# Thermodynamic Bases for Fatty Acid Ethyl Ester Synthase Catalyzed Esterification of Free Fatty Acid with Ethanol and Accumulation of Fatty Acid Ethyl Esters†

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ABSTRACT: Myocardial homogenates rapidly synthesize fatty acyl ethyl esters from nonesterified fatty acid and ethanol in the absence of coenzyme A or ATP, and the enzyme catalyzing this reaction, fatty acid ethyl ester synthase, has been purified 5400-fold to homogeneity [Mogelson, S., & Lange, L. G. (1984) *Biochemistry* (preceding paper in this issue)]. To define the factors permitting this de novo synthesis of ester bonds and the consequent accumulation of fatty acyl ethyl esters in myocardium, we determined thermodynamic parameters relevant to the kinetics and equilibria of this reaction and specifically characterized (1) the rates of synthesis of ethyl oleate, in both the presence and absence of purified enzyme catalyst, and (2) the physical properties of the product, ethyl oleate, in an aqueous milieu. Compared to the reaction of ethanol and oleate in the absence of catalyst, fatty acid ethyl ester synthase enhanced the rate of ethyl oleate synthesis by reducing the free energy of activation ($\Delta G^*$) from 32.5 to 19.9 kcal/mol, effected in large part by a positive entropy shift, $\Delta S^*_{enz} - \Delta S^*_{uncat} = 23.9$ cal/(mol·deg). Rate constants in the presence and absence of enzyme at 37 °C were $6 \times 10^{-2}$ $s^{-1}$ and $7.8 \times 10^{-11}$ $M^{-1}$ $s^{-1}$, respectively, indicating a catalytic power of at least $10^8$ M for this enzyme. Kinetic data indicated an enzymatic $V_{max}$ of 1.25 nmol/(mg·s) (37 °C). The equilibrium constant was calculated for the reaction oleate + ethanol ⇌ ethyl oleate and was 0.095 $M^{-1}$ at 37 °C. Since this equilibrium is slightly thermodynamically unfavorable ($\Delta G$ = +1.45 kcal/mol) even though ethyl esters are known to accumulate in myocardium, we considered the possibility that self-association of the lipid product, ethyl oleate, in an aqueous medium, is a thermodynamically more favorable step permitting net product accumulation. Indeed, aggregation of ethyl oleate occurred in water in a temperature-dependent manner between 20 and 44 °C, e.g., at and above 0.7 μM at 37 °C, associated with a $\Delta G°$ of −11.1 kcal/mol. These data indicate that (1) the synthesis of fatty acyl ethyl esters is rapidly facilitated by fatty acyl ethyl ester synthase, an augmentation of reaction rate that reflects a reduction in $\Delta G^*$ largely due to a positive entropy change, and (2) accumulation of product, fatty acyl ethyl ester, is a consequence of the thermodynamically favorable self-association of monomeric molecules into a hydrocarbon phase. These results elucidate an entropic rather than enthalpic basis for covalent bond formation in a biologic system that may bear importantly on the biosynthesis of other naturally occurring esters.

Ethanol incorporation into a family of fatty acid ethyl esters has been documented to occur in isolated rabbit heart homogenates incubated with ethanol (Lange et al., 1981) and human myocardium obtained at necropsy from ethanol imbibers (Lange & Sobel, 1983). The lipid precursor for this esterification with ethanol is nonesterified fatty acid (Lange, 1982), and heat-inactivation studies suggested the reaction was enzyme mediated. The existence of such enzymes was confirmed by the identification of two enzymes in rabbit myocardium capable of catalyzing the synthesis of fatty acid ethyl esters from nonesterified fatty acid and ethanol and the subsequent purification to homogeneity of the major fatty acid ethyl ester synthase (Mogelson & Lange, 1984). The availability of this homogeneous enzyme has permitted studies to elucidate the thermodynamic bases of the enzyme-catalyzed synthesis of fatty acid ethyl esters.

Nonesterified fatty acid in myocardium is generally considered to require activation by esterification to coenzyme A (with consumption of ATP) before undergoing further catabolic or synthetic reaction (Vary et al., 1981). A different fate for free fatty acids in myocardial homogenates, namely, esterification with short-chain alcohols to form fatty acid alcohol esters, is now known to be catalyzed by either of two soluble myocardial enzymes in the absence of ATP and coenzyme A (Mogelson & Lange, 1984). Since the uncatalyzed formation of fatty acid alcohol esters at neutral pH is exceedingly slow and it appeared unusual that ester bond formation should occur in myocardium independently of high-energy chemical intermediates, we undertook a study of the thermodynamics of the synthesis of fatty acyl ethyl esters catalyzed by purified rabbit myocardial fatty acyl ethyl ester synthase and contrasted the results to those obtained from observations of the uncatalyzed synthesis of fatty acyl ethyl ester from free fatty acid in ethanol. In addition, we have examined the physical properties of fatty acyl ethyl esters in an aqueous milieu that may contribute to their accumulation in tissues. The results provide a thermodynamic description of the catalytic mechanism for ester bond formation occurring in an aqueous environment at pH 7. Furthermore, we report the thermodynamic properties governing the intermolecular interactions of the neutral lipid product, fatty acyl ethyl ester, which contribute to the net synthesis and accumulation of fatty acyl ethyl esters in aqueous solutions. These data elucidate the mechanism, in macroscopic terms, for ester bond formation between ethanol and free fatty acid in the absence of cofactors.

## Materials and Methods

*Materials.* All materials used were the highest commercially available grade. [$^{14}$C]Oleic acid (59.9 Ci/mol) was obtained from Amersham. Ethyl [$^3$H]oleate was synthesized by acid-catalyzed esterification of [$^3$H]oleic acid in ethanol and isolated by preparative thin-layer chromatography (Mogelson & Lange, 1984).

*Uncatalyzed Synthesis of Fatty Acid Ethyl Esters.* To prepare fatty acid free of trace contaminating methyl esters

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present in commercially available fatty acid (99%, Sigma Chemical Co., St. Louis, MO), sodium oleate was dissolved in water (1.0 g in 50 mL) and extracted 3 times with ether. The aqueous solution was then acidified to pH 1.5 with HCl and extracted 3 times with glass-distilled benzene. The pooled benzene extracts were dried over $Na_2SO_4$, and the volume was reduced by evaporation under reduced pressure at 32 °C. Oleic acid thus obtained was chromatographically homogeneous on silica gel OF plates developed with petroleum ether/ether/acetic acid, 75/5/1 (Lange et al., 1981).

After removal of benzene by rotary evaporation, purified oleic acid was dissolved in either neat ethanol or a mixture of ethanol and aqueous tris(hydroxymethyl)aminomethane (Tris-HCl), pH 6.0 (13 M ethanol, 14 M $H_2O$, 0.1 M Tris-HCl), to give a final concentration of 5–25 mM. Aliquots of 10–20 mL were incubated at 20, 37, or 56 °C for 20 h. At the end of the incubation, 500 nmol of ethyl arachidate was added as an internal recovery standard, and the ethanolic solutions were evaporated under reduced pressure at 32 °C. Residual lipids were dissolved in acetone, and fatty acyl ethyl esters were isolated by thin-layer chromatography as previously described (Mogelson & Lange, 1984). Fatty acyl ethyl esters thus obtained were then quantitated by gas chromatography (Lange et al., 1981). Ethyl oleate concentration was determined by comparison of its peak area to that of the added internal standard, ethyl arachidate. Second-order rate constants were calculated from the initial concentrations of reactants, $a_0$ and $b_0$, and the concentration of product, ethyl oleate, $X$, at time $t$ (seconds) by using the relation (Laidler, 1965):

$$k = \frac{1}{t(a_0 - b_0)} \ln \frac{b_0(a_0 - X)}{a_0(b_0 - X)} \quad (1)$$

*Enzymatic Synthesis of Ethyl Esters.* Fatty acyl ethyl ester synthase was purified to homogeneity as described in the accompanying paper (Mogelson & Lange, 1984) and had a specific activity of 0.3 nmol/(mg·s) when assayed in the presence of 0.4 mM oleic acid and 0.2 M ethanol. Enzyme was incubated in sodium phosphate buffer, pH 7.2, with varying concentrations of [$^{14}$C]oleic acid and ethanol as indicated in the text in a total volume of 0.17 mL. Reactions were terminated by the addition of 10 volumes of cold acetone containing known amounts of ethyl [$^3$H]oleate and cold carrier ethyl oleate. Volumes were reduced by evaporation under nitrogen, and residual lipids in acetone were separated by thin-layer chromatography as described above. Fatty acyl ethyl ester spots were scraped, eluted, and assayed for radioactivity. $^{14}$C counts were adjusted for yield as determined by recovery of $^3$H. Results are expressed as nanomoles synthesized per milligram per second. The equilibrium constant, $k_{eq}$, for the reaction

$$FA + EtOH \rightleftharpoons FAEE_{mon} \quad (2)$$

(where the subscript mon denotes the monomeric form of fatty acyl ethyl ester in aqueous solution) was determined by using initial concentrations of substrates selected to yield product in equilibrum concentrations less than the solubility limit for ethyl oleate (vide infra) in water, e.g., 10 μM oleic acid and 0.2 M ethanol in a total volume of 2 mL. For these experiments, reaction products were extracted into petroleum ether/diethyl ether/acetic acid, 75/5/1, and then chromatographed as described previously (Mogelson & Lange, 1984). Equilibrium was assumed to exist when product concentration remained constant with time and did not change with addition of more enzyme.

*Ethyl Oleate Self-Aggregation.* Ethyl [$^3$H]oleate was periodically repurified by preparative thin-layer chromatography on silica gel H plates developed with petroleum ether/diethyl ether/acetic acid, 75/5/1, to maintain radiopurity >98% and stored in toluene over Amberlite IRA 400 OH.

The self-aggregation of monomeric ethyl oleate in water was assayed by two methods. In the first method, optically clear aqueous dispersions of ethyl [$^3$H]oleate were prepared by sonication. A toluene solution of ethyl [$^3$H]oleate was evaporated with a stream of nitrogen in a 30-mL Corex tube. Traces of residual solvent were removed under reduced pressure. All subsequent reactions were carried out with reactants and vessels equilibrated to the selected temperature. After addition of 12 mL of water, the sample was sonicated for 2 min at 15 W in a Branson Model 185 cell disrupter equipped with a microtip probe. Following sonication, the suspension was allowed to stand at the selected temperature for 15 min before being loaded into one chamber of 10-mL Lucite equilibrium dialysis cell fitted with a Spectra Por 1 dialysis membrane ($M_r$ cutoff of 1000). The opposite chamber contained water. At selected intervals, an aliquot from the water side was assayed for radioactivity. To eliminate errors introduced by loss of sample volume from the water side of the cell, a separate dialysis experiment was performed for each time interval. The increase in radioactivity in the water side of the dialysis cell with time was determined for a range of concentrations of ethyl [$^3$H]oleate. Diffusion rates, calculated as the slopes of the lines thus obtained, were then plotted as a function of ethyl [$^3$H]oleate concentration in the initial sonicate, yielding two lines (fitted by the least-squares method) indicating rates of diffusion of monomers across the membrane above and below the aggregating concentration for ethyl oleate. The aggregating concentration of monomeric ethyl oleate in water was taken as the concentration of ethyl [$^3$H]oleate at which the two lines intersected (Mukerjee & Mysels, 1970).

The second method of assaying aggregation of ethyl oleate was a fluorescent dye incorporation technique (Kovatchev et al., 1981). Sonicates of ethyl oleate ($10^{-5}$–$10^{-8}$ M) were prepared in aqueous solutions of *N*-naphthylaniline at selected temperatures. At each temperature, relative fluorescence emission intensity was determined over a range of ethyl oleate concentrations by irradiating samples at 350 nm and scanning for fluorescence emission from 390 to 500 nm on a Varian SF-330 fluorometer. Peak relative intensities of fluorescence were then plotted as a function of ethyl oleate concentration to yield two lines (fitted by the least-squares method) intersecting at the concentration at which aggregates form.

*Other Analytical Methods.* Protein was assayed by Lowry's technique (Lowry et al., 1956). Gas chromatography was performed as previously described (Lange et al., 1981).

*Thermodynamic Calculations.* Thermodynamic parameters characterizing rates of synthesis of fatty acyl ethyl ester were determined by using the relation (Jencks, 1969; Dixon & Webb, 1979):

$$k = \frac{k_B T}{h} e^{-\Delta G^*/(RT)} \quad (3)$$

where $k$ is the rate constant, $k_B$ is Boltzman's constant, $T$ is temperature (K), $h$ is Planck's constant, and $R$ is the gas constant, equal to 1.986 kcal mol$^{-1}$ deg$^{-1}$.

Energies of activation $E_a$ were calculated from the slope of the line relating ln $k$ and $T^{-1}$, from which enthalpies of activation $\Delta H^*$ were calculated (Dixon & Webb, 1979):

$$\text{slope} = \frac{-E_a}{R} \qquad \Delta H^* = E_a - RT \quad (4)$$

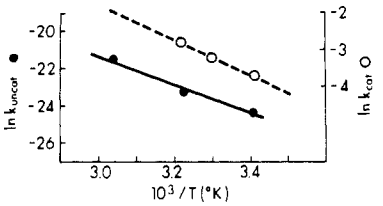


FIGURE 1: Rate constants for ethyl oleate formation as a function of 1/temperature. Rate constants for spontaneous formation of ethyl oleate from oleic acid in neat ethanol (●) and for enzyme-catalyzed formation of ethyl oleate in aqueous solution (○) were determined as described in the text.

Entropies of activation $\Delta S^*$ were determined from the general relationship between $\Delta G$ and $\Delta H$:

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

Thermodynamic parameters related to the equilibrium reaction (eq 2) were determined from $K_{eq}$ measured at selected temperatures and the relation between $\Delta G°$ and $K_{eq}$:

$$\Delta G° = -RT \ln K_{eq} \quad (6)$$

The energy changes associated with the equilibrium between monomeric fatty acid ethyl ester and aggregates of fatty acid ethyl ester in water

$$FAEE_{mon} \rightleftharpoons FAEE_{HC} \quad (7)$$

(where subscript HC denotes the hydrocarbon phase) were determined by using the relation:

$$\Delta G° = RT \ln C \quad (8)$$

where $C$ is the concentration of monomers in solution in the aqueous phase in mole fraction units. $\Delta G°$, $\Delta H°$, and $\Delta S°$ for reaction 7 were derived from $C$ measured at selected temperatures (Tanford, 1972).

## Results

*Uncatalyzed Synthesis of FAEE.* The rate of formation of ethyl [$^{14}$C]oleate in the standard enzyme assay mixture, but in the absence of enzyme (0.4 mM [$^{14}$C]oleic acid and 0.2 M ethanol in 0.06 M sodium phosphate buffer, pH 7.2), incubated for 24 h was so low as to be indistinguishable from blank (zero time) incubations. We therefore determined rates of ethyl oleate production, in this case, by incubating highly purified oleic acid dissolved in either neat ethanol or an ethanol/water mixture at selected temperatures. Product formation was quantitated by extraction of the reaction mixtures and analysis of extracted lipids by gas chromatography as described. Rate constants were calculated (eq 1) and used to derive the thermodynamic parameters characterizing the rate-limiting step in formation of active complex. When 24 mM oleic acid was incubated in a 14 M water and 13 M ethanol mixture at pH 6, no ethyl oleate was detectable after 20 h of incubation over the temperature range 20–56 °C. An upper limit for the rate constant for ethyl oleate formation in these conditions was calculated by assuming a maximal amount of product (2 nmol) that would still be just below the limits of detectability. The value thus obtained was $4.3 \times 10^{-12}$ $M^{-1}$ $s^{-1}$. In contrast, product formation was more easily detectable for the reaction in neat ethanol (16.8 M). Rates of ethyl oleate formation under these conditions were determined at 20, 37, and 56 °C. The respective rate constants ($k_{uncat}$) were $2.6 \times 10^{-11}$, $7.8 \times 10^{-11}$, and $4.1 \times 10^{-10}$ $M^{-1}$ $s^{-1}$, values that are 6–100-fold greater than the estimated constant for the aqueous system. The slope of the line relating ln $k_{uncat}$ to $T^{-1}$ (Figure 1) was used to calculate $E_a$ and $\Delta H^*$ (eq 4). $\Delta G^*$ and $\Delta S^*$ were calculated by using eq 3 and 5. Thus, $\Delta G^*$ was determined to be 32.5 kcal/mol, $\Delta S^*$ was −59.4 cal/(mol·deg), and $\Delta H^*$ was 14.1 kcal/mol (Table I).

Table I: Transition-State Thermodynamic Properties of Fatty Acid Ethyl Ester Synthesis[a]

| | kcal/mol | | $\Delta S^*$ [cal/ | |
|---|---|---|---|---|
| | $\Delta G^*$ | $\Delta H^*$ | (mol·deg)] | $k$ |
| uncatalyzed | 32.5 | 14.1 | −59.4 | $7.8 \times 10^{-11}$ $M^{-1}$ $s^{-1}$ |
| catalyzed | 19.9 | 8.9 | −35.5 | 0.060 $s^{-1}$ |

$k_{cat}/k_{uncat} = 7.7 \times 10^8$ M
$\Delta S^*_{cat} - \Delta S^*_{uncat} = 23.9$ cal/deg

[a] Comparison is made between the uncatalyzed reaction of 24 mM oleic acid in neat ethanol at 37 °C and the reaction of 0.4 mM oleic acid and 0.2 M ethanol at pH 7.2 catalyzed by fatty acid ethyl ester synthase at 37 °C.

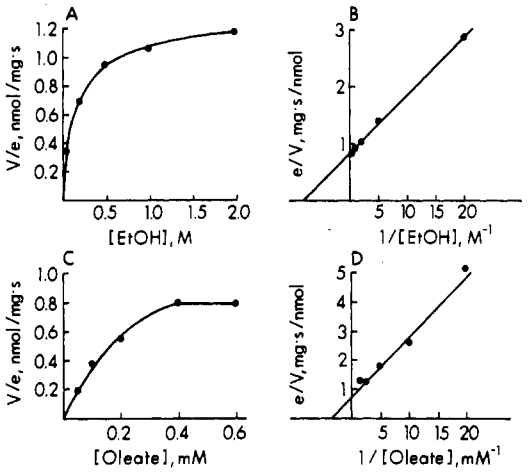


FIGURE 2: Kinetics of ethyl oleate formation catalyzed by purified fatty acyl ethyl ester synthase. Panels A and C are velocity vs. substrate concentration plots for substrates ethanol and oleic acid, respectively. Panels B and D are the respective double-reciprocal graphs. Enzyme (3 μg/mL) was incubated in 80 mM sodium phosphate, pH 7.2, at 37 °C for 1 h. For incubations with varying ethanol concentrations, the concentration of [$^{14}$C]oleate was 0.6 mM (panels A and B). For the experiments in panels C and D, the ethanol concentration was 1 M. Identical experiments were performed at 30 and 27 °C as described in the text.

*Enzyme-Catalyzed Formation of Fatty Acid Ethyl Esters.* Ethyl oleate synthesis catalyzed by fatty acyl ethyl ester synthase occurred readily in an aqueous milieu at neutral pH and at physiological concentrations of both substrates. Rates of synthesis of ethyl oleate catalyzed by the synthase were determined at selected temperatures, and $V_{max}$ was determined at each temperature. Velocity vs. substrate concentration plots were obtained with five concentrations of [$^{14}$C]oleate (0.04–0.60 mM) and five concentrations of ethanol (0.05–2.0 M). The results indicated that the maximal rate of product formation occurs in the presence of 0.4–0.6 mM oleate and 1–2 M ethanol. Two such plots are shown in Figure 2. Experiments performed at 37, 30, and 20 °C indicated maximal velocities of 1.25, 0.84, and 0.49 nmol/(mg·s), respectively, as determined from the double-reciprocal plots. The derived catalytic rate constant $k_{cat}$ at each temperature was then used to calculate thermodynamic parameters as described under Materials and Methods. For the formation of ethyl oleate at 37, 30, and 20 °C catalyzed by fatty acyl ethyl ester synthase, $k_{cat}$ was 0.060, 0.040, and 0.024 $s^{-1}$, respectively. The free energy of activation $\Delta G^*$ was 19.9 kcal/mol, the enthalpy component $\Delta H^*$ was 8.9 kcal/mol, and the entropy component $\Delta S^*$ was −35.5 cal/(mol·deg) (Table I).

*Equilibrium Thermodynamics.* Equilibrium concentrations of substrates and monomeric ethyl oleate product (reaction 2) were determined at three temperatures, 16.5, 30, and 37

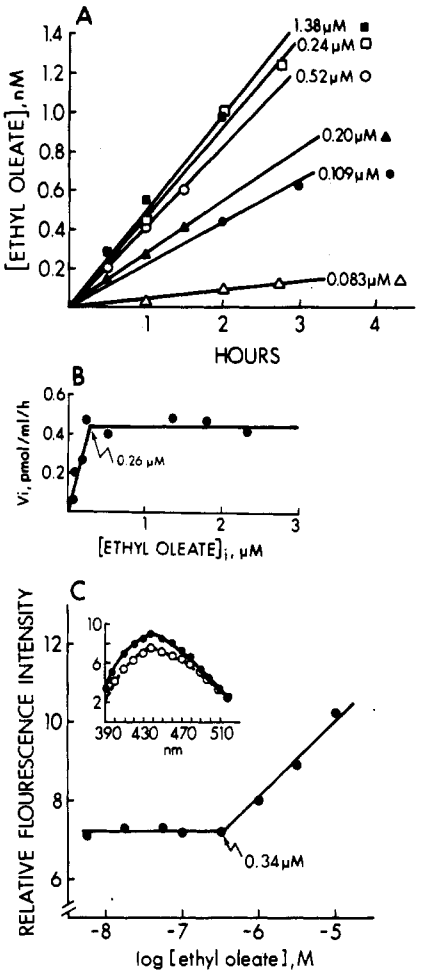


FIGURE 3: Aggregation of ethyl oleate in water. Aggregation concentrations were determined by two methods. Panels A and B depict results obtained with the membrane dialysis method at 22 °C. Panel A shows linear increases in [ethyl [$^3$H]oleate] in the receiving side of the dialysis chamber for a range of concentrations of ethyl [$^3$H]oleate in the starting sonicate [(△) 0.083 μM; (●) 0.109 μM; (▲) 0.20 μM; (□) 0.24 μM; (○) 0.52 μM; (■) 1.38 μM]. Lines for initial concentrations equal to 1.8 and 2.4 μM were omitted for clarity. Panel B shows the slopes of the lines obtained in (A), $V_i$ plotted against sonicate concentrations. Two lines intersect at the aggregating concentration, 0.26 μM. Identical experiments were performed at 30, 37, and 44 °C, with results as described in the text. Panel C depicts results obtained by the fluorescent dye incorporatiion method at 24 °C. A saturated aqueous solution of the dye, *N*-naphthylaniline, was used to prepare sonicates of ethyl oleate, $10^{-5}$–$10^{-8}$ M, at 24 °C. The samples were irradiated at 350 nm and scanned for emission fluorescence from 390 to 520 nm. Peak relative emission fluorescence intensity was then plotted against ethyl oleate concentration. Two lines intersecting at the aggregating concentration were obtained. The inset shows an example of fluorescence scans for $10^{-5.5}$ (●) and $10^{-7.25}$ M (○) sonicates. Identical experiments were performed at 31 and 37 °C.

°C, as described under Materials and Methods, and used to calculate values for $K_{eq}$ of 0.054, 0.093, and 0.095 $M^{-1}$, respectively. These data define a line relating ln $K_{eq}$ to $T^{-1}$:

$$\ln k_{eq} = -2600T^{-1} + 6.1 \qquad r = -0.95$$

where the slope d ln $k_{eq}/dT^{-1}$ is $-\Delta H/R$ and the intercept is $\Delta S/R$ for reaction 2. Thus, $\Delta H$ was 5.2 kcal/mol, and $\Delta S$ was 12.1 cal/(mol·deg). $\Delta G$, calculated as $\Delta G = -RT \ln K_{eq}$, was 1.45 kcal/mol at 37 °C.

*Aggregation of FAEE Molecules.* Rates of diffusion of monomeric ethyl oleate from sonicated aqueous suspensions across dialysis membranes (with pore size selected to exclude aggregates of more than three molecules) into water were determined for concentrations of ethyl oleate between 0.01 and

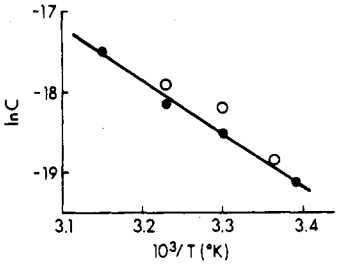


FIGURE 4: Effect of temperature on aggregation of ethyl oleate in water. Aggregation was determined at the indicated temperatures by either the diffusion rate (●) or dye incorporation (○) method, as described in the legend to Figure 3 and under Materials and Methods.

3 μM at four temperatures, 22, 30, 37, and 44 °C. Figure 3 shows the results of a typical experiment performed at 22 °C. At each sonicate concentration, the rate of diffusion of monomer was linear over the duration of the experiment, up to 3 h. Replotting the rates of diffusion, obtained as the slopes of the lines demonstrating increasing ethyl [$^3$H]oleate concentrations in the water side of the cell over time, vs. initial sonicate concentration yielded two lines intersecting at the critical aggregating concentration *C*. From this method, *C* was determined to be 0.26, 0.50, 0.70, and 1.4 μM at 22, 30, 37, and 44 °C, respectively. These data define a line (Figure 4) relating ln *C* (expressed as mole fraction units) and $T^{-1}$:

$$\ln C = -6800T^{-1} + 3.95 \qquad r = -0.99$$

where the slope d ln $C/dT^{-1}$ is $\Delta H°/R$ and the intercept is $-\Delta S°/R$ as derived from eq 5 and 8. Thus, for the formation of aggregates as described by eq 7, $\Delta H°$ was −13.5 kcal/mol, $\Delta S°$ was −7.8 cal/(mol·deg), and $\Delta G$ was −11.1 kcal/mol.

These conclusions were confirmed by determining concentrations of ethyl oleate that result in aggregation in water at selected temperatures with a second, independent assay employing a fluorescent dye incorporation method (Figure 3C). The values thus obtained agree well with those noted above, with aggregating concentrations of ethyl oleate at 24, 31, and 37 °C being 0.34, 0.72, and 0.93 μM, respectively (Figure 4).

## Discussion

Fatty acyl ethyl esters are now known to accumulate in myocardium after in vivo exposure to ethanol and have been shown to be enzymatic products of nonoxidative ethanol metabolism in myocardium (Lange et al., 1981; Lange, 1982; Lange & Sobel, 1983; Mogelson & Lange, 1984). The lipid precursor for the fatty acyl moiety of fatty acyl ether esters was identified as nonesterified fatty acid (Lange, 1982), thus establishing previously undescribed routes of metabolism for both ethanol and free fatty acid in myocardium. In general, the de novo formation of fatty acyl ester bonds is associated with a positive free energy of formation ordinarily available from ATP and thermodynamically expressed as exploiting a downhill reaction with respect to $\Delta H$. Activation of free fatty acids by esterification to coenzyme A, with the consumption of ATP, has been thought to be an essential prerequisite to further metabolism of free fatty acid by myocardium (Vary et al., 1981). In the case of fatty acyl ethyl esters, however, the occurrence of esterification of free fatty acid with ethanol catalyzed by fatty acyl ethyl ester synthase suggested the presence of another energy source for ester bond formation. The availability of purified fatty acid ethyl ester synthase permitted experiments to elucidate the thermodynamic bases for ester bond formation underlying enzymatic synthesis of fatty acyl ethyl esters. Additional experiments characterized some of the intermolecular interactions of the neutral lipid

product in an aqueous milieu that permit its accumulation.

Rates of formation of fatty acid ethyl esters were determined in the presence and absence of enzyme catalyst. The synthesis of ethyl oleate in standard aqueous assay medium but without added enzyme was undetectable with the methods used. Previous studies of the acid-catalyzed esterification of carboxylic acid with alcohol (reaction 9) established that the

$$RCOOH + R'OH_2^+ \rightleftharpoons RCOOR' + H_3O^+ \quad (9)$$

esterification process may be slowed by increasing concentrations of water (Smith, 1939). Thus, an equilibrium is established between protonated alcohol and water:

$$ROH_2^+ + H_2O \rightleftharpoons H_3O^+ + ROH \quad (10)$$

Since the rate of ester formation (reaction 9) is proportional to the concentration of $ROH_2^+$, increasing the concentration of water shifts the equilibrium for reaction 10 to the right, thereby reducing the concentration of $ROH_2^+$, with a resultant reduction in the rate of the acid-catalyzed esterification (reaction 9). In agreement with expectations based on this analysis, we were able to detect ester formation in the absence of enzyme catalyst and at neutral pH only when oleic acid was incubated in neat ethanol for prolonged periods.

In contrast, rates of ethyl oleate synthesis were easily assayable in the standard aqueous media in the presence of fatty acid ethyl ester synthase. Thus, incubations at selected temperatures under both these conditions permitted determination of thermodynamic parameters that allow comparison of the synthesis of ethyl oleate in the presence of enzyme catalyst to that in the absence of enzyme, though such estimates serve as a minimal evaluation. The data in Table I demonstrate that the reduction in the free energy of activation ($\Delta G^*$) attributable to the presence of enzyme catalyst is −12.6 kcal/mol (compared to the uncatalyzed reaction in ethanol), and of this difference, 8.3 kcal/mol is contributed by the entropy term $T\Delta S^*$. Thus, when the thermodynamic parameters for the synthesis of fatty acyl ethyl esters from free fatty acid in neat ethanol are contrasted with the enzyme-catalyzed reaction, the presence of fatty acyl ethyl ester synthase reduces $\Delta G^*$ largely by means of a positive entropy term, with $\Delta S^*_{cat} - \Delta S^*_{uncat}$ being 23.9 eu. This large positive entropy change is consistent with previously described reaction mechanisms for the formation of esters from alcohols and organic acids (Smith, 1939). In the absence of enzyme catalyst, it may be necessary for the alcohol and acid molecules to be oriented in a particular fashion upon collision in order to form ester and water, i.e., in a low-entropy state. The presence of enzyme may facilitate this orientation and permit the reaction of more randomly oriented molecules to form ester product (at a higher entropy level). This large positive entropy change accounts for the bulk (62%) of the reduction in $\Delta G^*$ attributed to the presence of enzyme catalyst, thereby permitting more rapid equilibrium between reactants and products and allowing in vivo synthesis of fatty acyl ethyl ester to occur at an appreciable rate.

A similar analysis contrasting enzyme-catalyzed fatty acyl ethyl ester synthesis with the uncatalyzed reaction in neutral aqueous media was not feasible (see above), but a quantitative comparison of the catalytic power of the enzyme for these conditions was obtained by using the estimated maximal rate constant for the uncatalyzed reaction in water, $4.3 \times 10^{-12}$ $M^{-1}$ $s^{-1}$, and the catalytic rate constant at 37 °C, 0.06 $s^{-1}$. The catalytic power of fatty acyl ethyl ester synthase thus obtained ($k_{cat}/k_{uncat}$) is at least $1.4 \times 10^{10}$ M.

To illustrate the catalytic power of this synthase in another way, we have used the rate constants measured experimentally for the uncatalyzed synthesis of ethyl oleate from oleic acid in ethanol to calculate the concentrations of substrate that would theoretically be required in the absence of catalyst to achieve the rates of synthesis of ethyl oleate observed in homogenates of rabbit myocardium. Ten percent myocardial homogenates (w/v) synthesized fatty acid ethyl esters from 0.04 M ethanol and 0.5 mM endogenous fatty acid at a rate of $1.6 \times 10^{-9}$ M $s^{-1}$ (Lange et al., 1981). For the rate of ethyl oleate synthesis in neat ethanol to be equal to the enzyme-catalyzed rate of fatty acid ethyl ester synthesis in homogenates, the product [ethanol][oleate] must equal 21.1 $M^2$ (rate = $7.75 \times 10^{-11}$ $M^{-1}$ $s^{-1}$ × [ethanol][oleate]). Thus, in neat ethanol (16.8 M), the oleate concentration must equal the unattainable concentration 1.14 M to achieve the rate of ethyl ester synthesis observed in assays of homogenates. The product of these substrate concentrations (16.8 M × 1.14 M) is 191 000 times greater than the corresponding product of substrate concentrations in homogenates achieving the same rate of ethyl oleate production ($5 \times 10^{-4}$ M × 0.2 M).

Equilibrium constants for reaction 2 were determined by incubating, at selected temperatures, purified fatty acyl ethyl ester synthase with oleic acid and ethanol in concentrations selected to yield equilibrium concentrations of ethyl oleate within its solubility limits in water. The equilibrium data indicate that the production of fatty acyl ethyl ester monomers is a thermodynamically unfavorable process, with a positive $\Delta G$ of 1.45 kcal/mol. However, it is apparent that, at concentrations of fatty acid and ethanol observed in myocardium in vivo, fatty acyl ethyl ester concentrations could readily exceed their solubility in water. For example, if fatty acid and ethanol concentrations were 0.3 mM and 0.05 M, respectively, at equilibrium the predicted fatty acyl ethyl ester monomer concentration would be 1.4 μM, a value in excess of the solubility of monomeric ethyl oleate in water at 37 °C (0.7 μM).

We therefore considered the possibility that net accumulation of fatty acyl ethyl esters is a consequence of a thermodynamically favorable self-association of fatty acyl ethyl ester molecules present in concentrations above the solubility limits of fatty acyl ethyl ester monomers in water. This thermodynamic analysis of the self-association of ethyl oleate in water treats the aggregates as a separate hydrocarbon phase, so that at each temperature studied the monomers comprise the "soluble" ethyl oleate in water and aggregates of ethyl oleate are considered to be that amount of hydrocarbon present in excess of solubility. We then analyzed thermodynamically the equilibrium between the saturated solution of monomeric ethyl oleate in water and the hydrocarbon, or aggregate phase (Tanford, 1980). The solubility of monomeric ethyl oleate molecules in water increased with rising temperature over the range 22–44 °C as assayed by two independent methods commonly used to determine critical aggregating concentrations of amphiphiles in aqueous solution. The negative $\Delta S°$ obtained represented an unanticipated observation that suggests that the solubilized ethyl oleate molecules in water comprise a state of maximal entropy compared to either free fatty acid or aggregated ethyl oleate. This likely reflects a greater dispersal of water molecules that occurs when going from the carboxylate anion to the monomeric form of ethyl ester than that which occurs when going from the neutral monomeric form to the neutral aggregated one. Corresponding data for other long-chain esters are not, to our knowledge, available for comparison. At 37 °C, $G°_{HC} - G°_{W}$, the unitary free-energy change associated with transfer of ethyl oleate from the water phase to the organic phase, is −11.1 kcal/mol. By comparison, the corresponding value predicted for a 20-carbon *n*-alkene [using the formula $\Delta G° = -1503 - 884n$ (Tanford,

Table II: Equilibrium Thermodynamics for Ethyl Oleate Synthesis and Accumulation[a]

| reaction | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ [cal/(mol·deg)] |
|---|---|---|---|
| (1) oleate + EtOH $\rightleftharpoons$ (ethyl oleate)$_{mon}$ | 1.45 | 5.2 | 12.1 |
| (2) (ethyl oleate)$_{mon}$ $\rightleftharpoons$ (ethyl oleate)$_{HC}$ | −11.1 | −13.5 | −7.8 |
| (1 + 2) oleate + EtOH $\rightleftharpoons$ (ethyl oleate)$_{HC}$ | −9.65 | −8.3 | 4.3 |

[a] Thermodynamic parameters for synthesis of ethyl oleate (1) and for aggregation of ethyl oleate (2) were determined at 37 °C as described in the text.

1972)], a less polar molecule than ethyl oleate, is −19.1 kcal/mol; for *n*-octadecylbetaine, a more polar compound than fatty acid ethyl ester [$\Delta G° = 2931 - 732n$; see Tanford (1980), p 67], the unitary free energy of micellization is −10.2 kcal/mol. Although the formulas used in calculating these comparative free energies are derived from data based on experimental determinations with smaller ($n < 11$) hydrocarbons, they serve as a useful comparison. It should be borne in mind that the hydrocarbon phase referred to here, for the aggregation of ethyl oleate, is not a bulk self-solvent phase but is contained within an optically clear aqueous sonicate. The thermodynamic parameters derived here for aggregation of ethyl oleate may be quantitatively different from observations of other systems, e.g., the transfer of hydrocarbon from water (where it may be present as both monomers and multimolecular aggregates) to a bulk organic phase. However, this analysis does not exclude the possible aggregation in vivo of fatty acid ethyl esters to form such a bulk hydrocarbon phase, and this thermodynamically favorable process would also be expected to promote further the accumulation of ethyl esters.

Reactions 2 and 7 can be rewritten to express a minimal reaction sequence required for synthesis and accumulation of fatty acyl ethyl ester product:

$$FA + EtOH \rightleftharpoons FAEE_{mon} \rightleftharpoons FAEE_{HC} \qquad (11)$$

The equilibrium thermodynamic parameters describing this reaction process are listed in Table II. Synthesis of monomeric fatty acyl ethyl esters in an aqueous milieu is thermodynamically unfavorable ($\Delta G° = 1.45$ kcal/mol) with equilibrium favoring reactants ($K_{eq} = 0.095$ M$^{-1}$). However, in conditions achievable in vivo, equilibrium concentrations of product may exceed its solubility, and fatty acyl ethyl esters would then accumulate in an organic phase, a process that is thermodynamically favorable ($\Delta G° = -11.1$ *kcal*/mol). This analysis indicates that equilibrium favors product accumulation largely as a consequence of the thermodynamically favorable transfer of monomeric ethyl oleate, which is sparingly soluble in water, to a self-aggregated phase, which comprises multimolecular aggregates of fatty acyl ethyl ester, an event that occurs above the solubility limits defined by our data.

To our knowledge, this may be the first description of a biological, chemical reaction that is dependent on the entropic and physical properties of the reactants and catalyst. Thermodynamic analyses of biochemical reactions characteristically depend on determination of enthalpy changes and energies of activation since changes in entropy usually are quantitatively small compared to changes in enthalpy. However, for the reaction considered here, the synthesis of fatty acid ethyl esters from fatty acid and ethanol, the physical properties of the reactants and product in aqueous media contribute in an imporant way to the catalytic mechanism and to the accumulation of product. This reaction mechanism may apply to other biological pathways involving neutral lipids, for example, the esterification of cholesterol with fatty acid to form cholesteryl esters and the synthesis of triglyceride from diglyceride and acyl-CoA, though clearly enthalpic contributions may also be crucial. The present system is most amenable to the study of considerations pertaining to such systems, however, since both substrates are soluble in water in physiologic concentrations, and thus may serve to study synthetic reactions in which the product, neutral lipid, is sparingly soluble in aqueous media.

The data presented elucidate a macroscopic description of a reaction mechanism for ester bond formation for free fatty acids in the absence of high-energy intermediates, e.g., acyl-CoA. Fatty acyl ethyl ester synthase permits rapid equilibrium between reactants (fatty acid and alcohol) and product (fatty acyl ethyl ester) by lowering the free energy of activation, $\Delta G^*$, a reduction largely the result of a positive entropy change associated with active complex formation. Consequently, fatty acyl ethyl ester product accumulates, eventually exceeds solubility, and aggregates into a hydrocarbon phase in a thermodynamically favorable process.

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