-position of NADPH showed an isotope effect of less than 2.0. We could not have hoped for more readily interpretable data at our disposal. It was deduced that the reduction process had in fact followed the simplest precept of mechanistic organic chemistry and could be rationalized by assuming that the reaction is initiated by the electrophilic addition of a proton to the C-7–C-8 double bond giving the preferred allylic carbonium ion which is then neutralized by the delivery of a hydride ion from NADPH to the 7α-position of the product (Scheme 3).

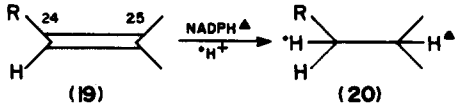
I have occasionally been asked, given that the reduction of the double bond under discussion occurs via an electrophilic addition but without the availability of the experimental results I have just described, would we have correctly predicted the positions in the product (**14**) occupied by the hydrogens originating from NADPH and the proton donating source. My answer clearly is, No. Although uncomplicated application of chemical knowledge dictates that an electrophilic addition to the 7,8-double bond should occur to produce an allylic carbonium ion in preference to the alternative addition mode producing the relatively less stable tertiary carbbonium ion, however, in a complex carbocyclic system such as that present in **12**, whether some unknown steric factors may alter the preferred stability predicted entirely on the basis of electronic considerations cannot be accurately assessed. Studies on a less ambiguous system were therefore needed.

Reduction of the 14,15-*double bond of steroid* 8,14- *and* 7,14-*dienes*

Some interesting observations on the involvement of a C-15 hydrogen atom in the conversion of lanosterol into cholesterol were made almost simultaneously in several laboratories,[6] which pointed to the possibility that the removal of a 14α-methyl group in sterol biosynthesis may occur through a novel mechanism.[7] Many of the details of the demethylation process have subsequently been worked out in our laboratory[8] and are illustrated in the sequence (**1**→**4**, Scheme 1). Of relevance in the present context is the reduction of 14,15-double bond of the 8,14-diene (**4**) to the Δ^8-compound during the biosynthesis of cholesterol (Scheme 1). The enzyme participating in the reduction of the 14,15-double bond is also located in the microsomal fraction[9a] and shows a broad specificity since it is effective with substrate containing the second double bond in either the 8,9 or the 7,8 position (**15** and **17** respectively, $R_1 = R_2 = Me$). The corresponding compounds lacking the 4,4'-dimethyl substituent (**15** and **17**, $R_1 = R_2 = H$) are also metabolized by the enzyme though with a somewhat diminished efficiency. The margin or error in predicting the orientation of addition, through an electrophilic addition mechanism, to the 14,15-double bonds of the four compounds was considerably reduced. Since in these cases an initial electrophilic addition of a proton must occur at C-15 to give the carbonium ion at C-14 which is both allylic and tertiary. We were gratified[9] to find that the prediction was born out by experiment when it was shown that in the reduction (**15**→**16**, $R_1 = R_2 = H$) the 15β-hydrogen atom of the compound **16**, ($R_1 = R_2 = H$) originated from the medium and its 14α-hydrogen from the 4-*pro* S position of NADPH.

Reduction of the side chain, C-24–C-25, double bond

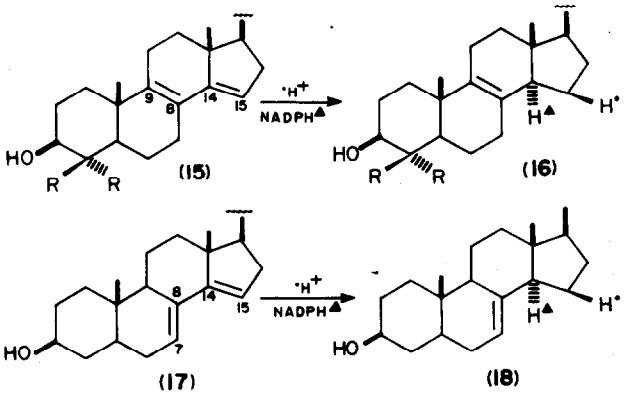
Both the examples discussed above involved the reduction of double bonds present in conjugated systems. The general application of the mechanistic principle required verification by studying the reduction of an unsymmetrical isolated double bond. Such an example was conveniently found in the conversion of desmosterol into cholesterol when the side chain 24,25-double bond is reduced. It was shown that in the reaction, (**19**→**20**), the medium derived hydrogen was incorporated at C-24 and the hydrogen from the 4-pro S position of NADPH was transferred to C-25 of the steroid side chain.[10] The results thus provided support for the hypothesis.



Scheme 5. *Orientation of addition to the* 24,25 *double bond through an electrophilic addition mechanism.*

GENERAL MECHANISTIC CONSIDERATIONS

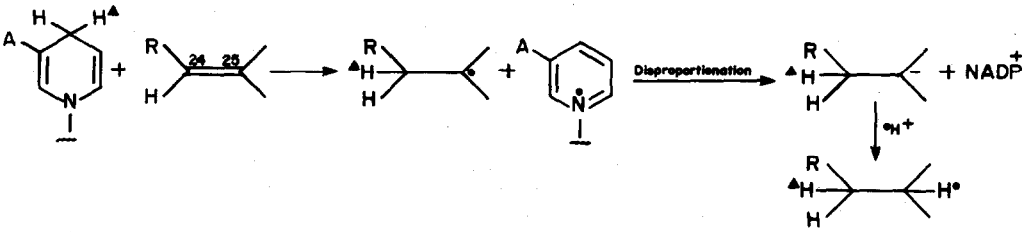
Radical mechanism. We have above considered the results only in terms of the Markownikoff type of process. The question arises, whether another mechanism could also explain the data. The possibility that reduced pyridine nucleotides may either function via hydrogen atom transfer or are endowed with the struc-



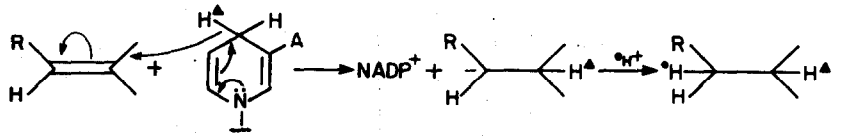
Scheme 4. *The reduction of the* 14,15 *double bond.* The stereochemistry of the reduction has been established only in the reduction of the compounds (15, R = H; also R = $-CH_3$).

tural features for the generation of a particularly powerful hydride for a direct nucleophilic attack on a double bond, though intuitively clumsy, deserves consideration. We must though make it clear from the outset that the theoretical framework for predicting the orientation of addition by the latter types of processes is not as clear-cut as for the electrophilic addition. Nevertheless, it is reasonable to assume that if the driving force for the reaction is the transfer of a hydrogen atom from the pyridine nucleotide to the olefin then the initial attack will occur in such a way as to produce the preferred carbon radical, which through a rather complex series of reactions will furnish the final product as shown in Scheme 6. Application of this principle to the three double bonds under discussion will give positions occupied by NADPH- and medium-derived hydrogen atoms as shown in Scheme 8. In none of the three cases are results expected from a radical type of mechanism consistent with the experimentally determined pattern.

Carbanion mechanism. Alternatively, if pyridine nucleotides are assigned the role of being unusually strong nucleophilic agents, so much so that they can overcome the energy barrier normally offered by the π electron system of the olefinic linkage to the approaching nucleophilic species, then the initial hydride transfer may be expected to produce the preferred carbanion species (Scheme 7) leading to products in which the position

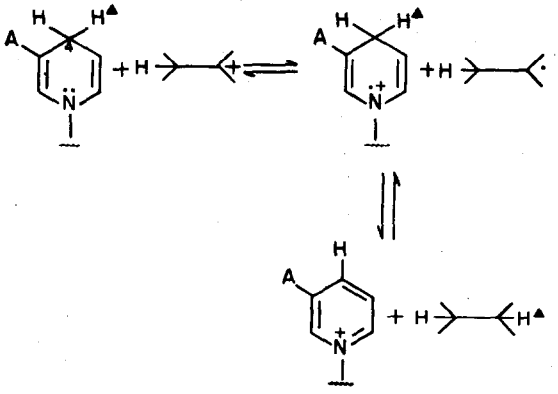


Scheme 6. *Orientation of addition to the* 24,25 *double bond via a radical mechanism.* For the other two double bonds the predicted positions are summerized in Scheme 8.



Scheme 7. *Orientation of addition to the* 24,25 *double bond via a carbanion mechanism.* For the other two double bonds the predicted positions are summarized in Scheme 8.

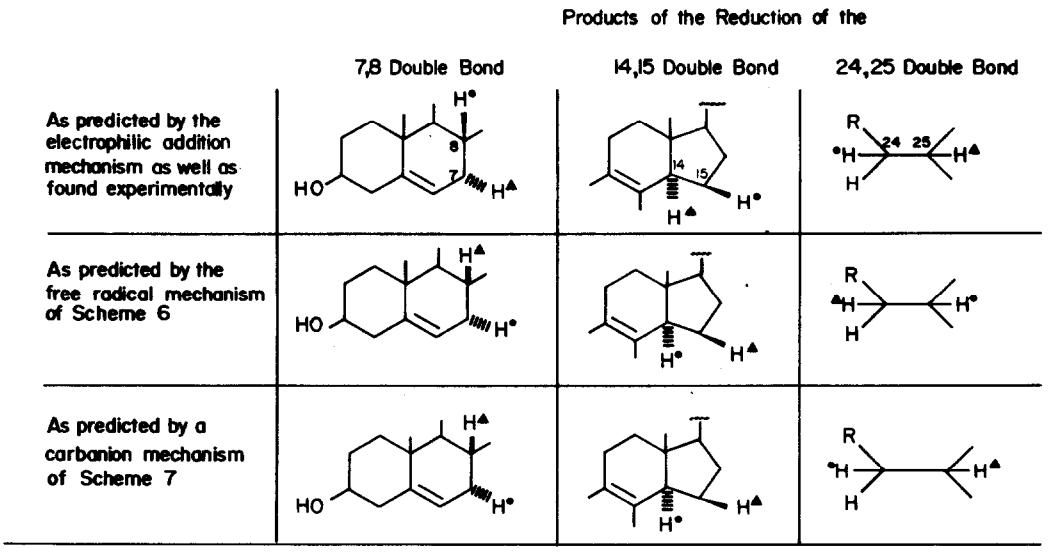
†It may be pointed out that the present work does not differentiate whether the C-4 hydrogen of pyridine nucleotides is transferred to the electron deficient carbon species directly as a hydride or if the process occurs in two steps involving first the transfer of an electron and then a hydrogen atom (H) as:



A stepwise mechanism of the type above has been considered for the reduction of formaldehyde by dihydroflavins and dihydropyridines [R. F. Williams, S. Shinkai and T. C. Bruice, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1763 (1975) and refs therin]. According to this mechanism, the energetically favourable path for the stepwise transfer of $le + H^{\cdot}$, involves protonated formaldehyde ($H_2C{=}\overset{+}{O}{-}H$) rather than the neutral species. Since the hypothesis advocated by us also requires the availability of an electron deficient species for hydrogen transfer from dihydropyridine, whithin the context of the present review, the hydride and $le + H^{\cdot}$ transfers are indistinguishable processes.

occupied by the two H atoms are as shown in Schemes 7 and 8. Thus only in one case, the reduction of the 24,25-double bond, the predicted results are in accordance with the experimental findings.

Concerted mechanism. We end this section by making a brief comment on the possibility of the participation of a truly concerted mechanism for the saturation of double bonds. In such an event the orientation of addition is unlikely to depend on the distribution of charge within the double bond, but on the positions occupied by NADPH and the proton donating group on the enzyme with respect to the two terminals of the olefinic linkage in the ternary complex. The fact that every one of the double bonds studied above possessed its own characteristic stereo-electronic environment, yet in every case the reduction reaction is readily explicable by a Markownikoff mode of addition, emphasises the importance of the distribution of charge within the bond to be reduced, thus making the involvement of a true concerted mechanism not impossible, but certainly unnecessary. The electrophilic mechanism favoured by us in which the first crucial event is the protonation of the olefin by an acidic group on the enzyme, and NADPH then participates to neutralize through hydride† transfer an already reactive carbonium ion intermediate assigns a more passive role to pyridine nucleotides. This role can be predicted with some justification from the knowledge of their weak reactivity in free solution (though a possible mechanism by which the pyridine nucleotides may be activated at the enzyme active site is considered later).



Scheme 8. *Orientation of addition to steroid double bonds as predicted by various mechanisms.* The sterochemistry of addition is *trans* in the case of 7, 8 & 14, 15 double bonds but *cis* with the 24,25 double bond. $H^{\blacktriangle}$, hydrogen from position 4 of NADPH; $H^{\bullet}$, hydrogen the medium.

SECTION 2. DEHYDROGENASES: REDUCTION OF CARBONYL GROUPS

Introduction

In the light of the results and conclusions described above with respect to the reduction of olefinic linkages it is of interest to consider the mechanism of the bond forming event in the enzymic reduction of carbonyl groups. The reversible reaction shown in eqn (1) is of wide occurrence in biological systems and is catalyzed by the group of enzymes collectively called dehydrogenases.

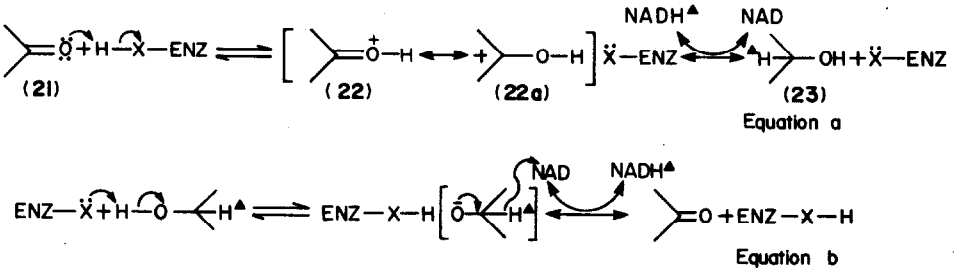
$$H^+ + \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!C{=}O + NADH^{\blacktriangle} \rightleftharpoons {}^{\blacktriangle}H\!-\!\begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!C\!-\!OH + NAD^+ \qquad (1)$$

Historically, the first step towards the elucidation of the mechanism of action of pyridine nucleotide dependent dehydrogenases was the observation by Westheimer and Vennesland,[11] using alcohol dehydrogenase, who showed that in the conversion of eqn (1) ($R_1 = CH_3$, $R_2 = H$), one of the hydrogen atoms from C-4 of the nicotinamide ring was stereospecifically transferred to the CO carbon atom. The generality of this observation was subsequently established by using numerous other dehydrogenases. The precise mechanism of, and the driving forces for, the transfer of hydrogen between the coenzyme and the substrate has been an area of intense research activity during the subsequent two decades.

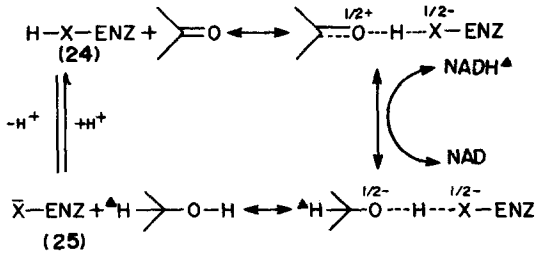
The importance of the protonation of carbonyl group prior to hydride transfer

We approached the problem from the standpoint of precedent and argued whether the mechanism of the reduction of olefinic linkages which fully took into consideration the relatively weak reactivity expected of the pyridine nucleotides as a hydride† donating agent may also be applicable to the reduction of carbonyl compound. A mechanism based on such a concept is displayed in Scheme 9 (eqn *a*). A problem posed by this mechanism is, that if in the forward direction the reaction occurs through the sequence (21)→(22; 22a)→(23), then the law of microscopic reversibility necessitates that in the reverse direction the first event is the abstraction of a hydride by the oxidized pyridinium ring to give the carbonium ion species (22a). What provides the driving force for such an unfavourable step is not readily obvious. An alternative possibility is that the reaction in the reverse direction may occur through a new series of intermediates as shown in Scheme 9 (eqn *b*), which provides activation for hydride transfer but contravenes the law of microscopic reversibility. The main features of the mechanisms of Scheme 9 (eqns *a* and *b*), however, could be married to suggest a compromise mechanism[12] of Scheme 10. The mechanism makes the important prediction that the carbonyl substrate will in

†See footnote on page 5.



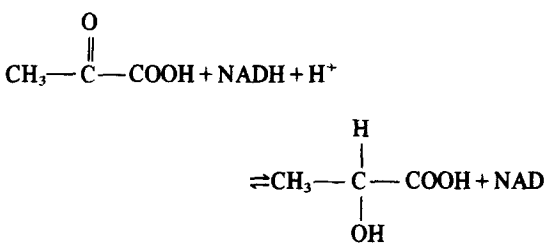
Scheme 9. *Consideration of a preliminary mechanism for dehydrogenase catalyzed interconversion of alcohols and carbonyl compounds.* Reduction of a carbonyl compound equation *a*; Oxidation of alcohol equation *b*.



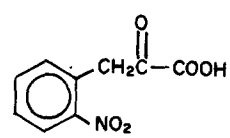
Scheme 10. *Mechanism postulating the sequence of bond forming events in pyridine nucleotide linked reactions.*

general bind to the protonated form of the enzyme (**24**) better than to its conjugate base (**25**). In view of the fact that at the present time the most convincing support for the mechanistic hypothesis is provided by studies on lactate dehydrogenase and also that the state of knowledge regarding its kinetic behaviour and tertiary structure are at a particularly advanced stage, we shall briefly consider the mechanism of dehydrogenases by reference to lactate dehydrogenase.

Lactate dehydrogenase consists of four identical subunits and catalyzes the reaction:[13]



At neutral pH, the reaction in both directions shows a compulsory binding order of coenzyme followed by substrate. Basic tenets of the mechanism were evaluated,[12b] though indirectly, by studying the affinity of 2,3-epoxybutyrate (**26**) and 2,3-epoxypropionate (**26a**) which are non-reducible analogues of pyruvate, for lactate dehydrogenase. It was found that both the compounds competitively inhibited the enzyme, and the inhibition was pH dependent. The binding of the epoxides to the enzyme being better at acidic than alkaline pH values. For each increase of one pH unit above the pKa of the relevant active site group (see below) the concentration of epoxide needed to produce the same extent of binding was approximately ten times higher. Inherent in the mechanism of Scheme 10 is the assumption that dehydrogenases consist of two types of catalytic species; the enzyme base (**25**) and its conjugate acid (**24**). The equilibrium between these species should depend on pH. The results with the epoxides therefore may be explained by suggesting that a decrease in pH increases the concentration of the protonated form of the enzyme (**27**) thus providing a favourable situation for the formation of the Enz-H^+-NADH-inhibitor complex (**27a**). With higher pH values, the concentration of the protonated form of the enzyme (**27**) progressively declines thus requiring increasing concentration of the inhibitor to push the equilibrium towards the formation of the complex (**27a**). From kinetic data the pKa of the group participating in the binding was estimated to be about 6.8. That the group may be histidine has been deduced from covalent modification studies taken in conjuction with the information on the tertiary structure of the enzyme.[13] Another important observation bearing on this facet emerged from the demonstration that O-nitrophenylpyruvate (**28**) is a weak[14] substrate for lactate dehydrogenase. This



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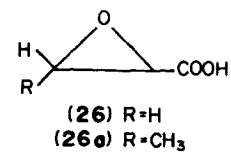
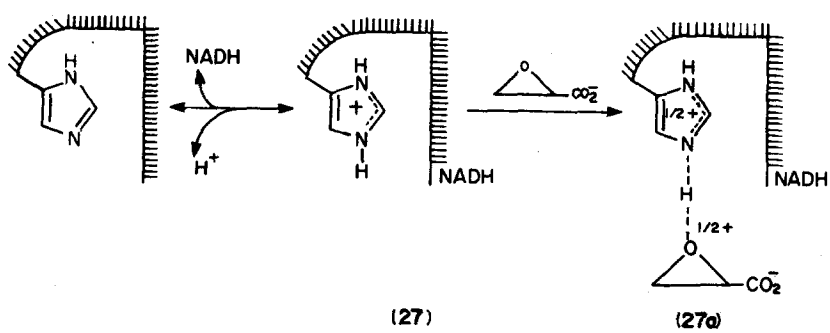
permitted a detailed analysis of the transient events which are normally too fast to be observed using the physiological substrate, pyruvate. It was found[14] that one proton was taken up from the solvent when O-nitrophenylpyruvate formed a ternary complex with lactate dehydrogenase and NADH, and that this event occurred before the complex rearranged to give the product. The group in the binary complex which received the proton had a pKa of 6.8.

A hypothetical mechanism for the activation of substrates in pyridine nucleotide linked reactions

In the light of the above evidence which admittedly is



(**26**) R=H
(**26a**) R=CH_3

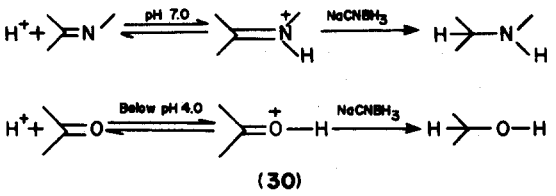


(**27**) (**27a**)

Scheme 11. *Mechanism for the binding of the epoxide inhibitor to the protonated-enzyme-NADH-complex.*

indirect, the mechanism of Scheme 10 may be extended to lactate dehydrogenase and enunciated as follows.[12b] An important step in the reaction in the forward direction prior to catalysis is the binding of pyruvate in a protonated[15] ternary complex (**29**, Scheme 12) via a H-bond. This results in the enhanced polarization of the CO group facilitating hydride transfer from the reduced pyridine nucleotide to the now more positively charged C atom. At this stage the lactate is still bound to the enzyme via H-bond (**29a**), full proton transfer to the -OH group of the lactate occurs only in the penultimate stage prior to its release from the enzyme. The reverse reaction by this mechanism will have a H-bond between the substrate, now lactate and the non protonated histidine, and substrate activation is achieved by partial deprotonation of the alcohol which facilitates hydride transfer to the oxidized coenzyme. Hence the enzyme could catalyze both forward and reverse reactions by a mechanism involving substrate activation by partial proton transfer due to H-bond formation.[12b] Evidence has been provided to suggest a similar mechanism may operate for β-hydroxybutyrate dehydrogenase.[12b]

anoborohydride has however brought to the surface some subtle facets of the reactivity of carbonyl compounds. This mild and relatively weak reducing agent reacts with imino compounds at pH 7.0, but reduction of carbonyl compounds occurs only at pH values below 4.5. The conclusion that the reagent can attack only activated polar double bonds is inescapable, since at pH 7 imino compounds exist in a protonated form whereas significant protonation of the CO groups to produce the reducible species (30) will only occur at low pH values. The need to activate CO groups in reactions when weak nucleophiles are involved is further emphasised by the fact that in non-enzymic model systems the only successful reduction of a carbonyl group by a 1,4-dihy-



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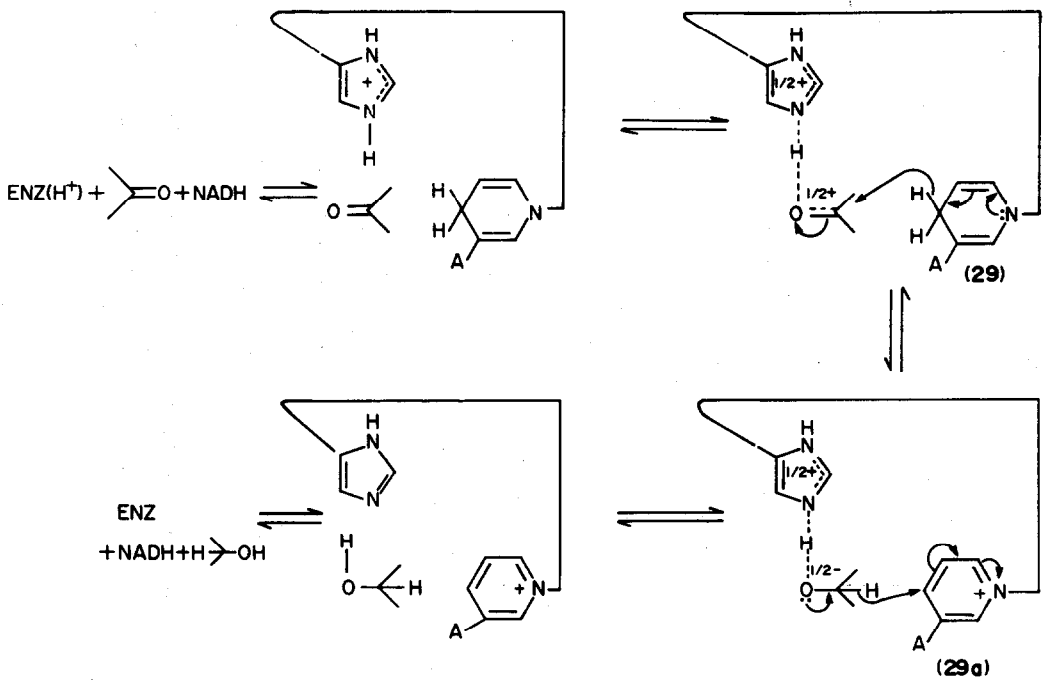


Scheme 12. *The sequence of bond forming events in the reversible reaction catalyzed by lactate dehydrogenase.* In both directions the substrates are activated by partial proton transfer processes. This Scheme also illustrated the Mechanism 2, which is discussed later.

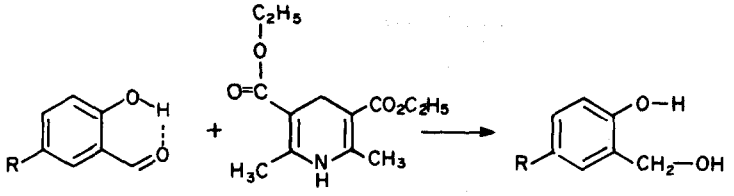
How important is the protonation of the carbonyl oxygen in non-enzymic additions to aldehydes and ketones?

Whereas the importance of protonation of olefins prior to hydride transfer in enzymic reactions proposed above must be readily acceptable to chemical logic, the stress we have laid on the adherence to a similar sequence for the reduction of CO groups, may however at first sight appear excessive. It is therefore desirable to consider some related problems regarding the reaction of carbonyl compounds in non-enzymic systems. It now appears to be well established that strong nucleophilic reagents like $LiAlH_4$ react with aldehydes and ketones by direct hydride transfer without the need for any prior activation of the CO group. The recent emergence of sodium cyanoborohydride has however brought to the surface some subtle facets of the reactivity of carbonyl compounds. This mild and relatively weak reducing agent reacts with imino compounds at pH 7.0, but reduction of carbonyl compounds occurs only at pH values below 4.5. The conclusion that the reagent can attack only activated polar double bonds is inescapable, since at pH 7 imino compounds exist in a protonated form whereas significant protonation of the CO groups to produce the reducible species (30) will only occur at low pH values. The need to activate CO groups in reactions when weak nucleophiles are involved is further emphasised by the fact that in non-enzymic model systems the only successful reduction of a carbonyl group by a 1,4-dihydropyridine was achieved in the reaction of **31** with **32**. It has been suggested[16] that H-bonding in O-hydroxybenzaldehyde activates the CO function towards reduction by Hantzsch ester (**32**). As expected, the presence of a nitro group in the *para* position as in **31** ($R = N_2O$) by increasing the acidity of the phenolic proton, favourably influenced the reduction process.

SECTION 3. ACTIVATION OF COENZYMES

Introduction

It is perhaps of interest at this stage to consider another aspect of biological chemistry. In order to carry out facile chemical transformations biological reactions require the availability of reactive reagents so that as far



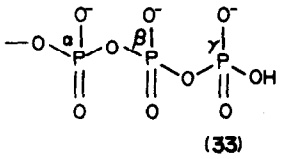
(31) (32)

as possible the overall conversion occurs exergonically and follows a low activation energy profile. But unlike laboratory chemistry, cellular chemistry obviously cannot cope with too reactive reagents, like acetic anhydride, alkyl lithium, etc.

Nature seems to have evolved the objective of "reactive-but-not-destructive" by one of two broad pathways. First, a chemically pleasing approach, in which substrate and/or coenzymes are covalently modified to produce reactive intermediates in an enzyme-bound form where they are available specifically for reaction at the active site. The generation of a variety of carbanion intermediates in pyridoxal phosphate and thiamine pyrophosphate linked reactions, the formation of enzyme-bound "formaldehyde" in tetrahydrofolate dependent reactions and the involvement of carboxy biotin in a number of carboxylation reactions are only some of the examples of a wide-spread phenomenon.

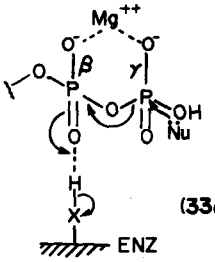
Activation of ATP

The second type of mechanism is that in which the reactants are activated, not by covalent modification, but by suitable charge manipulation. In our view such a mechanism is best illustrated by reference to ATP. In free solution the cleavage of one of the anhydride bonds of ATP through nucleophilic attack on phosphorous is prevented by the stabilization offered through resonance of the negative charges (33). During enzymic phosphorylation reaction however, the full potential of the anhydride linkage is exposed, at least partly, by forbidding resonance stabilization through the coordination of the negative charges with suitable electrophilic centres at the active site (33a), which allows nucleophilic attack on phosphorous via a low activation energy pathway.



(33)

Ionization state of ATP at pH ~7.0

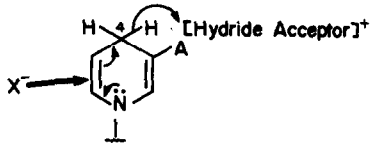


(33a)

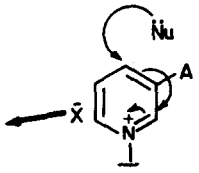
The suppression of resonance stabilization of ATP at an enzyme-active-site to allow nucleophilic attack at the γ-P atom

Hypothetical mechanism for the activation of pyridinium and dehydropyridine rings

We now consider the reaction of pyridine nucleotides and begin by drawing attention to the fact that in solution, both redox forms of pyridine nucleotides are only weakly reactive reagents. Thus dihydropyridines reduce only highly polarized double bonds and the 4-position of pyridinium ring undergoes attack by particularly strong nucleophilic reagents such as C≡N. The question now arises whether it is possible to activate pyridine nucleotides at the active-sites of enzymes by utilizing some features of their structures not fully realized in solution. Let us consider the reduction of a substrate occurring within the micro-environment of an enzyme active-site. The primary energy barrier to hydride transfer from the dihydropyridine ring to an oxidized substrate in the transition state will be development of an unneutralized positive charge on the pyridinium ring. The process however, may be greatly facilitated if prior to or during hydride transfer a strategically located negatively charged group approaches the pyridine ring thus reducing the energy barrier (Scheme 13). In the reverse direction the ground state structure of the pyridinium ring will have a closely associated negatively charged counter ion. The stability conferred on the molecule by the interaction will repel nucleophilic attack at C-4. The unfavourable energetics may be overcome by the removal of the counter ion from the enzyme-active-site during hydride transfer from the substrate to $NAD(P)^+$ (Scheme 13).



Approach of the counter ion towards the ring facilitating hydride transfer from C-4.

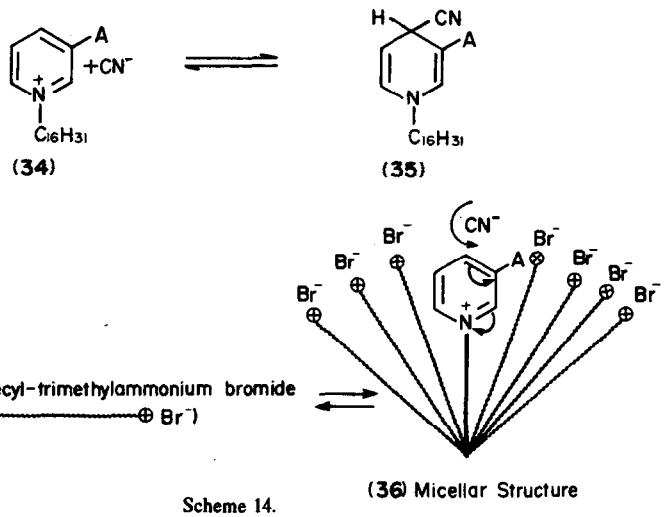


Removal of the counter ion from the vicinity of the ring facilitating nucleophilic attack at C-4

Scheme 13.

In support of the latter view the results of some recent model studies may be considered.[17] It has been shown that rates as well as equilibrium constants for the addition of cyanide ion to N-alkyl-pyridinium halides are greatly increased in the presence of detergent. The two parameters showed upward trends with increasing hydrophobicity in both the surfactants and the substrates. Most dramatic results were obtained in the reaction of N-hexadecyl-3-carbamoylpyridinium bromide with cyanide ion, in a dilute solution of hexadecyltrimethylammonium bromide, when the rate was increased by 950-fold and the association constant for the reaction (34)→(35) by 25,000-fold, compared to the reaction in the absence of the detergent. It is reasonable to

assume that the micelles contain the substrate molecules held by hydrophobic interactions, as shown in the hypothetical structure **36**, in which the pyridinium group is brought close to the micellar surface thus reducing the "solvation" of the positive charge by an external counter anion. The consequent electrostatic destabilization of the pyridine ring facilitates cyanide addition to C-4 producing the neutral dihydropyridine derivative which is attracted well within the micellar interior. Thus in our view the enhanced rate of reaction is the result of charge destabilization and the increased equilibrium constant for the addition process the consequence of the penetration of the product into the micelles.



Scheme 14.

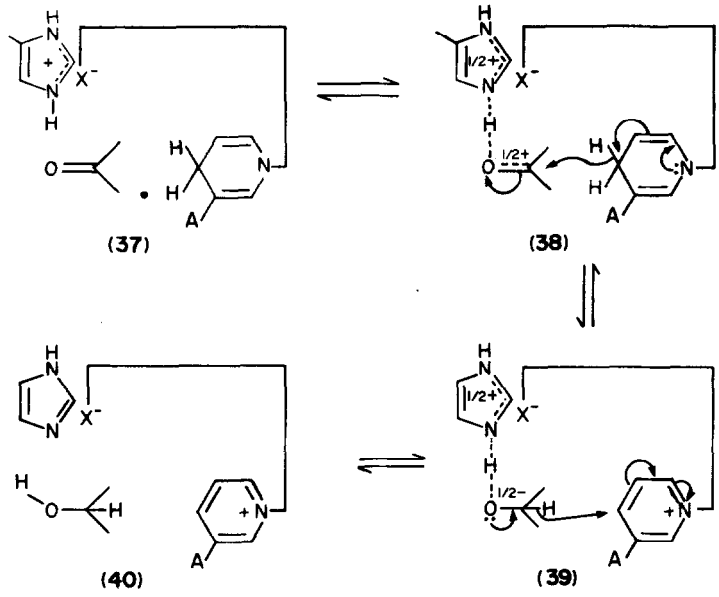
accompanying the conversion (**38**→**39**) is an energetically unfavourable process. Therefore the contribution which such a mechanism may make to facilitate hydride transfer from the dihydropyridine ring of NADH to the carbonyl C atom in the final stages of catalysis would be relatively small. Furthermore, it should be noted that according to this mechanism in the reverse reaction, the Michaelis complex (**40**) in the active-site region is electrostatically destabilized owing to the presence of two spatially separated charges; though if such an unstable complex (**40**) were to form then its rearrangement into the product (**37**) being attended by charge neutralization will be favourable.

SECTION 4. AN OVERALL VIEW OF CATALYSIS: CAN CHARGE-SEPARATION PROMOTE CATALYSIS IN PYRIDINE NUCLEOTIDE DEPENDENT ENZYMIC REACTIONS

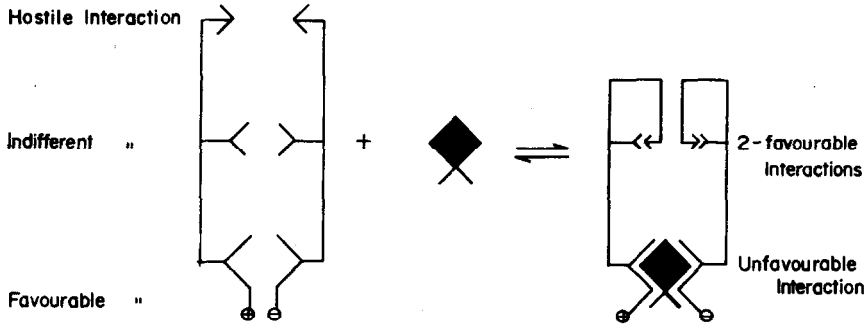
Mechanism 1 *involving a pseudo-neutral active-site.* Possible mechanisms for the activation of either the substrates or coenzymes in pyridine nucleotide linked reactions have been separately discussed above. We shall now integrate the information to develop an overall view of catalysis. Attention has already been drawn to work on lactate dehydrogenase which suggests[12b,13] that the ternary complex leading to the reduction of pyruvate must contain a protonated histidine (Scheme 12). The important role which protonated histidine may play in the reduction of carbonyl compounds has also emerged from work on β-hydroxybutyrate, malate and alcohol dehydrogenases. In considering the first mechanism we make the orthodox assumption that the micro-environment of the catalytic ternary complex for the reduction of carbonyl compounds is neutral. It therefore follows that during the formation of the latter complex the dehydrogenases must fold, trapping a counter anion to balance the charge on the imidazolium cation as shown in Scheme 15. From the mechanism outlined in the Scheme 12 it can be deduced that during catalysis the positive charge shifts from histidine via the carbonyl carbon to the pyridinium ring. The charge separation

Mechanism 2 *involving a "polar" active site.* Another view of the features of the active sites of enzymes which may effect catalysis may also be developed. The combination of an enzyme with its substrate to produce a competent catalytic complex, Michaelis complex, is always an exergonic process. The overall negative free energy change accompanying the complex formation may either represent the summation of several interactions, all of which are favourable, or more realistically a balance arising from many favourable, and a few unfavourable, interactions. In the latter event, the unfavourable interactions could exist primarily within the micro-environment of the active-site, where these are utilized for catalysis (Scheme 16). In the light of this comment, we may argue that electroneutrality at the active-site is not necessary, and in fact it may be advantageous to close the active-site without a counter ion as shown in Scheme 12 (this earlier Scheme is also used to illustrate the Mechanism 2), the price for the unfavourable energetics produced by a polar (charged) active-site being paid for by favourable interactions elsewhere (cf Scheme 16). Although with respect to catalysis, the relative merits of the two types of active-sites cannot be accurately assessed, however, on intuitive grounds it could be argued that in the forward reaction, the mechanism of type 2 (Scheme 12) compared to Mechanism 1 (Scheme 15) allows a more extensive proton transfer from the protonated histidine to the carbonyl oxygen.† Such a process will be expected to facilitate catalysis. On the other hand Mechanism 2, like Mechanism 1, makes no provision to facilitate electron release from the dihydropyridine ring, which is a prerequisite for hydride transfer. In the reverse reaction

†It is reasonable to assume that an imidazolium cation without its counter anion would be stabilized by deprotonation. In other words the concept provides a mechanism for the lowering of the pKa values of acids at the active-sites of enzyme-substrate complexes.



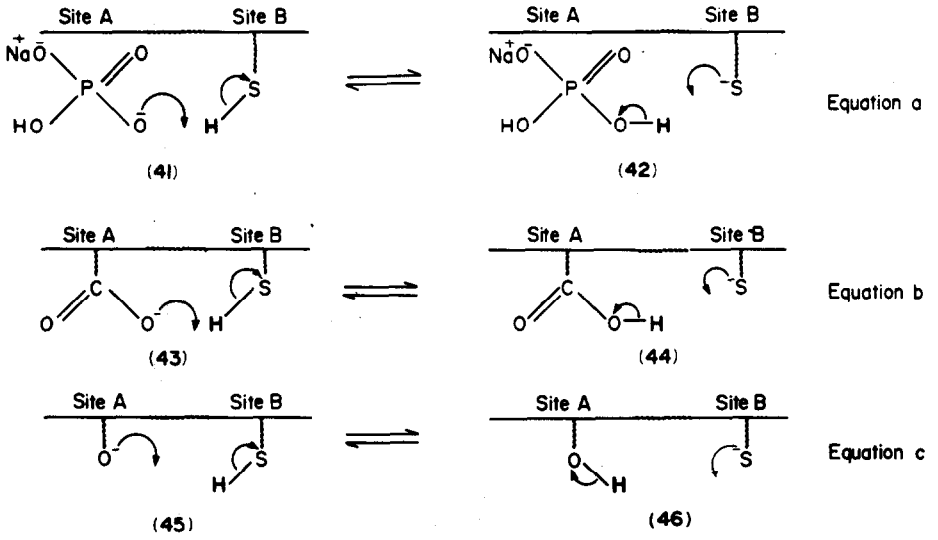
Scheme 15. *Mechanism* 1. Involves a pseudo-neutral active site with the positive change on the imidazolium cation neutralized by a counter ion. The group $-X^-$ either represents an ionizible group on the enzyme ($-COO^-$, $-S^-$) or an anion trapped during the formation of the ternary complex. The complex (38) represents the activation of the carbonyl by hydrogen bonding. As the reaction progresses the positive charge shifts from the carbonyl carbon (38) to the pyridinium ring (39). Thus although the active site of the Enz-carbonyl Compound-NADH-complex is truly neutral, in the Enz-alcohol-NAD^+ the two charges are spacially separated (hence the designation, pseudo-neutral).



Scheme 16. *The creation of unfavourable interactions in the active site region of the Michaelis-complex.* Although for the sake of illustrative convenience the unfavourable interaction is shown to arise from charge-separation, however other factors such as strain or charge-deprivation may also produce an unstable active-site, as indeed is inherent in many hypotheses.

according to Mechanism 2, the absence of a counter ion from the vicinity of the histidine may make the latter residue a less efficient base for proton abstraction from the –O–H group of the substrate. The consequences of the less extensive participation of the histidine in the reaction may though be partly compensated for by the fact that the hydride transfer from the substrate to NAD^+ now involves an activated pyridinium ring.

Mechanism 3, involving a neutral active-site with the counter anion "oscillating" between the histidine and the nucleotide sites. Finally, another version of the neutral active-site in which the enzyme also possesses a molecular architecture for the "oscillation" of the negative charge between the imidazolium ring and the nicotinamide binding site could combine the best features of both the previous mechanisms and permit the activation of the substrates as well as the coenzymes for reactions in both directions. This may be achieved either by a protein conformational change or through a charge-relay system. Three examples of the combination of groups which may form a suitable charge-relay system are shown in Scheme 17. In general, groups forming suitable charge-relay systems for the present purpose should meet two requirements. Firstly, that one of the groups in the charge-relay system should exist in an ionized form at the physiological pH values, 6.5–7.5, and indeed such is particularly the case with the carboxylate and to a lesser extent with sodium hydrogen phosphate ion. Secondly, that even the slowest rate of proton transfer between these groups, should still be sufficiently fast not to make this step rate limiting in the overall catalysis. From the extensive data now available[18] on the rates of proton transfer between acids and bases in solution, it may be assumed that groups whose pKa values do not differ by more than about 5 units will catalyze proton transfer, and hence by implication charge transfer, at rates considerably faster than the rate of overall catalysis. For

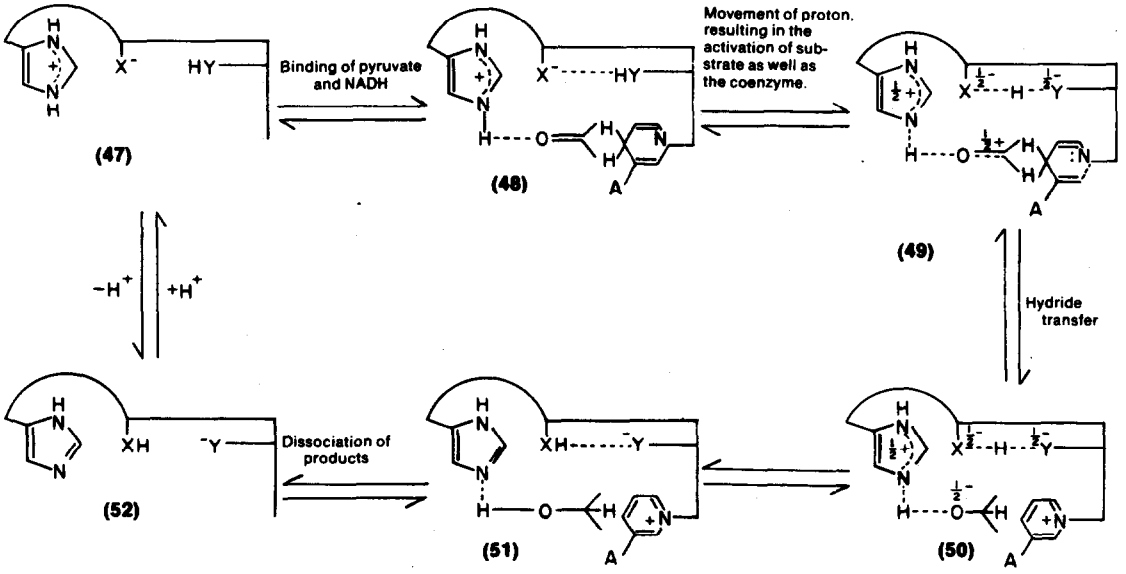


Scheme 17. *A hypothetical mechanism for the transfer of a negative charge between two remote sites A & B on an enzyme.* Although there is no theoretical limit regarding the number of groups which may form a charge-relay system, however for convenience in the Scheme the charge-relay system are shown to use only two groups. For the reactions of Equations (a) and (b) the rates of proton transfer will be expected to be fast (more than 10^7 M^{-1} Sec^{-1}) in both directions. For the reaction of Equation (c) the rate will be diffusion-controlled (about 10^{10} M^{-1} Sec^{-1}) in the forward direction but less than 10^2 M^{-1} Sec^{-1} in the reverse direction (pKa of –S–H being about 6–7 units lower than that of –O–H). The latter conclusion has been deduced from the discussion in Ref. 18.

convenience we illustrate the concept (Scheme 18) by using two ionizable groups to generate counter anions at sites which may be several Å apart.[19] The location of groups as shown in structure **49** will facilitate† reduction of the CO bond by allowing not only a more extensive proton transfer from the imidazolium cation to the substrate oxygen in the transition state, but also promote hydride transfer from the coenzyme by stabilizing the positive charge about to develop on the pyridinium ring. In the oxidation of the substrate the unneutralized positive charge on the pyridinium ring as in structure **50** will be particularly favourable for hydride transfer from the alcohol to C-4 of the pyridinium ring. In our view this Mechanism sets forth the optimal, and not the minimum, electronic requirement needed for catalysis in pyridine nucleotide dependent reactions. A partial fulfilment of these requirements as exemplified in Mechanisms 1 and 2 may in fact suffice in may cases.

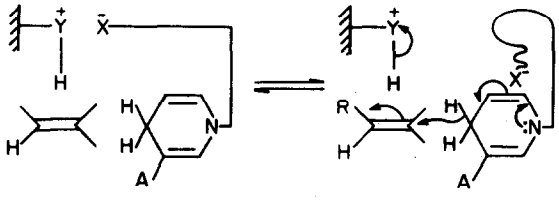
We side-stepped an important issue while discussing the reduction of C=C. The most acid group available for

†See footnote on page 822.



Scheme 18. *Mechanism* 3. This hypothesis assumes that the enzyme occurs in two forms, the enzyme base (52) and the conjugated acid (47) protonated on the imidazolium ring. The proton transfer between the groups, $-X^-$ and $-Y^-$, constituting the charge-relay system is assumed to be fast relative to other steps in the conversion. In the direction of the reduction of the substrate the migration of the proton towards $-X^-$ produces an anion near the dihydropyridine ring which activates it for hydride transfer. There may be conformational changes during the binding and disocciation of substrates and products, which are not shown here.

proton transfer to olefins in biological reactions in a carboxyl group and most likely is the imidazolium cation. In spite of much work on the mechanism of electrophilic addition to olefins in non-enzymic systems, quantitative information on the relationship between the pKa of an acid and its ability to protonate olefins is not available. It is however reasonable to assume that the rates of protonation of olefins by acids of pKa 4.5 (carboxyl) and 6.8 (imidazolium) will be very slow indeed. Once again the charge separation accompanying the rearrangement of a hypothetical Michaelis complex **53** to **54** could provide the driving force for the dissociation of the proton from the imidazolium cation and encourage the hydride transfer from the coenzyme by the presence of a counter ion to neutralize the positive charge on pyridinium ring.



(53) (54)

Scheme 19. The transfer of the negative charge to the coenzyme binding site may involve either a conformational change or occur through a charge relay system shown in Scheme 17.

SECTION 5. BIOLOGICAL ALKYLATION OF OLEFINIC LINKAGES: ELECTROPHILIC ADDITION OF "METHYL CATIONS" TO DOUBLE BONDS

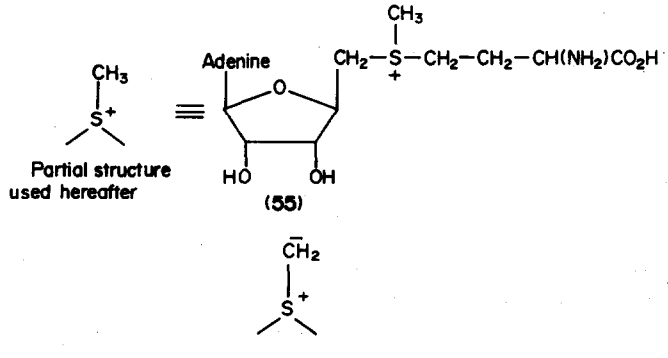
The ergosterol problem

An outstanding problem during the middle 1960s was the mechanism through which the "non-isoprenoid" derived carbon-28 was introduced in the ergosterol side chain (57, Scheme 21). It had been previously shown by Parks[20] that the C atom, C-28, originated from methionine‡ via S-adenosylmethionine (**55**) and that the presence of a 24,25-double bond in the steroid side chain was necessary for the alkylation reaction to occur.[21] In an elegant series of experiments using [CD_3] methionine, Lederer *et al.* showed that only two of the three original D atoms form the Me group of methionine were found at C-28 of ergosterol.[21a,21b,22] The discovery came at a time when a broad spectrum of carbanions generated from sulphonium compounds were finding wide-spread use in preparative organic chemistry. The loss of one of the D atoms from methionine during the alkylation reaction could be conveniently rationalized by invoking the intermediary of a species of the type **56**. However, the realisation that in the saturation of C=C in biological systems an electrophilic addition to the unsaturated linkage was the crucial primary event stimulated an extension of a similar principle to explain the mechanism for the addition of the "extra" C atom to double bond in ergosterol biosynthesis.[23] In the alkylation of the olefin of the type (57, Scheme 21) by an electrophilic mechanism,[21,23]§ the first step will be expected to be the transfer of a "methyl cation" from S-adenosylmethionine to C-24 of the steroid side chain giving a carbonium ion at C-25 (**58**, Scheme 21). Several mechanistic courses, such as hydrogen migration (**58–59**), hydrogen elimination (**58–58a**) and association with negatively charged species (**58–58b**) are now available for the neutralization of the carbonium ion. It must be immensely gratifying to the predictive sense of mechanistic organic chemists to find that all the theoretical alternatives are now known to operate somewhere in biosynthetic pathways. Returning to ergosterol, the experimental observations available at the time could be best rationalized by assuming that C-25 carbonium ion (**58**) through hydrogen migration rearranges to give a carbonium ion at C-24 (**59**) which then is neutralized through the loss of a hydrogen from the Me group. The mechanism made two predictions,[23] firstly that a 24,28-methylene sterol (**60**) must be the precursor of ergosterol side chain and second, that the H atom originally located at C-24 of the precursor of type **57** must be found at C-25 in the final product (**61**). In order to test the hypothesis several specifically labelled precursors, particularly species of lanosterol as shown in structures (**62a–c**) were synthesised.[23a,23b] A doubly labelled sample, [24-^{3}H; 26,27 $^{14}C_2$] lanosterol (**62b** + **62c**), was then incubated with *Saccharomyces cerevisiae* and the biosynthetic ergosterol when subjected to a

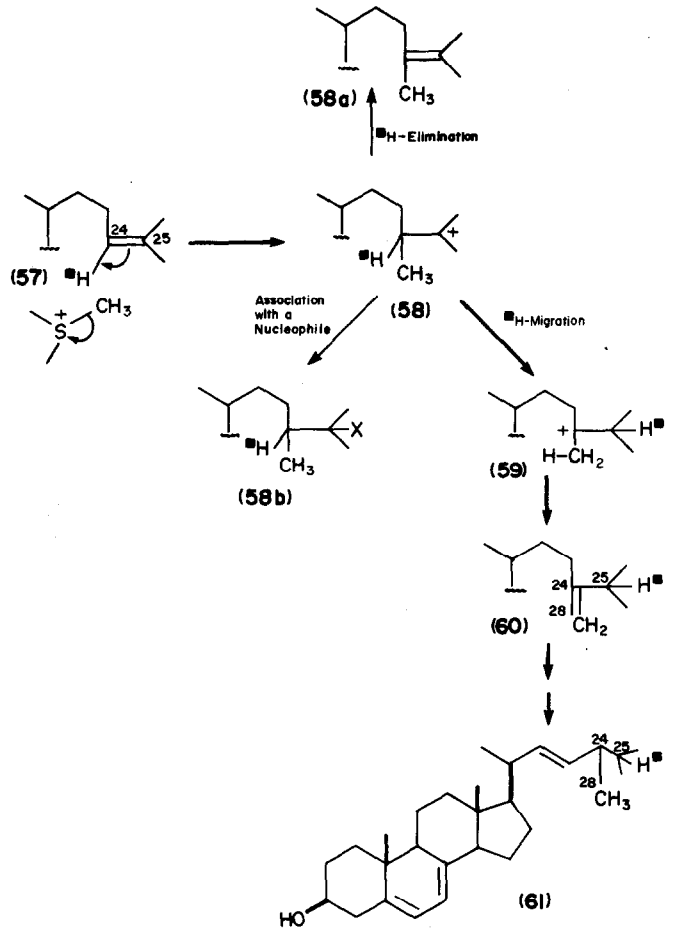
†See footnote on page 822.

‡The transfer of the Me group of methionine to an unsaturated C atom was first postulated by A. J. Birch, D. Elliott and A. R. Penford, *Austral. J. Chem.* **7**, 169 (1954) and confirmed soon after in the following papers: A. J. Birch, R. J. English, R. A. Massy-Westropp, M. Slaytor and H. Smith, *Proc. Chem. Soc.* 204 (1957); G. J. Alexander, A. N. Gold and E. Schwerk, *J. Am. Chem. Soc.* **79**, 2967 (1957); S. Badar, L. Guglielmethi and D. Arigoni, *Proc. Chem. Soc.* 16 (1964).

§It should be noted that a hypothesis similar to that in Scheme 21 was proposed as early as 1963 by Castle *et al.*[27] for the elaboration of phytosterol side chains and subsequently advocated with conviction in Ref. 21*c*, 21*d* and also by L. J. Goad, A. S. A. Hammam, A. Dennis and T. W. Goodwin, *Nature* **210**, 1322 (1966).



Scheme 20.



Scheme 21. Mechanism for the elaboration of ergosterol side chain (61). Nuclear changes not shown.

variety of degradations gave results showing that the H atom originally located at C-24 of lanosterol was present at C-25 of ergosterol thus confirming the hypothesis[23a,b]† outlined in Scheme 21 (heavy arrows). That a 24,28-methylene steroid side chain, as present in the compound **66**, was a precursor of ergosterol was shown indepentently[24] by us and also by Barton *et al.* at Imperial College, London.

Parallel studies at Gif by Lederer and his colleagues put the hypothesis on a firm footing, when they showed that a sequence identical to that described for ergosterol biosynthesis was also involved[25] in the alkylation of oleic acid (**67**) to give tuberculostearic acid (**68**).

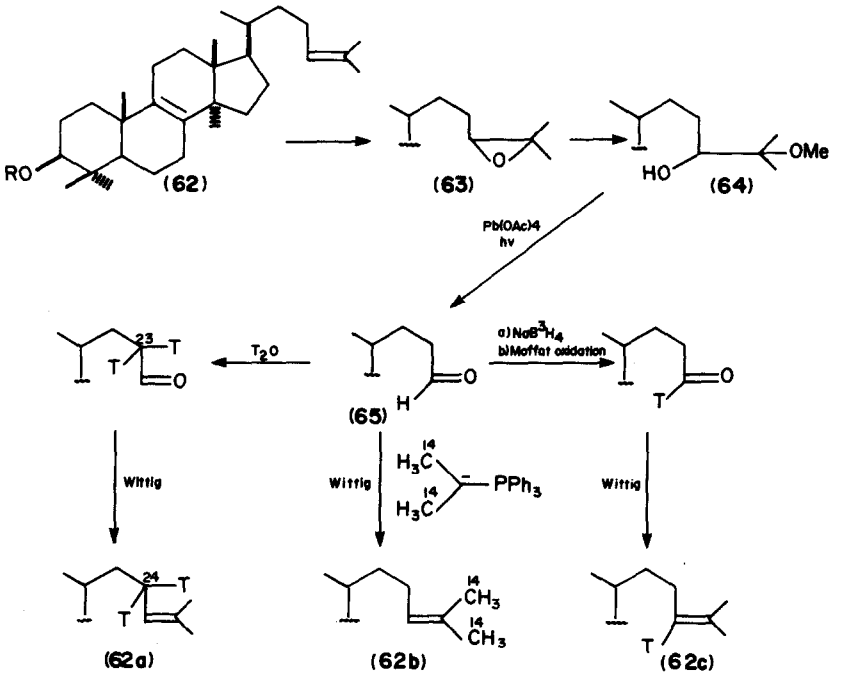
The biosynthesis of phytosterol side chains

The mechanisms for the addition of the extra C atoms in the elaboration of Me and Et side chains of phytosterol have been extensively studied and the literature in the area is covered in several excellent reviews.[21b,26] The main conclusion from these studies are that the Et side chains of phytosterols are formed through a double C-methylation process through a general mechanism originally proposed by Castle *et al.*[27] and shown in Scheme 24. It has been shown that the first methylation produces a 24,28-methylene intermediate which undergoes another electrophilic addition of the "methyl cation" to give a carbonium ion intermediate at C-24 (B, Scheme 24). Extensive and revealing studies of the Liverpool group[26] have shown that almost every theoretically conceivable mode of neutralization for the carbonium ion of the type (B, Scheme 24) operates somewhere in nature.

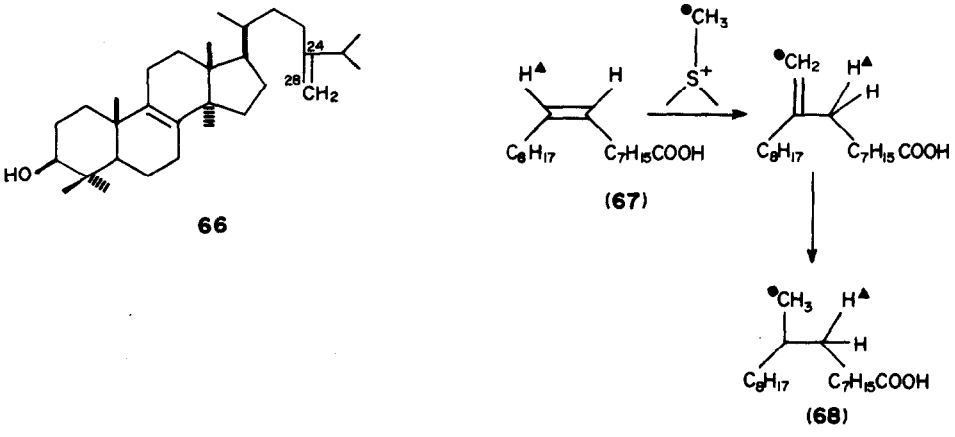
Thus in the biosynthesis of poriferasterol[28] (**69**; Scheme 25) by *Ochromonas melhamensis*, in the presence of [CD_3]methionine the ethyl side chain of **69** was found to contain 4 D atoms. This observation was rationalized[21b,28] by assuming that the addition of the first C_1-unit gives the 24,28-methylene intermediate containing 2 D atoms, and a second methylation produces a carbonium ion intermediate (B, Scheme 25) which is neutralized by hydrogen elimination from C-28 to give the ethylidene side chain containing 4 D atoms, Scheme 25. The conversion of the intermediate (C, Scheme 25) into poriferasterol then occurs by a two stage process involving the saturation of the 24–28 bond followed by a dehydrogenation reaction inserting a double bond between C-22–C-23. The mechanistic details for the latter two stages have not yet been elucidated.

On the other hand, in the biosynthesis of 5α-stigmast-22-en-3β-ol by *Dictyostelium discoideum* in the presence of [CD_3] methionine the Et side chain (**70**, Scheme 26) was found to contain 5 D atoms.[21b,29] Further experi-

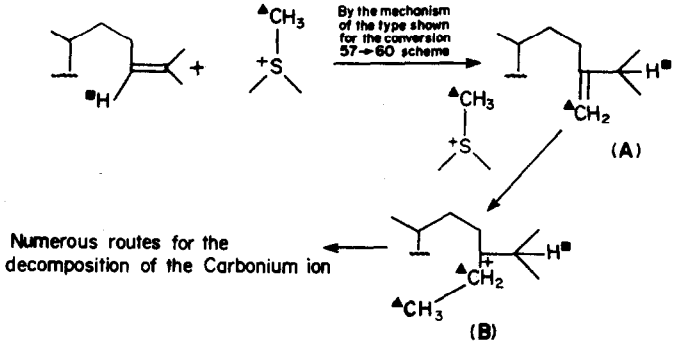
†In the steroid field other examples showing that the tritium at C-24 in the precursor is shifted to C-25 in the resulting sterol are: L. J. Goad and T. W. Goodwin, *Europ. J. Biochem.* 7, 502 (1969); K. H. Raab, N. J. De Souza and W. R. Nes, *Biochim. Biophys. Acta* **152**, 742 (1968).



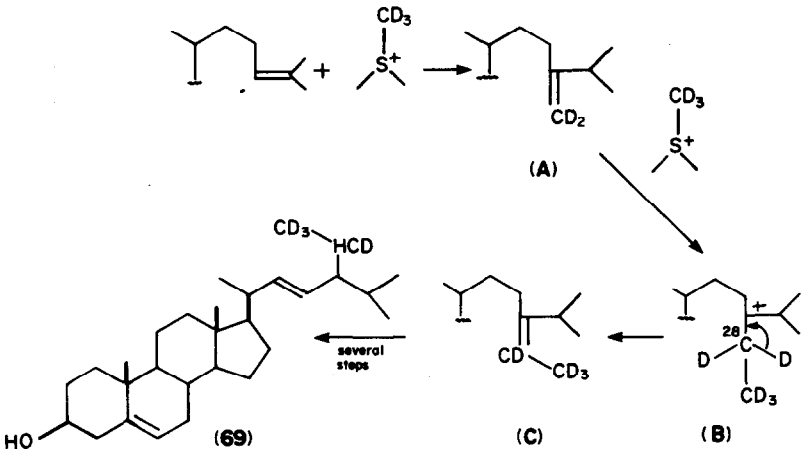
Scheme 22. Synthesis of variously labelled lanosterols



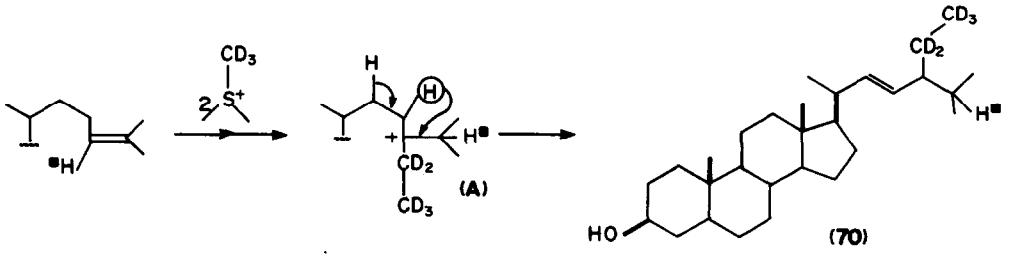
Scheme 23.



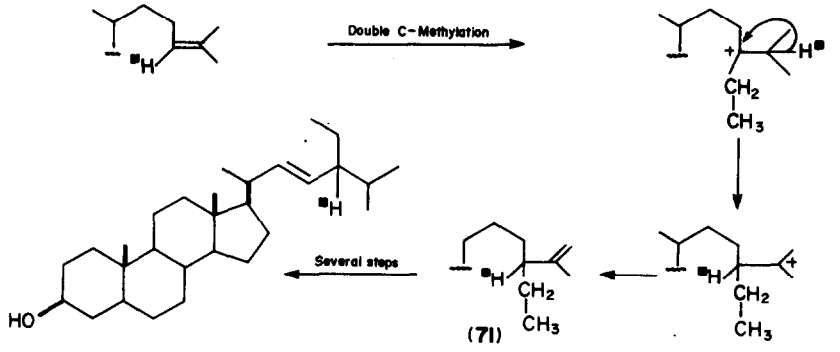
Scheme 24. The elaboration of ethyl side chains of phytosterol by double methylation.



Scheme 25. Mechanism for the formation of poriferasterol side chain.



Scheme 26. Biosynthesis of the side chain of 5α-stigmast-22-en-3β-ol.



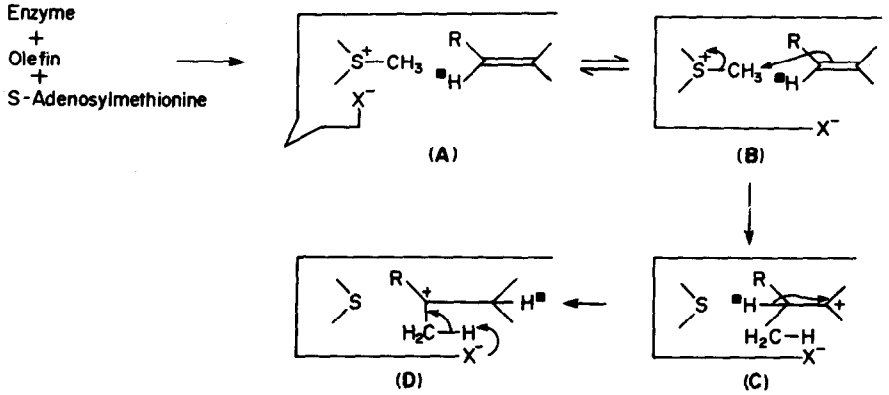
Scheme 27. Proposed mechanism for the biosynthesis of the side-chain of the type (71).

ments using 23-tritiated lanosterol as the precursor proved that in this case the side chain of stigmast-22-en-3β-ol (70) was formed from the 24-carbonium ion intermediate A, Scheme 26, by a hydrogen migration from C-23 followed by a hydrogen elimination from C-22. Yet another mode for the rearrangement of a 24-carbonium ion has been hypothesised[26c] for the elaboration of the 24-ethyl-25-methylene type of side chain (71) and is outlined in Scheme 27.

The mechanism of activation of S-adenosyl methionine through charge separation

In solution sulphonium salts are notoriously weak electrophilic reagents and with one exception[30] the alkylation of a double bond by a sulphonium group has not been observed in non-enzymic reactions. The possibility that in enzymic reactions the onus for the alkylation reaction falls entirely on the nucleophilic property of the olefin is unlikely, since it is difficult to envisage of a mechanism which would sufficiently activate an olefinic linkage to attack such a poor electrophile as a sulphonium compound, apart from some, but not enough, enhanced reactivity emerging from the binding of the olefinic linkage in a strained configuration. The possibility of activating S-adenosylmethionine for Me donation should therefore be examined. Once again, it is attractive to hypothesise that the Me donating capability of S-adenosylmethionine may be greatly enhanced through charge separation which may be achieved by one of two closely related processes. In solution the positively charged sulphonium cation must be associated with a counter anion. If the formation of the ternary complex, olefin-S-adenosylmethionine-Enzyme, occurred excluding the counter anion, then the electrostatic destabilization created by an unneutralized positive charge may promote Me transfer from the sulphonium group to

the π electron system of the double bond thus facilitating the crucial first step in the conversion. Alternatively the ternary complex may be formed including a negative counter ion at the active site, however a conformational change or a charge relay system removes the negative charge from the sphere of the sulphonium cation (conversion A→B, Scheme 28). It is reasonable to assume that the charge distribution as shown in structure *B* (Scheme 28) will promote not only "CH_3^+" transfer but also provide an anion for the removal of a proton in the terminal step of the overall reaction.



Scheme 28. The activation of S-adenosylmethionine for methyl donation through charge-separation as shown in A→B.

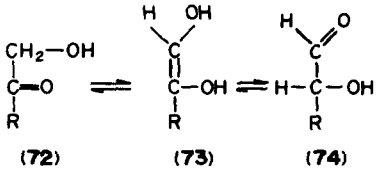
SECTION 6. ISOMERIZATION

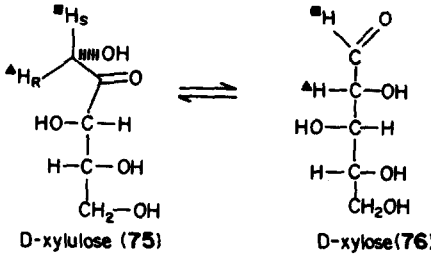
The activation of the CO group through protonation is an important first step not only in reactions catalyzed by dehydrogenases but may be a general feature of many other enzymic transformations in which aldehyde and ketones are used as substrates. Two examples of this phenomenon are found in aldose-ketose isomerizations and in the rearrangement of β,γ-double bond of β,γ-unsaturated ketones to produce the corresponding α,β-conjugated ketone. Enzymological information currently available in the first area is briefly summarized below.

Aldose-ketose isomerization

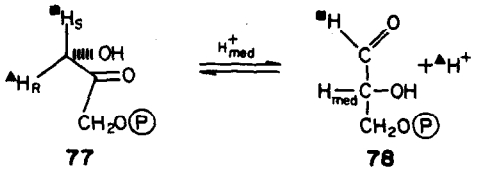
The enzymes catalyzing the reversible interconversion of aldoses into ketoses (72→74) are extensively distributed in nature and play crucial roles in carbohydrate metabolism in animals, plants and bacteria. Although it was recognized as early[31] as 1904 that isomerization reaction (72→74) may occur through the intermediary of an enediol (73), the origin and fate of the two H atoms participating in the reaction have been investigated[32] only in the last 15 years. From the viewpoint of conservation of H atoms in the overall reaction two extreme possibilities are represented by D-xylose and triose-3-phosphate isomerases which catalyze the reactions shown in Schemes 29 and 30 respectively. Isotopic studies using D-xylose isomerase have shown[33] that in this case the rearrangement involves an intramolecular hydrogen transfer between C-1 and C-2 and occurs without significant proton exchange with the medium. All isomerases studied to date are stereospecific with respect to the C-1 pro-chiral centre.[33] With the D-xylose isomerase it is the pro-R H atom which is transferred[33] to and from C-1 of the ketose in the interconversion of Scheme 29.

Triose phosphate isomerase from Rabbit muscle has been extensively studied from kinetic,[34] mechanistic[34] and X-ray crystallographic viewpoints.[35] With this enzyme reaction is not intramolecular[36] since the conversion of [1R-^{1}H] dihydroxy acetone phosphate into glyceraldehyde-





Scheme 29. The intramolecular hydrogen transfer in the reaction catalyzed by D-xylulose-D-xylose isomerase.

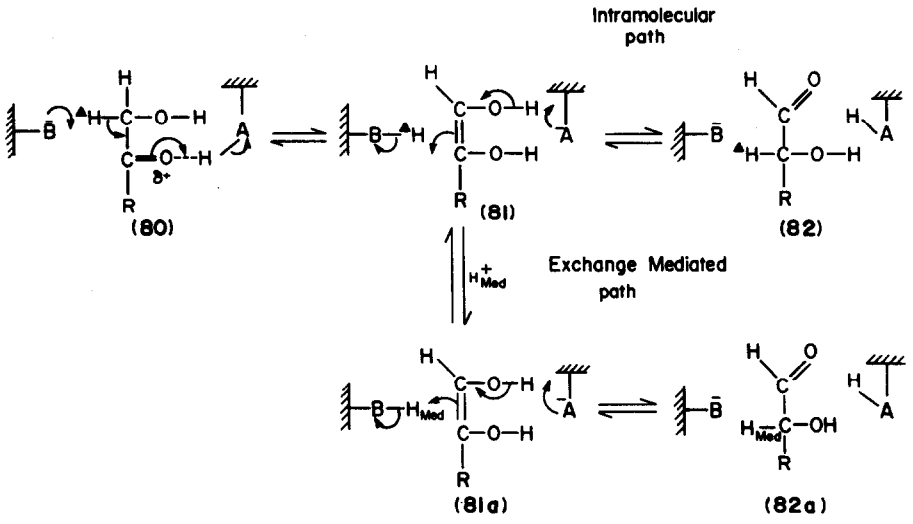


Scheme 30. *The exchange mediated interconversion catalysed by triose phosphate isomerase.* The status of hydrogen atoms in the conversion is shown for the reaction in the forward direction. H^+_{Med} is proton from the medium.

3-phosphate resulted[34] in the retention of less than 2% of the original tritium in the product (Scheme 30). In between these two extremes, with respect to intramolecular vs exchange mediated transfer, there are a range of possibilities. For example, in the case of Glucose-6-phosphate isomerase a high degree of intramolecular transfer observed at 0°C could be progressively decreased by carrying out the incubations at elevated temperatures.[37]

The mechanism of isomerization

Despite these apparent differences with respect to the degree of conservation of the participating H atoms in



Scheme 31. Postulated mechanism of aldose-ketose ismerases.

the aldose-ketose interconversion, there is a general agreement that all these isomerases have similar catalytic mechanisms (Scheme 31). The first step in the suggested mechanism[34] is the removal of one of the H atoms of the substrate by a basic group on the enzyme producing the enediol intermediate (**81**). Two possibilities then exist for the decomposition of the intermediary complex (**81**). Either the conjugate acid of the enzyme base in the complex (**81**) is completely shielded from the medium and the hydrogen removed in the deprotonation reaction is faithfully transferred to C-2 without exchange with the protons of the medium as must be the case with D-xylose isomerase (**81**→**82**). Alternatively, the group –BH in the complex is exposed to the medium and exchanges with the surrounding water molecules faster than the rate of collapse of the enediol intermediate, in which case the C-2 H atom in the product will originate from the protons of the medium (82a), as is observed in the triose phosphate isomerase reaction. With the latter enzyme, since less than 2% of the tritium from [1R-^{3}H] dihydroxyacetone phosphate was retained in the product, it has been estimated that the exchange reaction (**81**→**81a**) is at least 50 times faster than the reprotonation step (**81a**→**82a**).

The results with glucose-6-phosphate isomerase demonstrating a shift from an intramolecular to an exchange mediated transfer with increasing temperatures are consistent with the proposed mechanism. The conformational mobility within the Michaelis complex and hence the incidence of exposure of the catalytic group of the enzyme to the medium leading to proton exchange will be expected to be increased with increasing temperature.

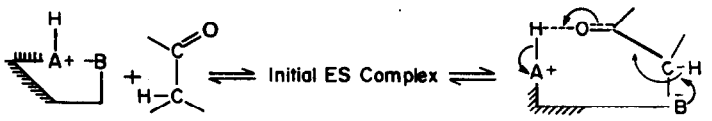
The question may be asked whether the formation of the enediol intermediate in reactions catalyzed by isomerases depends entirely on the presence of the CO group in the substrate coupled with the juxtaposition of a strategically located basic group in the Michaelis-complex or that an additional activation of the CO group is also required. Recent elegant studies of Webb and Knowles[38] have shed light on this facet. It was found that a mixture containing dihydroxyacetone phosphate and substrate quantities of triose phosphate isomerase when treated with $NaBH_4$ resulted in the formation of up to 95% of the L-isomer of glycerol 3-phosphate thus showing that the reduction involved the stereospecific trapping of the enzyme-bound dihydroxyacetone phosphate. Further experiments suggested that $NaBH_4$ reacted ten times faster with the enzyme-bound dihydroxyacetone phosphate than with the free substrate. The results are best rationalized by assuming that in the Michaelis-complex the CO group of the substrate is activated presumably via H-bonding as shown in structure **80**. Thus in the isomerase catalyzed reaction the H-bonding of the CO group must be aimed at facilitating the removal of the α-H atom, while we have already stressed in the previous section that in dehydrogenases the polarization of the CO group by H-bonding aids nucleophilic attack by the reduced coenzyme.

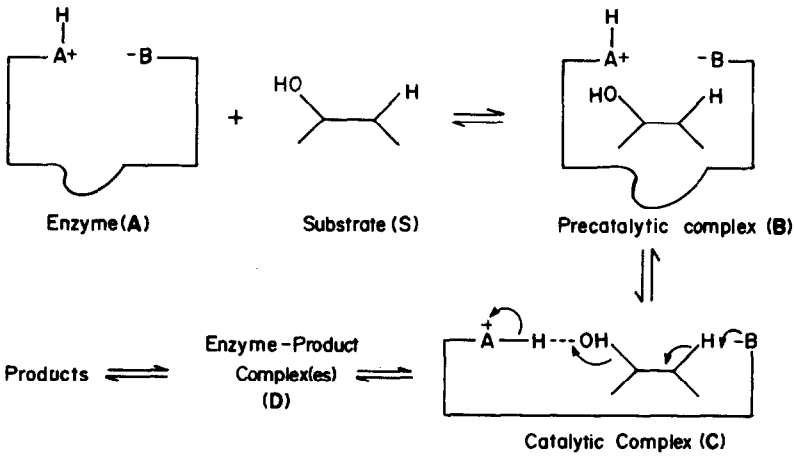
The application of the charge-separation hypothesis to isomerases

Two key processes involved in the reactions catalyzed by the isomerases discussed in section (7) are the protonation of the CO group and the cleavage of C–H bond. What provides the driving force for these steps deserves comment. Returning to the underlying theme in the review, once again one could argue that the active site of isomerases contains proton donating and basic groups ($-A^+-H$ and $-B^-$ of Scheme 32), which serve as counter ions for each other. The separation of these groups in the Michaelis-complex would be expected to enhance their reactivity as acids and bases thus facilitating the crucial steps in catalysis.

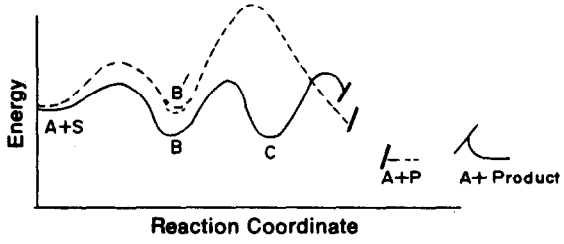
Concluding remarks

What makes enzymes such efficient catalysts has been an area of much speculation during the last 50 years.





Scheme 32. The Scheme shows the formation of an electrostatically destabilized active-site in (C) which possesses features suitable for catalysis. As emphasised in an earlier Scheme 16 the price for the unfavourable interaction in the active-site region of (C) being paid for by favourable interaction produced elsewhere in the Complex. Therefore with respect to *total structure* Complexes (B) and (C) *could* be isoenergetic, however the microenvironment of the active-site of (C) is of higher energy than that of (B). The steps in the conversion of (C) into product are neither shown above nor included in the diagram below.



For the reaction A + S → B → C → etc. → product(−). On the other hand a reaction path excluding an intermediate of the type (C) may follow a less favourable alternative path (----).

Consequently it is now recognized that factors such as precise orientation of the reacting centres at enzyme-active-sites, restriction of the rotational freedom of the reactants by "fixation" and substrate destabilization through conformational distortion, all make important contributions to the efficiency of enzymes. Jencks[39] has recently extended the ideas originally put forward by Pauling and Haldane into a semiquantitative theory which suggests that the driving force for the above processes may be provided through the utilization of binding energy. In the present review we have emphasized that a part of the binding energy may also be used to produce an electrostatically destabilized active-site through charge separation. For the sake of simplicity the concept is illustrated in Scheme 32 by reference to a hypothetical irreversible dehydration reaction. The Scheme 32 when taken in conjunction with the energy profile in the figure is intended to highlight that the overall transformation in an enzymic reaction is characterized by the involvement of several intermediary species, and that the structural features in each intermediate ensure that the succeeding species is formed via a low activation energy profile. Particularly relevant in the present context is the creation of an unstable active-site in the intermediate (C) which possesses the electronic environment required for the crucial catalytic steps leading to product formation (C → D). As has been emphasised earlier the charge separation process such as that shown in (C) provides a mechanism for the "transient" generation of strong acids and bases specifically at the active-site region of enzyme-substrate complexes.

Acknowledgements—I thank my colleagues Dr. D. C. Wilton and Dr. D. P. Bloxham who have contributed much to the work described in Sections 1 and 2 respectively and in discussion with whom I have formulated many of the concepts advocated in this report. Dr. Wilton was the first to point out the merits of involving a partial proton transfer in explaining the mechanism of dehydrogenases.

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