Volume 1
Number 1

Review

Biosynthesis of fatty acids and cholesterol considered as chemical processes

J. W. Cornforth

National Institute for Medical Research, London, N.W. 7, England

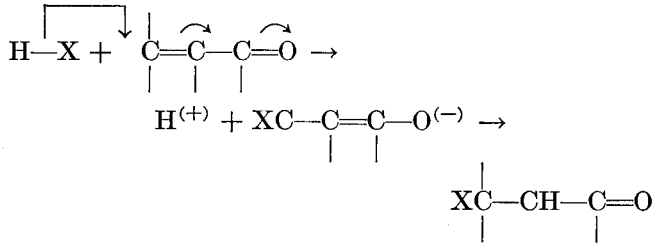
[Received for publication June 25, 1959]

The expansion of knowledge concerning biosynthesis of lipids has become almost explosive; and any review of its present state is likely to be overlaid by new developments by the time it is printed. Enough is already known, however, to show that lipids, in comparison with proteins and carbohydrates, are built up by an unusually wide variety of enzymic reactions representing many different chemical types. This article attempts a survey, from the viewpoint of an organic chemist, of the processes leading to biosynthesis of fatty acids and of cholesterol. It might be objected that enzymic transformation of a substrate can rarely be matched—for speed and adaptability to a cool substantially neutral aqueous medium—by the same transformation executed *in vitro* without enzymes; and therefore that procedures which are possible or impossible in a chemical laboratory have no necessary relation to the capabilities of a living cell. Yet few enzymic reactions are known which have no counterpart in organic chemistry: considered as chemical transformations of substrate into product, most are of quite familiar type. If enzymes, like other components of living matter, have undergone evolution and adaptation, they could be expected to mediate processes which are not too difficult to effect without specific catalysis. The apo-enzyme would have undergone progressive development of its power specifically to adsorb reactants and to facilitate the charge-transfers, in substrate and coenzyme, necessary for reaction. The entropy and energy of activation are thereby progressively lowered; but if they were initially too high, evolution could not have progressed. The function of an enzyme being so to provide an ideally favorable environment for an inherently facile chemical change, the ordinary laws of chemical reactivity can be used to discuss and predict the course of biosynthesis. The justification of this approach is found in its many successful predictions and in its power to limit the number of hypotheses to be tested by experiment; its dangers lie in the temptation to accept a chemically plausible mechanism without critical testing, and in the tendency to regard as a single chemical step an enzymic process which may prove to contain several.

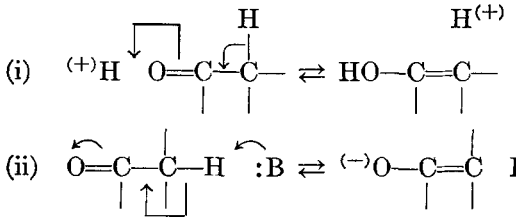
BIOSYNTHESIS OF FATTY ACIDS

The biosynthesis of fatty acids appears to be controlled almost entirely by one source of chemical reactivity: the permanent polarization of a carbonyl group, $\rangle C{=}O$. This polarization arises from the fact that although carbon and oxygen atoms are of the same size and have identical orbitals available to their electrons, oxygen has a higher nuclear charge than carbon and therefore tends to hold in its vicinity more than a half share in the electrons of a bond linking the two atoms. Thus the carbon atom has a fractional positive charge, and from this electron-deficiency comes its reactivity to nucleophilic reagents.

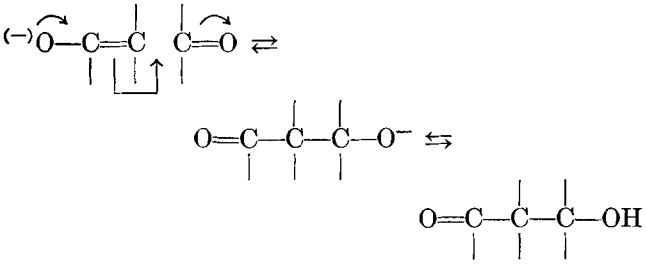
When a C=C double bond is conjugated with a carbonyl group, as in —CH=CH—C=O, the β-carbon also becomes electron-deficient and can add a nucleophilic reagent, generalized here as HX:



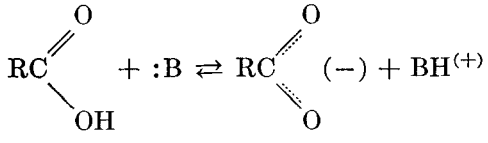
A further consequence of the polarization of a carbonyl group is that formation of an enol or its anion can easily be induced by (i) acidic or (ii) basic reagents, a hydrogen atom on the α-carbon being removed:



Both the enol and the enolate ion are *nucleophilic* reagents, and it is this easy transformation of an electrophilic carbonyl group to a nucleophilic enol which makes the group so important a generator of new carbon-carbon bonds. Enolization is a reversible reaction; but instead of recombining with the lost α-hydrogen atom, an enol or its anion may combine with other electron-deficient atoms, including a nonenolized carbonyl group:

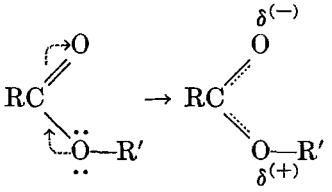


In carboxylic acids and their derivatives (esters, amides, anhydrides, and the like) the carbonyl group is attached to a heteroatom which can modify its reactivity profoundly. In a carboxylic acid, a base readily removes a proton to give the stable symmetrical anion in which, because of the distributed negative charge, the electron-deficiency at carbon is very slight.



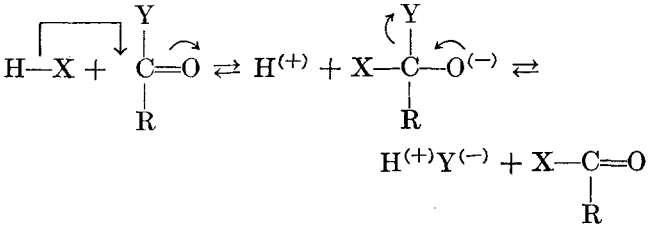
Thus bases add to or enolize the carboxyl group with great difficulty, and even when the undissociated form of the acid can react (as in acid-catalyzed esterification) a deactivation similar to that of esters (see below) makes addition more difficult.

In esters, RCOOR′, unshared electrons of the singly-bound oxygen atom tend to take part in the C—O bond, making it a partial double bond and largely neutralizing the positive charge on carbon:



This effect is strong enough to overcome the tendency, already mentioned, for oxygen to attract electrostatically the electrons of a carbon-oxygen bond, and it is even stronger in amides RCONR′R″, in which the carbonyl group is consequently even more unreactive.

A third class of carboxylic acid derivatives can be grouped as anhydrides. When the acyl group, RCO—, is attached to the anion of an acid HY, the group Y influences reactions at the carbonyl group in two related ways. Since HY is an acid (i.e., it tends to dissociate into $H^{(+)}Y^{(-)}$), the group Y necessarily is electron-attracting, and in the compound RCOY it withdraws electrons from the carbonyl carbon, accentuating its electron-deficiency. Thus, unless a deactivating effect like that shown by esters can occur, the electrophilic reactivity of the carbonyl group is actually greater than in an aldehyde or ketone. Further, when addition of a nucleophilic agent to the carbonyl group has occurred, elimination of the stable $Y^{(-)}$ anion can take place so readily that the replacement of Y by X may be virtually irreversible.

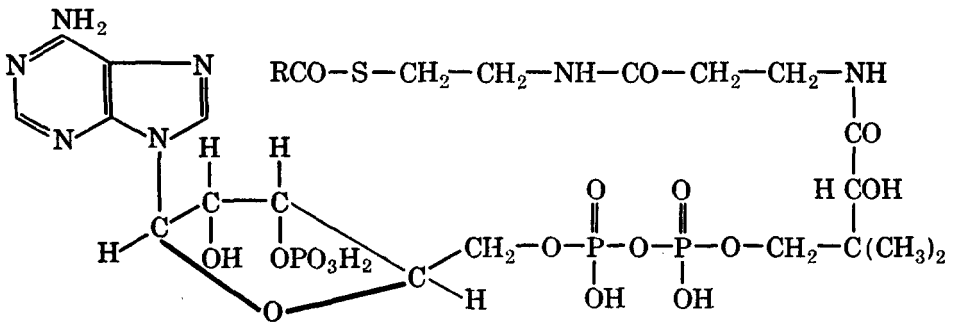


In an acid chloride, RCOCl, the electron-attracting power of the chlorine atom is little reduced by the slight tendency of its unshared electrons to participate in the C—Cl bond, and acid chlorides are among the most reactive of carbonyl compounds. In carboxylic acid anhydrides, RCOOCOR′, the deactivating effect seen in esters is lessened, since the same oxygen atom must supply two carbonyl groups. To acyl phosphates, $RCOOPO_3H_2$, similar considerations apply. Finally, in thiolesters, RCOSR′, the sulfur atom is electron-attracting and its unshared electrons have less tendency, relative to oxygen, to participate in the C—S bond. The carbonyl group in thiolesters is not so reactive as in acid chlorides or carboxylic acid anhydrides, but decidedly more reactive than in esters, amides, or acids.

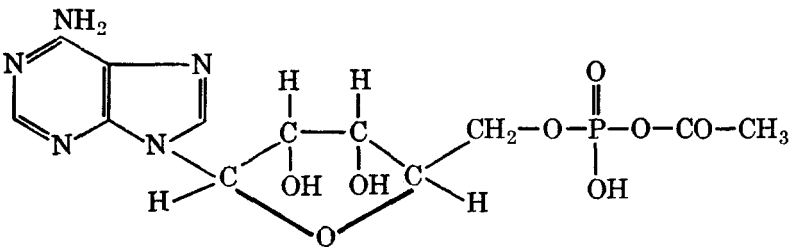
A chemist looking for a carboxylic acid derivative which, while being stable enough to persist in neutral aqueous solution, should undergo enolization and nucleophilic attack as readily as possible, could hardly find a better compromise than a thiolester offers, though perhaps he would consider the *p*-nitrophenyl esters (1) which have proved serviceable for peptide synthesis. The structure of coenzyme A thiolesters (I) appears complex: no doubt much of the molecule is adapted to specific adsorption on an enzyme; but it is the thiolester link which conveys the necessary reactivity to the acyl group COR.

The equilibrium constant of this balanced reaction is of the order of unity (5); Lynen and Decker (8) pointed out that pyrophosphatase is associated with acetate-thiokinase in practically all cells, and probably serves to displace the equilibrium in the direction of thiolester synthesis.

The experimental evidence, due principally to Berg (9), concerning the mechanism of this change may be summarized thus: (i) the reversible change is effected by a single enzyme; (ii) the only auxiliary factor known to be required is magnesium ion; (iii) synthetic acetyl adenylate (II) can replace adenosine



(I)



Acetyl adenylate (II)

Activation of Fatty Acids. Enzymic formation of coenzyme A thiolesters of fatty acids by direct combination of acid with coenzyme A is energetically unfavorable, the free energy change on esterification being (2) of the order of +8000 cal./Mol. Thiolester formation must therefore be coupled with a reaction yielding energy. Two systems for activation of acetate have been recognized. The first, mediated by the enzyme acetate-thiokinase, is the more widespread (3 to 7), occurring in animal tissues as well as in green plants and microorganisms; and here the necessary energy is supplied by the cleavage of adenosine triphosphate to adenylic acid and pyrophosphate.

$$CH_3CO_2H + CoASH + ATP \rightleftarrows CH_3COSCoA + AMP + PP$$

triphosphate plus acetate, and its addition to a complete system sharply reduces consumption of adenosine triphosphate; (iv) acetyl adenylate could not be shown to accumulate in the absence of coenzyme A; (v) exchange of C^{14}-acetate and acetyl-coenzyme A by the enzyme requires adenylic acid, pyrophosphate, and magnesium ions; (vi) exchange of C^{14}-adenylate with adenosine triphosphate requires acetate and coenzyme A; (vii) adenyl acetate is converted into adenosine triphosphate by the enzyme in the presence of pyrophosphate and magnesium ion; (viii) exchange of P^{32}-pyrophosphate with adenosine triphosphate by the enzyme requires acetate and magnesium ion but not coenzyme A; (ix) acethydroxamic acid is formed enzymically from adenosine triphosphate, magnesium ion, acetate, and hydroxylamine in

the absence of coenzyme A; (x) magnesium is not required for the formation of acetyl-coenzyme A from acetyl adenylate, coenzyme A, and the enzyme.

Except for the fact that its accumulation could not be induced, this is strong evidence that acetyl adenylate is an intermediate. This failure led Ingraham and Green (10) to formulate the change as a gathering of reactants within a single enzyme-magnesium complex in such a way that displacement reactions between them are favored by reduction of the entropy of activation: not acetyl adenylate, but its nondissociating magnesium chelate, is the intermediate. Berg (11) pointed out, however, that this view does not readily accommodate finding (x) quoted above.

To prepare a thiolester of acetic acid from the acid and the thiol, a chemist would first make from the acid an anhydride or mixed anhydride, and treat this with the thiol. His procedure seems analogous to the sequence induced by acetate-thiokinase; but having no enzymes, he might facilitate the second stage by use of a tertiary amine. A base such as pyridine greatly accelerates the acylation of an alcohol or phenol by an acyl chloride or anhydride; it does this by forming a saltlike complex in which the acyl cation is bound to the pyridine nitrogen and the chloride ion is free:

$$C_5H_5N + RCOCl \rightleftarrows C_5H_5\overset{(+)}{N}\text{—}COR\ Cl^{(-)}$$

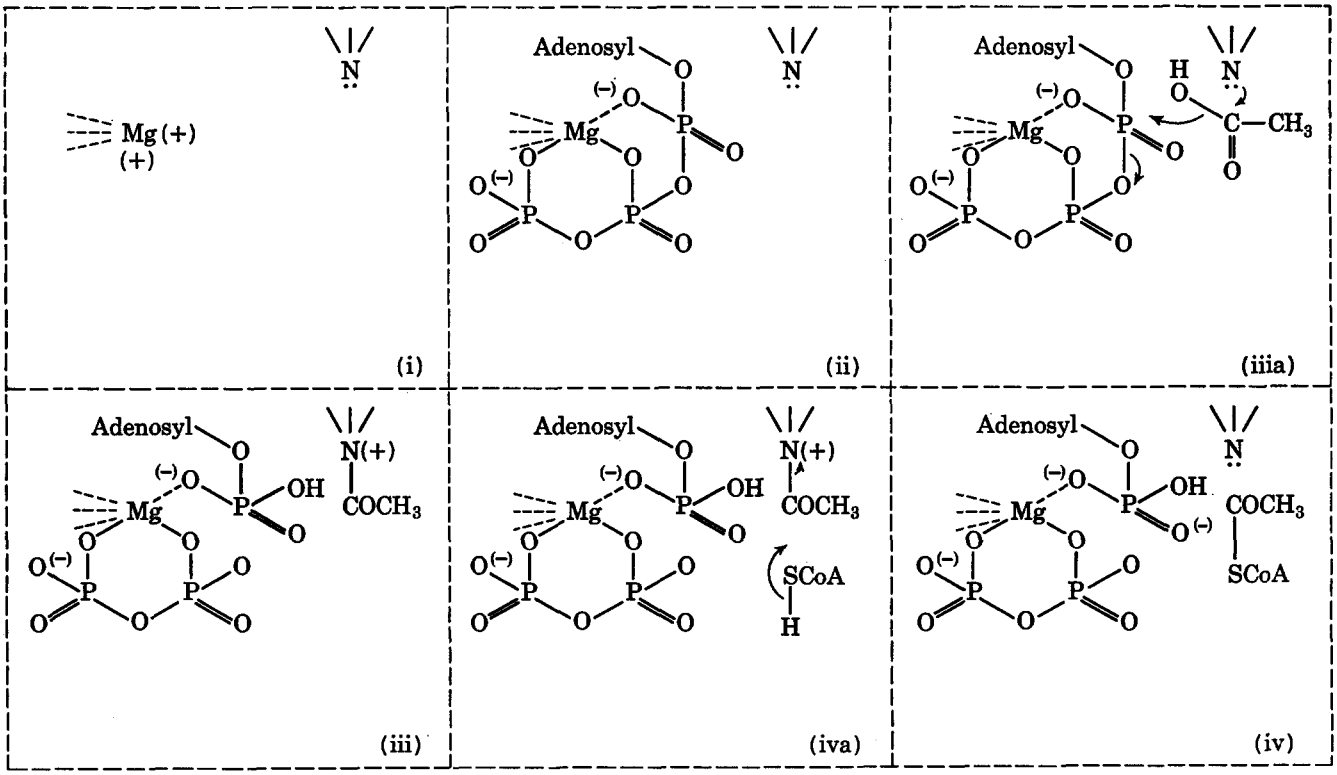
The reaction is reversible, and it is interesting that stabilization of similar acyl cations can be achieved by adding a complexing agent (antimony pentachloride) to remove chloride ion (12).

The cation is highly reactive to nucleophilic reagents (12, 13), e.g., it will form esters rapidly with alcohols, and anhydrides with acids (14):

$$C_5H_5\overset{(+)}{N}\text{—}\overset{\overset{O}{\|}}{\underset{R}{C}}\text{—}\overset{H}{O}\text{—}R' \rightarrow C_5H_5N + RCOOR' + H^{(+)}$$

By postulating that the change mediated by acetate-thiokinase may be base-catalyzed, a structure for an active center on the enzyme can be formulated; the mechanism is in some respects a reconciliation of the ideas of Berg and of Ingraham and Green. The active center would comprise a basic group (e.g., a tertiary amine) surrounded by receptors for (*a*) a magnesium ion; (*b*) adenylate; (*c*) acetate; (*d*) coenzyme A. It is assumed that (*b*) and (*c*) can act in concert as a receptor of acetyl adenylate, and (*c*) and (*d*) as a receptor of S-acetyl-coenzyme A; (*a*), occupied by magnesium, is a receptor for pyrophosphate or, in concert with (*b*), of adenosine triphosphate.

The sequence of events in the forward reaction



SCHEME 1

would then be (i) attachment of magnesium; (ii) attachment of adenosine triphosphate; (iii) attack by an acetate ion or acetic acid molecule to give pyrophosphate and a dissociated base-complex of acetyl adenylate; (iv) attack of a coenzyme A molecule to give S-acetyl-coenzyme A; (v) dissociation of products from the enzyme. All these stages, taken in their proper order, are assumed to be reversible. The state of the enzyme on completion of stages (i) to (iv) is illustrated (Scheme 1) as well as—in (iii*a*) and (iv*a*)—the electron-shifts of stages (iii) and (iv).

Acetyl adenylate is not an intermediate in this mechanism: when synthetic acetyl adenylate is attached to the enzyme, it passes to an ionized state, as in (iii). The acetyl-adenylate bond has high energy and its dissociation by the basic group could well be independent of the participation of magnesium.

A possible experimental test might be made of the dissociation of acetyl adenylate on the enzyme. Berg (9) found that ^{14}C-acetate did not exchange with acetyl adenylate when both were incubated on the enzyme, but if in the absence of magnesium the adenylate ion were not firmly bound, exchange of adenylate with acetyl ^{14}C-adenylate might be demonstrable.

A second activating system, found so far only in bacteria, consists of two enzymes, acetokinase (15) and phosphotransacetylase (16), which respectively catalyze the reversible changes:

$$\text{Acetate} + \text{ATP} \rightleftarrows \text{Acetyl phosphate} + \text{ADP}$$

$$\text{Acetyl phosphate} + \text{CoASH} \rightleftarrows \text{Acetyl—SCoA} + \text{phosphate}$$

Acetokinase requires magnesium or manganous ion; phosphotransacetylase does not; the two enzymes may in fact present, separately, functional sites similar to those found combined in acetic-thiokinase.

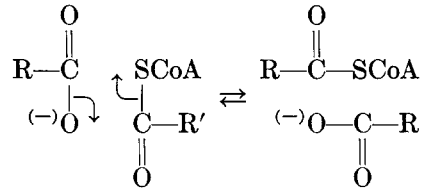
The thiophorases (CoA-transferases) effect activation of carboxylic acids by exchange with a molecule of an S-acyl-coenzyme A. Thus acyl-acetate-thiophorase from *Clostridium kluyveri* (17) is a relatively unspecific enzyme effecting the interchange:

$$\text{Acetyl—SCoA} + \text{carboxylic acid anion} \rightleftarrows \text{acyl—SCoA} + \text{acetate}$$

and succinyl-acetoacetate-thiophorase from mammalian heart muscle and kidney (18, 19) is important for the activation of acetoacetate:

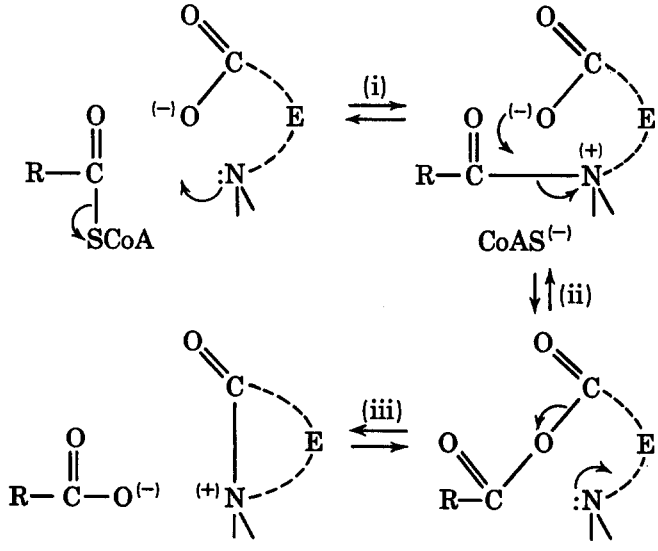
$$\text{Succinyl—SCoA} + \text{acetoacetate} \rightleftarrows \text{acetoacetyl—SCoA} + \text{succinate}$$

The free energy change in these reversible processes is naturally not large; but the mechanism, as affecting the energy of activation, is of some interest. The simplest mechanism which can be written is a cyclic one, e.g.,



Though this possibility cannot be dismissed, the four-membered cyclic transition state is unusual for single bonds. A tracer experiment (20) bearing on the question is the reported exchange of ^{14}C-succinate with S-succinyl coenzyme A on the enzyme. This is not compatible with direct exchange unless succinate can occupy the site adapted for acetoacetate. Ochoa (21) therefore suggested that the primary reaction is with a carboxyl group of the enzyme to form an "S-enzymyl" coenzyme A which then transfers coenzyme A to the receptor acid, the reaction thus being composed of two separate but similar exchanges of undetermined mechanism.

From the chemical standpoint it seems most likely that the anhydridelike character of an S-acyl coenzyme A permits a base-catalyzed equilibrium with a carboxylic anhydride, which in turn undergoes base-catalyzed reaction with coenzyme A. If the enzyme (E) contained a basic group and a carboxylate ion in suitable spatial relationship, a possible sequence would be as in Scheme 2.



SCHEME 2

The products of stage (iii) are the carboxylate anion of the acid originally esterified by coenzyme A, and an "activated enzyme," an acyl cation which could give a new S-acyl coenzyme A by attachment of a new carboxylate anion ($R'\text{-}COO^{(-)}$) and reversal of all three stages. Experimental testing of this and other possibilities would seem to be not too difficult, in view of the highly purified preparations available.

Formation of Carbon-Carbon Bonds. Until recently it was thought that synthesis and breakdown of coenzyme A thiolesters of fatty acids proceed by repetitions of a single sequence of reversible changes, the carbon chain being lengthened or shortened by two units at the completion of each sequence:

$$\text{RCOSCoA} + \text{CH}_3\text{COSCoA} \underset{\beta\text{-keto thiolase}}{\overset{(1)}{\rightleftharpoons}} \text{CoASH} + \text{RCOCH}_2\text{COSCoA}$$

$$\text{RCOCH}_2\text{COSCoA} \underset{\beta\text{-hydroxybutyryl dehydrogenase}}{\overset{(2)}{\rightleftharpoons}} \text{RCHOHCH}_2\text{COSCoA}$$

$$\text{RCHOHCH}_2\text{COSCoA} \underset{\text{enoyl hydrase}}{\overset{(3)}{\rightleftharpoons}} \text{RCH=CHCOSCoA}$$

$$\text{RCH=CHCOSCoA} \underset{\text{acyl dehydrogenase}}{\overset{(4)}{\rightleftharpoons}} \text{RCH}_2\text{CH}_2\text{COSCoA}$$

Most of the available evidence concerns the simplest case ($R = CH_3$), where all four reactions in both directions have been demonstrated with purified enzymes. For the higher fatty acids, evidence for the degradative sequence remains good, but evidence for step (1) of the synthetic sequence is lacking.

Step (1), chemically, is an acetoacetic ester condensation. This reversible reaction, when brought about between two ester molecules, requires a base which can enolize one molecule, the enolate ion then attacking the carbonyl group of the other. Thus the two molecules of S-acetyl coenzyme A which participate in the formation of S-acetoacetyl coenzyme A have necessarily different roles, and their environment on the enzyme must also be different: one site facilitating ionization of the α-hydrogen and the second the separation and reattachment of a thiol group. It has been suggested (22) that actual formation of an S-acetyl enzyme occurs at this second site:

$$\text{CH}_3\text{COSCoA} + \text{ESH} \rightleftarrows \text{CH}_3\text{COSE} + \text{CoASH}$$

$$\text{CH}_3\text{COSE} + \text{CH}_3\text{COSCoA} \rightleftarrows \text{CH}_3\text{COCH}_2\text{COSCoA} + \text{ESH}$$

Two pieces of evidence (22) support this view: (i) the enzyme, acetoacetyl-thiolase, is inhibited by iodoacetate, arsenoxide, etc.; (ii) S-propionyl coenzyme A exchanges rapidly with S^{35}-coenzyme A in presence of the enzyme. There is, however, no evidence about the actual location of the supposed sulfhydryl group in the enzyme, and in any case the site which necessarily facilitates separation and reattachment of coenzyme A in the enzymic reaction could be expected also to facilitate exchange. It was formerly thought that the asymmetric labeling observed in acetoacetate produced enzymically from 1-C^{14}-fatty acids or from C^{14}-acetyl coenzyme A was additional evidence for an intermediate S-acetyl enzyme, but the data seem to be explained more simply as an operation of the "hydroxymethylglutarate cycle" (23). The condensation with a second molecule of S-acetyl coenzyme A remains, of course, of the same type whether it is with S-acetyl coenzyme A or S-acetyl enzyme.

In the presence of acetoacetyl-thiolase, the cleavage of S-acetoacetyl coenzyme A is much faster than its synthesis, the equilibrium constant (19, 24, 8)

$$\frac{[\text{Acetoacetyl—SCoA}][\text{CoASH}]}{[\text{Acetyl—SCoA}]^2}$$

lying between 10^{-4} and 10^{-5} at physiological pH. In thiolesters of higher fatty acids, the carbonyl group is certainly less reactive to nucleophilic reagents (e.g., the S-acetyl coenzyme A enolate) than it is in an acetyl thiolester, and this is probably why the thiolase-catalyzed synthesis of a long-chain S-β-oxoacyl coenzyme A from S-acetyl coenzyme A has not been observed, the higher energy of activation making the energy barrier, already steep, almost impassable.

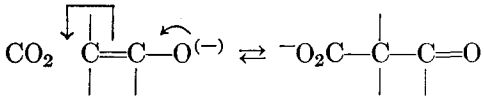
An important advance in understanding the biosynthesis of the higher fatty acids was the recent recognition of carbon dioxide as an essential cofactor. Gibson, Titchener, and Wakil (25, 26) obtained from avian liver a highly purified soluble enzyme system which synthesized long-chain fatty acids (chiefly palmitate) from S-acetyl coenzyme A in the presence of adenosine triphosphate, manganous ion, reduced triphosphopyridine nucleotide, and bicarbonate ion. Little synthesis of higher fatty acids occurred without carbon dioxide, but carbon dioxide was not a source of carbon for the product. Examination of the system failed to show the presence, in significant amount, of various enzymes mediating the breakdown of fatty acids.

Brady (27) prepared the mono-coenzyme A thiolester $HO_2C\text{—}CH_2\text{—}COSCoA$, by enzymic activation of malonate and showed that in the presence of acetaldehyde, manganous ions, and a soluble fraction

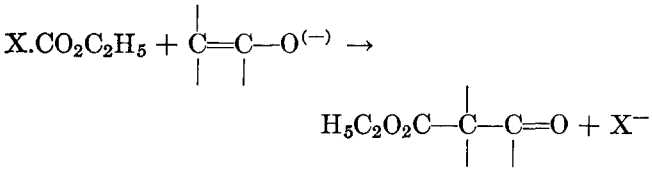
from pigeon liver this gave other coenzyme A thiolesters with mobility on paper chromatograms corresponding to chain-lengths of 8 to 12 carbon atoms. Wakil (28) and Formica and Brady (28*a*) later reported that S-malonyl coenzyme A could be obtained enzymically from S-acetyl coenzyme A in the presence of adenosine triphosphate, manganous or magnesium ion, and bicarbonate.

Recently Wakil and Ganguly (29) reported on two fractions from avian liver. The first of these contained biotin and synthesized S-malonyl coenzyme A from S-acetyl coenzyme A and bicarbonate in the presence of adenosine triphosphate and manganous ions; the second with S-malonyl coenzyme A gave intermediates which passed, on addition of reduced triphosphopyridine nucleotide, to carbon dioxide and fatty acids.

At the time of writing, evidence concerning the role of S-malonyl coenzyme A is still fragmentary, and a discussion of the chemical reactivities of the substance seems profitable; its formation from carbon dioxide and S-acetyl coenzyme A should be examined first. This reaction is of familiar type, the carboxylation of an enol:

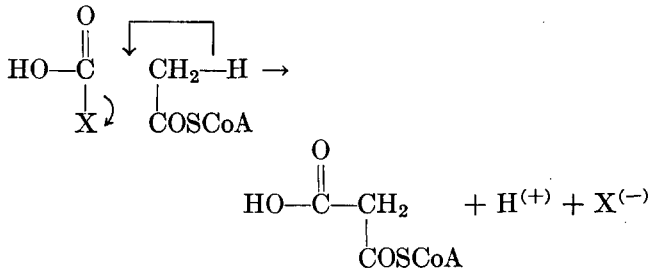


The oldest example is the classic synthesis (30) of salicylic acid from sodium phenoxide and carbon dioxide, but many others are known. Alternatively, the enolate ion can react with an appropriate derivative of carbonic acid, e.g., ethyl carbonate or ethyl chloroformate.

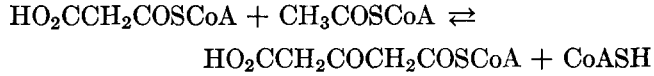


The course of carboxylations *in vitro* suggests that the principal energy barrier to be overcome is in the ionization of the carbonyl compound, e.g., a ketone enolized by reaction with a high-energy reagent such as sodium triphenylmethide can be carboxylated by pouring it over solid carbon dioxide (31).

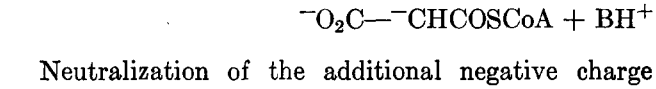
In the enzymic carboxylation of S-acetyl coenzyme A the adenosine triphosphate is presumably concerned with conversion of carbon dioxide to an intermediate of higher energy (HO.CO.X), which may then attack the methyl carbon of S-acetyl coenzyme A as the hydrogen atom is withdrawn, fission of C—H and C—X bonds being simultaneous:



S-Malonyl coenzyme A could *prima facie* be utilized in several ways for the biosynthesis of fatty acids. Extension of the carbon chain might be effected by condensation with (*a*) an S-acyl coenzyme A, or (*b*) an aldehyde. To discuss these possibilities it is necessary to consider how S-malonyl coenzyme A differs from an ordinary S-acyl coenzyme A in its reactivities. The electrophilic reactivity of the thiolester group would be that of an ordinary S-acyl coenzyme A: thus in the hypothetical condensation

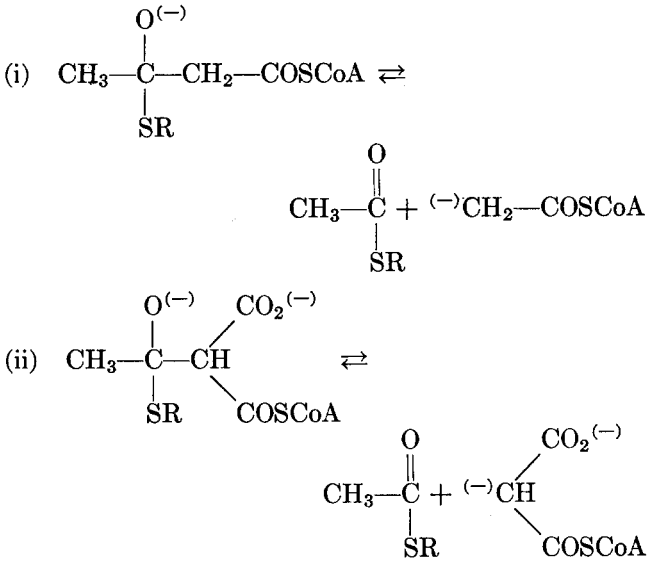


S-malonyl coenzyme A would have no advantage. The principal effect of the additional carboxyl group is to facilitate the enolization of the ester: the enolate ion would be formed with much greater ease than, for example, from S-acetyl coenzyme A, because the negative charge of the ion is distributed over a larger mesomeric system.

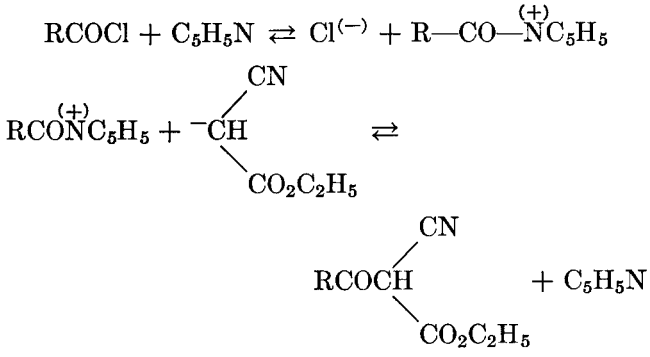


Neutralization of the additional negative charge on the carboxylate group, e.g., by complexing with a cation, would further stabilize the enolate.

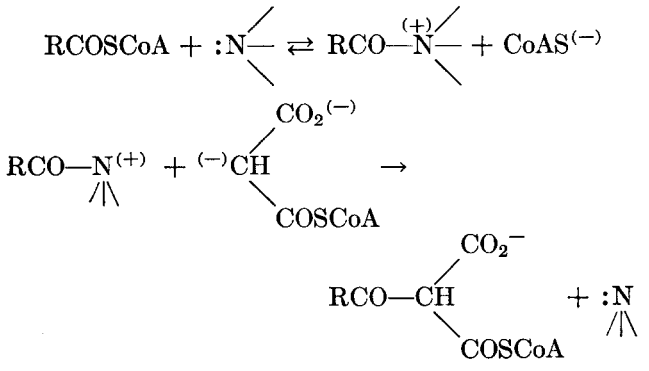
The condensation of this enolate with an S-acyl coenzyme A by an enzyme of the thiolase type does not seem likely to lead to a greater degree of synthesis than occurs in the condensation of two molecules of S-acetyl coenzyme A. Indeed, when the two equilibria (i) and (ii) are compared, the second should lie farther to the right because of the greater stability of the enolate ion; and in fact acylmalonic esters cannot be made *in vitro* by the usual acetoacetic ester condensation. It has been necessary, when making acylmalonic esters and their derivatives, to bring together an enolate of the malonic ester with an acyl derivative of the anhydride type, usually an acyl chloride. It is



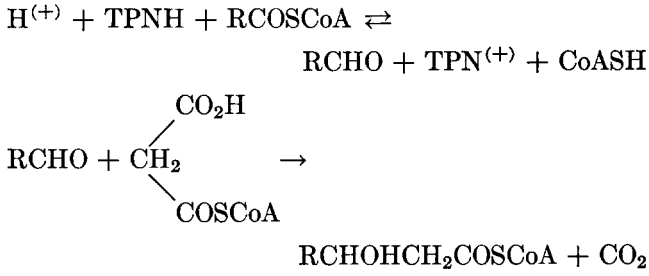
particularly interesting that C-acylation of a malonic acid derivative, e.g., ethyl cyanoacetate (32), can be effected by reaction in the cold with an acyl chloride and a tertiary base (pyridine or quinoline). The intermediate here would be an acylammonium cation of a type already discussed.



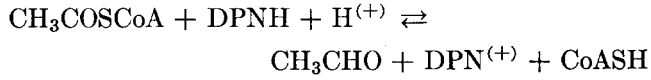
Thus it seems possible that the enzyme effecting condensation of an S-acyl coenzyme A with S-malonyl coenzyme A has a basic center which effects dissociation of the acyl derivative to a cation which then reacts with the malonyl enolate:



A different scheme, proposed by Brady (27), assumes reduction of an S-acyl coenzyme A to an aldehyde, which then undergoes condensation with S-malonyl coenzyme A.



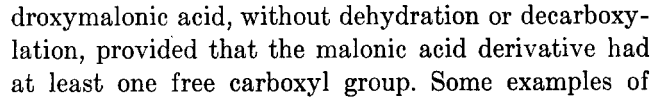
A system reducing S-acetyl coenzyme A in the presence of reduced triphosphopyridine nucleotide was present in Brady's fatty acid synthesizing system. Burton and Stadtman (33) found an enzyme in *Clostridium kluyveri* mediating the change:



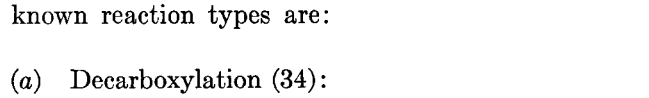
The reduction to acetaldehyde was endergonic to the extent of 4330 cal. per mol; with triphosphopyridine nucleotide as the reducing agent this figure would be only slightly lower.

Condensation of an aldehyde with a malonic acid derivative is easy to effect nonenzymically with relatively mild reagents, and the reaction has often been used preparatively; however, the initial condensation is often followed by dehydration or decarboxylation or both. Indeed, no example is known to the writer of the isolation of the simple addition product, a β-hydroxymalonic acid, without dehydration or decarboxylation, provided that the malonic acid derivative had at least one free carboxyl group. Some examples of known reaction types are:

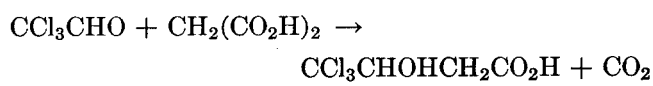
(*a*) Decarboxylation (34):



(*b*) Dehydration (35):



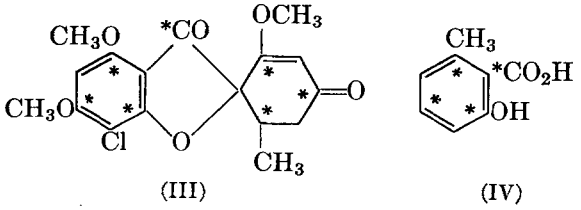
(*c*) Dehydration + decarboxylation (36):



If Brady's hypothesis is correct, the reaction which actually extends the carbon chain is facilitated by two energy-requiring steps: reduction of a thiolester to an aldehyde and carboxylation of the other reagent, S-acetyl coenzyme A. The stage at which the superfluous carboxyl group would be eliminated remains uncertain, but its continued presence would certainly facilitate reduction of the double bond formed by (also facile) dehydration. Chemically, the condensation is well supported by analogy; however, the actual utilization of a fatty aldehyde as such in the biosynthesis of a higher fatty acid has yet to be demonstrated.

All these schemes utilized, for extension of a carbon chain, the electrophilic nature of the thiolester group at the end of the existing chain; this is so whether direct formation of a C—C link occurs or whether reduction to an aldehyde comes first. Theoretically, it is not indispensable for this thiolester group to be attached to a saturated alkyl residue (methyl, propyl, etc.); the thiolester group in S-β-hydroxybutyryl coenzyme A, for example, should be as reactive as that in S-butyryl coenzyme A. In S-crotonyl coenzyme A, $CH_3CH{=}CHCOSCoA$, the electrophilic activity of the carbonyl group is decidedly lowered by the double bond; but in S-vinylacetyl coenzyme A, $CH_2{=}CHCH_2COSCoA$, there is no conjugation and the carbonyl group would be fully reactive. This is also true of S-malonyl coenzyme A, $HO_2CCH_2COSCoA$, and even of S-acetoacetyl coenzyme A, $CH_3COCH_2COSCoA$, if the more reactive keto group is prevented in some way from enolizing or otherwise reacting preferentially.

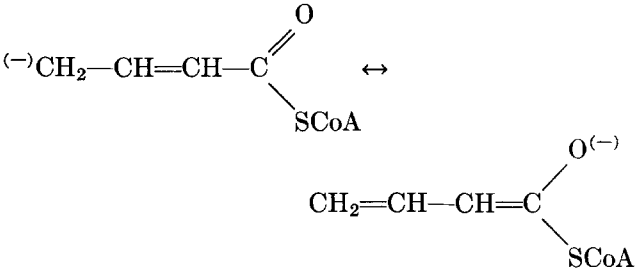
In recent years Birch (37, 38) has demonstrated that a considerable number of aromatic substances, most of them produced by fungi, are derived from acetic acid units arranged in head-to-tail sequence. In such compounds an oxygen atom is frequently found attached to a carbon originating from acetate carboxyl: a good example is griseofulvin (III). The labeling pattern in 6-methylsalicylic acid (IV) biosynthesized from 1-^{14}C-octanoic acid by *Penicillium griseofulvum* indicates dissociation of the fatty acid into one or more C_2 units before synthesis. No study has yet been made



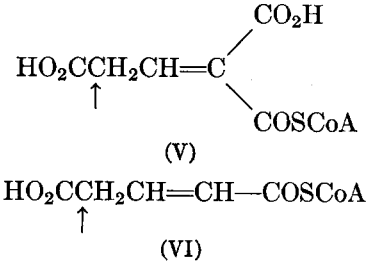
* Signifies carbon originating from acetate carboxyl.

of the enzymes effecting these biosyntheses, and it is still unknown whether coenzyme A plays its part in them, but it is at least possible that the processes are similar to fatty acid syntheses, the removal of oxygen sometimes being omitted before the next C_2 unit is added.

A further possibility for the building up of the carbon chain in fatty acids is the addition of four carbon atoms en bloc. S-Crotonyl coenzyme A could in theory form an enolate ion,



in which nucleophilic activity is transferred to C—4 via the double bonds. Lapworth (39) was able to condense ethyl crotonate with ethyl oxalate to give the straight-chain keto ester, $C_2H_5O_2CCOCH_2CH{=}CHCO_2C_2H_5$; a reaction in which a similar enolate ion must take part. Self-condensation of S-malonyl coenzyme A as part of the process of fatty acid synthesis could lead to intermediates (V and VI) from which more stable enolate ions could be formed, by loss of hydrogen at the arrowed positions, for reaction with a thiolester or an aldehyde; the intermediate (VI) could also arise by carboxylation of S-crotonyl coenzyme A.



The only experiments known to the writer which suggest that four-carbon addition may occur are those of Hele *et al.* (40), where a cell-free system utilizing acetate synthesized $C_{(4)}$ and $C_{(8)}$ acids rapidly, with $C_{(6)}$ only appearing at a later stage.

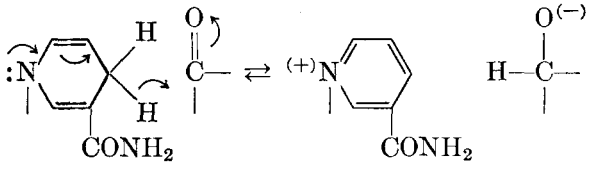
Hydrogen-transferring Processes. When fatty acids are built up from S-acetyl coenzyme A, two reductions necessarily occur for each $C_{(2)}$ unit added to the chain. In each of these reductions, two electrons are transferred; correspondingly, two oxidations are required

for each shortening of a fatty acid by two carbon atoms.

The reversible process whereby S-acetoacetyl coenzyme A is reduced to S-β-hydroxybutyryl coenzyme A in the presence of reduced diphosphopyridine nucleotide is representative of the first reducing step:

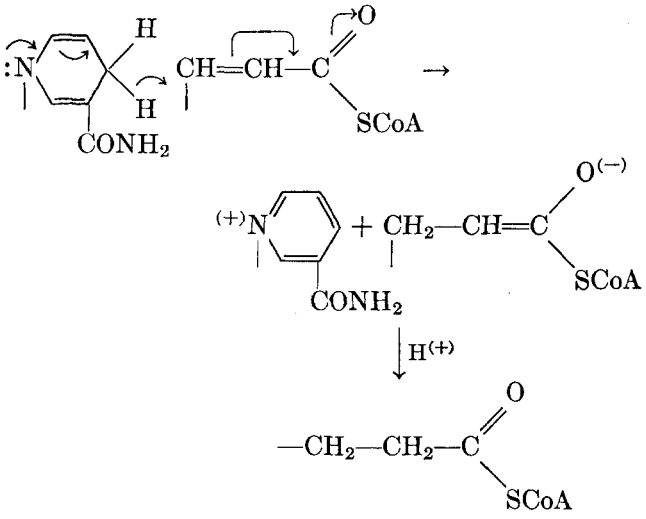
$$CH_3COCH_2COSCoA + DPNH + H^+ \rightleftarrows CH_3CHOHCH_2COSCoA + DPN^+$$

In general, reduction of a keto group to a secondary alcohol is easily effected nonenzymically by a considerable range of reducing agents. Enzymic reductions of this type are not confined to lipid biochemistry; and in this case (41), as with alcohol dehydrogenase (41*a*) and lactic dehydrogenase (42), it has been shown that reduction and oxidation are a direct transfer of "negative" hydrogen between the pyridine nucleotide and the substrate.



It should be pointed out that the various schemes for fatty acid synthesis via S-malonyl coenzyme A, discussed in the previous section, do not eliminate the need for a reduction of this type. The reduction of S-acyl coenzyme A to aldehyde required by Brady's hypothesis is a reduction of essentially the same kind. Certainly, the nonenzymic reduction of esters to aldehydes is difficult, but the thiolester group in S-acyl coenzyme A would facilitate nucleophilic attack by hydrogen. Also, attachment of the group —COSR to the enzyme in a particular configuration could facilitate reduction; a similar effect is seen in *cis*-lactones (43), which have a fixed configuration of the ester group and have greater electrophilic activity than esters. The reduction of gluconolactone to glucose (44) by so mild a reagent as sodium amalgam is a familiar example.

The second reducing step in fatty acid synthesis is presumably the reduction of an $\alpha\beta$-unsaturated coenzyme A ester, possibly carboxylated at the α-position. Formerly it was thought that reduction by one of the flavoprotein acyl dehydrogenases was on the principal pathway of synthesis, but this now appears doubtful, since these enzymes are absent from the synthesizing system of Gibson *et al.* (26). This system utilizes reduced triphosphopyridine nucleotide, as does also another (apparently different) system, found in extracts of liver, which reduces $\alpha\beta$-unsaturated coenzyme A esters (45, 46, 47). A reduction of this type can be regarded as similar to that of a ketone, but attack by the negative hydrogen would be on the β-carbon made electrophilic by transmission of the C=O polarization through the conjugated system.



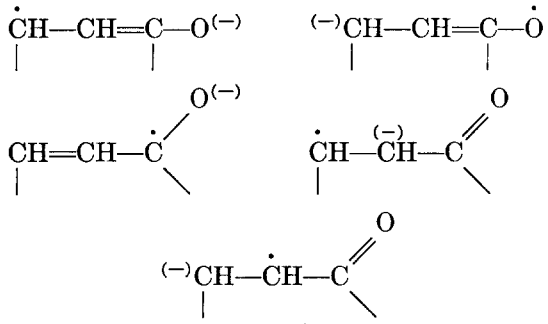
In this way the hydrogen transferred from the reducing agent would appear on the β-carbon of the saturated ester.

Chemical reduction of the C=C double bond in an $\alpha\beta$-unsaturated acid or ester can be brought about rather easily, e.g., by alkali metals and alcohols; and if $\alpha\beta$-unsaturated ketones are also considered (since coenzyme A thiolesters approach them in reactivity) the range of possible reducing agents is wider. Of the reverse process, dehydrogenation of a fatty ester or ketone to its $\alpha\beta$-unsaturated analogue, no example is known. Thus the reaction catalyzed by acyl-dehydrogenases is of considerable chemical interest; a discussion is therefore included despite the doubt concerning its role in biosynthesis of fatty acids. It has always been found difficult to drive this reaction in the direction of hydrogenation.

The mechanism of the dehydrogenation has been studied chiefly by Beinert and collaborators (48). Spectroscopic evidence indicates that an intermediate stage is produced by transfer of one electron from substrate to flavin. Beinert and Page's reasonable explanation of the considerable body of experimental fact is that the intermediate is in the nature of a bi-radical complex ($\cdot$YH.....HS$\cdot$) of partially reduced enzyme and partially oxidized substrate (enzyme and substrate are respectively Y and SH_2). This intermediate is relatively stable; the second electron-transfer needed to complete dehydrogenation requires an auxiliary factor, the "electron-transferring flavoprotein" (ETF).

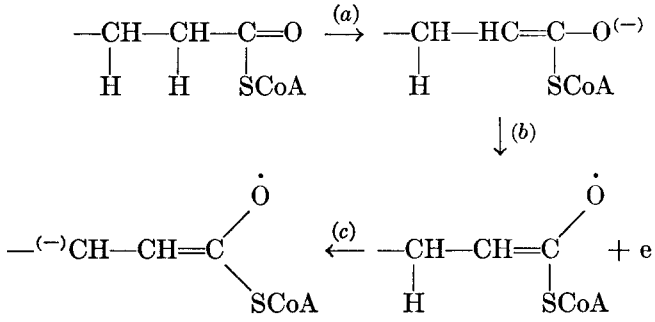
This reasoning can perhaps be given a more precise

formulation by considering the structure of the entity called (·SH). The relatively long life of this radical, even as a complex with the semiquinonoid flavin radical, argues a certain stability. Among possible half-reduction products of an $\alpha\beta$-unsaturated carbonyl compound the radical-ion (VII) produced by simple addition of one electron appears to be the most stable, since at least five canonical structures are possible between which resonance should occur.

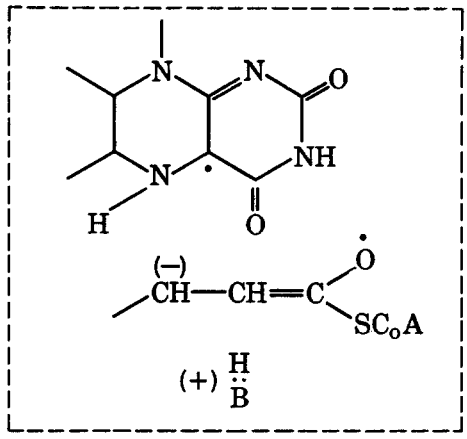


(VII)

On this view the radical derived from SH_2 is not ·SH but ·$S^{(-)}$. Its formation by oxidation of a saturated S-acyl coenzyme A can be formulated as follows:

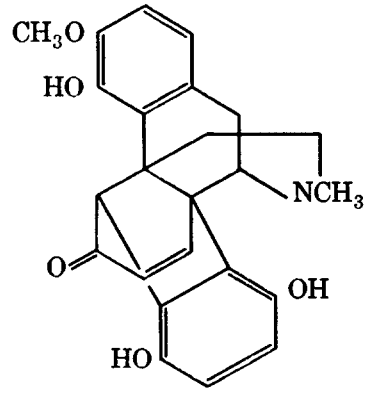


Stage (a) is a base-catalyzed enolization of the thiolester; in stage (b) the enolate ion donates one electron to the flavin, losing its negative charge and giving a free radical which passes in (c) to the stabilized radical-ion by loss of a proton from the β-carbon. The separation of this proton would be facilitated by a strong base, and it is interesting that the transfer of an electron in stage (b) would produce in the flavin a strongly basic radical-ion, which could accept the proton. The three stages can in fact be amalgamated as a continuous cyclic process: as abstraction of an α-hydrogen in the substrate by a basic center in the enzyme begins, the electronic excess so generated is transferred to the flavin, raising its basicity so that it can assist in abstraction of the β-hydrogen. The structure of the bi-radical complex would then be as (VIII). When an $\alpha\beta$-unsaturated thiolester reacts with the reduced enzyme (YH_2), removal of a proton from the reduced flavin is followed by transfer of one electron from flavin to substrate to produce the same intermediate.



(VIII)

The nature of the electron-transfer is of particular interest; most probably, it would be the result of orbital overlap between the two mesomeric systems in flavin and substrate. This overlap would persist in the complex and help to stabilize it. It is not easy to find a known chemical model, but a parallel can perhaps be seen in the alkaloid derivative flavothebaone (49, 50). In this substance (IX) a hydroquinone ring and an $\alpha\beta$-unsaturated keto group, though not in conjugation, are fixed at a separation of about 3 Å. Flavothebaone is yellow and its alkaline solution is deep red, though both should be colorless if no interaction occurred between the two chromophores. Electronic interaction between the two components of the bi-radical (VIII) might thus occur over a distance greater than the length of an ordinary covalent bond. The transfer of a second electron after oxidation or reduction of the flavin radical in the complex (VIII) would lead to the fully reduced or oxidized product.



(IX)

Enoyl Hydrase (*Crotonase*). This enzyme, which has been crystallized (51), catalyzes the reversible addition of water to $\alpha\beta$-unsaturated S-acyl coenzyme A. The $\beta\gamma$-unsaturated analogues are also hydrated, but proof is lacking that the process is reversible. As with the acyl-dehydrogenases, the part played by crotonase in the synthesis, as opposed to the degradation, of fatty acids, is at present uncertain. However, some type of dehydration of an S-β-hydroxyacyl coenzyme A or its α-carboxylated analogue would seem to be essential to most of the possible schemes involving S-malonyl coenzyme A.

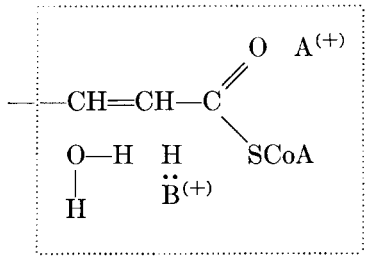
With crotonase, the known relationships in the four-carbon series can be summarized (52, 53):

$$CH_3CH{=}CHCOSCoA \rightleftarrows L(+)\text{-}CH_3CHOHCH_2COSCoA$$
$$\uparrow$$
$$CH_2{=}CH{-}CH_2COSCoA$$

The reversible hydration of $\alpha\beta$-unsaturated carbonyl compounds is a well-known process in organic chemistry; with $\alpha\beta$-unsaturated ketones it may occur under very mild conditions. That this intrinsic facility includes coenzyme A thiolesters is shown by Rendina and Coon's observation (54) that S-acrylyl coenzyme A, $CH_2{=}CH{-}COSCoA$, and S-methacrylyl coenzyme A, $CH_2{=}C(CH_3){-}COSCoA$, are hydrated spontaneously in aqueous solution. With $\alpha\beta$-unsaturated ketones the most effective catalysts for hydration are acids, and for dehydration bases. The electron-shifts would be as shown in Scheme 3.

No comparable mechanisms exist for the $\beta\gamma$-unsaturated isomers.

Crotonase could thus be pictured as having an acidic group (A^+) near the carbonyl group of the attached substrate, a basic group (B:) near an α-hydrogen atom, and possibly a group such as a metallic cation near the β-carbon atom for addition of water molecules and abstraction of hydroxyl ions. (Stern and del Campillo suggested (52) that a sulfhydryl group adds to the β-carbon and is later exchanged for hydroxyl; but a chemical parallel for the second stage is hard to find.) In view of the fact that both *cis*- and *trans*-isomers of S-crotonyl coenzyme A and S-2-hexenoyl coenzyme A can be hydrated, and that crotonase will accept coenzyme A thiolesters having a wide range of chain-lengths (52), it would seem that carbon atoms beyond the β-carbon are not specifically bound to the enzyme. The structure of the enzyme-substrate complex could then be pictured as (X).



(X)

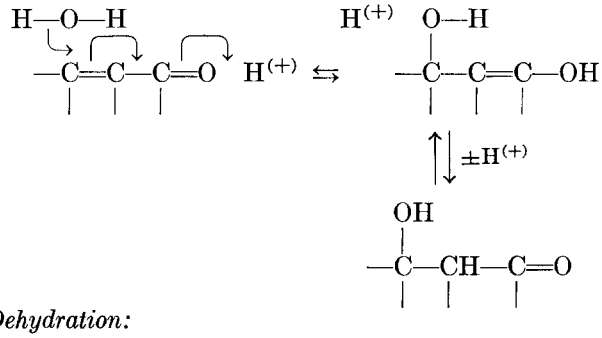
The sequences for hydration and dehydration then proceed as in Scheme 3. On this view, hydration of S-vinylacetyl coenzyme A is not direct but is preceded by isomerization to S-crotonyl coenzyme A (Scheme 4).

Whether the change (ii) is reversible in the absence of a site on the enzyme for removal of a proton from the γ-carbon may be doubted, and the absence of such a site would also hinder the formation of S-vinylacetyl coenzyme A from S-β-hydroxybutyryl coenzyme A. More light, it seems, could be thrown on the nature of crotonase by determining whether S-vinylacetyl coenzyme A is actually in equilibrium, in presence of the enzyme, with the $\alpha\beta$-unsaturated and β-hydroxy thiolesters.

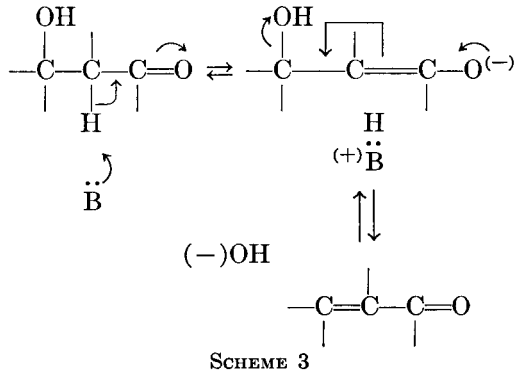
BIOSYNTHESIS OF CHOLESTEROL

The individual enzymes mediating the biosynthesis of cholesterol have not yet received intensive study;

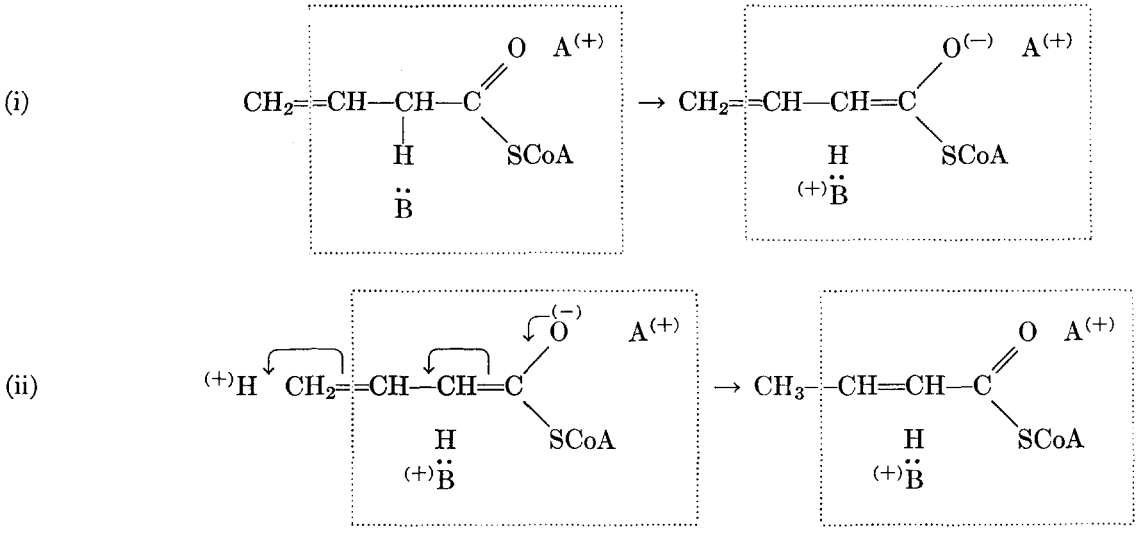
Hydration:



Dehydration:



SCHEME 3



SCHEME 4

none, if we except acetoacetyl thiolase, has been obtained pure, and most of the evidence has been secured with rather complex preparations of living tissue. The principal purpose of this section is to relate to organic chemistry as a whole the very interesting and varied changes proceeding during biosynthesis. The chain of transformations leading from acetate to cholesterol is thought, on the basis of evidence now available, to be as shown in Scheme 5 (pp. 16-17).

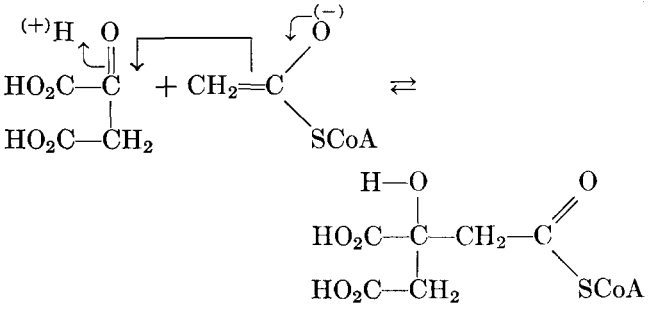
The successive discoveries of acetate (55), squalene (56), lanosterol (57), and mevalonate (58) as precursors of cholesterol provided four signposts on the way; the paths between them are being mapped out in rapidly increasing detail.

Acetate to Mevalonate. The actual transformation of acetate to mevalonate in one operation has only recently been demonstrated (59) in a preparation from rat liver of microsomes and soluble enzymes supplemented by cofactors (CoASH, ATP, Mg^{++}, GSH, TPNH). However, processes which almost certainly represent successive stages in this transformation had already been studied. The activation of acetate by acetic-thiokinase and the reversible condensation of two molecules of S-acetyl coenzyme A to give S-acetoacetyl coenzyme A can be accepted as reactions common to other systems.

The next step is the condensation of S-acetoacetyl coenzyme A with S-acetyl coenzyme A to give one molecule each of coenzyme A and S-3-hydroxy-3-methylglutaryl coenzyme A. The enzyme mediating this change ("HMG-CoA condensing enzyme") has been partially purified from yeast (23, 60); and its presence, apparently in a more labile form, in liver microsomes is also indicated.

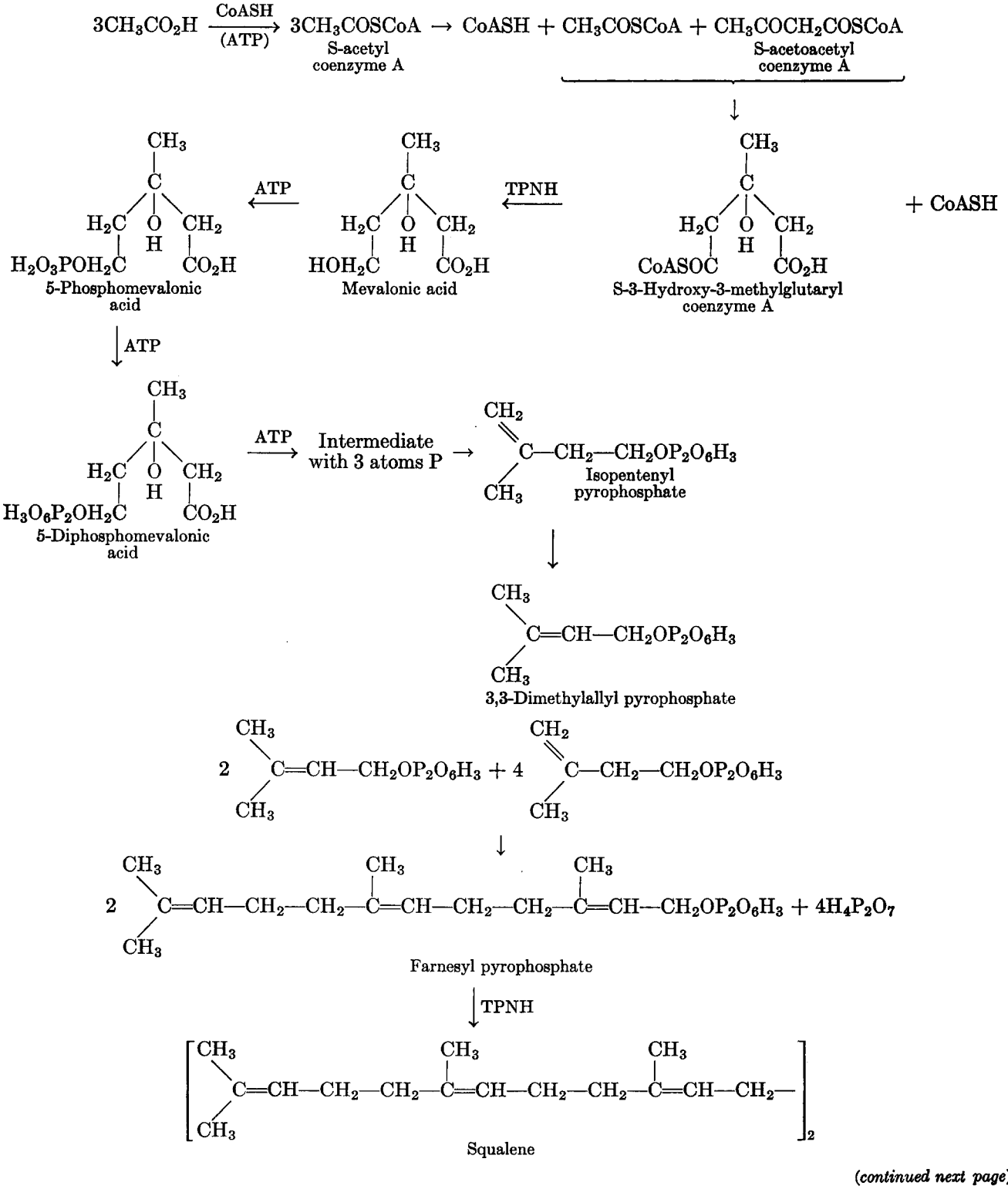
The chemical change effected by this enzyme is formally of the same type as the well-known synthesis of citrate from oxaloacetate and S-acetyl coenzyme A by the crystalline citrate condensing enzyme (61, 62); and since this enzyme has been purified and extensively studied, its mechanism can be discussed here as a close parallel. There are two interesting features of this change: the liberation of coenzyme A accompanying condensation, and the reversibility of the process (though the equilibrium lies heavily on the side of synthesis, $K \approx 10^4$ at pH 7.2).

Superficially, it would appear that the thiolester group executes its known role of facilitating the enolization of the acetyl group, the enol or enolate ion then attacking the carbonyl group of oxaloacetate:



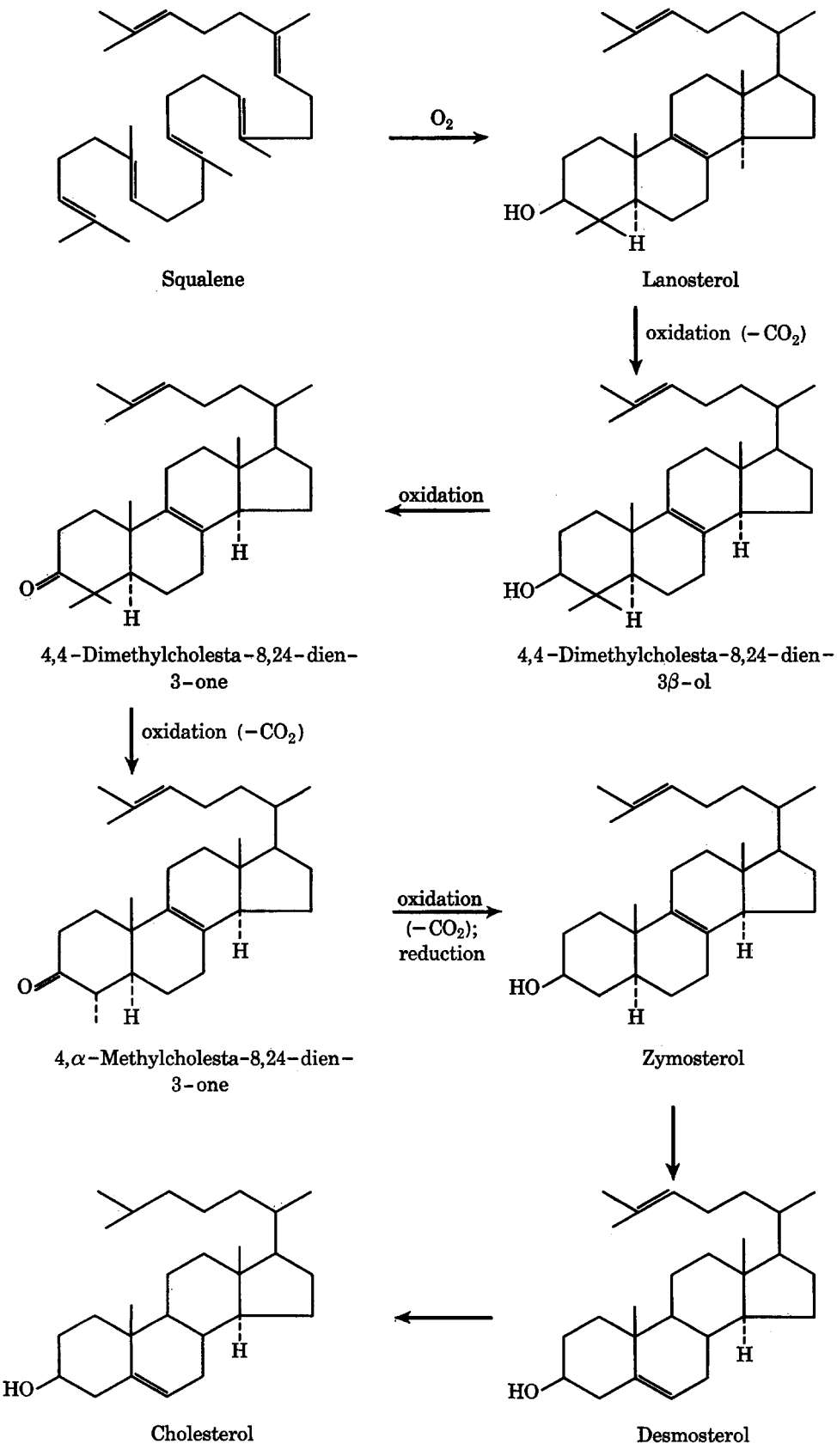
This leads to an S-citryl coenzyme A which would have to be hydrolyzed, reversibly, in a second step. There are two objections to this idea. First, S-acetyl coenzyme A does not exchange hydrogen with the aqueous medium in the presence of the enzyme and the absence of oxaloacetate (62*a*). This argues against enolization as a first step. The second objection is even more weighty: reversal of the hydrolysis would amount to the formation of a coenzyme A thiolester from a car-

SCHEME 5



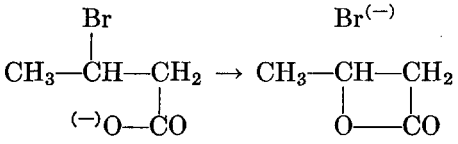
(continued next page)

SCHEME 5 (*continued*)

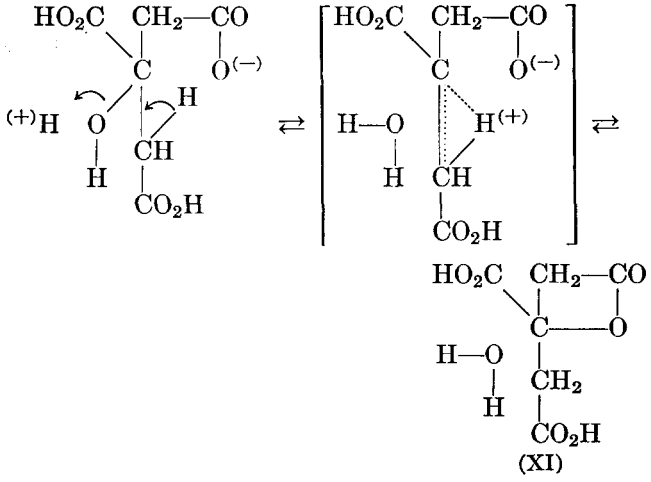


boxylic acid and coenzyme A without an external source of energy. The energy required to form the CO—S bond is so high that such a process appears most unlikely in the absence of some special characteristic of the acid concerned. In fact, S-acyl coenzyme A deacylases appear not to catalyze resynthesis; a recent example (63) is S-palmityl coenzyme A deacylase.

A possible explanation is that citric acid can be converted on the enzyme into the β-lactone (XI). Ordinary lactones are formed, nonenzymically, from hydroxy-acids by attack of the alcoholic hydroxyl group on the carbonyl group of the acid, but formation of β-lactones in this manner has never been observed. However, if an electron-deficiency can be produced on the β-carbon the carboxylate ion can attack it: thus sodium 3-bromobutyrate produces a β-lactone in neutral aqueous solution (64):

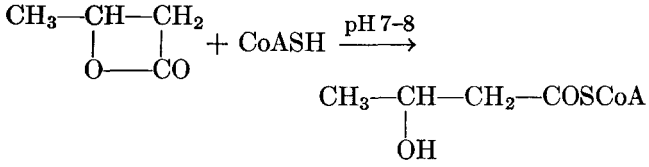


An electron-deficiency on the central carbon of citric acid could be produced by a process corresponding to the initial stage of a dehydration to aconitic acid, and the β-lactone could then be formed, thus:

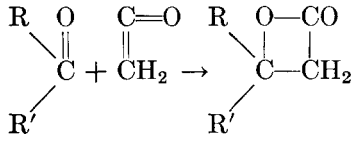


It is a peculiarity of β-lactones that in addition to the usual reaction with nucleophilic reagents by attack on the carbonyl group, they can also be attacked at the β-carbon atom; indeed, in the hydrolysis of β-butyrolactone by molecular water this is the predominant mode (65). Thus the over-all reaction pictured above is reversible, though naturally the equilibrium would lie heavily on the side of citrate.

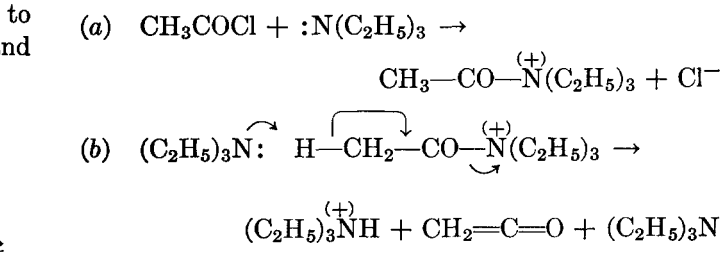
β-Lactones will, as indicated, also react with nucleophilic reagents at the carbonyl group, and indeed reaction of β-butyrolactone with coenzyme A in this sense has been demonstrated (8) *in vitro:*



The next question is whether the β-lactone (XI) could be formed from oxaloacetate and S-acetyl coenzyme A. β-Lactones can, in fact, be formed with very mild catalysis from ketones and keten (66):



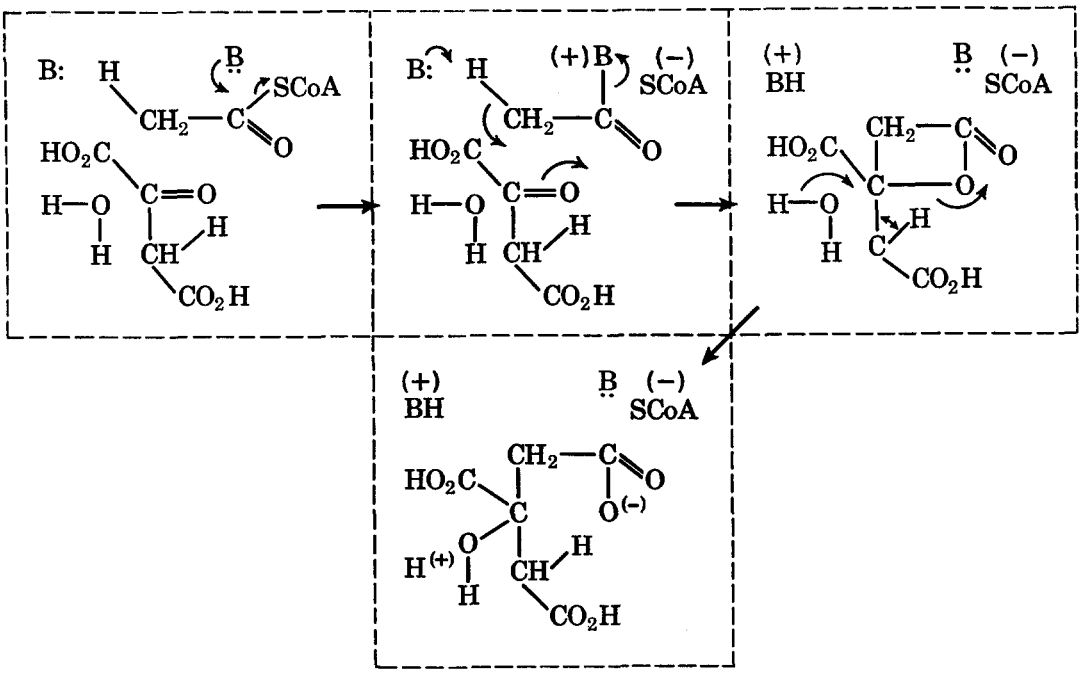
Keten is, of course, a high-energy compound, usually produced by pyrolytic methods; but it is also apparently formed from acetyl chloride and triethylamine (67), though the dimer is the product actually isolated. The mechanism of the process is presumably



two molecules of base taking part. Free keten could hardly be formed enzymically from S-acetyl coenzyme A, but it does seem possible that two basic centers on the enzyme could induce similar electron-shifts and facilitate a direct transformation of oxaloacetate to the β-lactone. These considerations lead to the following imaginary picture of the enzyme and the synthesis thereon of a molecule of citrate (Scheme 6).

Since in this mechanism little change of position is required in the atoms concerned, the entire movement of electrons may be a concerted process, the stages not being sharply divisible. In the reverse process, the electron-shifts are in the opposite direction. If this mechanism is substantially correct, the oxygen atom of the keto group in oxaloacetate becomes part of one of the carboxyl groups in citrate; a prediction which may be amenable to experimental test.

Similar mechanistic arguments apply to the condensation of S-acetyl coenzyme A with S-acetoacetyl coenzyme A to give S-3-hydroxy-3-methylglutaryl coen-

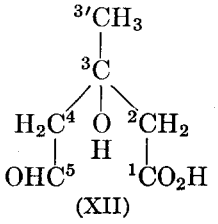


SCHEME 6

zyme A, a process which is, however, not known at present to be reversible. An enzyme (68) cleaving the product certainly exists, but it is not identical with the condensing enzyme and its products are free acetoacetate and S-acetyl coenzyme A.

Reduction of S-3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid has been reported by Lynen *et al.* (69) and by Durr *et al.* (70). The enzyme preparations were derived from yeast. Reduced triphosphopyridine nucleotide was required.

Reduction of an S-acyl coenzyme A by reduced pyridine nucleotides has been mentioned earlier (p. 10); in these examples an aldehyde was the product. The further reduction of an aldehyde to a primary alcohol is unremarkable chemically; but here both groups reported that the aldehyde concerned, mevaldic acid (XII), could not be detected as an intermediate. Mevaldic acid is, however, reduced to mevalonic acid by



(XII)

reduced triphosphopyridine nucleotide and yeast enzymes (71). A system from pig liver also effecting this reduction utilizes di- or tri-phosphopyridine nucleotide (72).

Another remarkable feature of the reduction of S-3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid is its relative irreversibility. Attempts to reverse the reduction by removing the product with the "HMG-CoA cleavage enzyme" have been reported (71), but the total conversion was very small. One would not expect this oxidation, an energy-yielding process, to be so difficult; and it is clear that this step, which seems as it were a one-way street connecting biosynthesis of cholesterol with general metabolism, will prove of unusual interest when studied in detail.

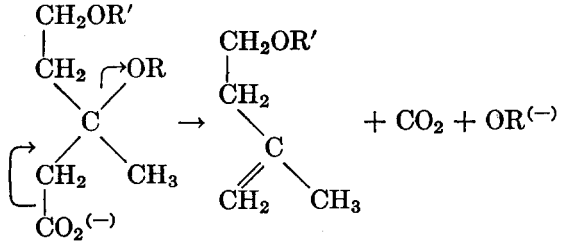
Mevalonate to Squalene. Most of the progress in this section has been made by use of preparations from yeast, the systems in animal tissues offering more resistance to purification. The first three stages happen to be phosphorylations, and have been studied in the laboratories of Bloch and of Lynen; Popják has reported complementary studies with liver preparations.

All three phosphorylations require adenosine triphosphate and a divalent cation, and thus appear to be of a normal type. The enzyme effecting the first step leading to 5-phosphomevalonic acid is called mevalonic kinase and has been partially purified from yeast autolysate (71, 73) and from pig liver (74). It phosphorylates only "natural" (+)-mevalonic acid.

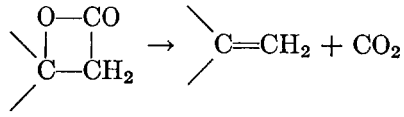
The second phosphorylation apparently produces the pyrophosphate, 5-diphosphomevalonic acid (75); this substance has not yet been well characterized, nor has the product of the third phosphorylation (76). The next identifiable intermediate is isopentenyl pyrophos-

phate (75, 77), $CH_2{=}C(CH_3)CH_2CH_2OP_2O_6H_3$. This has been identified by enzymic hydrolysis to isopentenol, $CH_2{=}C(CH_3)CH_2CH_2OH$, and recently by synthesis (77).

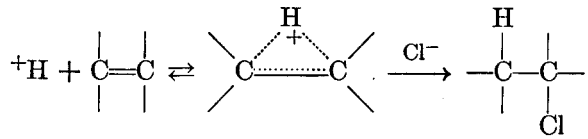
In isopentenyl pyrophosphate, the carboxyl carbon (C-1) of mevalonate (numbering as in XII) has been lost: it had already been shown (78) that this carbon of mevalonate is not incorporated into cholesterol. There is evidence, however, against the process being a simple decarboxylation. Rilling *et al.* (79) studied the biosynthesis of squalene from mevalonate and yeast enzymes in a heavy water medium: 3.5-3.9 atoms of deuterium then entered each molecule of squalene. Now if a simple decarboxylation of a mevalonic acid derivative occurred in this medium, the group $—CH_2—CO_2H$ would become $—CH_2D$. Squalene is certainly built up from some six units of mevalonate, and in the four central units this group $—CH_2D$ would appear as a $—CH_2—$ group linking the next mevalonate unit (80, 81). Thus in these four units one hydrogen atom has been lost from each $—CH_2D$, and in the absence of an isotope effect the average composition of the resulting methylene group would be $—CH_{4/3}D_{2/3}—$ and the total number of D atoms in squalene $2 + 4 \times \frac{2}{3} = 4.67$, if no further deuterium were introduced during biosynthesis. The discrepancy between this and the experimental value is not conclusive—if small amounts of exchangeable protium were available, they could, aided by the large kinetic isotope effect, depress incorporation of deuterium; and degradation of the deuterated squalene gave succinic acid containing as much as 0.5D, an observation difficult to reconcile with the presence of deuterium at only 3 or 4 positions of the molecule—but if it is accepted as valid, the decarboxylation is not a replacement of $—CO_2H$ by —H but accompanies an elimination of some other group. One such elimination, postulated by Bloch (79), is



Here $OR^{(-)}$ may be a phosphate ion; the process is analogous to the known loss of carbon dioxide and halide ion from some β-halogeno acids in weakly alkaline solution (32). β-Lactones also can lose carbon dioxide to give olefins (66).

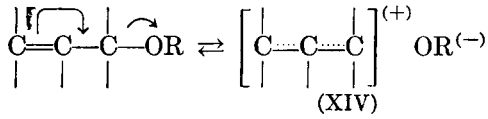


With the formation of isopentenyl pyrophosphate, the biosynthesis enters a series of transformations dominated by the nucleophilic activity of the C=C double bond. Four electrons participate in such a bond, and two of them are readily available for reaction with an electrophilic species, such as a proton or a Lewis acid. The initial attack of, e.g., a proton is thought to produce a charged complex (XIII) which may stabilize itself either by expulsion of a proton (not necessarily the same proton) or by accepting an anion, e.g., chloride ion.

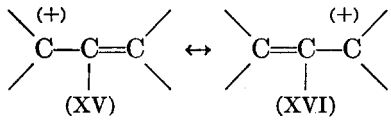


(XIII)

When such a group as halide or hydroxyl or esterified hydroxyl (e.g., a phosphate ester) is on a carbon atom α to the double bond, an elimination can take place rather easily, for the resulting cation (written as XIV) is stabilized by resonance between two equivalent forms (XV) and (XVI).

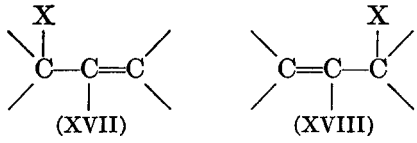


(XIV)



(XV) (XVI)

In this cation, attack of a nucleophilic species ($X^{(-)}$) can be at either end of the three-carbon system, to give (XVII) or (XVIII). Similarly, expulsion of a proton

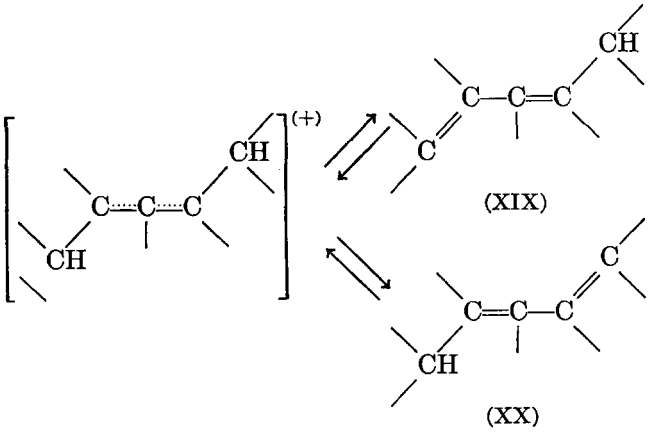


(XVII) (XVIII)

from the cation can also be from either end of the chain, leading to different conjugated dienes (XIX) and (XX).

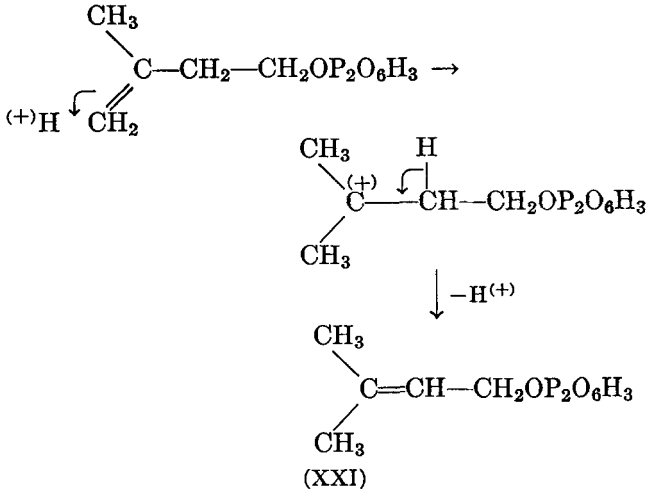
The direct enzymic conversion of isopentenyl pyrophosphate, both natural and synthetic, into squalene has been demonstrated (75, 77). If one accepts the view that six molecules of this substance, as of mevalonate, are required to furnish one molecule of squal-

ene, it follows that a shift in the position of the double bond in each unit must accompany the synthesis. Squalene terminates at both ends in the group $(CH_3)_2C{=}CH$-; thus it is reasonable to suppose that the initial condensation of two $C_{(5)}$ units is preceded by an isomerization of one of them to give 3,3-dimethylallyl pyrophosphate (XXI). This can be done by an addition, of the type discussed above, of a proton to the terminal methylene group, followed by elimination of a different proton.



(XIX)

(XX)

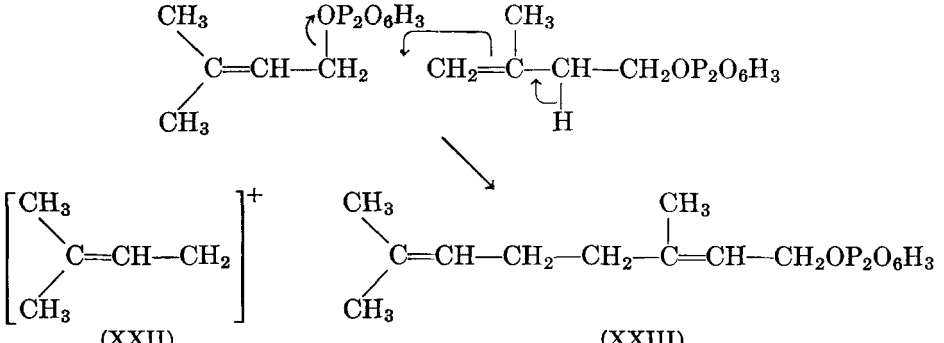


(XXI)

Lynen and his colleagues have recently reported (83) that this transformation can indeed be effected enzymically by a fraction from yeast.

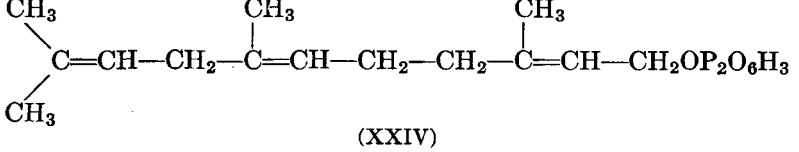
Dimethylallyl pyrophosphate is a substance, of the type discussed above, which can readily give an allylic cation (XXII) by loss of pyrophosphate ion. This cation, an electrophilic agent, can then add to the double bond of isopentenyl pyrophosphate in the same manner as a proton added in the first step; a similar sequence then leads to geranyl pyrophosphate (XXIII), which can itself give a cation and react in the same way with another molecule of isopentenyl pyrophosphate to give farnesyl pyrophosphate (XXIV). The formulae show the electron-shifts without intermediate stages.

The identification of farnesyl pyrophosphate as an intermediate in the biosynthesis of squalene from isopentenyl pyrophosphate is described by Lynen and colleagues (77), who put forward the synthetic scheme formulated above. An essentially similar scheme had previously been proposed by Bloch (84). The condensation may in other biosyntheses proceed to the addition of further $C_{(5)}$ units; this is presumably so with carotenoids (85, 86) and with coenzyme Q (87), both of which can be built up from mevalonate. The biosynthesis of the rubber hydrocarbon (XXV) from synthetic isopentenyl pyrophosphate is said to occur (88) in the latex of *Taraxacum kok-saghyz;* here the



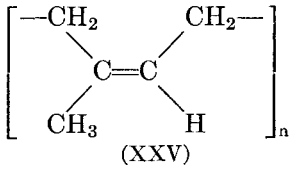
(XXII)

(XXIII)



(XXIV)

double bonds have the opposite geometry from that in geraniol, farnesol, and squalene, a difference which could be the result of different orientation of $C_{(5)}$ units for polymerization.



(XXV)

Farnesyl pyrophosphate (XXIV) is converted (77) into squalene by a particulate fraction of yeast in the presence of reduced triphosphopyridine nucleotide. This process is a reductive coupling of two $C_{(15)}$ units at the carbon atoms bearing the ester groups, and thus has a superficial resemblance to the chemical synthesis of squalene (89) by reaction of farnesyl bromide with lithium. Two mechanisms have been tentatively suggested (76, 77) for the coupling of the $C_{(15)}$ units. In the first, loss of phosphate from both units is assumed, the hydrocarbon farnesene then coupling with uptake either of two protons and two electrons or of one proton and a "negative" hydrogen such as is supplied by reduced pyridine nucleotides. The evidence concerning the participation of "negative" hydrogen is thus far indecisive, DPND introducing only 0.3 atom of deuterium, at undetermined positions, into squalene during biosynthesis from mevalonate. The second mechanism imagines the participation of the reduced form of a quinone, e.g., coenzyme Q. This forms a tertiary ether with one molecule of farnesyl pyrophosphate; a concerted reaction then occurs, liberating the quinone and providing electrons for nucleophilic attack on a second molecule of farnesyl pyrophosphate. The role of the reduced pyridine nucleotide is then to regenerate the hydroquinone, and none of its "negative" hydrogen enters squalene.

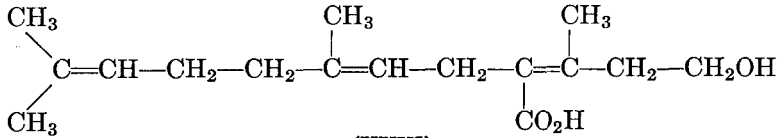
These two mechanisms are illustrated in Scheme 7. Neither of them can at present be supported by chemical analogies; fortunately, the current rapid progress should soon permit more detailed study of this highly interesting coupling. It is worth remarking that a number of chemical reductive couplings, e.g., of ketones and esters, are thought to proceed via free radicals.

Most of the information concerning the stage mevalonate → squalene has so far been obtained by the use of yeast enzymes. The existence of a parallel sequence in mammalian tissues is not yet completely proved. Conversion of mevalonate to squalene in such systems is of course established (80), and the conversion of 5-phosphomevalonate (90) and of synthetic isopentenyl pyrophosphate (91) into cholesterol by preparations of rat liver is also indicated. However, the formation of considerable amounts of higher carboxylic acids from mevalonate in incomplete systems (90) has not yet been explained. An acid recently obtained (92) from such a system was examined chemically (93) and the structure (XXVI) was assigned; it is, however, a poor precursor of unsaponifiable lipids in a complete system.

Squalene to Lanosterol. This cyclization is thought to take place as a single concerted reaction. If this is so, it is the most complex chemical reaction yet known, no fewer than sixteen centers participating. Since the conversion of squalene to lanosterol was originally proved (94), the transformation has been demonstrated (95) with pure, synthetic all-*trans* squalene, and it has been shown (96) that the aqueous medium provides neither oxygen nor stably bound hydrogen to the product. Chemically, the process is oxidative, and O^{18} from the oxygen gas necessarily present during incubation has been shown (96) to enter the hydroxyl group of lanosterol. The cyclizing enzyme has been tentatively named squalene oxidocyclase I.

Despite its complexity, the process depends on a relatively simple property: the nucleophilic activity of a double bond. As explained earlier, the electrons available at a double bond can attack an electron-deficient species, say $X^{(+)}$. The product (XXVII) is itself electron-deficient and can attack another double bond to give a new species (XXVIII); and if this second double bond is in the same molecule, the process is a cyclization.

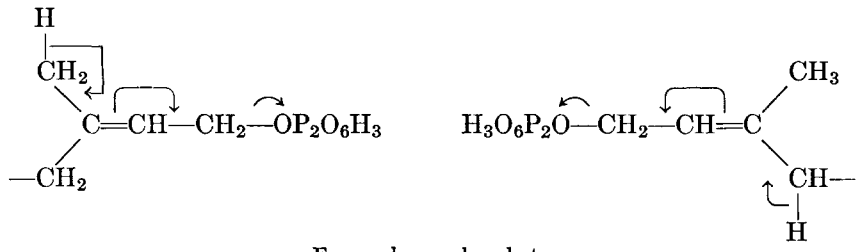
"Carbonium ions" such as (XXVII) or (XXVIII) are highly unstable intermediates, and their presence can rarely be demonstrated unless exceptional stabilizing structures are also present. Indeed, the sign (+) on (XXVII) and (XXVIII) may mean no more than a developing electron-deficiency which induces the next step in a concerted reaction. This next step can be any of three types, all of which are found in the cyclization of squalene: (i) reaction with a new nu-



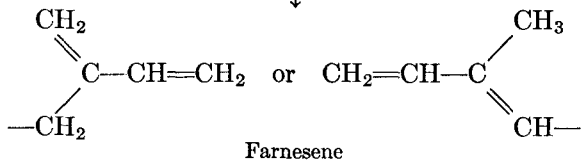
(XXVI)

SCHEME 7

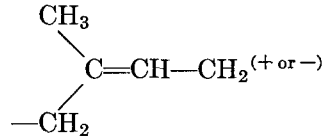
(a)

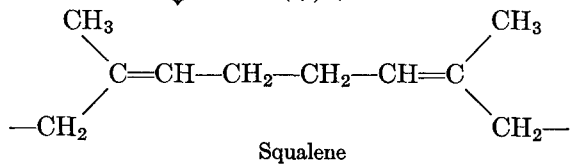


Farnesyl pyrophosphate



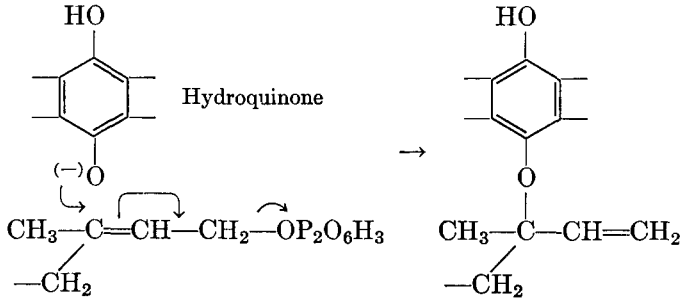
Farnesene





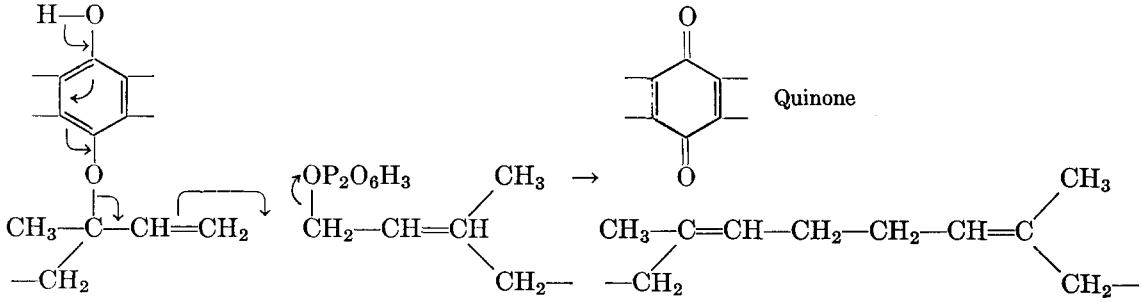
Squalene

(b) (i)



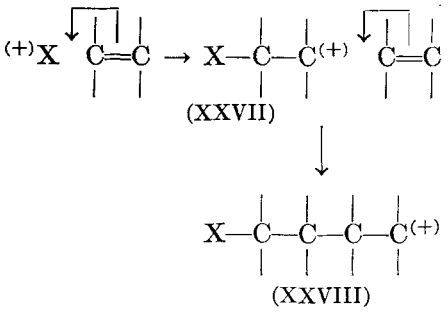
Farnesyl pyrophosphate Tertiary ether

(ii)



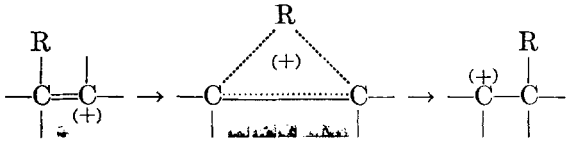
Quinone

Squalene



(XXVII) (XXVIII)

cleophilic species; (ii) elimination of a cation, usually a proton, to give a neutral molecule; (iii) a *1,2-shift* transferring the electron-deficiency to an adjacent carbon, from which a group migrates (XXIX → XXX).



(XXIX) (XXX)

A detailed theory of the chemical change leading from squalene to cholesterol has been formulated by the Zürich school (97). It is possible to fold the long squalene molecule in such a way that an electron-shift, assumed to be initiated by an electron-deficient oxygen atom such as ^{+}OH, can be transmitted in a concerted manner through five double bonds (Scheme 8). A tetracyclic system is thus formed, with an electron-deficient center at the carbon atom which is to become C-20 of lanosterol. A series of 1,2-shifts then occurs; four groups migrate, and the electron-deficiency thus transferred back to the junction of the two central rings is finally eliminated by expulsion of a proton from C-9.

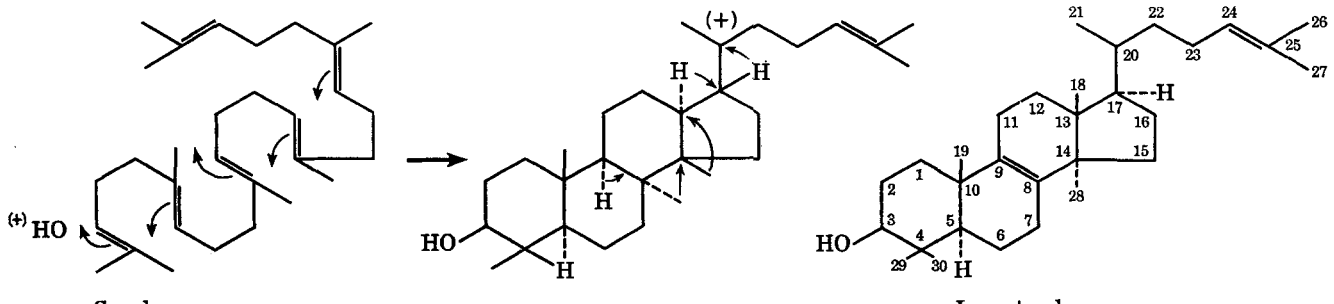
The Swiss authors showed that by slightly different foldings of the squalene molecule, and slightly different sequences of the same types of nucleophilic attack and rearrangement, it was possible to formulate all known types of triterpenes as being derived from squalene. This is the most elaborate formulation of Ruzicka's "isoprene rule," which has helped in the interpretation of so many terpenoid structures and has been vindicated by the discovery of the basic building unit isopentenyl pyrophosphate, a substance formally derivable from isoprene by addition of pyrophosphoric acid. The mode of cyclization is supported by the labeling patterns found by total degradations of squalene (98) and of cholesterol (99 to 103) biosynthesized from acetate. The proof (95, 105) that both the angular methyl groups 18 and 28 in lanosterol have indeed reached their positions by 1,2-shifts has provided further detailed support for the mechanism proposed.

A concerted cyclization of the type illustrated can be regarded, as explained above, as a series of nucleophilic attacks by electrons of double bonds on electron-deficient centers, and it is not logically necessary to assume that these should all occur simultaneously. The novelty of the process, chemically speaking, is the stereochemical control, eight new centers of optical asymmetry being fixed in their proper mutual relationships when cyclization is complete. Attempts to achieve similar cyclizations nonenzymically have had some success, e.g., the transformation (XXXI → XXXII) effected by Eschenmoser (105) in 5 to 10 per cent yield.

The available evidence is against a concerted mechanism for such multiple cyclizations in solution, but given a surface, such as the enzyme presumably provides, for attachment of the substrate in the most favorable configuration, a concerted reaction might well occur. By contrast, when a rigid system of carbon rings is provided and the necessary centers are already fixed in proper relationship, concerted *rearrangements* quite as complex as that leading to lanosterol can be achieved nonenzymically, e.g., (106), (XXIII → XXXIV).

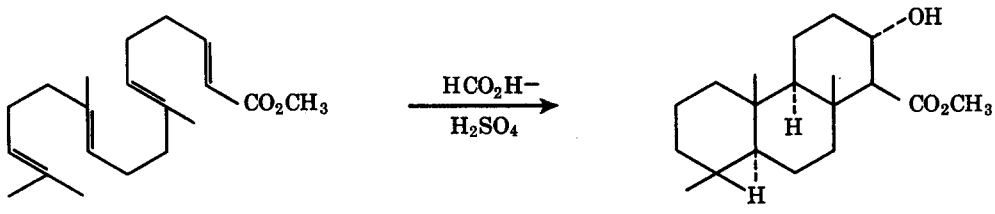
Lanosterol to Cholesterol. Since the original demonstration of the over-all process (107), the course of the transformation has been deduced in more detail (see Scheme 5).

The necessary removal of three methyl groups is an

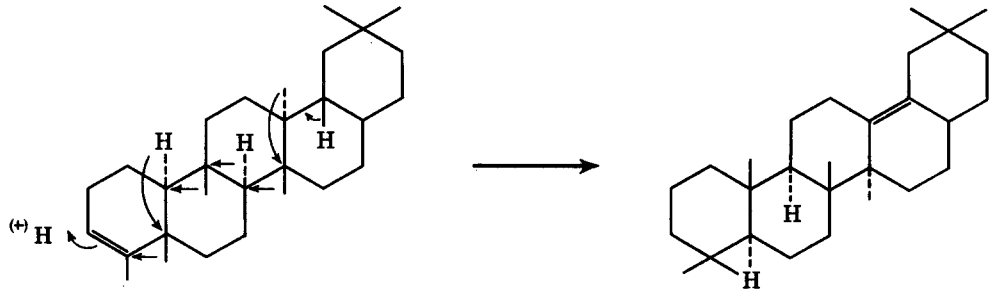


Squalene Lanosterol

SCHEME 8



(XXXI) (XXXII)



(XXXIII) (XXXIV)

oxidative process. Studying the conversion, by a particulate preparation of rat liver, of lanosterol to cholesterol, Olson *et al.* (108) found that aerobic conditions were necessary, that the three carbon atoms eliminated appeared as carbon dioxide, and that formaldehyde could not be demonstrated as an intermediate. This lends support to the view that the actual eliminations are decarboxylations.

Oxidations of the sterol skeleton at specific positions necessarily takes place in the biogenesis of, e.g., bile acids, adrenal hormones, and many other types, and similar specific oxidations of steroid substrates artificially supplied can be effected by micro-organisms. It is convenient to consider these oxidations in conjunction with the oxidative demethylation of lanosterol.

Enzymic oxidations of this sort are difficult to classify chemically, because of the lack of analogous non-enzymic processes. When one uses in homogeneous solution an oxidizing agent powerful enough to attack nonactivated C—H bonds at an appreciable rate, it is rare for the product to escape further extensive oxidation. How an enzyme can attack these bonds while leaving untouched more sensitive groups in the same molecule is still unknown, but the mechanism must surely depend on the structure of the enzyme-substrate complex: the substrate may be attached to the enzyme in such a way as to leave exposed to an oxidizing agent only the group to be oxidized, or the enzyme itself may carry an oxidizing group correctly oriented to oxidize one part of the attached substrate and no other.

Three types of chemical mechanism can be envisaged for the direct replacement of a hydrogen by hydroxyl: nucleophilic, homolytic, and electrophilic. In a nucleophilic mechanism, the hydrogen would be removed along with both its bonding electrons and would be replaced by an hydroxyl ion. Since it has been shown (109) that isotopic oxygen from the atmosphere, not from water, provides the 11β-hydroxyl group introduced by adrenal 11β-hydroxylase, and hydroxyl groups introduced at several other positions by micro-organisms (110), this type of mechanism can probably be excluded. In addition, this and other experimental evidence (111) also excluded an indirect hydroxylation via an olefin first formed by dehydrogenation.

By labeling specific hydrogen atoms in various steroids with tritium or deuterium it has been possible to show that the 11β-hydroxylation of progesterone by perfusion through bovine adrenals (112), the microbiological 11α-hydroxylation of pregnane-3,20-dione (112, 113), and the 7α-hydroxylation occurring during biosynthesis of cholic acid from cholesterol in the living rat (114), all proceed by direct replacement of hydrogen by hydroxyl without change in configuration. It has been pointed out (113, 114) that this retention of configuration is consistent with what little is known of the stereochemistry of electrophilic displacements at a saturated carbon atom. The evidence therefore favors the view that the attacking species is electrophilic, like the $^{(+)}$OH postulated as the initiator of squalene cyclization, and that the departing hydrogen

leaves behind the electrons which bound it to carbon. A homolytic mechanism, with free radicals as intermediates, is not excluded by the evidence but requires additional assumptions to explain retention of configuration.

Oxidation of the three methyl groups eliminated during conversion of lanosterol to cholesterol evidently proceeds farther than hydroxylation, and the evidence is clear that the methyl group C-28 is eliminated first. The ingenious demonstration (115, 116, 117), that 4,4-dimethylcholesta-8,24-dien-3β-ol is an intermediate, overcame great difficulties occasioned by the minute amounts of available radioactive material. An interesting feature of the elimination of the two methyl groups at C-4 is the demonstration that lanosterol labeled in the 3α position with tritium loses the isotope completely on the way to cholesterol, whereas zymosterol similarly labeled does not; and that 4,4-dimethylcholesta-8,24-dien-3-one is converted enzymically into cholesterol as efficiently as is the corresponding alcohol, whereas lanostadienone, the ketone of lanosterol, is inert. As Bloch (84) pointed out, oxidation to carboxyl of the 4-methyl groups in a 3-oxo-4,4-dimethyl-steroid would give β-keto acids which would decarboxylate with ease. In comparison, any assistance given by the 8,9-double bond in lanosterol to decarboxylation of a 14-carboxylic acid would be rather slight.

The first $C_{(27)}$ intermediate appears to be zymosterol (118, 119) or possibly the corresponding ketone. Of the transformation of this into desmosterol (120)—for formulae see Scheme 5—or of the mechanism of reduction of the 24,25-double bond, nothing is at present known. Chemically, neither process is without analogy, and light can perhaps be thrown on the mechanism by suitable labeling of hydrogen atoms.

REFERENCES

1. Bodánszky, M. *Nature* **175:** 685, 1955.
2. Burton, K. *Biochem. J.* **59:** 44, 1955.
3. Chou, T. C., and F. Lipmann. *J. Biol. Chem.* **196:** 89, 1952.
4. Beinert, H., D. E. Green, P. Hele, H. Hift, R. W. Von Korff, and C. V. Ramakrishnan. *J. Biol. Chem.* **203:** 35, 1953.
5. Hele, P. *J. Biol. Chem.* **206:** 671, 1954.
6. Millerd, A., and J. Bonner. *Arch. Biochem. Biophys.* **49:** 343, 1954.
7. Eisenberg, M. A. *J. Biol. Chem.* **203:** 815, 1953.
8. Lynen, F., and K. Decker. *Ergeb. Physiol.* **49:** 327, 1957.
9. Berg, P. *J. Biol. Chem.* **222:** 991, 1956.
10. Ingraham, L. L., and D. E. Green. *Science* **128:** 310, 1958; **129:** 896, 1959.
11. Berg, P. *Science* **129:** 895, 1959.
12. Klages, F., and E. Zange. *Ann. Chem. Liebigs* **607:** 35, 1957.
13. Sonntag, N. O. V. *Chem. Revs.* **52:** 294, 1953.
14. Adkins, H., and Q. E. Thompson. *J. Am. Chem. Soc.* **71:** 2242, 1949.
15. Lipmann, F. *J. Biol. Chem.* **155:** 55, 1944.
16. Stadtman, E. R., G. D. Novelli and F. Lipmann. *J. Biol. Chem.* **191:** 365, 1951.
17. Stadtman, E. R. *J. Biol. Chem.* **203:** 501, 1953.
18. Green, D. E., D. S. Goldman, S. Mii, and H. Beinert. *J. Biol. Chem.* **202:** 137, 1953.
19. Stern, J. R., M. J. Coon and A. del Campillo. *J. Am. Chem. Soc.* **75:** 1517, 1953.
20. Gilvarg, C. Quoted in Reference 21.
21. Ochoa, S. *Advances in Enzymol.* **15:** 183, 1954.
22. Lynen, F. *Federation Proc.* **12:** 683, 1953.
23. Lynen, F., U. Henning, C. Bublitz, B. Sörbo, and L. Kröplin-Rueff. *Biochem. Z.* **330:** 269, 1958.
24. Goldman, D. S. *J. Biol. Chem.* **208:** 345, 1954.
25. Gibson, D. M., E. B. Titchener and S. J. Wakil. *J. Am. Chem. Soc.* **80:** 2908, 1958.
26. Gibson, D. M., E. B. Titchener and S. J. Wakil. *Biochim. et Biophys. Acta* **30:** 376, 1958.
27. Brady, R. O. *Proc. Natl. Acad. Sci. U.S.* **44:** 993, 1958.
28. Wakil, S. J. *J. Am. Chem. Soc.* **80:** 6465, 1958.

28*a*. Formica, J. V., and R. O. Brady. *J. Am. Chem. Soc.* **81:** 752, 1959.

29. Wakil, S. J., and J. Ganguly. *Federation Proc.* **18:** 346, 1959.
30. Kolbe, H. *J. prakt. Chem.* **10:** 89, 1874.
31. Baumgarten, E., R. Levine and C. R. Hauser. *J. Am. Chem. Soc.* **66:** 862, 1944.
32. Michael, A., and O. Eckstein. *Ber. deut. chem. Ges.* **38:** 50, 1905.
33. Burton, R. M., and E. R. Stadtman. *J. Biol. Chem.* **202:** 873, 1953.
34. Doebner, O., and L. Segelitz. *Ber. deut. chem. Ges.* **38:** 2733, 1905.
35. Knoevenagel, E. *Ber. deut. chem. Ges.* **31:** 2596, 1898.
36. Mannich, C., and K. Ritsert. *Ber. deut. chem. Ges.* **57:** 1116, 1924.
37. Birch, A. J. *Fortschr. Chem. org. Naturstoffe* **14:** 186, 1957.
38. Birch, A. J., and H. Smith. In *Chemical Society Symposia, Bristol 1958*, London, The Chemical Society, 1958, p. 1.
39. Lapworth, A. *J. Chem. Soc.* **79:** 1265, 1901.
40. Hele, P., G. Popják and M. Lauryssens. *Biochem. J.* **65:** 348, 1957.
41. Marcus, A., B. Vennesland and J. R. Stern. *J. Biol. Chem.* **233:** 722, 1958.

41*a*. Fisher, H. F., E. E. Conn, B. Vennesland, and F. H. Westheimer. *J. Biol. Chem.* **202:** 687, 1953.

42. Loewus, F. A., P. Ofner, H. F. Fisher, F. H. Westheimer, and B. Vennesland. *J. Biol. Chem.* **202:** 699, 1953.
43. Huisgen, R., and H. Ott. *Tetrahedron* **6:** 253, 1959.
44. Fischer, E. *Ber. deut. chem. Ges.* **23:** 799, 1890.
45. Langdon, R. G. *J. Biol. Chem.* **226:** 615, 1957.
46. Gibson, D. M., and M. I. Jacob. *Federation Proc.* **15:** 261, 1956.
47. Seubert, W., G. Greull and F. Lynen. *Angew. Chem.* **69:** 359, 1957.

48. Beinert, H., and E. Page. *J. Biol. Chem.* **223:** 479, 1957 (and earlier references cited therein).
49. Meinwald, J., and G. A. Wiley. *Chem. & Ind. (London)*, 957 (1956).
50. Bentley, K. W., J. Dominguez and J. P. Ringe. *J. Org. Chem.* **22:** 418, 1957.
51. Stern, J. R., A. del Campillo and I. Raw. *J. Biol. Chem.* **218:** 971, 1956.
52. Stern, J. R., and A. del Campillo. *J. Biol. Chem.* **218:** 985, 1956.
53. Wakil, S. J. *Biochim. et Biophys. Acta* **19:** 497, 1956.
54. Rendina, G., and M. J. Coon. *J. Biol. Chem.* **225:** 523, 1957.
55. Bloch, K., and D. Rittenberg. *J. Biol. Chem.* **145:** 625, 1942.
56. Langdon, R. G., and K. Bloch. *J. Biol. Chem.* **200:** 129, 135, 1953.
57. Clayton, R. B., and K. Bloch. *J. Biol. Chem.* **218:** 305, 1956.
58. Tavormina, P. A., M. H. Gibbs and J. W. Huff. *J. Am. Chem. Soc.* **78:** 4498, 1956.
59. Witting, L. A., H. J. Knauss and J. W. Porter. *Federation Proc.* **18:** 353, 1959.
60. Rudney, H., and J. J. Ferguson, Jr. *J. Biol. Chem.* **234:** 1072, 1076, 1959.
61. Ochoa, S., J. R. Stern and M. C. Schneider. *J. Biol. Chem.* **193:** 691, 1951.
62. Stern, J. R., B. Shapiro, E. R. Stadtman, and S. Ochoa. *J. Biol. Chem.* **193:** 703, 1951.
62*a*. Marcus, A., and B. Vennesland. *J. Biol. Chem.* **233:** 727, 1958.
63. Srere, P. A., W. Seubert and F. Lynen. *Biochim. et Biophys. Acta* **33:** 313, 1959.
64. Johansson, H. *Ber. deut. chem. Ges.* **48:** 1262, 1915.
65. Olson, A. R., and R. J. Miller. *J. Am. Chem. Soc.* **60:** 2687, 1938.
66. Zaugg, H. E. In *Organic Reactions,* edited by R. Adams *et al.,* New York, Wiley, 1954, vol. 8, p. 305.
67. Sauer, J. C. *J. Am. Chem. Soc.* **69:** 2444, 1947.
68. Bachhawat, B. K., W. G. Robinson and M. J. Coon. *J. Biol. Chem.* **216:** 727, 1955.
69. Lynen, F., J. Knappe, H. Eggerer, U. Henning, and B. W. Agranoff. *Federation Proc.* **18:** 278, 1959.
70. Durr, I. F., H. Rudney and J. J. Ferguson, Jr. *Federation Proc.* **18:** 219, 1959.
71. Lynen, F. In *Biosynthesis of Terpenes and Sterols* (Ciba Foundation Symposium), edited by G. E. W. Wolstenholme and Maeve O'Connor, London, J. & A. Churchill, Ltd., 1959, p. 95.
72. Coon, M. J., F. P. Kupiecki, E. E. Dekker, M. J. Schlesinger, and A. del Campillo. Reference 71, p. 62.
73. Tchen, T. T. *J. Biol. Chem.* **233:** 1100, 1958.
74. Levy, H. R., and G. Popják. *Biochem. J.* **72:** 35 p, 1959.
75. Chaykin, S., J. Law, A. H. Phillips, T. T. Tchen, and K. Bloch. *Proc. Natl. Acad. Sci. U.S.* **44:** 998, 1958.
76. Bloch, K., S. Chaykin and A. H. Phillips. *Federation Proc.* **18:** 193, 1959.
77. Lynen, F., H. Eggerer, U. Henning, and I. Kessel. *Angew. Chem.* **70:** 738, 1958.
78. Tavormina, P. A., and M. H. Gibbs. *J. Am. Chem. Soc.* **78:** 6210, 1956.
79. Rilling, H., T. T. Tchen and K. Bloch. *Proc. Nat. Acad. Sci. U.S.* **44:** 167, 1958.
80. Cornforth, J. W., R. H. Cornforth, G. Popják, and I. Y. Gore. *Biochem. J.* **69:** 146, 1958.
81. Dituri, F., S. Gurin and J. L. Rabinowitz. *J. Am. Chem. Soc.* **79:** 2650, 1957.
82. Johansson, H., and S. M. Hagman. *Ber. deut. chem. Ges.* **55B:** 647, 1922.
83. Agranoff, B. W., H. Eggerer, U. Henning, and F. Lynen. *J. Am. Chem. Soc.* **81:** 1254, 1959.
84. Bloch, K. Reference 71, p. 4.
85. Grob, E. C. *Chimia Switz.* **11:** 338, 1957.
86. Braithwaite, G. D., and T. W. Goodwin. *Biochem. J.* **66:** 31P, 1957.
87. Gloor, U., and O. Wiss. *Experientia* **14:** 410, 1958.
88. Arreguin, B. Quoted in Reference 77.
89. Isler, O., R. Rüegg, L. Chopard-dit-Jean, H. Wagner, and K. Bernhard. *Helv. Chim. Acta* **39:** 897, 1956.
90. Popják, G. Reference 71, p. 148. See also Popják, G., M. Horning, N. L. R. Bucher, and Rita H. Cornforth. *Biochem. J.* **72:** 34p, 1959.
91. Bucher, N. L. R. Quoted in Reference 77.
92. Ogilvie, J. W., Jr., and R. G. Langdon. *J. Am. Chem. Soc.* **81:** 754, 1959.
93. Ogilvie, J. W., Jr. *J. Am. Chem. Soc.* **81:** 756, 1959.
94. Tchen, T. T., and K. Bloch. *J. Biol. Chem.* **226:** 921, 1957.
95. Maudgal, R. K., T. T. Tchen and K. Bloch. *J. Am. Chem. Soc.* **80:** 2589, 1958.
96. Tchen, T. T., and K. Bloch. *J. Biol. Chem.* **226:** 931, 1957.
97. Eschenmoser, A., L. Ruzicka, O. Jeger, and D. Arigoni. *Helv. Chim. Acta* **38:** 1890, 1955.
98. Cornforth, J. W., and G. Popják. *Biochem. J.* **58:** 403, 1954.
99. Wüersch, J., R. L. Huang and K. Bloch. *J. Biol. Chem.* **195:** 439, 1952.
100. Cornforth, J. W., G. D. Hunter and G. Popják. *Biochem. J.* **54:** 590, 597, 1953.
101. Bloch, K. *Helv. Chim. Acta* **36:** 1611, 1953.
102. Dauben, W. G., and K. H. Takemura. *J. Am. Chem. Soc.* **75:** 6302, 1953.
103. Cornforth, J. W., I. Y. Gore and G. Popják. *Biochem. J.* **65:** 94, 1957.
104. Cornforth, J. W., R. H. Cornforth, A. Pelter, M. G. Horning, and G. Popják. *Tetrahedron* **5:** 311, 1959.
105. Eschenmoser, A., D. Felix, M. Gut, J. Meier, and P. Stadler. Reference 71, p. 217.
106. Corey, E. J., and J. J. Ursprung. *J. Am. Chem. Soc.* **78:** 5041, 1956.
107. Clayton, R. B., and K. Bloch. *J. Biol. Chem.* **218:** 319, 1956.
108. Olson, J. A., Jr., M. Lindberg and K. Bloch. *J. Biol. Chem.* **226:** 941, 1957.
109. Hayano, M., M. C. Lindberg, R. I. Dorfman, J. E. H. Hancock, and W. von E. Doering. *Arch. Biochem. Biophys.* **59:** 529, 1955.
110. Hayano, M., A. Saito, D. Stone, and R. I. Dorfman. *Biochim. et Biophys. Acta* **21:** 380, 1956.
111. Hayano, M., and R. I. Dorfman. *J. Biol. Chem.* **211:** 227, 1954.

112. Hayano, M., M. Gut, R. I. Dorfman, O. K. Sebek, and D. H. Peterson, *J. Am. Chem. Soc.* **80:** 2336, 1958.
113. Corey, E. J., G. A. Gregoriou and D. H. Peterson. *J. Am. Chem. Soc.* **80:** 2338, 1958.
114. Bergstrom, S., S. Lindstredt, B. Samuelson, E. J. Corey, and G. A. Gregoriou. *J. Am. Chem. Soc.* **80:** 2337, 1958.
115. Schneider, P. B., R. B. Clayton and K. Bloch. *J. Biol. Chem.* **224:** 175, 1957.
116. Gautschi, F., and K. Bloch. *J. Am. Chem. Soc.* **79:** 684, 1957.
117. Gautschi, F., and K. Bloch. *J. Biol. Chem.* **233:** 1343, 1958.
118. Schwenk, E., G. J. Alexander, C. A. Fish, and T. H. Stoudt. *Federation Proc.* **14:** 752, 1955.
119. Johnston, J. D., and K. Bloch. *J. Am. Chem. Soc.* **79:** 1145, 1957.
120. Stokes, W. M., F. C. Hickey and W. A. Fish. *J. Biol. Chem.* **232:** 347, 1958.