Mechanism of Action of \( \beta \)-Hydroxydecanoyl Thioester Dehydrase*

(Received for publication, July 10, 1968)

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SUMMARY

Studies on the mechanism of the reversible enzymatic dehydration of \( D(-)\)-\( \beta \)-hydroxydecanoyl-\( N \)-acetylcysteamine to \( \text{trans-2-decenoyl-N-acetylcysteamine} \) and \( \text{cis-3-decenoyl-N-acetylcysteamine} \) are presented. They include determination of kinetic isotope effects with deuterium-labeled substrates and labeling experiments designed to follow the fates of the \( \alpha \) - and \( \gamma \)-hydrogen atoms during the course of the reactions. The results show that \( \text{trans-2-decenoyl-N-acetylcysteamine} \) is formed directly by dehydration of \( \beta \)-hydroxydecanoate, whereas \( \text{cis-3-decenoyl-N-acetylcysteamine} \) arises only by isomerization of the conjugated enoate. The present findings, in conjunction with earlier kinetic data, lead to the conclusion that interconversion of the three substrates involves a common intermediate, presumably enzyme-bound \( \alpha,\beta \)-decanoate.

The multifunctional enzyme, \( \beta \)-hydroxydecanoyl thioester dehydrase, isolated from \( E. coli \) is unusual in that it possesses both dehydrase and isomerase activities (1). Under the agency of this enzyme, equilibrium among \( D(-)\)-\( \beta \)-hydroxydecanoate, \( \text{trans-2-decenoyl-N-acetylcysteamine} \), and \( \text{cis-3-decenoyl-N-acetylcysteamine} \) is readily attained.

The initial velocities for interconversion of the three substrates have been reported (2). It was shown that isomerization of \( \beta,\gamma \)-decanoate to \( \alpha,\beta \)-decanoate was by far the most rapid of the reactions catalyzed by the enzyme. It could be inferred from the data that a hydration step is not obligatory for interconverting the two unsaturated thioesters, and hence that the dehydrase can also function as a double bond isomerase.

The present mechanistic studies were undertaken to uncover the sequence of events occurring during the interconversions of the three substrates. Specifically, we wished to know whether \( \text{trans-\( \alpha,\beta \)- and cis-\( \beta,\gamma \)-decanoates} \) can be formed independently from \( \beta \)-hydroxydecanoate or whether they arise by way of a common intermediate. The problem was approached by (a) labeling the substrates with tritium at \( C_\alpha \) and \( C_\gamma \) and following the fate of labeled hydrogen early in the reaction, (b) measuring the influence of deuterium substitution at \( C_\alpha \) and \( C_\gamma \) on the rate of dehydration of \( \beta \)-hydroxydecanoate, and (c) examining the enzymatic hydration of \( \text{cis-\( \beta,\gamma \)-decanoate} \) in the presence of a trapping reagent which removes \( \alpha,\beta \)-decanoate irreversibly from the system.

The thioester substrates for these experiments were in all cases the \( N \)-acetylcysteamine derivatives.

EXPERIMENTAL PROCEDURE

Materials

\( \text{H}_2\text{O} \) (1 C per g) and \( \text{NaB}_3\text{H}_4 \) (100 mC per mmole) were purchased from New England Nuclear, 99% acetic acid-d\(_4\) from Volk Radioisotopes Corporation, and 99.8% \( \text{D}_2\text{O} \) from Merck. Unisil silicic acid (100 to 200 mesh) was a product of Clarkson Chemical Company, and Silica Gel HF\(_{254}\) was a product of Brinkmann Instruments, Inc., Westbury, New York. \( \text{DL-3-}^{14}\text{C}-\beta \)-Hydroxydecanoyl-\( N\)-AC, \( \text{1-}^{14}\text{C-cis-3-decenoyl-NAC} \), \( \text{2-}^{14}\text{C-trans-2-decenoyl-NAC} \), and \( \text{trans-2-decenoyl-NAC} \) were gifts of Dr. Leon Kass. \( N \)-Acetylcysteamine was prepared by a published procedure (3). Pronase, grade B, was purchased from Calbiochem. A fatty acyl thioesterase from \( E. coli \) was a gift of W. Bonner. Highly purified dehydrase (1200-fold and 2000-fold) was provided by Dr. Leon Kass and G. Helmkamp. The dehydrase was purified by the published procedure (1). Specific enzyme activity is expressed as units per mg of protein; one unit of activity is defined as the formation of 1 mmpmole of \( \alpha,\beta \)-decanoate from \( \beta,\gamma \)-decanoate per min under specified conditions. A 1000-fold purified enzyme preparation has a specific activity of 3000.

Synthesis of Substrates

\[
\begin{align*}
\text{Methyl } \alpha\text{D}-\beta \text{-Hydroxydecanoate} & \quad \text{O} \\
\text{CD}_3\text{-C-OD} + \text{Br}_2 & \quad \text{P} \\
\text{Br-CD}_3\text{-C-Br} & \quad \text{MeOD}
\end{align*}
\]
Methyl α-dideuterobromocetate was prepared by brominating 2.0 g (31 mmole) of tetradecanoic acid with 23 g of dry bromine in the presence of 0.35 g of red phosphorus followed by the addition of 3.21 g of deuterated methanol (MeOD). A 50% yield (16 mmole) of product was obtained after distillation.

The brominated dideuterate ester (0.50 g; 3.2 mmole) was allowed to react in the usual manner (4) with n-octanal (0.43 g; 3.2 mmole) in the presence of zine (0.24 g; 3.7 mmole) to afford 0.16 g of αD2-β-hydroxydecanoic acid after hydrolysis and several crystallizations from pentane (m.p. 55–55.2°). The infrared and nuclear magnetic resonance spectra of this compound were consistent with the assigned structure.

The mixed anhydride of αD2-octanoic acid (1.8 g; 12.2 mmole) was prepared in the usual manner to afford the αH-β-hydroxydecanoate. The procedure used for the preparation of αH-β-hydroxydecanoate-NAC has been described (1). The product had a specific activity of 0.2 C per mole.

αD2-Octanoic acid was prepared by exchanging to completion (5) the acidic protons of n-hexylmalonic acid in 99.8% D2O (Merck) and decarboxylating the resultant deuterated malonic acid derivative at 150°.

The mixed anhydride of αD2-octanoic acid (1.8 g; 12.2 mmole) was prepared in the usual manner by allowing the acid to react with 1.3 g (12.2 mmole) of ethyl chloroformate in the presence of 1.1 g (12 mmole) of triethylamine. The resultant anhydride was combined with the magnesium complex of monoethyl malonic acid, which was prepared by allowing 1.6 g (13.5 mmole) of the monoethyl ester of malonic acid to react with 0.3 g of magnesium (6). After acidification, crude ethyl αD2-β-keto-decanoate was obtained. Reduction of this compound with sodium borohydride followed by hydrolysis afforded, after chromatography on silicic acid and recrystallization from pentane, 250 mg of pure γD3-β-hydroxydecanoic acid, m.p. 55–55.2°.

The mass spectrum of this compound showed that it contained 63% α-dideuterated and 37% α-monodeuterated species. Hence, the fraction of molecules susceptible to deuterium removal by the enzyme is 81.5% (63 + 37/2). Some deuterium exchange occurs during the preparation of the thioester under the slightly basic conditions (pH 8) used.

Ethyl γD2-β-hydroxydecanoate

2-Nonyl aldehyde (7) was reduced with NaB3H4 in the usual manner to afford the αH-β-alcohol. The procedure used for the conversion of this alcohol to cis-3-decenoic-NAC has been described (1). The product had a specific activity of 6.6 C per mole.

αH-β-hydroxydecanoate-NAC—The procedure was the same as described for the preparation of the αD3 compound except that n-hexylmalonic acid was exchanged with 3H2O (1 C per g). The product had a specific activity of 6.6 C per mole.

αH-β-hydroxydecanoate-NAC—The procedure was the same as described for the preparation of the γD3-NAC except that n-hexylmalonic acid was exchanged with 3H2O (1 C per g).

The product had a specific activity of 2.4 C per mole.

Enzyme Assays

Incubations were carried out at 30° in 0.01 m potassium phosphate, pH 7.0, unless otherwise noted. The radioactive assay procedure has been described in detail (2). Briefly, it consisted of extracting substrates and products from the incubation mixture with ether. After drying and evaporating the ether, the mixture of thioesters was applied to Unisil silicic acid (100 to 200 mesh) columns. The nonpolar fraction (trans-α, β- and cis-β, γ-decenoates) was eluted with 0.75% methanol in CH2Cl2. The polar fraction (β-hydroxydecanoate) was eluted with 10% methanol in CH2Cl2. After evaporation of the solvent the β-hydroxydecanoate fraction was counted. The α, β-trans- and β, γ-cis-decenoates were separated after hydrolysis of the NAC derivatives with Pronase B. The resultant acids were converted to the methyl esters with diazomethane; carrier methyl esters were added and separations were performed by gas-liquid chromatography (2). All the fractions were counted in toluene-
2,5-diphenyloxazole (PPO). The production of \( \text{trans-} \alpha,\beta \)-decanoate could be measured conveniently by spectrophotometry; \( \lambda_{\text{max}} \) at 263 nm; \( e = 6.7 \times 10^4 \).

In all experiments nonenzymatic blanks were subtracted prior to final calculation.

**RESULTS**

*Rates of Dehydration of \( \alpha D_2\beta \)-Hydroxydecanoyl-NAC and \( \gamma D_2\beta \)-Hydroxydecanoyl-NAC*

Solutions of \( DL-\beta \)-hydroxydecanoyl-NAC, \( DL-\alpha D_2\beta \)-hydroxydecanoyl-NAC, and \( DL-\gamma D_2\beta \)-hydroxydecanoyl-NAC (all \( 3.7 \times 10^{-4} \) M) were prepared in 0.01 M potassium phosphate, pH 7.0. On the addition of purified (1) dehydrase (specific activity, 100) the change in absorption at 263 nm was measured with each of the substrates on a Cary model 14 recording spectrophotometer. The time course of the reactions is given in Fig. 1. The measured kinetic isotope effect for the \( \alpha \)-deuterated substrate was 1.84. After correction for the amount of \( \alpha \)-hydrogen present in the deuterated substrate, \( k_{\text{H}}/k_{\text{D}} = 1.84/0.815 = 2.25 \). There was no measurable isotope effect when the substrate was \( \gamma D_\beta \)-hydroxydecanoyl-NAC.

**Enzymatic Dehydration**

\( DL-\gamma^3H-3^14C-\beta \)-Hydroxydecanoyl-NAC

To a solution containing 130 mmoles of \( \gamma^3H-\beta \)-hydroxydecanoyl-NAC (1.9 \( \times 10^4 \) dpm) and 130 mmoles of \( 3^14C-\beta \) hydroxydecanoyl-NAC (5.8 \( \times 10^4 \) dpm) in 1 ml of 0.01 M potassium phosphate, pH 7.0, 2 \( \mu \)g of dehydrase (specific activity, 2000) were added. After 4 min of incubation the reactions were terminated by the addition of 1 drop of concentrated HCl. The polar and nonpolar fractions were separated and their \( ^3H:14C \) ratios were determined. The results are given in Table I, along with the results of experiments with larger amounts of more highly purified enzyme incubated for longer time periods. When the \( ^3H:14C \) ratios for \( \alpha,\beta \)-decanoate and \( \beta,\gamma \)-decanoate were determined separately, the NAC derivatives were hydrolyzed with Pronase B, converted to the methyl esters, and analyzed by gas-liquid chromatography.

**Enzymatic dehydration of \( \gamma^3H-3^14C-\beta \)-hydroxydecanoyl-NAC**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( (\text{H}:14C)_{\beta-\text{OH}} )</th>
<th>( (\text{H}:14C)_{\beta'\text{OH}} )</th>
<th>( (\text{H}:14C)_{\beta,\gamma'} )</th>
<th>( (\text{H}:14C)_{\beta,\gamma} )</th>
<th>Reaction time</th>
<th>Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.0</td>
<td>10.0</td>
<td>9.1</td>
<td>4.0</td>
<td>240</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>10.6</td>
<td>10.6</td>
<td>10.5</td>
<td>220</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13.6</td>
<td>11.6</td>
<td>10.6</td>
<td>163</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14.7</td>
<td>9.0</td>
<td>8.3</td>
<td>645</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

* Early in the reaction, the extent of conversion is small and therefore the radioactivity in products is too low for separate analysis of the two isomeric decanoates.

| Substrate reisolated after incubation. | Average \( (\text{H}:14C)_{\text{recovered } \beta \text{-hydroxy acid}} = 12.48 \) | Average \( (\text{H}:14C)_{(\alpha,\beta;\beta,\gamma')-\text{decanoates}} = 4.94 \) | Average \( (\text{H}:14C)_{(\alpha,\beta+\beta,\gamma) \text{-decanoates}} = 12.82 \) | Average \( (\text{H}:14C)_{(\alpha,\beta,\gamma')-\text{decanoates}} = 5.13 \)

**Analysis of Polar and Nonpolar Fractions—Solutions containing 350 mmoles of \( DL-\alpha^3H-3^14C-\beta \)-hydroxydecanoyl-NAC (9.1 \( \times 10^4 \) dpm) and 350 mmoles of \( DL-3^14C-\beta \)-hydroxydecanoyl-NAC (2.9 \( \times 10^4 \) dpm) in 1 ml of 0.01 M potassium phosphate, pH 7.0, were incubated with \( 5 \mu \)g of dehydrase (specific activity, 1800) for 20 min. Reactions were terminated by the addition of 1 drop of HCl. The thioesters were extracted into ether, dried, and chromatographed on Unisil silicic acid (100 to 200 mesh). The \( ^14C \) ratios for the recovered \( \beta \)-hydroxydecanoyl thioester and the mixture of unsaturated thioesters were determined. The results of quadruplicate experiments are shown in Table II.

**Analysis of Separated \( \alpha,\beta \)- and \( \beta,\gamma \)-Decanoates—A solution of 484 mmoles of \( \alpha^3H-\beta \)-hydroxydecanoyl-NAC (1.3 \( \times 10^4 \) dpm) and 316 mmoles of \( 3^14C-\beta \)-hydroxydecanoyl-NAC (2.6 \( \times 10^4 \) dpm...
from E. coli (1). After hydrolysis of the thioesters, the methyl hydrolyzed by incubation with a fatty acyl thioesterase isolated for 20 min. The polar and nonpolar fractions were separated by gas-liquid chromatography. The results are given in Table III.

**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nonpolar fraction (mg + βH)</th>
<th>α,β-Decenolate</th>
<th>β,γ-Decenolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>13C, cpm</td>
<td>60,115</td>
<td>3,106</td>
<td></td>
</tr>
<tr>
<td>2H, cpm</td>
<td>488,310</td>
<td>10,920</td>
<td></td>
</tr>
<tr>
<td>1H+13C</td>
<td>7,29</td>
<td>3.51</td>
<td>3.22</td>
</tr>
</tbody>
</table>

* 4 x 10^-4 M.  
* Percentage conversion based on active α( ) isomer = 0%.  
* Analyzed by gas-liquid chromatography.

The α,β- and β,γ-unsaturated thioesters were hydrolyzed in the usual manner, and the unsaturated thioesters were separated by gas-liquid chromatography. The results are given in Table III.

**Enzymatic Hydration of αH-1-14C-cis-3-decenoyl-NAC**

A solution containing 150 mmoles of αH-cis-3-decenoyl-NAC (6.3 x 10^4 dpm) and 100 mmoles of L-14C-cis-3-decenoyl-NAC (2.4 x 10^4 dpm) in 1 ml of 0.01 M Tris-HCl, pH 7.0, was incubated with purified dehydrase (4 μg, specific activity, 300) for 5 min. The polar fraction was isolated and the 14C to 3H ratio was determined in the usual manner. The results of a typical experiment are given in Table IV.

**Table IV**

<table>
<thead>
<tr>
<th>Time</th>
<th>Substrate</th>
<th>α-Hydroxydecanoate</th>
<th>Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>14C</td>
<td>14C</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>13,644</td>
<td>15,200</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>6,82</td>
<td>295</td>
<td>0.433</td>
</tr>
</tbody>
</table>

* 2.5 x 10^-4 M.  

Enzymatic Hydration of αH-1-14C-cis-3-decenoyl-NAC

A solution containing 150 mmoles of αH-cis-3-decenoyl-NAC (6.3 x 10^4 dpm) and 100 mmoles of L-14C-cis-3-decenoyl-NAC (2.4 x 10^4 dpm) in 1 ml of 0.01 M potassium phosphate, pH 7.0, was incubated with purified dehydrase (specific activity, 1800) for 5 min. The polar fraction was isolated and the 14C to 3H ratio was determined in the usual manner. The results of a typical experiment are given in Table III.

**Enzymatic Isomerization of cis-3-Decenoyl-NAC in Presence of N-Acetylcysteamine**

A solution containing 125 mmoles of cis-3-decenoyl-NAC in 1 ml of 0.1 M Tris-HCl, pH 7.95, was transferred to a cuvette and after the addition of 2 μg of dehydrase (specific activity, 1500) the change of optical density was measured with a Cary model 14 spectrophotometer. In parallel experiments these rates were measured in the presence of NAC (final concentrations, 1.84 x 10^-4 M and 5.52 x 10^-4 M, respectively). Plots of absorbance at 263 μM versus time are given in Fig. 2.

**Enzymatic Hydration of 13C-cis-3-Decenoyl-NAC in Presence of NAC**

Solutions containing 47 mmoles of 13C-cis-3-decenoyl-NAC (25,420 cpm) in 1 ml of 0.1 M Tris-HCl, pH 7.95, were incubated with or without NAC (final concentration, 5.52 x 10^-4 M). In all cases 2 μg of dehydrase (specific activity, 1500) were added. Incubations were allowed to proceed at 30°C for 20 min. The reactions were quenched and the products were extracted in the usual manner. The polar products (β-hydroxy esters + Michael addition product) were separated from the unsaturated thioesters on silicic acid (Unisil, 100 to 200 mesh). The Michael product is eluted with 10% methanol in CH2Cl2 along with the β-hydroxythioesters. This was shown by preparing radioactive addition product from 13C-trans-Δ5-decenoyl-NAC and free NAC as described above and determining its chromatographic behavior.

The α,β- and β,γ-unsaturated thioesters were hydrolyzed with Pronase B. The liberated acids were esterified and the products were analyzed by gas-liquid chromatography in the usual manner. The β-hydroxysterthioesters and Michael decrease in absorbance at 263 μM was measured in a Gilford model 210 spectrophotometer. Under these conditions the sulfhydryl reagent was present in a 20 fold molar excess. At pH 7.0 the initial rate of α,β-decenolate consumption was 3.74 μmoles per min and at pH 7.95, 13.8 μmoles per min. The initial substrate concentration was 1.25 x 10^-4 M in 0.1 M Tris-HCl, pH 7.95; 2 μg of dehydrase (specific activity, 1500) were used.

**Fig. 2.** Initial rates of α,β-decenolate formation from cis-3-decenoyl-NAC in the absence of NAC (○), in the presence of 1.8 x 10^-3 M NAC (●), and in the presence of 5.52 x 10^-4 M NAC (△). The initial substrate concentration was 1.25 x 10^-4 M in 0.1 M Tris-HCl, pH 7.95; 2 μg of dehydrase (specific activity, 1500) were used.
product were effectively separated by thin layer chromatography on HF2s4 silicic acid with 3.5% methanol in CH$_2$Cl$_2$ as eluent. Under these conditions the Michael product did not migrate from the base-line. When the total radioactivity in polar and nonpolar fractions, the relative amounts of radioactivity in the $\alpha$, $\beta$- and $\beta$, $\gamma$-enoates, and the relative amounts of Michael product and $\beta$-hydroxythioesters are known, the absolute amounts of each product can be calculated. As expected, no $\alpha$, $\beta$-decenoate could be detected by gas-liquid chromatography when the trapping agent (NAC) was present during incubation. The results of a typical experiment are given in Table V.

**DISCUSSION**

The enzyme $\beta$-hydroxydecanoyl thioester dehydrase has been purified to near homogeneity (1); it has a molecular weight of approximately 28,000. It requires neither organic cofactors nor metal ions for activity. Its function is to introduce a double bond $\beta$, $\gamma$ to the thioester moiety at the C10 level in the course of the synthesis of long chain unsaturated fatty acids by certain bacteria. The enzyme is multifunctional, catalyzing the reversible interconversions of the thioesters of $\nu(-)$-$\beta$-hydroxydecanoate, trans-2-decenoate, and cis-3-decenoate.

The primary goal of any mechanistic investigation is to deduce from experimental evidence the potential energy surface that connects the various reacting entities. In the current investigation we sought to determine whether the potential energy surface for the reactions could best be represented by diagrams A, B, or C (Fig. 3). Mechanism C seems the least likely of the three, since it would appear kinetically unreasonable to arrive at $\alpha$, $\beta$-decenoate from $\beta$-hydroxydecanoate via the relatively unstable, unconjugated $\beta$, $\gamma$-decenoate.

In their kinetic study on the initial rates of interconversion of $\beta$-hydroxydecanoyl-NAC, trans-2-decenoyl-NAC, and cis-3-decenoyl-NAC, Brock, Kass, and Bloch (2) found the values shown below.

These values are not rate constants, but a composite of both initial rate constants and binding constants. In the same report the equilibrium concentrations of the three products were given as 67 to 73% $\beta$-hydroxydecanoate, 24 to 29% $\alpha$, $\beta$-decenoate, and 2 to 4% $\beta$, $\gamma$-decenoate, and were thus shown to lie in a narrow range, irrespective of the starting substrate (2).

Since the various reaction profiles depicted in Fig. 3 can be differentiated experimentally by following the fate of the $\alpha$ and $\gamma$ hydrogen atoms of some of the substrates during the course of the reactions, we have sought to elucidate the reaction mechanism with the aid of appropriately deuterated and tritiated substrates. First, the effect of deuterium substitution on the rate of $\alpha$, $\beta$-decenoate formation from $\beta$-hydroxydecanoate was investigated. The reaction rates of both $\alpha$D$_2$-$\beta$-hydroxydecanoyl-NAC and $\gamma$D$_2$-$\beta$-hydroxydecanoyl-NAC were compared with that of nondeuterated substrate by following $\alpha$, $\beta$-decenoate production spectrophotometrically (Fig. 1).

From these data the following relative rates are calculated.

$$R-\text{CH}_2-\text{CH}-\text{CD}_2-C-SX \rightarrow R-\text{CH}_2-\text{CH}=\text{CD}-C-SX$$

$k_H/k_D = 2.25$

$$R-\text{CD}_2-\text{CH}=\text{CH}-C-SX \rightarrow R-\text{CD}_2-\text{CH}-\text{CH}=C-SX$$

$K_D/K_H = 1.0$

The distinct kinetic isotope effect in the dehydration of $\alpha$D$_2$-$\beta$-hydroxydecanoyl-NAC means that stretching of the $\alpha$ $C$–$H$ bond is rate limiting in the formation of $\alpha$, $\beta$ decenoate.$^3$ By the same token the unchanged reaction rate with substrate

$^3$ In an independent experiment the apparent $K_a$ for $\alpha$D$_2$-$\beta$-hydroxydecanoyl-NAC was determined and found to be identical, within experimental error, with that of the nonlabeled substrate ($K_a = 1.7 \times 10^{-3}$ M (1)).
labeled at C7 excludes any reaction at C7 in the rate-limiting step for α,β-decenoate formation. These results are consistent with direct α,β-decenoate formation either by a carbanion or an E2 elimination mechanism (8).

Further evidence for direct α,β-decenoate formation comes from studies on the dehydration of γ3H-β-hydroxydecanoyl-NAC. If conversion of this substrate to α,β-decenoate is direct, no tritium should be lost at C7. The 3H:14C ratio in α,β-decenoate early in the reaction should be identical with that of the starting material. If, alternatively, cleavage of the γ C-H bond is involved in α,β-decenoate formation, then the 3H:14C ratio in α,β-decenoate should be one-half that in the starting material. This case would obtain either if α,β-decenoate were an obligate intermediate in α,β-decenoate formation or if the β,γ and α,β-decenoates arose via a common intermediate.

The potential energy surface interconnecting the three substrates is now defined in part. It is clear that β-hydroxydecanoyl and α,β-decenoate are directly connected. The question to be asked next is whether or not β,γ-decenoate can also be formed by direct dehydration. For this purpose the enzymatic conversions of the two labeled substrates, α3H-β-hydroxydecanoyl-NAC and α3H-β-hydroxydecanoyl-NAC, were investigated. If the reversible conversion of β,γ-decenoate and β-hydroxydecanoyl is direct, i.e. if the α-hydrogen atom is not involved, then the 3H:14C ratio in the product should be the same as in the starting substrate (β-hydroxydecanoate and β,γ-decenoate, respectively) provided there is no rapid tritium exchange. However, if α,β-decenoate is an obligate intermediate then the 3H:14C ratio in the product should be half or less of that in the starting substrate.

The results obtained for the reverse process, i.e. the enzymatic hydration of α3H-3-decenoyl-NAC, are also consistent with the obligate intermediacy of α,β-decenoate. The 3H:14C ratio decreased to slightly less than half (Table IV), showing that the addition of water to the β,γ double bond is not direct but proceeds with removal of hydrogen at Cα.

The obligatory removal of α-hydrogen in the production and in the transformation of β,γ-decenoate allows the further conclusion that an intramolecular 1,3-hydrogen transfer does not occur during the α,β- and β,γ isomerizations. Shifts of this type are known to occur with a variety of double bond isomerases (9, 10).

4 All of the substrates used in these labeling experiments were mixtures containing 14C as well as 3H. Also, the β-hydroxydecanoate substrates were racemic. For the sake of brevity these notations are omitted in the remainder of the text.

5 The resultant ratio in the product can be less than one-half the ratio in the starting substrate if there is a significant secondary isotope effect in the reaction.

6 A relevant experiment was carried out by Dr. S. Matsumura previously (unpublished). α3H β-Hydroxydecanoyl-NAC was incubated with enzyme for 2 hours. The isolated α,β- and β,γ-decenoates both had a 3H:14C ratio approximately one-half that of the starting substrate. Since later kinetic experiments showed that the three thioester substrates are readily interconverted by the enzyme (2), the interpretation of this early finding was equivocal.
Moreover, the fact that a significant secondary isotope effect is observed in the interconversion of $\beta,\gamma$-decenoate and $\beta$-hydroxydecanoate eliminates the possibility that the lowered $^3$H/$^1$C ratio in the product is simply the result of rapid tritium exchange at the $\beta,\gamma$-decenoate level. If it were, the $^3$H/$^1$C ratio in product would necessarily be $\geq$ $^3$H/$^1$C/2 of the starting material.

Based on all the isotope data presented so far the action of the dehydrase may be formulated as follows.

$$\text{CH}_{3}CH_{2}CH_{2}CH=CH-C-SX \rightarrow \text{CH}_{3}CH_{2}CH_{2}CH=CH-C-SX$$

This mechanism satisfies the energy relationships shown in Diagram A of Fig. 3.

The postulation of $\alpha,\beta$-decenoate as an obligatory stage in the reversible $\beta$-hydroxydecanoate to $\beta,\gamma$-decenoate transformation raises the following problem. Studying the kinetics of the dehydrase-catalyzed reactions, Brock, Kass, and Bloch (2) found that the initial rate of hydration of $\beta,\gamma$-decenoate was 3.6 times as fast as the rate of hydration of $\alpha,\beta$-decenoate. Moreover, starting with either $\beta,\gamma$-decenoate or $\beta$-hydroxydecanoate, product formation commenced without a lag. The kinetic evidence therefore excluded free $\alpha,\beta$-decenoate as an obligate intermediate on the path to and from $\beta,\gamma$-decenoate. In support of this conclusion we now have independent and direct chemical proof ruling out the intermediacy of $\alpha,\beta$-decenoate as such.

It was reasoned that if free $\alpha,\beta$-decenoate were an intermediate then trapping it would quench irrevocably the formation of $\beta,\gamma$-decenoate from $\beta$-hydroxydecanoate and vice versa. We have found that such trapping can be achieved with the mercaptan $N$-acetylcycteamine, which reacts with $\text{trans-2-decenoyl-NAC}$ by Michael addition to afford, presumably, a $\beta$-substituted thioether. We have chosen $N$-acetylcycteamine for this purpose in order to avoid possible complications arising from thioester exchange at the carbonyl group. The formation reaction is pH dependent, proceeding more rapidly at higher pH values. This is expected, since the sulfhydryl anion is much more nucleophilic than its conjugate acid. The reaction of NAC with $\alpha,\beta$-decenoyl-NAC can be followed spectrophotometrically by measuring the decrease in absorption at 263 nm.

The free NAC is not expected to react with either the $\beta,\gamma$-decanoate or $\beta$-hydroxydecanoate except in a virtual fashion. Under the conditions used, the addition reaction is sufficiently rapid to trap any $\alpha,\beta$-decenoate instantaneously. When $\beta,\gamma$-decenoate was incubated with enzyme and $10^{-1}$ to $10^{-4} \text{ M NAC}$, the ordinarily rapid formation of product absorbing at 263 nm could not be detected (Fig. 2). Control experiments had shown that the Michael addition product can be easily separated chromatographically from all other reactants. It was therefore possible to demonstrate that free $\alpha,\beta$-decenoate, once formed, was immediately diverted to the trapped, non-absorbing “product” during the enzymatic run.

In order to test the effect of the trapping agent on product formation, $14C-\beta,\gamma$-decenoate was incubated with enzyme both in the presence and in the absence of NAC. It was noted that substrate was consumed enzymatically at approximately the same rate whether the “trapping” agent was present or not. Moreover, radioactivity ordinarily associated with the free $\alpha,\beta$-decenoate fraction appeared instead in the polar Michael product when NAC was present. If free $\alpha,\beta$-decenoate were a compulsory intermediate in the interconversion of $\beta,\gamma$-decenoate and $\beta$-hydroxydecanoate, then the effective and irreversible trapping of the intermediate should abolish the formation of any other product. Yet, as shown by the data in Table V, the presence of trapping agent affects the yield of $\beta$-hydroxy product only slightly and it is therefore clear that the trapping of $\alpha,\beta$-decenoate did not interfere with the normal reaction path. Free $\alpha,\beta$-decenoate therefore cannot be an intermediate. To reconcile this observation with the results showing a compulsory loss of hydrogen at $C_\alpha$ during the $\beta$-hydroxydecanoate to $\beta,\gamma$-decenoate interconversion, we are driven to conclude that the intermediate is enzyme-bound $\alpha,\beta$-decenoate and not the free thioester. It would then follow that, in the enzyme-bound form, $\alpha,\beta$-decenoate reacts only sluggishly if at all with the mercaptan (NAC). This may be so for one or both of two reasons: (a) when enzyme-bound, the intermediate is all set up to isomerize to $\beta,\gamma$-decenoate or to be hydrated to $\beta$-hydroxydecanoate, and (b) it is sterically inaccessible to NAC.

A modified reaction scheme, which rationalizes the results of the trapping experiments as well as the labeling experiments, is shown below.

$$\beta,\gamma \text{ hydration}$$

$$\beta,\gamma \rightarrow (\alpha,\beta)-E \rightarrow \text{free} (\alpha,\beta) \rightarrow X-SH \rightarrow \alpha,\beta \text{ adduct}$$

The mechanism that we are proposing for the dehydrase-catalyzed reactions is the one that might have been predicted a priori on chemical grounds. Direct formation of $\beta,\gamma$-decenoate from $\beta$-hydroxydecanoate is not kinetically favorable in the light of the negligible acidity of the $C=H$ bond at $C_\gamma$ compared to the $C=H$ bond at $C_\alpha$. Even if the transition state for the reaction resembled the products more than the reactants (11), the unfavorable stability of the unconjugated $\beta,\gamma$ system compared to the conjugated $\alpha,\beta$ system would still argue against a direct $\beta,\gamma$ dehydration (12). Portsmouth, Stoolmiller, and Abeles (13) have uncovered a similar situation in the mechanism of action of $\alpha$-ketoacylase of dehydrase, in which a formal $\beta,\gamma$ dehydration is mediated by an $\alpha,\beta$-unsaturated, conjugated system.
that hydrogen exchange at Cα with protons in the medium is not rapid, so that a carbanion, if formed, should immediately collapse to α,β-decenoate. A concerted or near concerted elimination could profit from the classical "push-pull" catalysis described by Swain and Brown (14), and in that sense might be favorable.

The physiological role of the enzyme is now well understood. As a component of the fatty acid synthetase system of E. coli, the dehydrase functions at the point where the pathways to long chain saturated and unsaturated fatty acids diverge. By generating cis-3-decenoate, the enzyme furnishes the first specific intermediate for the branch that leads eventually to palmitoleate and vaccenate. The nonconjugated double bond introduced by the dehydrase-catalyzed reactions remains intact throughout elongation.

In E. coli the dehydrase is essential for the formation of unsaturated fatty acids. Treated with the powerful and dehydrase-specific inhibitor, 3-decynoyl-NAC (15), the bacterial fatty acid synthetase no longer produces unsaturated fatty acids. It is curious that this acetylenic substrate analogue does not diminish but in fact enhances the production of palmitate and stearate. This occurs in spite of the fact that 3-decynoyl-NAC quenches all dehydrase-catalyzed reactions, including the formation of α,β-decenoate, an essential intermediate on the way to long chain saturated acids. This apparent paradox suggested that the ability of the isolated dehydrase to produce α,β-decenoate is not expressed when the enzyme acts in concert with the elongating system and that it is the function of another E. coli dehydrase to furnish α,β-decenoate for further conversion to saturated fatty acids (15). The same inference was made by Silbert and Vagelos (16) on the basis of studies with a dehydraseless mutant of E. coli. The recent isolation of an enzyme (or enzymes) (17, 18) from E. coli capable of dehydrating β-hydroxydecanoyl-ACP specifically to trans-α,β-decenoyl-ACP confirms these expectations. Thus, the ability of the isolated β-hydroxydecanoyl thioester dehydrase to form α,β-decenoate in quantity cannot be crucial for the synthesis of long chain unsaturated acids. This being the case, the α,β-decenoate-forming capacity of this enzyme has to be rationalized on chemical rather than physiological grounds. It may be the only mechanism for producing the β,γ-decenoate by a chemically favorable pathway.

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