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Enzymatic Synthesis of Monounsaturated Fatty Acids

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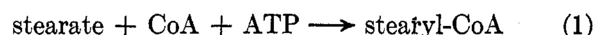
One of the striking disclosures of modern biochemical research is the unity of metabolic and especially biosynthetic processes. By this we mean that the vast majority of pathways are chemically unique and, moreover, that they are universal for all forms of life.

However, in a few instances identical molecules are known to be of dual origin. Two pathways, totally different chemically, have been described for the biosynthesis of the amino acid lysine, for nicotinic acid, and for long-chain monounsaturated fatty acids. Multiple or alternate biosynthetic pathways are probably a consequence of evolution and can be so rationalized. For example, the existence of dual pathways for the biosynthesis of monounsaturated fatty acids is taken to mean that one is more primitive than the other. When life was primitive and the life processes were entirely anaerobic, unsaturated fatty acids could only be formed by anaerobic reactions. With the advent of atmospheric oxygen and aerobic metabolism, novel biosynthetic reactions evolved.[1] Thus, aerobic desaturation superseded the process of anaerobic olefin formation. As this Account will show, the oxygen-dependent and the oxygen-independent biosyntheses of monoolefinic fatty acids are distinct and totally unrelated chemical processes.

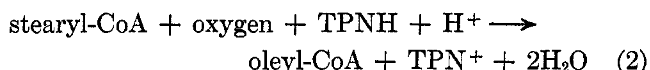
Oxidative Desaturation

Long-chain fatty acids are ubiquitous constituents of natural fats. In a given glyceride molecule saturated and unsaturated fatty acids occur generally side by side, their structures varying widely in chain length and in degree of unsaturation. Among the monounsaturated acids, *cis*-9-hexadecenoic (palmitoleic) and *cis*-9-octadecenoic (oleic) acids are by far the most common. Alternate double bond structures which are relevant to the present review will be discussed in a later section.

The early enzymatic studies on the biosynthesis of monounsaturated fatty acids could draw on the knowledge that (1) in animals oleic acid (*cis*-9-octadecenoic acid) arises from stearic acid (octadecanoic acid) by direct transformation of the C_{18} carbon chain,[2] and that (2) yeast growing under strictly anaerobic conditions fails to synthesize oleic acid even though chain elongation *per se* occurs at the normal rate.[3] Taken together, these two findings suggested that the conversion of stearate to oleate is effected by an oxygen-mediated abstraction of hydrogen. In confirmation of this inference, cell-free extracts of yeast were shown to catalyze this transformation provided oxygen and TPNH (triphosphopyridine nucleotide) were supplied to the system.[4] Moreover, the coenzyme A (CoA) thioester (see Figure 1) of the fatty acid was the substrate for the reaction. The over-all process was separable into two steps



$$\text{stearate} + \text{CoA} + \text{ATP} \longrightarrow \text{stearyl-CoA} \quad (1)$$



$$\text{stearyl-CoA} + \text{oxygen} + \text{TPNH} + \text{H}^+ \longrightarrow \text{oleyl-CoA} + \text{TPN}^+ + 2H_2O \quad (2)$$

Analogous transformations occurred with palmitate. Reaction 1 represents the well-known carboxyl activation of fatty acids. Reaction 2, the desaturase reaction, was pursued further with the objective of clarifying (a) the requirement for a fatty acyl thioester substrate; (b) the roles of oxygen as an electron acceptor and of TPNH as an electron donor; (c) the stereospecificity of the hydrogen abstraction reaction; and (d) chain-length specificity.

Oxidative Desaturation, an Oxygenase Reaction? One may formulate the generation of an olefin from a saturated precursor as a consecutive removal of proton and hydride ion from adjacent carbon atoms. This must be facilitated by an electron acceptor of suitable electrode potential. In numerous well-studied dehydrogenation reactions, the oxidized forms of flavin coenzymes serve in this capacity. These, however, involve chemically plausible dehydrogenations α,β to carbonyl systems and may therefore not be relevant. The mechanistically remarkable insertion of an isolated double bond at the nonactivated 9,10 positions of the aliphatic chain has no analogy, chemically or enzymatically.

Oxygen and TPNH serve characteristically as the electron acceptor–donor pair in enzyme-catalyzed

(1) K. Bloch, *Fed. Proc.*, **21**, 1058 (1962).
(2) R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **113**, 505 (1936).
(3) A. A. Andreasen and T. J. B. Stier, *J. Cellular Comp. Physiol.*, **43**, 271 (1954).
(4) D. K. Bloomfield and K. Bloch, *J. Biol. Chem.*, **235**, 337 (1960).

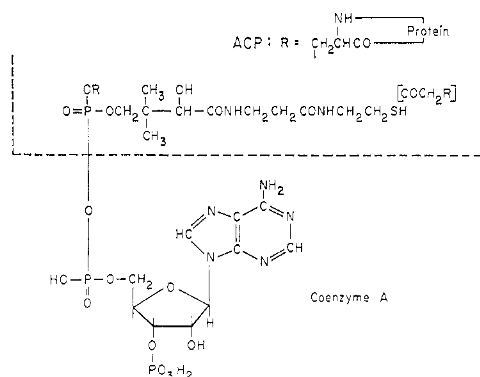


Figure 1. Coenzyme A is the entire structure as given (where R = H). The portion set off by the dotted lines is the structure of acyl carrier protein (see ref 10).

hydroxylations of aliphatic, hydroaromatic, and aromatic substrates, atmospheric oxygen furnishing the entering hydroxyl oxygen. Since the stearate → oleate conversion also requires O_2 and TPNH, one component of the system seems to be a typical oxygenase. A two-step sequence, consisting of a hydroxylation of the saturated chain at C_9 or C_{10} followed by dehydration of a hydroxy derivative to form the olefin, was therefore considered. However, neither direct nor indirect experimental evidence for a sequential process has been obtained. Synthetic 9- or 10-hydroxystearates, either as the free acids or as thioesters, are inert as substrates for oleate-forming enzyme systems,[5,6] nor have oxygen-containing intermediates been encountered in the over-all conversion. Such intermediates may have a transient existence on the enzyme, in which case only the isolation of highly purified desaturating enzymes will furnish the answer. It is worth noting that in the one well-studied case, the oxygen-linked desaturase appears to be a single catalytic entity.

Whether or not oxygenated intermediates take part in the Δ^9 desaturation of fatty acids, the resemblance with the true oxygenases (or mixed-function oxidases) certainly holds as far as the electron-transfer chain for oxygen activation is concerned. Some of the *bona fide* aliphatic or hydroaromatic oxygenases (hydroxylation of steroids, ω oxidation of hydrocarbons or of fatty acids, hydroxylation of camphor derivatives) are linked enzyme systems consisting of an oxygenating enzyme proper and two or more electron carriers intervening between TPNH and oxygen.[7] Electrons are thought to pass from reduced pyridine nucleotide to the oxygen-activating enzyme proper by way of flavoproteins and nonheme iron proteins. Active oxygen may exist only as a ternary complex with enzyme and substrates. The nature of oxygen activation and the details of the interaction between oxygen and substrate are still concealed in a black box.

Studies with enzyme systems present in the phytoflagellate *Euglena gracilis* have yielded most of the information which relates fatty acid desaturation to the authentic oxygenases.[8] Unlike the desaturases from other sources,[9] the *Euglena* enzymes are soluble and therefore susceptible to conventional fractionation. Three catalytic proteins comprising the desaturase system have been obtained from this source. One of the fractions contains flavin and oxidizes TPNH in the presence of various electron acceptors. The second fraction is a nonheme iron protein (ferredoxin) of the type found in other plants. These two enzymes constitute the electron-transport chain for reducing molecular oxygen to the level at which it can accept hydrogen from the substrate. Fraction 3, which appears to be the desaturase proper, has no measurable activity of its own; in conjunction with fractions 1 and 2 it catalyzes oleate formation from stearyl-CoA or stearyl-ACP.[10] Unfortunately, the desaturating enzyme is relatively unstable and the least well-characterized of the three components. It does not appear to contain cytochrome P_{450}, the CO-sensitive hemoprotein which is associated with various aliphatic and hydroaromatic hydroxylation systems.[7] The current information on the flow of electrons in the desaturase reaction is summarized in Figure 2.

Whether the soluble stearate desaturase of *Euglena* should be classified as an oxygenase is a matter of definition. If oxygenases (or hydroxylases) are enzymes which bring about the covalent attachment of oxygen to carbon, then fatty acid desaturases do not belong to this category, at least on the basis of present evidence. On the other hand, they would so qualify if we define oxygenases as catalysts which effect an oxygen-dependent conversion of a substrate to a more highly oxidized (or less reduced) state.

Stereospecificity. Ever since Ogston introduced the concept of multipoint attachment of substrates to enzymes,[11] the stereospecific course of reactions at *meso* carbon atoms has become an accepted and well-documented biochemical phenomenon. Without exception, enzymes catalyzing the making and breaking of carbon–hydrogen bonds at *meso* carbon atoms have been found to do so stereospecifically. The hydroxylases which catalyze substitution reactions at methylene groups in the formation and transformation of bile acids and steroids are relevant examples.[12] Since the

(5) R. J. Light, W. J. Lennarz, and K. Bloch, *J. Biol. Chem.*, **237,** 1793 (1962).
(6) M. I. Gurr and K. Bloch, *Biochem. J.*, **99,** 16C (1966).
(7) This subject is reviewed in "Biological and Chemical Aspects of Oxygenases," K. Bloch and O. Hayaishi, Ed., Maruzen Co., Ltd., Tokyo, 1966.
(8) J. Nagai and K. Bloch, *J. Biol. Chem.* **243,** 4626 (1968).
(9) The desaturases from yeast, mammalian liver, and some microorganisms are associated with membranous cell fractions and have not yet been obtained in soluble form.
(10) In some plant and bacterial systems, ACP (acyl carrier protein), the 4-phosphopantetheine derivative of a noncatalytic protein, serves in lieu of CoA as the thioester moiety of "activated" carboxyl groups: P. W. Majerus, A. W. Alberts, and P. R. Vagelos, *Proc. Natl. Acad. Sci. U. S.*, **51,** 1231 (1964); S. J. Wakil, E. L. Pugh, and F. Sauer, *ibid.*, **52,** 106 (1964). See Figure 1.
(11) A. G. Ogston, *Nature*, **162,** 963 (1948).
(12) For an early example, see S. Bergström, S. Lindstadt, B. Samuelsson, E. J. Corey, and G. A. Gregoriou, *J. Am. Chem. Soc.*, **80,** 2337 (1958).

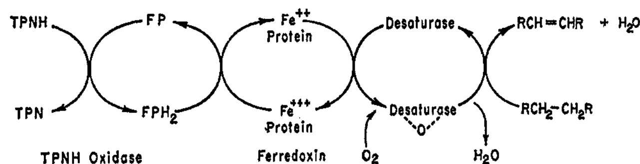


Figure 2. Electron-transport chain for oxygen activation in the desaturase reaction.

hydroaromatic ring system is conformationally restrained, the stereospecific course of these reactions is unexceptional. In a fatty acid every methylene carbon is of the *meso* type, and therefore an enzyme-catalyzed breaking of C–H bonds, *e.g.*, at C_9 and C_{10}-could, in principle, proceed stereospecifically. Yet in view of the small rotational barrier between carbon atoms in a polymethylene chain, a stereospecific event can be visualized only if the reacting groups are held firmly by the enzyme so that only one hydrogen atom of each pair at C_9 and C_{10} is exposed at the catalytic site. Stereospecific 9–10 desaturation would require a strongly preferred or frozen conformation for at least a portion of the methylene chain in the enzyme–substrate complex.

The usual procedure of labeling substrate with isotopic hydrogen was employed to test the stereospecificity of the stearate–oleate transformation.[13] Starting from the available optically active D- and L-9- and 10-hydroxystearates, four "isomeric" monotritio derivatives were prepared by the reactions shown in Figure 3. The configuration of the labeled atoms in each of the four monotritiostearates was presumed to be opposite to that of the tosylate that had been reduced. For obvious reasons the stereoselectivity of the chemical operations had to be assumed and chemical precedent had to serve as the basis for configurational assignment. Only in the event that the enzymatic transformation proceeded stereospecifically—the point that was to be proven—could one be certain that the chemical procedure had afforded stereospecifically labeled products.

Corynebacterium diphtheriae was the organism chosen to test the stereospecificity of the biological process. These bacteria incorporate stearate from the culture medium and convert it effectively to oleate. Four parallel experiments were run, each with one of the four "isomeric" substrates. Prior to use the tritiated stearate samples were combined with stearate-1-^{14}C so that the removal or retention of hydrogen associated with olefin formation could be accurately calculated from the change of isotope ratio. Two of the four monotritiostearates, the 9-"D" and the 10-"D" species, lost 70–90% of the carbon-bound tritium on conversion to oleate in the bacterial culture. By contrast, the 9-L and 10-L isomers retained 85–90% of the tritium radioactivity. Thus there was a clear preference for removal of D hydrogen atoms both at C_9 and C_{10}. Absolute stereospecificity of the enzyme-catalyzed

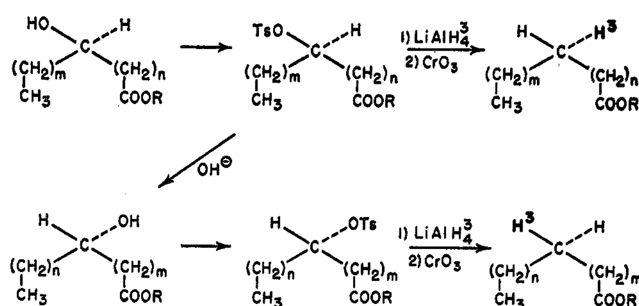


Figure 3. Synthesis of monotritiostearates from 9-D-, 9-L-, 10-D-, and 10-L-hydroxystearic acids.

process is likely on principle, but could not be proven since the stereochemical purity of the synthetic substrates was unknown. The fact that removal or retention of labeled hydrogen was not complete may reasonably be blamed on partial racemization of the substrate during the chemical labeling.

Under the chosen experimental conditions only part of the stearic acid was consumed by the bacterial cultures, and the remainder could therefore be reclaimed from each of the four experiments. Isotopic analysis showed that the recovered stearic-9-D-^{3}H acid—one of the two substrates that lost ^{3}H on conversion to oleate—contained significantly more ^{3}H with respect to ^{14}C ($^3H/^{14}C$ = 1.3–1.6) than it did at the start ($^3H/^{14}C$ = 1). Clearly the $^3H/^{14}C$ ratio rose because H-containing molecules were consumed in preference to the ^{3}H-containing species. Such tritium enrichment failed to occur with the tritiated 9-L-, 10-D-, and 10-L-stearates. The abstraction of the 9-D-hydrogen must therefore be the initial and rate-limiting step in the over-all dehydrogenation. It further follows that the removal of hydrogen at C_9 and C_{10} is not a synchronized process.

The geometry of the double bond entering the hydrocarbon chain is *cis*. As for the configuration of the leaving hydrogen atoms, this was known to be D at C_9 but only inferred to be D at C_{10}. One could therefore not state explicitly that the stereospecific H removal occurred by a *cis*-elimination process. In order to settle this point, 9,10-dideuteriostearate containing the two labeled atoms in *cis* relationship was prepared by the unambiguous diimide procedure.[14] Reduction of the cisoid oleic acid by dideuteriodiimide is expected to yield two species of *cis*-9,10-dideuterated molecules in equal proportions, hydrogen adding to the double bond either frontally or from the back. Since hydrogen removal at C_9 and C_{10} was already known to be stereospecific, *cis* elimination of hydrogen from the dideuterated substrate should either release both deuterium atoms or none. Mass spectrographic analysis of the resulting oleate population did indeed show a great preponderance of dideuterated and undeuterated species. Monodeuterated molecules expected to arise from *trans* elimination amounted to only 16%

(13) G. J. Schroepfer and K. Bloch, *J. Biol. Chem.*, **240**, 54 (1965).

(14) E. J. Corey, W. L. Mock, and D. J. Pastro, *Tetrahedron Letters*, 347 (1961).

of the total.[15] The configuration of the two leaving hydrogen atoms is therefore the same, and since the reactive hydrogen at C_9 was already known to be D, the same configuration must be assigned to the hydrogen removed from C_{10}.[16]

Granting that the two departing hydrogens are *cis* positioned at the start of the reaction, one must nevertheless ask whether the formation of a *cis* product unequivocally establishes a *cis*-elimination mechanism. Usually *trans* eliminations are preferred, both in chemical and enzymatic systems. The problem in the present instance is that we do not know the conformation of stearate in the enzyme–substrate complex, nor do we have evidence for or against stabilized intermediates. If the two leaving D hydrogen atoms assume an eclipsed conformation in the enzyme–substrate complex, then desaturation would indeed proceed by *cis* elimination. Yet the reaction may involve removal of *trans* hydrogens in a staggered conformation at the active enzyme site and concurrently a conformational change from staggered to eclipsed. The actual formation of a *trans* olefin followed by isomerization to the *cis* isomer while the fatty acid is still enzyme bound is another possibility. It is true that an enzyme-catalyzed isomerization of elaidic acid (*trans*) to oleic acid is not known. Nevertheless, configurational changes of enzyme-bound intermediates during the desaturation reaction cannot be excluded, and hence what appears grossly as a *cis*-elimination mechanism may not, in fact, be so.

The stereospecific removal of hydrogen in the formation of oleate, although predictable on principle grounds, would seem to approach the limits of the discriminatory power of enzymes. The phenomenon can be understood only on the assumption that the flexible hydrocarbon chain becomes conformationally locked in the enzyme–substrate complex. To achieve a rigidity comparable to that of cyclohexane systems in solution, strong nonpolar interactions between the flexible hydrocarbon chain and hydrophobic amino acid side chains in the enzyme must be invoked. The strength of these interactions is perhaps as remarkable as the stereospecificity for which these cohesive forces are clearly a determinant.

The Activation of the Carboxyl Group. For the oxidative insertion of the 9,10 double bond into palmitate or stearate fatty acyl thioesters are required substrates. One of two thiols can serve in this capacity. Coenzyme A derivatives are active in desaturation by enzyme systems of yeast,[4] animal tissues,[17] and at least one bacterial organism (*M. phlei*).[18] On the other hand, the two plant systems that have been studied (spinach and *Euglena gracilis*[8]) use the ACP thioesters[19] in the same reaction.

While thioesterification facilitates the stretching of adjacent C–H bonds, in saturated chains this activation does not extend beyond the α carbon. It is therefore not apparent how thioesterification of stearate or palmitate can assist in activating C–H bonds at C_9 or C_{10}. According to a suggestion of Richards and Hendrickson,[20] the hydrocarbon chain may not be fully extended in the enzyme–substrate complex but may assume a pseudoannular conformation allowing the reacting carbon atoms to approach the thioester grouping. These authors also raise the possibility that oxygen may attack sulfur and not the carbon chain. The resulting perthioester would provide "active" oxygen for withdrawal of hydrogen from a suitably positioned methylene group (C_9 or C_{10}) to yield oleate and an oxidized derivative of CoA. The scheme has appeal on several grounds; it rationalizes the apparent failure of oxygen to become covalently linked to the carbon, it assigns a nontrivial function to the thioester group, and finally it furnishes a plausible explanation for the positional (Δ^9) specificity of dehydrogenation.[21]

One argument that can be made against this scheme is the experimental finding that the enzymatic product is oleyl-CoA and not the free fatty acid, *i.e.*, the thioester linkage remains intact throughout desaturation.[4,8] Moreover, it seems to us that the requirement for a thioesterified carboxyl group may have a more trivial explanation that is unrelated to the mechanism of the hydrogen abstraction reaction. Viewing metabolic relationships more broadly, one can reason as follows. In the cell the biosynthesis of the major lipids proceeds in three stages, all of which involve thioester derivatives of carboxylic acids. First, fatty acid chains are assembled from acetyl-CoA and malonyl-CoA to yield palmityl and stearyl thioesters. In the next phase, these products, carrying an activated carboxyl group already, can be utilized as such and esterified to form triglycerides and phospholipids. However, a certain portion of the saturated acyl thioester will undergo desaturation to the corresponding olefins before entering into glyceride linkage. Thus, desaturation occurs at an intermediary stage; it follows a process that produces and precedes reactions which consume fatty acyl thioesters. Preservation of the energy-rich thioesters emerging from chain elongation will allow the next steps, *i.e.*, desaturation and esterification, to proceed directly without additional expenditure of

(15) The slight deviation from the values expected for a rigorously stereospecific elimination of cisoid hydrogens may again be attributed to the partial racemization of label during preparation of the substrate.

(16) This result allows one to deduce the previously unproven L configuration for the (+)-10-hydroxystearic acid which was the ultimate source of the putative 10-D- and 10-L-monotritiostearates.

(17) Y. Imai, *J. Biochem.* (Tokyo), **49**, 642 (1961).

(18) A. J. Fulco and K. Bloch, *J. Biol. Chem.*, **239**, 993 (1964).

(19) Acyl carrier protein functions as a substrate carrier, not as a catalytic protein. It contains the 4-phosphopantetheine moiety of CoA in phosphodiester linkage to a serine residue of the polypeptide chain. As in CoA, the terminal SH group of the mercaptoethanolamine moiety is the site of attachment for acyl groups.[10]

(20) J. H. Richards and J. B. Hendrickson, "The Biosynthesis of Steroids, Terpenes and Acetogenins," W. A. Benjamin, Inc., New York, N. Y., 1964, p 135.

(21) In view of what is now known about the strictly circumscribed dimensions of "active" enzyme sites the positional specificity of the desaturase reaction is, in fact, no longer conceptionally difficult. Positional specificity is no more remarkable than stereospecificity even for a fully extended hydrocarbon chain.

energy. In the absence of any obvious mechanistic need for the thioester function in the desaturation reaction we favor this argument of cellular economy.

Anaerobic Synthesis of Monounsaturated Fatty Acids

Monounsaturated fatty acids occur in all forms of life, and since life is possible without oxygen, the oxidative mode of forming olefinic acids as described above cannot be universal.[22] Among the early clues for the existence of an oxygen-independent pathway, Hofmann's isolation of *cis*-vaccenic acid (Δ^{11}-octadecenoic acid) from *Lactobacillus* species was of special importance. This unusual double bond structure and various other lines of evidence prompted Hofmann to propose that "The biosynthesis of unsaturated fatty acids in bacteria may . . . involve elongation of an already unsaturated or potentially unsaturated precursor."[23] This idea proved valuable not only for the *Lactobacillus* case, but applicable also to the double bond patterns of various other bacterial fatty acids. For example, it readily explained the simultaneous occurrence of oleic acid and vaccenic acid in certain strains of *E. coli* and of Δ^7- and Δ^9-C_{16} and Δ^9- and Δ^{11}-C_{18} acids in *C. kluyveri*.[24] Granting that the general process involved elongation of already unsaturated precursors and not dehydrogenation of completed carbon chains, then olefin formation was likely to occur at the level of C_{10} or C_{12} acids. A process yet to be specified would furnish Δ^3-decenoic acid and Δ^3-dodecenoic acid, the requisite precursors for the series terminating in Δ^9-C_{16}, Δ^{11}-C_{18} and Δ^7-C_{16}, Δ^9-C_{18}, respectively. Appropriately labeled enoic acids underwent these transformations in bacterial cultures as predicted.[25] Bacterial fatty acid synthetases operating in this manner might be expected to produce saturated and unsaturated long-chain acids side by side, provided the formation of Δ^3 olefins is coupled to the elongation process. The first experiments with cell-free extracts of *E. coli* showed this to be the case.[26,27] Palmitoleate and vaccenate were formed in this system along with palmitate and stearate. A biosynthetic scheme incorporating the various experimental observations is shown in Figure 4.[25] The reactions are assumed to be the same as in the normal elongation process with one major modification. At the stage of

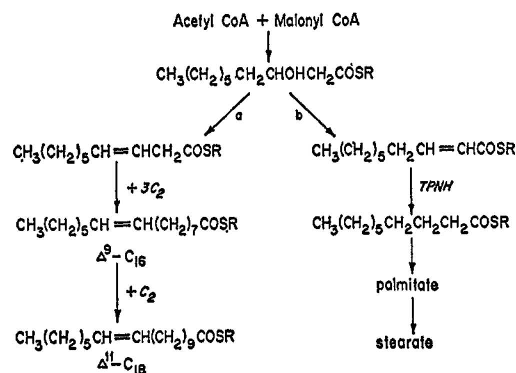


Figure 4. Fatty acid biosynthesis in *E. coli*. Reaction a initiates the pathway to unsaturated acids and reaction b, the branch to saturated acids.

β-hydroxydecanoate, the pathways diverge, one branch leading to α,β-decenoate and thence to palmitate and stearate, and the other to β,γ-decenoate, the precursor for palmitoleate and vaccenate. The novel reaction was the generation of the β,γ-unsaturated intermediate.

The postulated dehydration of β-hydroxydecanoyl thioesters was then shown to be effected by the same *E. coli* extracts which catalyzed the over-all elongation.[26] Eventually the dehydrating enzyme was isolated in pure form. Several thousandfold purification of this activity yielded a homogeneous protein whose properties are described below.[28–30] Notably, the pure enzyme (β-hydroxydecanoyl thioester dehydrase, called "dehydrase" from here on) catalyzed the formation of β,γ-decenoate and of α,β-decenoate as well. So far the dehydrase has been found only in bacteria which produce long-chain unsaturated fatty acids by the anaerobic pathway.[31]

Properties of β-Hydroxydecanoyl Thioester Dehydrase. At the start of bacterial fatty acid synthesis acetyl and malonyl units are transferred from CoA to acyl carrier protein (ACP). Throughout the reactions that follow, the fatty acyl intermediates remain attached to the acyl carrier protein.[10,32] The dehydrase-catalyzed reactions are no exception. β-Hydroxydecanoyl-ACP is readily transformed to the isomeric decenoates at a rate which exceeds that for any other thioester derivatives tested.[33] The N-acetylcysteamine, pantetheine, and coenzyme A derivatives, being artificial substrates, react more slowly, but the rates are sufficient for purposes of enzyme assay. Because of easy access, the N-acetylcysteamine (NAC) derivatives were used in all subsequent studies.

(22) This statement is true only in the sense that olefinic fatty acids are found in representatives of all phylogenetic groups. Exceptions are certain halophilic bacteria which apparently contain no fatty acids at all, and also several species of *Clostridium* and *Bacillus* which seem to produce saturated fatty acids only (M. Kates, *Advan. Lipid Res.*, **2**, 17C (1964)). In the latter cases, however, the saturated fatty acids are either branched (iso or anteiso) or of short chain length. Olefinic acids are therefore not universally essential.

(23) K. Hofmann, W. M. O'Leary, C. W. Yoho, and T. Y. Liu, *J. Biol. Chem.*, **234**, 1672 (1959).

(24) G. Scheuerbrandt and K. Bloch, *ibid.*, **237**, 7 (1962).

(25) G. Scheuerbrandt, H. Goldfine, P. E. Baronowsky, and K. Bloch, *ibid.*, **236**, PC71 (1961).

(26) W. J. Lennarz, R. J. Light, and K. Bloch, *Proc. Natl. Acad. Sci. U. S.*, **48**, 840 (1962).

(27) P. Goldman and P. R. Vagelos, *Biochem. Biophys. Res. Commun.*, **7**, 414 (1962).

(28) A. T. Norris and K. Bloch, *J. Biol. Chem.*, **238**, PC3133 (1963).

(29) A. T. Norris, S. Matsumura, and K. Bloch, *ibid.*, **239**, 3653 (1964).

(30) L. R. Kass, D. J. H. Brock, and K. Bloch, *ibid.*, **242**, 4418 (1967).

(31) The distribution of this pathway is curious. It is not confined to anaerobic bacteria. Some facultative anaerobes and even obligate aerobes (*Pseudomonas* sp.) synthesize long-chain unsaturated acids by the dehydration–elongation mechanism (J. Erwin and K. Bloch, *Science*, **143**, 1006 (1964)).

(32) J. Nagai and K. Bloch, *J. Biol. Chem.*, **242**, 357 (1967).

(33) D. J. H. Brock, L. R. Kass, and K. Bloch, *ibid.*, **242**, 4432 (1967).

The fairly broad thiol specificity suggests relatively weak or secondary binding of this moiety to the enzyme. On the other hand, the length of the carbon chain is crucial for the rate of the dehydrase-catalyzed reaction. Peak activity is shown by the ten-carbon chain; it declines somewhat for the C_9 and C_{11} compounds and can barely be measured with the C_8 and C_{12} homologs. Substrate specificity of enzymes does not follow any general rules, but the present case is certainly not common. In most known cases enzyme activity progressively increases or declines as a function of carbon chain length. The unusually narrow specificity of the dehydrase is, however, reasonable in terms of function, C_{10} being the requisite chain length for the β,γ-unsaturated precursor of palmitoleate and vaccenate. If the chain length specificity were broad enough to allow dehydration of C_8 and C_{12} β-hydroxy acids, then the enzyme would also furnish β,γ-dodecenoate and β,γ-octanoate. Elongation of these intermediates would ultimately furnish two series of isomeric long-chain acids (Δ^7-C_{16}, Δ^9-C_{18} and Δ^{11}-C_{16}, Δ^{13}-C_{18}). The fact that only traces of these compounds are found in the lipids of *E. coli* reflects and confirms the narrow chain-length specificity of the dehydrase. The *in vitro* dehydration rates of the C_9 and C_{11} β-hydroxy acid derivatives, while appreciable, are physiologically without consequence. In the cell the acetyl-CoA-initiated synthetase produces only even-numbered fatty acids.

It is of considerable interest that all the other enzymes participating in bacterial fatty acid synthesis show a different specificity pattern. Their function, in contrast to that of the dehydrase, is to repeat the same operations (carbon–carbon condensations, reductions, etc.) each time the fatty acid is lengthened by two carbon atoms, and hence it is reasonable that their chain-length specificity should be very much broader. However, the same elongating enzymes have a nearly absolute requirement for ACP derivatives as substrates. It thus appears that in fatty acid synthesis only one of the major structural features in the substrate is critical for productive binding and catalysis. One may further speculate that, when chain-length specificity dominates, as in the dehydrase case, a hydrophobic pocket of defined dimensions provides the major binding site for substrate. On the other hand, for the elongating enzymes, contact with complementary or matching regions of the polypeptide chain of ACP would appear to be more critical than binding to the fatty acyl portion of the substrate.

Mechanism of the Reaction. Kinetic Studies. The dehydrase not only catalyzes the dehydrations shown in Figure 4a, b but also the reversal of these reactions. In addition, the enzyme functions as a double bond isomerase, interconverting *cis*-β,γ-decenoyl-NAC and *trans*-α,β-decenoyl-NAC at a rapid rate (Figure 5). At equilibrium, the relative proportions of β-hydroxy, α,β-enoate, and β,γ-enoate are 70:27:3. Initial velocity measurements of the forward reactions also show a much more rapid rate for

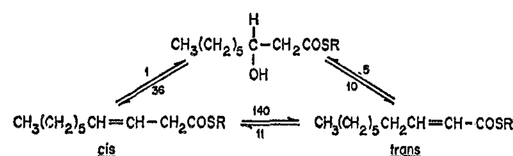
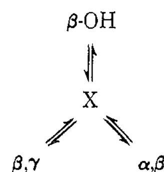


Figure 5. Reactions catalyzed by β-hydroxydecanoyl thioester dehydrase and their initial velocities.

the formation of α,β- than of β,γ-decenoate. That the β,γ isomer is not a major product under any conditions is somewhat of a problem. Long-chain unsaturated fatty acids predominate among the ultimate elongation products, and this requires that the dehydrase produce more of the β,γ-decenoate intermediate than of the α,β isomer (see Figure 4). An explanation of this anomaly has been attempted.[34]

Figure 5 also summarizes the initial rates of product formation at linear velocity conditions (no mechanism is implied). In the present discussion, comments on the kinetics will be limited to a few essential points. Of the dehydrase-catalyzed reactions, the transformation of β,γ-decenoate to α,β-decenoate is by far the most rapid. This indicates direct isomerization of the double bonds rather than interconversion of the enoates by way of β-hydroxy acid. The reversible "dehydrase" therefore functions as a double bond isomerase as well. The further and main objective of the kinetic analysis was to ascertain whether β,γ-decenoate is formed directly by elimination of the β-hydroxyl group and of hydrogen at C_γ, or whether it arises by isomerization of α,β-decenoate. The kinetic data are clear on this point. The rate of hydration of the β,γ-enoate is more than three times as rapid as the rate of hydration of the α,β isomer, ruling out free α,β-decenoate as an obligate stage in the reversible β-hydroxy–β,γ-enoate transformation. Kinetic arguments[33] tend to exclude also the intermediacy of β,γ-enoate in the transformation of hydroxy acid to α,β-decenoate. The one model that was still compatible with the kinetic data involved two independent and reversible dehydrations and an associated isomerization of the two enoates. By introducing an intermediate X which connects all three reactants, one arrives at a kinetically satisfactory model



Experiments designed to identify this central intermediate are described below.

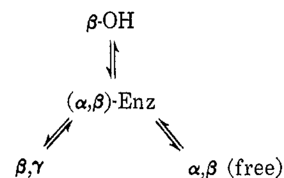
Labeling Experiments.[35] The fate of hydrogen at C_α and C_γ during the dehydrase-catalyzed reactions is of obvious interest since these atoms are necessarily removed and added regardless of mechanism. Sub-

(34) L. R. Kass and K. Bloch, *Proc. Natl. Acad. Sci. U. S.*, **56**, 1168 (1967).
(35) R. R. Rando and K. Bloch, *J. Biol. Chem.*, **243**, 5627 (1968).

stituting hydrogen by deuterium at C_α ($CH_3(CH_2)_5$-$CH_2CHOHCD_2CONAC$) caused the substrate to be dehydrated much less rapidly ($k_H/k_D = 2.25$). Deuterium substitution at C_γ ($CH_3(CH_2)_5CD_2CHOHCH_2$-$CONAC$) did not change the dehydration rate. Clearly therefore, stretching of the C_α–H bond is rate limiting in decenoate formation. In a series of labeling (in contrast to rate) experiments, the fate of hydrogen at C_α and C_γ was followed by isotope analysis of the dehydration products. Under initial velocity conditions the doubly labeled $CH_3(CH_2)_5C^3H_2{}^{14}CHOHCH_2$-$CONAC$ afforded α,β-decenoate without change in the ratio of the two isotopes. Hydrogen at C_γ is therefore not involved in the β-hydroxy $\rightarrow$ α,β-enoate transformation. Analogous experiments with $CH_3(CH_2)_5$-$CH_2{}^{14}CHOHC^3H_2CONAC$ revealed the expected loss of one atom of hydrogen from C_α in the formation of α,β-decenoate. Surprisingly though, the same loss of tritium at C_α occurred in the reversible dehydration to β,γ-enoate. In fact, early in the reaction, the isotope ratios in α,β-decenoate and β,γ-decenoate were identical. Clearly, the reversible removal of water from β-hydroxy acid to form β,γ-enoate is not a direct process. The interpretation of this result is obvious; a product containing only one hydrogen at C_α lies on the path connecting β-hydroxydecanoate and β,γ-decenoate. For reasons discussed elsewhere,[35] we conclude that the intermediate on the path to β,γ-decenoate is, in fact, α,β-decenoate and that it arises by concerted dehydration.[36] While the sequence β-OH $\rightleftharpoons$ α,β $\rightleftharpoons$ β,γ is mechanistically sound, an obligatory intermediacy of α,β-decenoate in the β-hydroxy $\rightarrow$ β,γ-enoate conversion is in clear conflict with the kinetic data presented above. As will be recalled, these data had ruled out *free* α,β-decenoate as a compulsory intermediate. The emphasis here should be on *free* α,β-decenoate, *i.e.*, product not associated with any other component of the system. (When the molar ratio of substrate to catalyst is very large, as is ordinarily the case in enzyme assays, the measured concentrations are of course those of the free reactants.) An escape from the dilemma may be the assumption that the chemical intermediate in question is indeed α,β-decenoate, but that it is enzyme bound when first formed. The complex could then either isomerize to β,γ-enoate or dissociate to enzyme and free α,β-decenoate.

Trapping Experiments. Our evidence for the existence and identity of X is so far indirect. In the experiments to be described, we present chemical evidence that excludes free α,β-decenoate and thereby implicates enzyme-bound α,β-decenoate as the intermediate in question.

If free α,β-decenoate were a compulsory intermediate, then any reaction removing it irreversibly from the system should quench the formation as well as rehydration of β,γ-decenoate. Michael addition of a mercaptan to the conjugated α,β-decenoyl-NAC to form a β-substituted thioester proved suitable for the purpose of trapping α,β-decenoate. At pH 8, 5×10^{-3} M N-acetylcysteamine reacts rapidly with α,β-decenoyl-NAC as judged by the disappearance of material absorbing at 263 mμ. When β,γ-decenoyl-NAC is incubated with enzyme, α,β-decenoate absorbing at 263 mμ appears rapidly (isomerase reaction). Addition of N-acetylcysteamine (5×10^{-3} M) to the same enzymatic system suppresses these spectral changes. An equivalent amount of product (radioactive) is now associated with a highly polar fraction which is identical chromatographically with Michael adduct formed nonenzymatically from NAC and α,β-decenoyl-NAC. Thus, the mercaptan did not interfere with the enzyme-catalyzed β,γ $\rightarrow$ α,β isomerization. More significantly, it failed to reduce the rate of conversion of β,γ-decenoate to β-hydroxy acid. Since the latter reaction proceeded normally even though α,β-decenoate had been trapped irreversibly and diverted to the Michael adduct, *free* α,β-decenoate cannot lie on the path from β,γ-enoate to β-hydroxy acid. This conclusion, of course, reinforces the kinetic results and adds weight to the argument that the intermediate in question is enzyme-bound and not free α,β-decenoate. The model for the dehydrase-catalyzed reaction is therefore modified to



It seems quite remarkable that α,β-decenoate, when enzyme bound, appears to be protected from reacting with mercaptans.

By postulating an α,β-decenoate–enzyme complex as the common intermediate for connecting the three reactants in the dehydrase-catalyzed reaction, we can harmonize all our kinetic, chemical, and labeling data. It is clear that the enzyme adds a special dimension to an otherwise unremarkable set of chemical reactions.

Active-Site Studies. A complete understanding of an enzyme mechanism must include a description of the events occurring at the active enzyme site. The minimal chemical events in the dehydrase-catalyzed system are hydrogen addition and abstraction at C_α and C_γ and the reversible removal of OH^-. Organic cofactors or metal ions are not involved in the catalysis, and therefore amino acid side chains of the polypeptide structure must serve as general acid and general base catalysts. The effects of a number of chemical reagents on dehydrase activity are shown in Table I.[37]

(36) One argument against the alternative possibility of a C_α carbanion intermediate—which could be stabilized by the enzyme—is the lack of appreciable H exchange at this position under normal assay conditions.

(37) G. M. Helmkamp, unpublished; some recent evidence suggests that one or possibly two tyrosine residues participate in the catalysis (see Table I).

Table I

Chemical Modifications of β-Hydroxydecanoyl Thioester Dehydrase

Reagent	Target amino acid(s)	Inactivation	Modified amino acid
Iodoacetate	Cysteine	None	
Iodoacetamide	Cysteine	None	
N-Ethylmaleimide	Cysteine	None	
p-Hydroxymercuribenzoate	Cysteine	None	
Phenylmethanesulfonyl chloride	Serine	None	
Acetic anhydride	Lysine	None	
2,4-Dinitrofluorobenzene	Lysine	None	
Bromoacetate (5×10^{-2} *M*, pH 7)	Histidine	$k = 1.50\ M^{-1}\ \text{hr}^{-1}$	One 3-carboxymethylhistidine
3-Decynoyl-N-acetylcysteamine (10^{-6} *M*, pH 7)	Histidine?	$k = 0.14\ \text{min}^{-1}$	Loss of one histidine
Tetranitromethane (10^{-3} *M*, pH 7)	Tyrosine (tryptophan, cysteine)	+	Four 3-nitrotyrosines
3-Decynoyl-N-acetylcysteamine followed by tetranitromethane	Tyrosine (tryptophan, cysteine)	+	Two 3-nitrotyrosines

Reagents directed at reactive serine or cysteine residues or ε-amino groups of lysine are without effect on enzyme activity. However, there is clear-cut evidence that one histidine residue participates in the catalysis. The enzyme is sensitive to photooxidation in the presence of methylene blue and to alkylation by bromoacetate at pH 7.0. Alkylation reduces the number of histidine residues recoverable in the protein hydrolysate from 4 to 3 and results in the appearance of 3-carboxymethylhistidine. Since alkylation blocks all the reactions catalyzed by the dehydrase (*i.e.*, isomerization as well as dehydration), the susceptible histidine residue is most likely concerned with proton abstraction and addition at C_α of the substrate. A second nucleophilic site on the enzyme is probably needed as a proton acceptor and donor at C_γ. This second site has not yet been identified.[37] Hydrogen at C_γ does not participate in the interconversion of β-OH acid and α,β-enoate, and hence the blocking of this presumed second site could conceivably convert the multifunctional enzyme into a conventional α,β-dehydrase. For steric reasons, the selective inactivation of one of the enzyme activities may be difficult to achieve.

Enzyme Inhibition. 3-Decynoyl-NAC, the acetylenic analog of one of the substrates, is a potent (apparent $K_I = 2 \times 10^{-7}\ M$) and highly specific inhibitor of the dehydrase.[33,37,38] The fact that all the reactions catalyzed by the enzyme are affected equally adds another strong argument in favor of catalysis by a single multifunctional enzyme. The acetylenic inhibitor inactivates the enzyme more rapidly than bromoacetate by a factor of 10^6 and forms a stoichiometric complex with protein in a ratio of 1 mol/28,000 g of enzyme. The acid hydrolysate of this complex contains one histidine less than the native enzyme. When the enzyme is first alkylated with bromoacetate and treated with acetylenic inhibitor afterwards, the number of derivatized histidine residues remains at one. This is true also when the inhibitor sequence is reversed, *i.e.*, enzyme is first exposed to the acetylenic inhibitor and then to bromoacetate. Thus the same histidine residue appears to be the target for both reagents.[37]

The chemistry of the interaction between enzyme and acetylenic inhibitor is not known. Radioactive enzyme–inhibitor complexes are formed whether 3-decynoyl-NAC is labeled in the fatty acyl chain or in the NAC portion. Enzyme acylation with elimination of the thioester function is therefore ruled out. These findings and the evidence that the interaction is covalent narrow the possibilities to a reaction between histidine and some element of the $-C{\equiv}CCH_2-$ grouping.

In consequence of its irreversible covalent binding, 3-decynoyl-NAC blocks the action of the enzyme noncompetitively.[38] Yet the close structural resemblance between substrate and inhibitor would suggest that the two molecules compete initially for the same enzyme binding site. The observed lack of competition in the kinetic sense arises from the extraordinary chemical reactivity of the β-acetylenic grouping which leads to irreversible enzyme inactivation. On the other hand, in the encounter between substrate and enzyme covalent bonds do not appear to be formed at any time. The substrate therefore provides no protection against the inhibitor.

As already mentioned, there is strong evidence for the identity of one of the catalytic sites with the site at which the acetylenic inhibitor attaches. Other data make it quite clear that the inhibitor molecule as a whole occupies the same region which ordinarily accommodates the substrate. As is seen in Figure 6, the plots of apparent K_I for inhibitors and of $1/V$ or K_m for substrates as a function of chain length are nearly coincident. For both substrate and inhibitor chain length is optimal at C_{10}, the activities declining slightly for C_9 and C_{11} and to almost zero for C_8 and C_{12}. Furthermore, the free acid and the methyl ester are without effect on enzyme activity. Thus the acetylenic compounds inhibit only if they have the same overall dimensions as the substrates. As structural substrate analogs they are highly specific active-site directed agents, yet they show noncompetitive behavior because of their chemical reactivity.

A second determinant of inhibitor activity is the

(38) G. M. Helmkamp, R. R. Rando, D. J. H. Brock, and K. Bloch, *J. Biol. Chem.*, **243**, 3229 (1968).

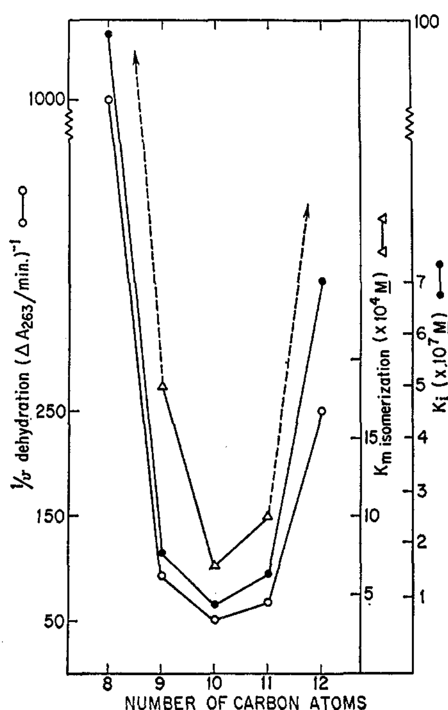


Figure 6. Kinetic parameters of dehydrase substrates and inhibitors as a function of chain length.[38]

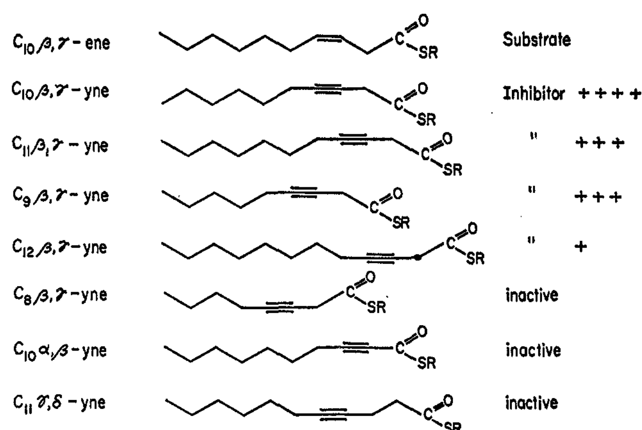


Figure 7. Effect of chain length and triple bond position on inhibitor potency of alkynoic acid thioesters.

location of the triple bond[38] (Figure 7). Unless this bond is in β,γ position to the thioester function enzyme activity is not affected. Neither 2-decynoyl-NAC nor 4-undecynoyl-NAC interferes with the catalytic process even in relatively high concentration. The inactivity of the C_{11}-γ,δ-acetylene is noteworthy since the alkyl portion of the molecule extending from the terminal methyl group through the triple bond is identical with that of the potent 3-decynoyl-NAC. A methylene group spaced between the acetylenic bond and the carbonyl group appears essential for the covalent attachment to enzyme. Whether the presumably facile isomerization of the β,γ-acetylene to an allene plays a role in this interaction remains to be ascertained.

The combination of two properties makes the acetylenic compound an unusually selective inhibitor: (1) irreversible attachment to a single amino acid side chain in the dehydrase and (2) failure to inactivate any other enzyme, except in very much higher concentrations. The response of the fatty acid synthetase of *E. coli* to 3-decynoyl-NAC illustrates the second point.[34] As noted, this system consists of several enzymes which cooperatively catalyze chain elongation of acetyl-CoA to a mixture of long-chain saturated and unsaturated acids. In the presence of 1×10^{-5} *M* 3-decynoyl-NAC this product pattern changes drastically.[39] The elaboration of olefinic acids is completely blocked, while the synthesis of saturated end products continues unimpaired. A selective inactivation of the β-hydroxydecanoyl thioester dehydrase would account for these results. Indeed, the "poisoned" fatty acid synthetase can be reactivated by supplementation with fresh dehydrase. The system then reverts to normal and fully regains the ability to synthesize unsaturated fatty acids. The same results settle one troublesome question which was raised by the kinetic properties of the dehydrase. When studied as an isolated process, the dehydration of β-hydroxydecanoate is found to yield primarily α,β- rather than β,γ-decenoate. Yet in the complete fatty acid synthetase system unsaturated fatty acids, the elongation products of β,γ-decenoate, predominate. The dehydrase would therefore seem to behave differently under these two circumstances. Whatever the explanation of this phenomenon, the supplementation experiments just mentioned clearly prove that the dehydrase alone is responsible for generating the olefinic precursor of palmitoleate and vaccenate.[34] The same point has been elegantly demonstrated with the aid of the mutant technique.[40]

The selective action of 3-decynoyl-NAC on bacterial fatty acid synthesis requires one further comment. Since 3-decynoyl-NAC blocks all the dehydrase-catalyzed reactions, including the formation of α,β-decenoate, and since α,β-decenoate is an obligatory precursor for saturated acids, one would expect the inhibitor to abolish fatty acid synthesis altogether. This puzzle has recently been resolved by the finding that *E. coli* contains additional β-hydroxyacyl dehydrases. These enzymes have a broader chain-length specificity but appear to produce α,β-enoates only.[41,42] The α,β-enoate forming ability of the C_{10}-specific dehydrase is therefore physiologically gratuitous. The reason why it occurs nevertheless appears to be mechanistic: the sequence β-OH acid $\rightarrow$ α,β-enoate $\rightarrow$ β,γ-enoate is chemically the most favorable.

Selective inhibition of fatty acid synthesis by acetyl-

(39) Certain microbial fatty acid synthetases which are enzymatically quite similar to the *E. coli* system but synthesize only saturated acids (*e.g.*, those from *B. subtilis* and *E. gracilis*) are completely resistant to 3-decynoyl-N-acetylcysteamine (P. Butterworth and J. Delo, unpublished).

(40) D. F. Silbert and P. R. Vagelos, *Proc. Natl. Acad. Sci. U. S.*, **58**, 1579 (1967).
(41) C. H. Birge, D. F. Silbert, and P. R. Vagelos, *Biochem. Biophys. Res. Commun.*, **29**, 808 (1967).
(42) M. Mizugaki, A. C. Swindell, and S. J. Wakil, *ibid.*, **33**, 520 (1968).

enic substrate analogs is observed not only *in vitro* but also in cultures of *E. coli*.[43] What appears to be an interference with a single step in a metabolic pathway leads to an early arrest of bacterial growth. Oleic and vaccenic acids, products whose synthesis is blocked by the acetylene, overcome the action of the inhibitor and restore bacterial growth to normal. These results prove two points: (1) a long-chain unsaturated fatty acid is essential for the growth of *E. coli* (see also ref 40), and (2) the dehydrase is the only inhibitable enzyme in the bacterium. Other bacterial species which synthesize unsaturated fatty acids by the dehydrase-mediated mechanism are equally sensitive to the acetylenic analogs.[44] However, the effect is one of selective bacteriostasis and not of general toxicity. Yeast and animal cells in tissue culture, which produce long-chain unsaturated fatty acids by oxidative desaturation and hence lack the target for the inhibitor, grow normally in the presence of the acetylenic compounds.[43]

A powerful and selective inhibitor of a bacterial enzyme is potentially a useful antibacterial agent. Unfortunately, however, 3-decynoyl-NAC does not promise to be of practical value for chemotherapy. It fails to protect animals against bacterial infections, presumably because of rapid inactivation by serum.[44] Nevertheless, the case of 3-decynoyl-NAC is an example of a more systematic approach to the development of antibacterial agents which utilizes the greatly expanded knowledge of intermediary metabolism and of comparative enzymology to pinpoint appropriate targets for antimetabolites.

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(43) L. R. Kass, *J. Biol. Chem.*, **243**, 3223 (1968).
(44) Some of these tests were performed in the Medical Research Laboratories, Charles Pfizer & Co., Groton, Conn., through the courtesy of Dr. Arthur English.

The Role of Antimetabolites in Immunosuppression and Transplantation

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Immunological reactions are an essential part of an organism's defense mechanisms against foreign invaders. These reactions work to the advantage of the individual in some situations, *e.g.*, the synthesis of antibodies to combat infectious disease, but may have undesirable and even fatal consequences in others. Examples of the latter are the anaphylactic shock produced in sensitized individuals on further exposure to the antigen which produced the sensitization, *e.g.*, horse serum proteins, and the "autoimmune" diseases (such as autoimmune hemolytic anemia and lupus erythematosus) in which the organism seemingly becomes confused in his discrimination between self and nonself. Tissue and organ transplantation have been technically feasible for many years, as shown by the indefinite survival of autografts (reimplanted in the same individual). However, allografts (from another individual of the same species) are rejected rapidly and become nonviable within a matter of days. It is evident that the ability to control the immune response would enable the physician to deal effectively with a number of serious medical problems including, but by no means limited to, the rejection of a transplanted organ.

Considerable progress has been made in the discovery of drugs to control the immune response. Some of the most effective drugs are antimetabolites which interfere with the normal metabolic pathways involving nucleic acids. This Account will concentrate on the clues that studies with antimetabolites, and in particular with thiopurine derivatives, have provided with respect to immune mechanisms. These compounds have provided the point of departure for transplantation and for much investigative work on basic immunology. A number of reviews that cover immunosuppressive agents more broadly are available.[1-3]

Features of the Immune Response

The small lymphocytes make up about 20% of the white cells in the circulating blood of man. They are the major cellular type in lymph nodes, thymus, and spleen (lymphoid tissues) and are also distributed diffusely in other tissues, such as bone marrow and intestinal mucosa. Unlike other leukocytes, they are neither phagocytic nor chemotactic, nor do they divide continuously.

(1) G. H. Hitchings and G. B. Elion, *Pharmacol. Rev.*, **15**, 365 (1963).
(2) R. S. Schwartz, *Progr. Allergy*, **9**, 246 (1965).
(3) M. C. Berenbaum, *Brit. Med. Bull.*, **21**, 140 (1965).