The Conversion of Long Chain Saturated Fatty Acids to Their \(\alpha,\beta\)- Unsaturated, \(\beta,\gamma\)-Unsaturated, and \(\beta\)-Hydroxy Derivatives by Enzymes from the Cellular Slime Mold, *Dictyostelium discoideum*

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A major problem in the study of fatty acid metabolism at the subcellular level has been the demonstration of the individual steps of both oxidation and synthesis (1, 2). Oxidation of long and short chain fatty acids by intact mitochondria is considered to proceed directly to acetate, without the accumulation of intermediates (3). Synthesis of fatty acids from acetyl coenzyme A and malonyl-CoA by the soluble "synthetase complex" likewise occurs as a concerted process, and significant amounts of intermediates have not been detected (4). Only in the incompletely defined synthetic system from mammary gland have such intermediates been directly demonstrated, and these are limited to compounds of chain length \(C_2\) to \(C_4\) (5).

Each of the enzymes of \(\beta\) oxidation has been highly purified and studied in detail as the catalyst of a single step (6). Most of these studies have been carried out with fatty acids of chain length \(C_{12}\) or less, and a number of them have relied on spectrophotometric and enzymatic assays for characterization of the products (7–9). The structures of the long chain fatty acid intermediates have generally been inferred from the identification of the \(C_4\), \(C_6\), and \(C_8\) intermediates.

In the biosynthetic system, a number of separate intermediate steps have also been studied, using chemical, spectrophotometric, and exchange tracer techniques (2, 10). As in \(\beta\) oxidation, only the short chain fatty acid intermediates have been isolated and characterized. Using substrates of \(C_4\) chain length, Brady and his coworkers (11, 12) have shown the participation of synthetic \(\alpha,\beta\)-unsaturated, \(\beta\)-keto, and \(\alpha,\beta\)-hydroxyacyl-CoA derivatives in the biosynthesis of fatty acid chains (11, 12).

The inability to detect long chain fatty acid intermediates in these intact systems may have several causes. Until the advent of gas-liquid chromatography and thin layer chromatography, these compounds were difficult to isolate and characterize. Also, there is evidence that, at least in some systems, the intermediates may exist only bound to enzyme sulfhydryls as thiol esters (2), and, therefore, never would accumulate unless the enzymes were used in substrate concentrations. Such a phenomenon could also explain the difficulty of demonstrating the entry of synthetic intermediates into the biosynthesis of fatty acids (4).

Whatever the explanation, the long chain fatty acid derivatives presumed to be the intermediates in fatty acid oxidation and synthesis appear never to have been isolated and characterized. It is therefore of interest that enzymes from the cellular slime mold *Dictyostelium discoideum* have been found which transform saturated long chain fatty acyl-CoA derivatives to a series of long chain intermediates, each of which accumulates in considerable quantity. These compounds include long chain \(\alpha,\beta\)-unsaturated and \(\beta,\gamma\)-unsaturated fatty acids which are the postulated intermediates in fatty acid oxidation and synthesis, long chain \(\delta(-)\)-\(\beta\)-hydroxy acids which are the presumed intermediates in fatty acid synthesis, but not in \(\beta\) oxidation, and long chain \(\alpha,\beta\)- and \(\alpha,\beta\)-\(\gamma\)-unsaturated fatty acids for which a metabolic role is not clear. The same compounds are formed during the incubation of palmitoyl-CoA with the mitochondrial fraction of guinea pig liver, and, therefore, the reactions described in this paper are not unique to the cellular slime mold.

**EXPERIMENTAL PROCEDURE**

*Materials—Radioactive fatty acids were obtained commercially. The 1-\(^{14}\)C-palmitic acid used in several experiments was contaminated with 1.8% of fatty acids having the same retention time as palmitoleate on gas-liquid chromatography, but giving rise on oxidative cleavage (13) to approximately equal quantities of radioactive dicarboxylic acids of chain lengths \(C_3\) to \(C_5\). Appropriate corrections were made for the presence of these contaminants. Subsequent batches of 1-\(^{14}\)C-palmitate were hydrogenated before use. 16-\(^{14}\)C-Palmitic acid was radiochemically pure, as determined by gas-liquid chromatography. 2-Pentadecanone and lauric, myristic, and palmitic aldehydes were obtained from K and K Laboratories. CoA, NAD, and ATP were obtained from Pabst Laboratories.

Synthesis of \(\beta\)-Hydroxy Acids—\(\beta\)-Hydroxymyristate, \(\beta\)-hydroxypalmitate and \(\beta\)-hydroxystearate were synthesized by the Reformatsky procedure (14). Each product was purified either by crystallization of the free acid from n-hexane, or chromatography of the methyl ester on silicic acid. Analysis of each compound by gas-liquid chromatography revealed only one peak. Synthetic \(\beta\)-hydroxypalmitate was optically inactive; the optical rotations of the other hydroxy acids were not determined.

The \(\beta\)-hydroxy acids were further characterized by their infrared spectra, and by identification of the unsaturated derivatives in the biosynthesis of fatty acid chains (11, 12). The inability to detect long chain fatty acid intermediates in these intact systems may have several causes. Until the advent of gas-liquid chromatography and thin layer chromatography, these compounds were difficult to isolate and characterize. Also, there is evidence that, at least in some systems, the intermediates may exist only bound to enzyme sulfhydryls as thiol esters (2), and, therefore, never would accumulate unless the enzymes were used in substrate concentrations. Such a phenomenon could also explain the difficulty of demonstrating the entry of synthetic intermediates into the biosynthesis of fatty acids (4).

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acids formed by dehydration. Methyl esters of the β-hydroxy acids were saponified in 0.5 M NaOH in 50% methanol for 2 hours at 85°. The β-hydroxy acids were stable under these conditions.

**Synthesis of Unsaturated Acids**—A mixture of α, β, and γ-\(\text{\(\beta\)}\)-hexadecenoic acids was prepared from β-hydroxypalmitic acid by reaction with \(\text{\(p\)}\)-toluenesulfonyl chloride in pyridine, followed by treatment with potassium t-butoxide. The products were methylated, and separated by preparative gas-liquid chromatography. Alternatively, more efficient separation of larger quantities of α, β- and γ-unsaturated fatty acids was achieved by chromatography, as methyl esters, on a column of silicic acid impregnated with silver nitrate, according to the technique of de Vries (15). The \(cis\) and \(trans\) isomers of the β, γ-hexadecenoic acid fraction were separated by rechromatography on a smaller silicic acid-silver nitrate column. The final preparation of \(trans\)-β, γ-hexadecenoic acid contained 1.5% of the \(cis\) isomer, and the \(cis\) preparation contained 0.6% of the \(trans\) isomer. The proportions of each isomer were determined by thin layer chromatography of the purified compounds, as methyl esters, on silica gel G impregnated with silver nitrate, a technique that separates \(cis\) and \(trans\) isomers (16). The spots corresponding to each isomer were eluted with diethyl ether, and the fatty acid determined quantitatively by analytical gas-liquid chromatography.

Dehydration of β-hydroxypalmitate produced about equal amounts of \(cis\) and \(trans\)-β, γ-hexadecenoate, but only the \(trans\)-α, β-isomer could be detected among the products. The synthetic α, β- and γ-unsaturated compounds were identified by their infrared spectra, specifically the absorption of the \(trans\)-α, β-hexadecenoic acid at 965 cm\(^{-1}\), and the absorption of \(trans\)-α, β-hexadecenoic acid at 965 cm\(^{-1}\) (17). Further confirmation of structure was achieved by oxidative cleavage of the unsaturated fatty acids by the MnO\(_4\) -- 10\(^{-4}\) procedure of von Rudloff (18), and identification of the resultant aliphatic monocarboxylic acids by gas-liquid chromatography. Myristic acid was formed by reaction with \(p\)-toluenesulfonyl chloride in pyridine, followed by treatment with potassium \(t\)-butoxide. The products were methylated, and separated by preparative gas-liquid chromatography. Alternatively, more efficient separation of larger quantities of α, β- and γ-unsaturated fatty acids was achieved by chromatography, as methyl esters, on a column of silicic acid impregnated with silver nitrate, according to the technique of de Vries (15). The \(cis\) and \(trans\) isomers of the β, γ-hexadecenoic acid fraction were separated by rechromatography on a smaller silicic acid-silver nitrate column. The final preparation of \(trans\)-β, γ-hexadecenoic acid contained 1.5% of the \(cis\) isomer, and the \(cis\) preparation contained 0.6% of the \(trans\) isomer. The proportions of each isomer were determined by thin layer chromatography of the purified compounds, as methyl esters, on silica gel G impregnated with silver nitrate, a technique that separates \(cis\) and \(trans\) isomers (16). The spots corresponding to each isomer were eluted with diethyl ether, and the fatty acid determined quantitatively by analytical gas-liquid chromatography.

Free \(cis\)- and \(trans\)-β, γ-unsaturated acids were formed from their methyl esters by saponification in 0.5 M NaOH in 50% methanol for 2 hours at 85°. However, under these conditions, the α, β-unsaturated fatty acid underwent addition to form the β-methyl ether, and was partially isomerized to the β, γ-unsaturated acid. Hence, methyl-α, β-hexadecenoate was saponified in 0.5 M NaOH in a mixture of tert-butyl alcohol and water.

**Synthesis of Acyl-CoA Derivatives**—Before synthesizing the CoA derivatives, the purity of each fatty acid was checked by gas-liquid chromatography. Pasty acyl-CoA derivatives were prepared according to the method of Goldman and Vagelos (18). The reaction between CoA and mixed anhydride was continued aerobically in test tubes. The reaction was stopped by addition of an equal volume of 1 M NaOH. In experiments...
with intact subcellular particles, the samples were then saponified for 1 hour at 70°C. Following incubations with soluble enzymes, hydrolysis was performed at room temperature for 15 minutes (7). The mixture was then acidified with 10 N H₂SO₄, and extracted three times with diethyl ether. Ether was evaporated under an air stream, and the free acids converted to the methyl esters using BF₃-methanol reagent (24), with heating to 100°C for 2 to 4 minutes. Neither isomerization of unsaturated acids nor dehydration of hydroxy acids occurred under these conditions.

Analysis of the reaction products from incubations with non-radioactive fatty acyl-CoA derivatives was carried out directly by gas-liquid chromatography on ethylene glycol succinate polyester. The area under each peak was quantitated by triangulation. Detector response was calibrated by analysis of known quantities of fatty acid methyl esters. In each analysis, the concentration of each component was calculated as a percentage of the total fatty acids present in the chromatogram. The absolute quantity of each product was then calculated from these percentages and the known amount of substrate added to the original incubation mixture. The soluble enzyme preparations did not contribute significant amounts of fatty acids.

When radioactive saturated acyl-CoA derivatives were used as substrates, the products of reaction were first separated as mercuric acetate adducts by chromatography on silicic acid (25). Carrier methyl esters were added when appropriate. Methyl esters of saturated fatty acids were eluted with 10 ml of benzene; adducts of the unsaturated fatty acids, and the hydroxy fatty acids were eluted together with 2 ml of benzene-5% acetic acid in methanol, 1:1, followed by 8 ml of 5% acetic acid in methanol. Adducts were then decomposed by addition of 0.5 ml of concentrated HCl directly to the acetic acid-methanol solution. After 1 hour, water was added, and the methyl esters of the unsaturated and hydroxy acids were extracted with petroleum ether.

Direct analysis and quantitation of the radioactive products was accomplished with either the continuous combustion-continuous counting gas-liquid chromatographic technique of Karnen, McCaffrey, and Bowman (26), or fractional collection of samples as they emerged from a preparative gas-liquid chromatogram onto anthracene coated with silicone oil, followed by direct counting in a scintillation spectrometer (27). For most analyses, ethylene glycol succinate polyester served as the liquid phase for chromatography, but analysis of the radioactive products was also carried out with SE 30 silicone rubber as the liquid phase.

In some experiments, after removal of the methyl esters of the saturated fatty acids by the adducts technique, the radioactive products were quantitated after separation of the components, as methyl esters, by thin layer chromatography on silica gel G impregnated with silver nitrate. This technique provides separation of trans-α, β-unsaturated, cis- and trans-β, γ-unsaturated, Δ2-unsaturated, and β-hydroxy long chain fatty acid methyl esters (16). The developing solvent was 5% ether in petroleum ether. Spots were detected by spraying with dichlorofluorescein and viewing under ultraviolet light. The silica gel was then either scraped into counting vials containing 0.4% diphenyloxazole in toluene and the radioactivity determined, or the esters were eluted with diethyl ether and an aliquot counted. In control experiments, recovery of radioactivity from the plates was 80%.

Characterization of Products—In order to obtain sufficient material for complete identification, the fatty acid methyl esters were separated and isolated by preparative gas-liquid chromatography (20) or thin layer chromatography. Unsaturated fatty acid methyl esters were subjected to oxidative cleavage by a modification of the technique of von Rudloff (18). Oxidation products were then identified by gas-liquid chromatography. When it was desired to collect radioactive CO₂ produced by this reaction, samples of radioactive fatty acids were incubated with the oxidative reagents in flasks fitted with center wells and sealed with serum caps. After completion of the reaction, a methanolic solution of Hyamine was injected into the center well, and the reaction mixture was acidified with 0.2 ml of 10 N H₂SO₄; in this case sodium metabisulfite was not added. The flasks were then incubated for 1 hour at 37°C, the Hyamine was transferred to a scintillation vial, 0.4% diphenyloxazole in toluene was added, and the radioactivity determined in a scintillation spectrometer. In control experiments, no radioactive CO₂ was produced from 1-14C-linoleic acid.

β-Hydroxy acids were degraded by the procedure of van Slyke (28). Fatty acids were heated to 100°C in capped, center-well flasks with 1 ml of 1.4 N H₂SO₄ and 0.3 ml of tert-butyl alcohol. Then 0.65 ml of 5% K₂Cr₂O₇ was injected into the reaction mixture, and the flasks were shaken at 100°C for 1 hour. The flasks were then chilled, methanolic Hyamine was added to the center well, and the CO₂ was collected and counted as above. In control experiments with 1-14C-linoleic acid, no radioactive CO₂ was formed. Oxidations and degradations were performed at least in duplicate.

Hydroxy fatty acids were acetylated with isoamyl alcohol-acetanilide, according to the micro technique of Kishimoto and Radin (29). Infrared spectra of the synthetic methyl esters were determined in carbon tetrachloride in a Beckman IR 7 or Perkin-Elmer 21 spectrophotometer. Spectra of the enzymatically produced fatty acids were obtained with a micro-KBr pellet in the Beckman IR 7. Optical rotations were obtained in ethanol, in a Rudolph polarimeter, with a 0.75-ml cell.

**RESULTS**

Identification of Products of Palmityl-CoA Metabolism

A gas-liquid chromatogram of the products of the incubation of 1-14C-palmityl-CoA with the light particle fraction of the homogenate of D. discoideum is shown in Fig. 1. Essentially identical results were obtained with 16-14C-palmityl-CoA. In both cases, the light particle fraction converted approximately 30 to 45% of the substrate to the three products A, B, and C. In a control experiment, only 3% of sodium 1-14C-palmitate was metabolized.

**Compound A**—Compound A was identified by the following procedures as a mixture of methyl trans-β, γ-hexadecenooate (75%) and methyl cis-β, γ-hexadecenoate (25%). Its retention time in gas-liquid chromatography was very close, but not identical, to that of methyl palmitoleate (Table I). Hydrogenation
of Compound A changed its retention time to that of methyl palmitate. Position of the double bond was established by analysis of the products of oxidation with MnO4-104. When Compound A derived from 14C-palmityl-CoA was oxidized, 69% of the radioactivity was recovered as CO2. This is consistent with the production of malonic acid by cleavage of the original olefin, since under these conditions malonate is degraded to CO2 (30). When radioactive Compound A produced from 1614C-palmityl-CoA was isolated and degraded in similar fashion, the only radioactive compound produced was tridecanoic acid. Compound A, therefore, is a 16 carbon fatty acid with a double bond at the 3,4-position.

Synthetic methyl cis- and trans-β,γ-hexadecenoate and Compound A all had identical retention times on gas-liquid chromatography (Table I) and, therefore, this technique could not be used to determine stereochemistry. The proportions of cis and trans isomers in the enzymatic product were established in several ways. The products formed from incubation of light particles with 14C-palmityl-CoA were separated by thin layer chromatography on silicic acid impregnated with AgNO3 (Table II). The spots corresponding to trans-β,γ-hexadecenoate and cis-β,γ-hexadecenoate were found to contain radioactivity in the ratio of approximately 3 to 1. Also, methyl β,γ-hexadecenoate was isolated by preparative gas-liquid chromatography of the products of incubation of soluble enzyme with nonradioactive α,β-hexadecenoyl-CoA. Details of this enzymatic reaction will be described later. The infrared spectrum of the methyl β,γ-hexadecenoate indicated a preponderance of the trans isomer. The two isomers were then separated by thin layer chromatography on AgNO3 silicic acid. The compounds active fatty acids from D. discoideum were added as carrier. The radioactive peaks were identified (see text) as Palmilate, methyl palmitate; A, methyl cis- and trans-β,γ-hexadecenoate; B, methyl trans-α,β-hexadecenoate; and C, methyl β-hydroxy-palmitate. Nonradioactive peaks are the methyl esters of 1, palmitoleate; 2, 5,9-hexadecadienoate; 3, oleate and vaccenate; and 4, 5,9-octadecadienoate and 5,11-octadecadienoate.

TABLE I
Gas-liquid chromatographic retention times of substituted fatty acid methyl esters

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time relative to methyl stearate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl β,γ-tetradecenoate</td>
<td>0.41*</td>
</tr>
<tr>
<td>Methyl α,β-tetradecenoate</td>
<td>0.57*</td>
</tr>
<tr>
<td>Methyl palmitoleate</td>
<td>0.67*</td>
</tr>
<tr>
<td>Methyl cis- and trans-β,γ-hexadecenoate</td>
<td>0.71</td>
</tr>
<tr>
<td>Methyl trans-α,β-hexadecenoate</td>
<td>0.93</td>
</tr>
<tr>
<td>Methyl β,γ-octadecenoate</td>
<td>1.22</td>
</tr>
<tr>
<td>Methyl α,β-octadecenoate</td>
<td>1.62</td>
</tr>
<tr>
<td>Methyl β-hydroxymyristate</td>
<td>2.12</td>
</tr>
<tr>
<td>Methyl β-hydroxypalmitate</td>
<td>3.61</td>
</tr>
<tr>
<td>Methyl β-hydroxystearate</td>
<td>5.95</td>
</tr>
</tbody>
</table>

* The retention times marked with an asterisk were obtained at 183° and 15 p.s.i.; the remainder were obtained at 193° and 15 p.s.i.

were eluted from the silicic acid, and the amounts of each quantitated by analytical gas-liquid chromatography. Again the ratio of trans to cis was 3 to 1. The products of incubation of soluble enzyme with α,β-hexadecenoyl-CoA were also separated by thin layer chromatography and trans-β,γ-hexadecenoate and cis-β,γ-hexadecenoate were both demonstrated to be present.
TABLE II
Comparison of the conversion of myristyl-CoA, palmitoyl-CoA, and stearyl-CoA to α,β-ununsaturated, γ-unsaturated, and β-hydroxy fatty acids

Light particle fraction of D. discoideum homogenate at a final dilution of approximately 1.2 mg of protein per ml (optical density, 0.48 at 600 ma) was incubated with each radioactive long chain fatty acyl-CoA in 0.5 ml of 0.1 m phosphate buffer, pH 7.25. Substrates were 1-14C-stearyl-CoA, 3.5 × 10^4 c.p.m. (162 mmoles); 1-14C-palmitoyl-CoA, 4.1 × 10^4 c.p.m. (75 mmole); and 1-14C-myristyl-CoA, 4.6 × 10^4 c.p.m. (101 mmoles). After 3 hours of incubation at 31°, fatty acid esters were saponified, and the fatty acids were extracted and cotedified, and carrier methyl esters were added. The mercuric acetate adducts were made and separated as described in “Experimental Procedure.” Saturated fatty acids were separated from unsaturated and hydroxy acids on small columns of silicic acid, and an aliquot of each eluate counted. Recovery of radioactivity from the incubation mixture was quantitative. Regenerated methyl esters of the products were then analyzed by thin layer chromatography on silicate nitrate-imregnated silicic gel. Individual spots were scraped into counting vials, and radioactivity was determined in diphenoxyaceta-toluene in a scintillation counter. Recovery of radioactivity from the plates was 65 to 80%.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products</th>
<th>α,β-Ununsaturated fatty acid</th>
<th>γ-Ununsaturated fatty acid</th>
<th>cis,γ-Ununsaturated fatty acid</th>
<th>β-Hydroxy fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-14C-Myristyl-CoA</td>
<td></td>
<td>4.9</td>
<td>2.9</td>
<td>1.2</td>
<td>28.4</td>
</tr>
<tr>
<td>1-14C-Palmitoyl-CoA</td>
<td></td>
<td>3.0</td>
<td>1.7</td>
<td>0.7</td>
<td>11.9</td>
</tr>
<tr>
<td>1-14C-Stearyl-CoA</td>
<td></td>
<td>1.3</td>
<td>0.6</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* The remainder of the radioactivity was in the saturated fatty acid (unreacted substrate).

Compound B—Compound B was identified as methyl trans-α,β-hexadecenoate. Hydrogenation of this compound converted it to methyl palmitate. Upon MnO2-O2 oxidation of Compound B enzymatically produced from 1-14C-palmitoyl-CoA, 78% of the radioactivity was recovered as CO2. This is consistent with the production of oxalic acid from the original unsaturated fatty acid, since oxalate (like malonate) is degraded to CO2 under these conditions (31). Oxidative cleavage of Compound B produced enzymatically from 1-14C-palmitoyl-CoA gave rise only to radioactive myristic acid. Compound B is thus a 16 carbon fatty acid with a double bond at the 9,10-position.

The infrared spectrum of methyl α,β-hexadecenoate isolated as the enzymatic product derived from nonradioactive β-hydroxypalmitoyl-CoA had the adsorption band at 985 cm-r characteristic of trans olefins conjugated with carbonyl groups (17). No evidence was found for the presence of the cis isomer. The retention time in gas-liquid chromatography of the enzymatic product from palmitoyl-CoA, β-hydroxypalmitoyl-CoA, and γ-hexadecenoyl-CoA was identical with the retention time of synthetic methyl trans-α,β-hexadecenoate. A sample of synthetic methyl cis-α,β-hexadecenoate was not available, but cis and trans isomers of α,β-ununsaturated fatty acids are reported to have very different retention times (32) and the cis isomer should have been detected if it were present. The α,β-hexadecenoate produced enzymatically had the same mobility in thin layer chromatography as synthetic trans-α,β-hexadecenoate.

Compound C—Compound C was characterized as methyl n-(-)-β-hydroxypalmitate. Its retention time in gas-liquid chromatography (Table I) and its mobility in thin layer chromatography were identical with those of synthetic methyl n-(-)-β-hydroxypalmitate. The chromatographic behavior of Compound C did not change upon hydrogenation. However, acetylation of Compound C changed its retention time in a manner analogous to the effect of acetylation on the retention time of synthetic methyl 9(10)-hydroxypalmitate.

Upon van Slyke oxidation of radioactive Compound C enzymatically produced from 1-14C-palmitoyl-CoA, 72% of the radioactivity was recovered as CO2. To characterize the other fragment of the molecule resulting from such oxidation, radioactive Compound C from 16-14C-palmitoyl-CoA was used. The oxidation mixture was extracted with petroleum ether, unlabeled carrier 2-pentadecanone was added, and the mixture was analyzed by mass and radioactivity by gas-liquid chromatography. Radioactivity cochromatographed exactly with the carrier 2-pentadecanone.

In order to obtain sufficient β-hydroxyvalpimate to determine its optical activity, 36.5 μmoles of synthetic trans-α,β-hexadecenoyl-CoA were incubated for 2 hours at 31° with 24 mg of soluble enzyme from the acetone powder of light particles. After hydrolysis of the thiol esters for 15 minutes at room temperature in 0.6 N NaOH, the solution was acidified and extracted with ether. The products were methylated with BF3-methanol.

Gas-liquid chromatographic analysis of an aliquot of this preparation revealed 45% conversion of the substrate to β-hydroxyvalpimate. Methyl β-hydroxyvalpimate was then isolated by chromatography on a column of silicic acid. Its specific rotation in ethanol was [α]D^29 = −10.39°. The rotation was increasingly negative with decreasing wave length.

The specific rotation of methyl n-(-)-β-hydroxybutyrate is −21.09° (33). If one assumes the same rotation on a molar basis for the two compounds, the expected specific rotation for methyl n-(-)-β-hydroxyvalpimate would be −8.71°, a value in good agreement with that found.

Metabolism of Saturated Long Chain Fatty Acyl-CoA Derivatives

With a given homogenate preparation, there were consistent differences between subcellular fractions in total conversion and ratios of products formed with 1-14C-palmitoyl-CoA as substrate (Table III). With light particles (the most active fraction), total conversion of radioactive palmitoyl-CoA averaged about 30%, with maximum conversion of about 45%. Analysis of the products by gas-liquid chromatography and thin layer chromatography gave comparable ratios of products (Tables II and III). Recombination of the enzyme fractions caused no increase in the total conversion, and no significant shifts in the ratios of products.

The light particle fraction was also assayed for activity against myristoyl-CoA and stearyl-CoA. Somewhat more myristoyl-CoA was converted to products than either stearyl-CoA or palmitoyl-CoA. However, the ratio of products formed from each substrate was quite similar (Table II).

To investigate the relationship of the formation of the long chain fatty acyl derivatives to fatty acid degradation, particulate and supernatant fractions of homogenates of D. discoideum were incubated with 1-14C-palmitoyl-CoA, with and without cofactors (Table III). The radioactivity of each of the fatty acid products and of CO2 was determined.

Although the oxidation of 1-14C-palmitoyl-CoA to CO2 was not
Radioactive products from incubation of D. discoideum homogenate fractions with 1-14C-palmityl-CoA

Particulate fractions diluted to a final optical density of 0.32 at 600 mg (approximately 0.8 mg of protein per ml) or supernatant fraction (approximately 1 mg of protein per ml) were incubated with 8.4 X 10^4 c.p.m. (73 mmoles) of 1-14C-palmityl-CoA. All flasks contained 100 mmoles of phosphate buffer, pH 7.25; flasks with cofactors contained 1 mmoles of CoA, 10 mmoles of ATP, 1 mmoles of NAD, and 5 mmoles of Mg<sup>2+</sup> in a final volume of 1.0 ml. Incubations were carried out in sealed flasks equipped with center wells containing 0.5 ml of Hyamine in methanol to trap COS. At the end of 2 hours of incubation at 30°, 0.2 ml of 10 N H<sub>2</sub>S<sub>2</sub>O was added to the reaction compartment, and the flasks incubated for 1 hour more. The Hyamine solution was then counted in diphenyloxazole-toluene in a scintillation spectrometer. Unsaturation and hydroxy fatty acids were extracted and separated by chromatography, as in Fig. 1. Total recovery of radioactivity was quantitative by continuous combustion-counting gas-liquid chromatography, as described in "Experimental Procedure," and determined quantitatively by continuous combustion-counting gas-liquid chromatography as in Fig. 1. Total recovery of radioactivity was from 75 to 95%.

<table>
<thead>
<tr>
<th>Enzyme and additions</th>
<th>Product</th>
<th>β,γ-Hexadecenoate</th>
<th>α,β-Hexadecenoate</th>
<th>β-Hydroxy-palmmitate</th>
<th>CO&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy particles</td>
<td>% recovered radioactivity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>6</td>
<td>10</td>
<td>0.04</td>
</tr>
<tr>
<td>Heavy particles + cofactors</td>
<td>% added radioactivity</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>3.1</td>
</tr>
<tr>
<td>Light particles</td>
<td>7</td>
<td>10</td>
<td>20</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Light particles + cofactors</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>3</td>
<td>4</td>
<td>17</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Supernatant + cofactors</td>
<td>2</td>
<td>3</td>
<td>9</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The remainder of the recovered radioactivity was in palmitate.

great in any fraction, the heavy particle fraction was by far the most active. Oxidation to CO<sub>2</sub> was completely dependent on the presence of cofactors. The presence of oxaloacetate did not affect the results. It is also apparent that the accumulation of long chain acyl intermediates was only moderately affected by the presence of cofactors, and that in this regard, cofactors had similar effects whether or not the enzyme fraction was capable of degrading fatty acid to CO<sub>2</sub>. Thus, the light particle and supernatant fractions, although very active in transforming palmitoyl-CoA to the several intermediate fatty acids, were virtually unable to oxidize 1-14C-palmitoyl-CoA to CO<sub>2</sub>.

Interconversions of Four Intermediates

To delineate more clearly their metabolic relationships, trans-α,β-hexadecenoyl-CoA, cis-β,γ-hydroxy-palmmitoyl-CoA, trans-β,γ-hexadecenoyl-CoA, and cis-β,γ-hexadecenoyl-CoA were each incubated separately with soluble enzymes extracted from the acetone powder of light particles. The soluble enzymes catalyzed the conversion of each substrate to all of the other compounds with one exception; there was only slight conversion of trans-β,γ-hexadecenoyl-CoA to cis-β,γ-hexadecenoyl-CoA and cis-β,γ-hexadecenoyl-CoA was not converted to the trans isomer. Details of each incubation are presented below.

Metabolism of trans-α,β-Hexadecenoyl-CoA—Only the trans isomer of this compound was available for study as substrate, but as discussed before, this appears to be the isomer produced enzymatically from the other substrates. trans-α,β-Hexadecenoyl-CoA was converted to β-hydroxy-palmmitate much more rapidly than it was converted to β,γ-hexadecenoate (Fig. 2). Control incubation with boiled enzyme showed no nonenzymatic conversion to either compound.

When α,β-hexadecenoyl-CoA was incubated with excess enzyme (Table IV), the relative amounts of products were similar to those formed when intact particulate enzyme was incubated with radioactive palmitoyl-CoA (Tables II and III). Qualitative analysis of the β,γ-hexadecenoic acid produced from α,β-hexadecenoyl-CoA showed both cis and trans isomers to be

![Fig. 2. Conversion of trans-α,β-hexadecenoyl-CoA to β-hydroxy-palmmitate and β,γ-hexadecenoate. The incubation mixture contained 100 μg of soluble enzyme protein and 560 mmoles of substrate per ml of 0.1 mM phosphate buffer, pH 7.25. The reaction was started by addition of enzyme; incubation was at 31°. Aliquots of 1.0 ml were removed at increasing time intervals, and analyzed for substrate and products by gas-liquid chromatography as described in "Experimental Procedure."](http://www.jbc.org/)

### TABLE IV

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final proportions of compounds</th>
<th>α,β-Hexadecenoate</th>
<th>β-Hydroxy-palmmitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-α,β-Hexadecenoyl-CoA</td>
<td>8.7</td>
<td>42.3</td>
<td>49.0</td>
</tr>
<tr>
<td>cis-β,γ-Hexadecenoyl-CoA</td>
<td>7.4</td>
<td>10.5</td>
<td>82.1</td>
</tr>
<tr>
<td>trans-β,γ-Hexadecenoyl-CoA</td>
<td>71.3</td>
<td>8.3</td>
<td>19.4</td>
</tr>
<tr>
<td>cis-β,γ-Hexadecenoyl-CoA</td>
<td>68.5</td>
<td>8.6</td>
<td>20.8</td>
</tr>
</tbody>
</table>
present. As noted before, the β-hydroxypalmitate formed was the \(\alpha(-)\) isomer.

**Metabolism of DL-β Hydroxypalmitoyl-CoA**—When \(\alpha\beta\) hydroxy-palmitoyl-CoA was incubated with soluble enzyme under conditions identical with those employed for the incubation of \(\alpha\beta\)-hexadecenoyl-CoA, the products were trans-\(\alpha\beta\)-hexadecenoate, and \(\beta\gamma\)-hexadecenoate, of which 75% was the trans isomer and 25% the cis isomer (Table IV). \(\alpha\beta\)-Hexadecenoate was formed more rapidly than was \(\beta\gamma\)-hexadecenoate (Fig. 3). The large proportion of β-hydroxypalmitate remaining when the substrate was incubated with excess enzyme (Table IV) is probably because the \(\alpha\alpha\) isomer was not metabolized.

**Metabolism of cis- and trans-\(\beta\gamma\)-Hexadecenoyl-CoA**—Since both stereoisomers of \(\beta\gamma\)-hexadecenoate were formed enzymatically from all the other substrates, it was of interest to study the behavior of both isomers as substrate. Both cis- and trans-\(\beta\gamma\)-hexadecenoyl-CoA were converted to β-hydroxypalmitate and to \(\alpha\beta\)-hexadecenoate (Fig. 4). The rate of conversion of cis-\(\beta\gamma\)-hexadecenoyl-CoA to β-hydroxypalmitate was about twice the rate of conversion of the trans isomer. Despite the differences in the rates of reaction, the equilibrium concentrations of products and substrates appeared to be about the same for both isomers (Table IV).

The \(\alpha\beta\)-hexadecenoate produced from both cis- and trans-\(\beta\gamma\)-hexadecenoyl-CoA had the same mobility on thin layer chromatography and gas-liquid chromatography as the \(\alpha\beta\)-hexadecenoate formed from β-hydroxypalmityl-CoA, and is presumed to be the trans isomer.

**Isomerization of cis- and trans-\(\beta\gamma\)-Hexadecenoyl-CoA**—The foregoing studies demonstrated the reversible interconversion of all of the compounds except for cis- and trans-\(\beta\gamma\)-hexadecenoate. To test for this, an aliquot of each of the samples analyzed in Fig. 4 was chromatographed on thin layer plates of AgNO\(_3\)-silica gel. The areas corresponding to cis and trans methyl \(\beta\gamma\)-hexadecenoate were eluted and quantitatively analyzed by gas-liquid chromatography. No production of the trans isomer from the cis substrate was observed. However, the cis isomer comprised about 5 to 6% of the total \(\beta\gamma\)-unsaturated fatty acid remaining at the end of the experiment in which the trans isomer was substrate.

The reasons for the absence or very low level of "cis-trans isomerase" activity are not clear. Thiol esterase activity has been detected in these enzyme extracts and may partially account for the results. For example, interconversion of the cis and trans isomers might occur only through the intermediate formation of \(\beta\)-hydroxy- or \(\alpha\beta\)-unsaturated fatty acids. If these were hydrolyzed as rapidly as they were formed, "isomerase" activity would not be observed.

Alternatively, both stereoisomers may bind to the same catalytic site on the enzyme, in competitive fashion. Again assuming that interconversion of the \(\beta\gamma\) isomers can occur only through the intermediate formation of \(\beta\)-hydroxy- or \(\alpha\beta\)-unsaturated acyl thiolesters. If these were hydrolyzed as rapidly as they were formed, "isomerase" activity would not be observed.

Evidence for Pathway of \(\beta\gamma\)-Hexadecenoate Production—Both dehydration of a β-hydroxy acyl-CoA (34, 35) and direct isomerization of the double bond of an \(\alpha\beta\)-unsaturated acyl-CoA (36) have been postulated as mechanisms for the enzymatic formation of \(\beta\gamma\)-unsaturated fatty acyl-CoA in other systems. To investigate this problem, the rates of production of the \(\beta\gamma\)-
Many points remain to be clarified, however; for instance, it is not known if both stereoisomers of \( \beta,\gamma \)-hexadecenoate are made by the same reaction, or that the reactions occur at the CoA level. There remains the real possibility that the acyl moiety must be transferred from CoA to a sulphydryl group on the enzyme and that this is the rate-determining step. Purification of the enzymes should be helpful in elucidating the mechanisms involved.

**Chain Length Specificity towards Hydroxy Substrates**

Incubations with \( \beta \)-hydroxymyristyl-CoA, \( \beta \)-hydroxypalmityl-CoA, and \( \beta \)-hydroxyoctadec-9-enoyle-CoA were carried out to determine the relative rates of reaction with each substrate. \( \beta,\gamma \)-Unsaturated fatty acids were produced at comparable rates from all three substrates (Fig. 7), although \( \beta,\gamma \)-octadecenoate was formed somewhat more rapidly. The \( \alpha,\beta \)-unsaturated acid was formed more more

---

**Fig. 5.** Comparison of the rates of production of \( \beta,\gamma \)-hexadecenoate from \( \alpha,\beta \)-hexadecenoyl-CoA and \( \beta \)-hydroxypalmityl-CoA. This figure is partially a composite of the data from Figs. 2 and 3. In addition, data are shown from an experiment using as substrate 870 mmoles of \( \beta \)-hydroxypalmityl-CoA per ml. This was done to adjust the concentration of the D isomer of \( \beta \)-hydroxypalmityl-CoA to a level more comparable to that of \( \alpha,\beta \)-hexadecenoyl-CoA.

**Fig. 6.** Comparison of the rates of conversion of trans-\( \beta,\gamma \)-hexadecenoyl-CoA and cis-\( \beta,\gamma \)-hexadecenoyl-CoA to \( \beta \)-hydroxypalmityl and \( \alpha,\beta \)-hexadecenoate. Conditions of incubation were the same as in Fig. 2 except that the substrate was 364 mmoles of trans-\( \beta,\gamma \)-hexadecenoyl-CoA per ml or 315 mmoles of cis-\( \beta,\gamma \)-hexadecenoyl-CoA per ml.

**Fig. 7.** Conversion of \( \beta \)-hydroxymyristyl-CoA, \( \beta \)-hydroxypalmityl-CoA, and \( \beta \)-hydroxyoctadec-9-enoyle-CoA to the corresponding \( \beta,\gamma \)-unsaturated fatty acids. Conditions of incubation were the same as in Fig. 2, except that substrates were 183 mmoles of \( \beta \)-hydroxymyristyl-CoA, 117 mmoles of \( \beta \)-hydroxypalmityl-CoA, or 163 mmoles of \( \beta \)-hydroxyoctadec-9-enoyle-CoA per ml. Enzyme concentration was 320 \( \mu \)g of protein per ml.
were cr, & hexadecenoate and a, , octadecenoate. The interconversions demonstrated in this paper are most readily explained by the following reactions:

\[
\text{Saturated fatty acid} \rightarrow \text{trans-} \alpha, \beta-
\]

unsaturated or cis- or trans- \alpha, \beta-unsaturated fatty acid (1)

\[
\text{trans-} \alpha, \beta-\text{Unsaturated fatty acid} \rightleftharpoons \text{d(-)-} \beta-\text{hydroxy fatty acid} \quad (2)
\]

\[
\text{trans-} \alpha, \beta-\text{Unsaturated fatty acid} \rightleftharpoons \text{cis- and trans-} \beta, \gamma-\text{unsaturated fatty acid} \quad (3)
\]

These reactions occur either as the CoA derivatives or possibly as thiol esters bound directly to sulfhydryl groups on the enzymes; there are no data to exclude either possibility. Although both Reactions 2 and 3 have been shown to be reversible, measurement of equilibrium constants has been impossible in the crude system because of the presence of thiolesterase activity, and contamination of the substrates by inactive acyl thioesters (18). Each of the above reactions will now be discussed in some detail.

The evidence for Reaction 1 is indirect, depending entirely upon the demonstration of conversion of saturated fatty acyl-CoA to all the intermediates. Direct dehydrogenation of the saturated fatty acid is the simplest explanation. This reaction is presumably analogous either to that catalyzed by the acyl dehydrogenase of fatty acid synthesis (37), or the reversal of the reaction catalyzed by the ethylene reductase of fatty acid synthesis (2).

The hydration of trans- \alpha, \beta-unsaturated fatty acid (Reaction 2) is similar to the hydration step in fatty acid synthesis in that the \( \text{d(-)-} \beta-\text{hydroxy acid} \) is formed (2). The product of the analogous step in fatty acid oxidation is the \( \text{l(+)-} \) isomer when trans- \alpha, \beta-unsaturated fatty acid is the substrate (38). The cellular slime mold enzyme appears to form only trans- \alpha, \beta-unsaturated fatty acids from \( \beta-\text{hydroxy acids} \). The enoyl hydrase of \( \beta-\text{oxidation} \), although not specific for the trans- \alpha, \beta-unsaturated isomer as substrate (38), synthesizes only the trans isomer from \( \text{l(+)-} \beta-\text{hydroxy fatty acids} \), for which substrate the enzyme is specific (34, 35, 39). The stereochemistry of the \( \alpha, \beta-\text{unsaturated fatty acid} \) produced by the dehydrase of fatty acid synthesis is apparently also trans (2). The stereochemistry of the intermediates in fatty acid synthesis and oxidation have not been determined directly for compounds of chain length greater than \( \text{C}_{12} \).

The evidence for Reaction 3 is the kinetics of the interconversions of \( \alpha, \beta-\text{hexadecenoate} \), \( \beta, \gamma-\text{hexadecenoate} \), and \( \beta-\text{hydroxy-} \)palmitate when each is added as the CoA derivative. The data strongly suggest that \( \alpha, \beta-\text{hexadecenoate} \) is the intermediate between \( \beta, \gamma-\text{hexadecenoate} \) and \( \beta-\text{hydroxy-} \)palmitate. The alternative pathway with \( \beta-\text{hydroxy-} \)palmitate as the intermediate between the two unsaturated fatty acids cannot be definitely excluded by these data, however, especially if the interconversions occur as enzyme-bound substrates rather than as the free CoA derivatives. There are, in fact, some possible precursors for the direct interconversion of \( \beta-\text{hydroxy acids} \) and \( \beta, \gamma-\text{unsaturated acids} \). Enoyl hydrase appears to catalyze the hydration of \( \beta, \gamma-\text{unsaturated acids} \) although at a much slower rate than the hydration of \( \alpha, \beta-\text{unsaturated acids} \) (34, 35). However, the possibility exists that this hydration actually follows isomerization of the \( \beta, \gamma-\text{isomer} \) to the \( \alpha, \beta-\text{isomer} \) (36). Bloch et al. (32, 40) have studied a bacterial enzyme that converts \( \beta-\text{hydroxydecanoyl-CoA} \) to \( \beta, \gamma-\text{decanoate} \). (The stereochemistry of the product has not been reported.) These investigators have suggested that the reaction is a direct dehydrogenation, but the possible presence of an \( \alpha, \beta, \gamma-\text{isomerase} \) in their preparations has not been eliminated. The reaction is specific for \( \text{d(-)-} \beta-\text{hydroxydecanoyl-CoA} \).

There are also precedents, however, for the direct isomerization of \( \alpha, \beta- \) and \( \beta, \gamma-\text{unsaturated fatty acids} \) as postulated in Reaction 3. Such reactions have been described previously in bacterial (41, 42) and mammalian systems (36), but only for short chain fatty acids. In these cases, vinylacetate has been shown to be converted to crotonate without the intermediate formation of \( \beta-\text{hydroxybutyrate} \). With increasing chain length much less activity was found. The data reported in this paper indicate that this type of isomerization also occurs with long chain fatty acids, and are also the first demonstration of the formation and conversion of both cis and trans isomers of \( \beta, \gamma-\text{unsaturated fatty acids} \). The apparent lack of stereospecificity is unexplained. It is perhaps surprising that the cellular slime mold enzyme forms \( \alpha, \beta-\text{hexadecenoate} \) and \( \beta, \gamma-\text{hexadecenoate} \) in the ratio 3:1. The reaction catalyzed by vinylacetly isomerase, for example, leads to 98%-crotonate at equilibrium (41). The differences may be attributable to the difference in chain length of the substrates. The proportions of crotonate and vinylacetate produced by alkali-catalyzed isomerization (43) are identical with those produced enzymatically. Elongation of the fatty acid chain beyond 4 carbons, however, appears to change the ratio of products formed by alkali isomerization, so that 30% of the \( \beta, \gamma-\text{isomer} \) is found at equilibrium (43). The influence of the thiol ester bond on the enzyme-catalyzed reaction cannot be quantitatively evaluated, but by analogy to ketones (44, 45), one might expect the equilibrium mixture to contain relatively more of the \( \alpha, \beta-\text{unsaturated} \) compound than is found with the free acids.

The metabolic role of these fatty acid intermediates is not obvious; they may function in oxidation, synthesis, or elongation of fatty acids. The data cannot be consistently interpreted to favor any one of these possibilities.

The least likely role for these reactions would seem to be in oxidation of fatty acids, for the following reasons. (a) Growing cells of \( D. \text{ discoideum} \) do not degrade palmitate or stearate to any significant extent (46), although laurate and myristate are partially degraded. (b) There was no correlation (Table III) between the ability of subcellular fractions to oxidize \( 1-\text{H}-\)palmityl-CoA to \( \text{CO}_2 \), and to convert it to any of the intermediates under consideration. (c) The \( \text{d(-)-} \)hydroxy acids are formed, rather than the \( \text{l(+)-} \)isomer thought to be the intermediate in fatty acid oxidation. (d) It is difficult to envision a role for \( \beta, \gamma-\text{unsaturated fatty acids} \) in \( \beta \) oxidation unless they be intermediates in the oxidation of unsaturated fatty acids. During \( \beta \) oxidation of oleate, for example, \( \beta, \gamma-\text{dodecenoate} \) would presumably be formed. The naturally occurring unsaturated fatty acids of \( D. \text{ discoideum} \) have double bonds in positions that similarly would lead to the formation of \( \beta, \gamma-\text{unsaturated fatty acids} \).
acids upon β oxidation (21). The lack of stereospecificity with regard to synthesis of the β,γ-unsaturated acids in vitro suggests that Reaction 3 might serve to consume, rather than to produce, β,γ-unsaturated fatty acids in vivo.

In this connection it is pertinent to note that although there is indirect evidence that the acyl dehydrogenases of mitochondrial fatty acid oxidation dehydrogenate short chain fatty acids to α,β-unsaturated fatty acids, the possible formation of a β,γ-unsaturated product has not been eliminated, especially for long chain fatty acids. In fact, β,γ-unsaturated acyl-CoA binds to acyl dehydrogenase and produces the same spectral change in the flavoprotein as does α,β-unsaturated acyl-CoA (47).

Bloch (48) has proposed that the formation of β,γ-unsaturated fatty acids in certain bacteria is a necessary step in the synthesis of unsaturated fatty acids. A similar mechanism does not seem likely in D. discoideum despite the fact that the dimethylated fatty acids of the cellular slime mold, 5,9-hexadecadienoate, 5,9,11-octadecadienoate, and 5,11-octadecadienoate, all have a double bond at C-5. One might suppose that these acids arise from 5-unsaturated fatty acids (49) to form the trans isomer which is then chlorinated to the cis isomer (50).

The predominant fate of long chain fatty acids (C18 and C16) in growing cells of D. discoideum is elongation (and desaturation). The predominant product of the enzymatic reactions described in this paper. The major objection, however, is that experiments in the intact organism have demonstrated that desaturation at the 5-position occurs after the carbon chain is completed (46).

Formation of cis-β,γ-unsaturated acids might be incidental to the formation of the saturated fatty acid chain, but serve, in some systems, for formation of unsaturated fatty acids. Although it is believed that in the usual fatty acid synthetase the intermediate which is reduced is the α,γ-bisubstituted acid (2), there is no direct evidence on this point for long chain fatty acids, and the intermediates in the chain elongation reactions of mitochondria are unknown as yet (49). We have no data on which unsaturated fatty acid is reduced in D. discoideum.

SUMMARY

Subcellular fractions from homogenates of the cellular slime mold, Dictyostelium discoideum, convert saturated C16a, C16b, and C18 fatty acyl coenzyme A to the trans-α,β-unsaturated, d(-)-β-hydroxy, and cis- and trans-β,γ-unsaturated fatty acids of the same chain length. Soluble enzymes, derived from acetone powders of such subcellular fractions, catalyze the interconversion of all of these products. Evidence is presented that the reactions involved include the reversible hydration of α,β-unsaturated acids, and the direct isomerization of α,β- and β,γ-unsaturated acids.

REFERENCES

The Conversion of Long Chain Saturated Fatty Acids to Their $\alpha,\beta$ -Unsaturated, $\beta,\gamma$-Unsaturated, and $\beta$-Hydroxy Derivatives by Enzymes from the Cellular Slime Mold, *Dictyostelium discoideum*  
Frank Davidoff and Edward D. Korn  


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