Critical micelle concentrations (CMCs) of palmitoyl-CoA were determined by surface tension, conductivity, and fluorimetric measurements in a variety of buffers at several pH values and ionic strengths. They ranged from 7 to 250 \( \mu M \) and were frequently an order of magnitude higher than most reported values. The CMCs of stearoyl-CoA and oleoyl-CoA, determined fluorimetrically, were also high and consistent with the expected effects of chain length and unsaturation. The effects of ionic strength and temperature were analyzed to obtain the extent of counterion binding and the thermodynamic parameters of micellization. The values of \( \Delta H^0, \Delta G^0, \) and \( \Delta S^0 \) obtained in 0.011 M Tris, pH 8.3, are \(-6 \) KJ mol\(^{-1}\), \(-64 \) KJ mol\(^{-1}\), and \(+193 \) J mol\(^{-1}\)-K\(^{-1}\), and the average number of univalent ions bound per molecule in the micelles is 1.4. These values are within the range of those obtained for other univalent and polyvalent detergents. Analyzed by sedimentation and diffusion, the micelles are approximately spherical with an anhydrous mass of 50,000 daltons but with dimensions inconsistent with fully extended molecules. Correlation of the information obtained from the present physical studies with kinetic studies using long-chain fatty acyl-CoAs as enzyme substrates may be helpful for understanding the enzymology of these compounds, and some previously published kinetic studies of membrane-bound and soluble enzymes may bear reinterpretation.

As key intermediates in fatty acid catabolism and lipid biosynthesis, the long-chain fatty acyl-CoAs are substrates for a variety of membrane-bound and soluble enzymes (1, 2). These amphiphilic compounds, like other detergents, form molecular solutions only at low concentrations. As concentration is increased, the critical micelle concentration (CMC) is reached and association into micelles begins; above the CMC, the concentration of free molecules in equilibrium with micelles remains nearly constant and independent of total concentration providing mixed micelles are not present (3). It would not be surprising if the pronounced change in physical state at the CMC were reflected as changes in kinetic parameters, and in fact anomalous kinetic inhibition is frequently encountered when fatty acyl-CoAs are used as substrates (1, 4, 5). Such phenomena are often ascribed to the formation of micelles. However, increasing evidence suggests that enzyme inhibition by fatty acyl-CoAs can occur at concentrations well below the CMC (6–9) and supports a regulatory role for these substrates rather than nonspecific effects due to their detergent properties. Moreover, since the CMC of ionic surfactants can depend strongly upon pH, ionic strength, and temperature, it is possible that variations in the substrate concentration at which inhibition is observed (4–200 \( \mu M \)) might be partly explainable in terms of the effect of ambient conditions upon the CMC of the substrate. In order to clarify the role of fatty acyl-CoAs in the various enzyme systems, more extensive and dependable estimates of CMCs in biochemically relevant buffers are needed, as well as a better understanding of the effects of ambient conditions upon the CMC.

Until recently, the CMC of palmitoyl-CoA has been taken to be invariant at about 4 \( \mu M \), an estimate obtained by using the cationic dye pinacyanol chloride (10) and subsequently verified by using the same probe or rhodamine 6G (11). In contrast, alternative methods such as analytical ultracentrifugation, spin labeling with 6-doxylstearoyl-CoA, gel permeation chromatography (6), and ultrafiltration (7) suggest that the CMC of palmitoyl-CoA may be more than an order of magnitude higher than previously determined. Smaller discrepancies in the measured CMCs might be accounted for by variations in ionic strength, but the rather large and consistent disparity between the results from these latter techniques and those from pinacyanol chloride might arise from methodology. The combination of a dye and detergent of unlike sign has been shown to introduce artifacts when used to determine CMCs (12, 13), and in the case of SDS-pinacyanol chloride the formation of a salt of the dye and detergent has been demonstrated to take place at detergent concentrations well below the CMC (12). On the other hand, the use of a dye and detergent of like sign such as SDS-ANS (14, 15) or cetyltrimethylammonium bromide-rhodamine 6G (15) does not result in salt formation, and CMCs obtained with such probes agree well with those determined by techniques which introduce no foreign probes at all. The use of anionic dyes to evaluate the CMCs of fatty acyl-CoAs has not been reported. We have, therefore, employed this last method, together with the direct and relatively nonperturbing methods of surface tension and conductivity, to monitor the CMC of fatty acyl-CoAs and have obtained values under various conditions which are indeed higher than previously reported.

**MATERIALS AND METHODS**

Palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA, and CoASH were purchased from P-L Biochemicals (Milwaukee, WI). Less than 1% free fatty acids in all three fatty acyl-CoAs were revealed by xanthic acid dichromate charring of silica gel plates (16) developed in hexanes/diethyl ether/acetic acid (9:9:1, v/v). Paper chromatograms developed in 1-butanol/acetic acid/water (5:2:3, v/v) and examined under...
ultraviolet light or sprayed with nitroprusside (17) showed a single
absorbing spot. The ratio of the absorbance at 232 nm to the abso-
bance at 260 nm was 0.52-0.53, a value which meets the established
criteria for pure thioester (18), but Ellman's reagent (19) revealed 4%
free CoA. Similarly, 96% of the commercial product was precipitated
by 5 M KCl. The nonprecipitated material produced a UV spectrum
characteristic of free CoA or oxidized CoA, which are spectrally
indistinguishable. Because of the lability of the thioester bond furth-
ner purification was not pursued, and the compound was used as supplied
by the manufacturer.

The stability of fatty acyl-CoAs in the various buffers used in the
present studies was examined as a function of time, either by mea-
suring the ratio of absorbance at 232 nm to that at 260 nm or by
using Ellman's reagent to monitor the formation of free sulfhydryl
groups. Solutions of fatty acyl-CoAs in all of the buffers, both below
and above the CMC, showed negligible hydrolysis in 24 h at room
temperature; frozen at -20 °C they were stable for several weeks. All
solutions used for experiments were made fresh or taken from frozen
stocks. Loss of the fatty acyl-CoAs by adsorption to cuvettes and
other glass containers used for CMC determinations was shown to be
negligible by comparing the absorbances of aliquots removed from
the test apparatus with absorbances calculated from the dilution of stock
material.

Surface tensions of aqueous solutions of palmitoyl-CoA were mea-
sured at 25.0 ± 0.1 °C with a 1-cm sand-blasted platinum plate
coupled to a Cahn recording electrobalance (20). The container was
a Pyrex cylinder, 3.5 cm in diameter, siliconized with chlorotrimethy-
ysilane as a surfactant to the glass surface. The equip-
ment was calibrated with methanol at the desired temperature. Water
used to prepare buffers was doubly distilled from Pyrex and gave
surface tensions within 0.4 dyne cm⁻¹ of the accepted value, while
Tris buffer lowered the surface tension by about 1 dyne cm⁻¹. Con-
centrations were varied either by successively adding small aliquots
of a concentrated stock solution until the highest needed concentra-
tion was obtained or by diluting the highest concentration by succes-
sive additions of buffer; both methods yielded identical results. Each
dilution was stirred with a Teflon-coated magnetic stirrer for 1 h and
surface tensions were monitored unstirred for another 60 min.
At this point, no further change in the surface tension was taken as
an indication that an equilibrium distribution of palmitoyl-CoA had
been achieved.

For the conductivity measurements, a Copenhagen type CDM 2d
conductivity meter equipped with a dip cell was used; for scale
expansion, a mercury battery and potentiometer were added to the
recorder circuit to suppress the recorder zero. Water for the measure-
ments was distilled twice from Pyrex and passed through a mixed
ion-exchange resin bed to produce a conductance of 1.5-2.5 μmho, a
value frequently reported for water used in other conductivity studies
(21). A nitrogen purge excluded atmospheric CO₂. Solutions of KCl
at different concentrations were used to calibrate the instrument.
Ross's rule, successive aliquots of the concentrated palmitoyl-CoA
stock solution were added to water or 0.011 M Tris buffer in a Pyrex
tube thermostated to the desired temperature, and specific conduc-
tances were recorded after gentle mixing.

Fluorescence measurements were made on an Aminco-Bowman
spectrofluorometer with ANS and TNS as fluorescent probes. ANS
ammonium salt was purchased from Eastman Kodak and TNS potas-
sium salt from Aldrich. Titrations were done manually by adding
microliter quantities of a concentrated fatty acyl-CoA solution to a
buffer solution containing ANS or TNS. Relative fluorescence
was recorded after thorough mixing. As a control, fluorescence inten-
sities were recorded on separate solutions of palmitoyl-CoA prepared
at a fixed dye concentration before transferring to the cuvette; both
methods gave identical CMCs. The excitation wavelength for ANS
was 390 nm and for TNS 360 nm; the emission wavelengths were 505
and 460 nm. Intensities were recorded until a constant value was ob-
erved (22).

For the determination of the sedimentation and diffusion coeffi-
cients of palmitoyl-CoA, a Beckman model E analytical ultracentri-
fuge with Schlieren optics and a synthetic boundary cell was used.
An AD-2 rotor was employed at 50,740 rpm for the sedimentation
efficiency measurements and at 4086 rpm for the diffusion coeffi-
cients. A difference in the sedimentation coefficients of CoA between
both at concentrations above the CMC (22), formed in a 1-cm alu-
num centerpiece synthetic boundary cell with quartz windows.
The values of D and S were evaluated from the diffusion and sedi-
mantion patterns following well-established procedures (23).

RESULTS

Surface Tension—Palmitoyl-CoA in 0.10 M Tris-HCl at pH 8.5 and 25 °C produced the surface tension concentration plot shown in Fig. 1. The minimum is characteristic of surfactants containing small amounts of surface-active impurities (13, 24), sometimes comprising less than 0.1% of the total surfac-
tant, which preferentially adsorb at the air-water interface when the concentration of the surfactant is below the CMC and depress the surface tension below the value expected for the pure compound. When micelles form, the impurities re-
distribute from the surface to the micelles and the surface tension rises again. As determined by surface tension measure-
ments, the CMCs of surfactants which show such minima are usually below the CMCs obtained after the compound is purified (24). The 150-200 μM range (Fig. 1) can, therefore, be taken as a lower limit of the CMC of palmitoyl-CoA in
0.10 M Tris-HCl, pH 8.5. Curves analogous to Fig. 1 were also obtained in distilled water at pH 4.0 and showed CMCs in the range of 50-60 μM. A lower CMC in the distilled water might be expected, since ionization of the phosphate groups would be suppressed at low pH.

Because of the minimum in the surface tension plots, surf-
tace tension was used as an auxiliary method to obtain a prelimi-
ary estimate of the CMC range. For more accurate values conductivity and fluorescence, which are not sensitive
to trace contaminants, were used.

Conductivity—Fig. 2 shows the specific conductance of a solution of the dipotassium salt of palmitoyl-CoA in distilled
water at pH 4.0, plotted as the deviation from the straight
line in order to expand the scale. The two extrapolated
straight lines intersect at 60 ± 2 μM, which can be taken as the
CMC. The same experiment carried out in 0.011 M Tris
at pH 8.3 gave a CMC of 221 ± 20 μM. Attempts to measure
the CMC by conductivity in 0.1 M Tris-HCl at pH 8.5 gave un-
reliable data and were, therefore, abandoned. The presence
of salt reduces the precision of CMC determinations by con-
ductivity (13, 21) because the change in slope of the specific
conductance versus concentration becomes less pronounced as the salt concentration is increased (21). CMCs in 0.1 M
Tris-HCl, pH 8.5, with and without added salt were success-
fully determined by fluorescence.

The CMCs reported for both the conductivity and fluo-

![Fig. 1. Determination of the CMC of palmitoyl-CoA by surface tension measurements.](image-url)
metric methods are the intercept of the least-square straight line at concentrations below the slope change with the least-square straight line at concentrations above the slope change. The reported errors are the standard deviation of this intercept, calculated from the separate standard deviations of each line. Points in the immediate vicinity of the slope change are ignored, since curvature in that region arises from the gradual appearance of micelles and not from experimental error (13, 21).

Fluorescence—Measurements of CMCs made by monitoring spectral changes in added dyes must be interpreted cautiously. Serious artifacts may be introduced, possibly because of salt formation (12, 13), if anionic surfactants are combined with cationic dyes such as pinacyanol chloride, which has been the dye most commonly used to determine the CMC of palmitoyl-CoA. The formation of a salt with pinacyanol chloride and SDS can be identified by an absorption band near 485 nm at concentrations well below the CMC of the surfactant (12). Spectroscopic evidence for the formation of a similar salt of pinacyanol chloride and palmitoyl-CoA in 0.1 M Tris-HCl, pH 8.5, is shown in Fig. 3. The stoichiometry of the salt appears to be 3–4 dye molecules/palmitoyl-CoA molecule, a composition which seems reasonable since palmitoyl-CoA is fully ionized at pH 8.5.

The formation of a water-insoluble micelle-soluble salt is avoided if the charges of the surfactant and dye have a like sign (13, 15). Using ANS and TNS with palmitoyl-CoA in 0.050 M KPi at pH 7.4 and 23 °C, we obtained the results shown in Fig. 4. The CMC lies in the 70–80 μM range, and the following features can be noted. First, the measured CMC is independent of the dye used; both ANS and TNS give approximately the same intersection. Second, for the two dye concentrations tested, 10 and 40 μM, the measured CMC is independent of the dye concentration. Third, TNS exhibits greater fluorescence enhancement above the CMC than does ANS and hence provides a much more sensitive measure of the CMC. Similar differences in the fluorescent intensities of ANS and TNS have been reported from studies of SDS-dye combinations (14) and may occur because TNS more effectively samples the micelle interior.

The CMCs defined as the intersection of fluorimetric titrations were, within experimental error, quite reproducible and independent of the way in which the titrations were performed. That is, for palmitoyl-CoA identical CMCs were obtained when the concentration of surfactant was increased by consecutively adding small aliquots of the acyl-CoA to a solution of ANS or TNS in the cuvette or alternatively when separate solutions were prepared at a fixed dye concentration before transferring to the cuvette. Moreover, no time-dependent change in the CMC was observed upon measuring the CMC of palmitoyl-CoA within a few minutes after preparing the solutions or after overnight incubation with the dye, suggesting that unlike similar measurements with lyssolecithin (25), the micelle-monomer-dye equilibrium for fatty acyl-CoA is rapid compared to the time required for the usual experiments. A contaminant that could give rise to errors in the CMC determinations is the 4% free CoA present in the fatty acyl-CoA samples we employed. However, since an extra 4% exogenously added CoA had no effect upon the CMC of palmitoyl-CoA measured by fluorescence (Table I), free CoA can be discounted as a source of error at the levels encountered in the present investigation.

Fluorimetric titrations were also carried out in a variety of buffers at several pH values and ionic strengths; the results are summarized in Table I. In 10 mM KCl buffered at pH 6.7 with 6.7 mM phosphate, the value of the CMC of palmitoyl-CoA obtained with 10 μM TNS was 37 ± 6 μM, approximately 10 times higher than earlier reported for the same buffer system using pinacyanol chloride (10). In 0.1 M Tris-HCl at pH 8.5, a buffer frequently used in cell-free acyltransferase studies (26), the CMC was 202 ± 5 μM, whereas in 0.1 M Tris-HCl at pH 8.5 containing 0.4 M KCl, which gives optimal acyltransferase activity with reconstituted systems (27), the CMC was 6 ± 2 μM. From the CMC values listed in Table I it is clear that the CMC decreases upon increasing the ionic strength at constant pH, probably because at higher ionic strengths the decreased electrostatic repulsion between molecules favors micellization (28, 29). The lower CMC of palmitoyl-CoA in 0.050 M KPi at pH 7.4 (ionic strength 0.11 M)
Physical Properties of Fatty Acyl-CoA

for the oleoyl compound and 12 ± 2 µM for the stearoyl (Fig. 5). The changes seen when compared to the behavior of palmitoyl-CoA would be expected from an introduction of unsaturation or an increase in saturated chain length (29) and parallel quite precisely the CMCs of the potassium salts of the corresponding free fatty acids (potassium palmitate 2.2 mM, potassium oleate 1.2 mM, potassium stearate 0.4 mM) determined by refractive index measurements (30).

Salt and Temperature Effects on the CMC of Palmitoyl-

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**TABLE I**

Critical micelle concentrations of palmitoyl-CoA determined by fluorimetry

| Buffer                  | TNS µM | ANS µM |
|------------------------|--------|--------|
| 6.7 mM KPi, pH 6.7     | 36 ± 6 (10) |        |
| 0.050 M KPi, pH 7.4    | 77 ± 2 (10) | 71 ± 7 (10) |
| 0.050 M KPi, pH 7.4 + 4% CoASH  | 67 ± 3 (10) |        |
| 0.10 M Tris-HCl, pH 8.5 | 202 ± 5 (10) |        |
| 0.10 M Tris-HCl, pH 8.5 + 50 mM KCl | 55 ± 4 (10) |        |
| 0.10 M Tris-HCl, pH 8.5 + 200 mM KCl | 13 ± 1 (10) |        |
| 0.10 M Tris-HCl, pH 8.5 + 400 mM KCl | 6 ± 2 (10) |        |

*All values were corrected for 4% free CoA; CMC values were calculated through a least-square fit.

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FIG. 4. Determinations of the CMC of palmitoyl-CoA by fluorescence measurements at 23 °C. Titrations were performed by adding 20.0-µl aliquots of a stock solution of palmitoyl-CoA in 0.050 M KPi, pH 7.4, to 2.0 ml of the same buffer in the fluorimeter cuvette at room temperature containing: ▲, 10 µM ANS; or △, 40 µM ANS; or ●, 10 µM TNS; or ○, 40 µM TNS. The excitation wavelength was 390 nm for ANS and 360 nm for TNS, while the emission wavelength was 505 nm for ANS and 460 nm for TNS.

compared to the CMC obtained in 0.1 M Tris-HCl at pH 8.5 (ionic strength 0.03 M) must be explained primarily as a salt effect rather than a pH effect, since at both pH values palmitoyl-CoA is expected to be fully ionized.

Fluorimetry was also used to determine the CMCs of oleoyl-CoA and stearoyl-CoA at 23 °C in 0.050 M KPi, pH 7.4. Corrected for 4% free CoA, a value of 33 ± 1 µM was obtained.
The CMC of palmitoyl-CoA in 0.10 M Tris-HCl at pH 8.5 is strongly dependent upon salt concentration (Fig. 6). A linear relationship is observed between the log of the CMC and the log of the total ionic concentration. Pronounced salt effects and linear log-log plots have been reported for other ionic surfactants (28, 29, 31). Linearity is predicted thermodynamically for ionic surfactants if $\Delta G^0$ is independent of salt concentration (31). A useful parameter needed to estimate the thermodynamic parameters of micellization is $\alpha$, which can be estimated directly from the slope of the log-log plot and is a measure of the magnitude of the salt effect. It can be defined as the average number of counterions bound by each molecule in the micelle (28, 29). The liponucleotide is a polyvalent ionic surfactant, and the effect of salt upon the CMC of this class of amphiphiles should be considerably greater from that observed with uni-univalent ionic surfactants (28, 31, 34). The value of $\alpha$ of 1.4 obtained for palmitoyl-CoA is consistent with similar values observed with other polyvalent surfactants (28, 31, 34).

Conductivity was used to study the effect of temperature upon the CMC of palmitoyl-CoA in 0.011 M Tris at pH 8.3, a pH at which the molecules are fully ionized. Temperatures were controlled within 0.1 °C, and deviation plots such as the one shown in Fig. 2 were used to obtain the CMC as a function of temperature. A plot of log CMC against reciprocal temperature is shown in Fig. 7. Although such plots for surfactants are frequently curved and often pass through a minimum (32, 33), for palmitoyl-CoA in 0.011 M Tris a straight line is obtained. If the CMC is expressed as a molar concentration, in the presence of excess salt the thermodynamic parameters for micellization can be estimated from the following equations (34, 35).

$$\Delta G^0 = RT \ln \text{CMC} + \alpha RT \ln C_x - (1 + \alpha) RT \ln w$$

$$\frac{\partial \ln \text{CMC}}{\partial (1/T)} = \frac{\Delta H^0}{R} - \frac{\partial \alpha}{\partial (1/T)} (\ln C_x - \ln w)$$

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T}$$

In these equations, which are based upon the pseudo phase-separation model (28, 34, 35), $C_x$ is the molarity of added salt, $w$ is the concentration of water (55.5 M), and $\alpha$ is the extent of counterion binding given as 1.4 by the slope of Fig. 6. If the conventional assumption that $\alpha$ is independent of temperature is made, the standard Gibbs function, enthalpy, and entropy of micellization are $\Delta G^0 = -64 \text{ KJ mole}^{-1}$ at 25 °C, $\Delta H^0 = -6 \text{ KJ mole}^{-1}$, and $\Delta S^0 = 193 \text{ J K}^{-1} \text{ mole}^{-1}$. Although $\Delta G^0$ is a very large negative number because of the large entropic contribution, the values estimated for the thermodynamic parameters are within the bounds of those obtained for other univalent and polyvalent surfactants (28, 29).

Size and Shape of Palmitoyl-CoA Micelles—Diffusion and sedimentation velocity measurements were carried out on palmitoyl-CoA in 0.1 M Tris-HCl, pH 8.5, at 25 °C to estimate the aggregation number and shape of the micelles (22, 23). Fig. 8 shows the dependence of $s$ and $D$ upon palmitoyl-CoA concentration. While $s$ decreases with increasing concentration, $D$ increases with concentration in agreement with similar studies on SDS micelles (22). The extrapolated values are $s^0 = 4.6 \pm 0.4 \text{ S}$ and $D^0 = 8.2 \pm 0.1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. When corrected for 4% free CoA by assuming independent diffusion of micelles and free CoA, the diffusion coefficient is $7.7 \pm 0.1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. Both $s^0$ and the corrected $D^0$ were then normalized to the viscosity and density of water at 20 °C to give $s^0_{uw} = 4.2 \pm 0.3 \text{ S}$ and $D^0_{uw} = 6.8 \pm 0.1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. Used together with a partial specific volume of 0.70 ml g$^{-1}$ (6), in the Svedberg equation (36) these parameters give an anhydrous mass of $(50 \pm 4) \times 10^2$ daltons for the palmitoyl-CoA micelles, a value within the range recently determined by sedimentation equilibrium (6) and much lower than the $1000 \times 10^2$ daltons estimated earlier by light scattering (10). An anhydrous mass of 50,000 daltons strongly suggests that the particle is spherical or nearly spherical, a conclusion which can be demonstrated by calculating the anhydrous mass of a

![Fig. 7. The effect of temperature upon the CMC of palmitoyl-CoA](image)

![Fig. 8. The concentration dependence of the sedimentation (○) and diffusion (○) coefficients of palmitoyl-CoA in 0.10 M Tris-HCl at pH 8.5. Both $s$ and $D$ were measured at 25.0 °C with a 4° synthetic boundary cell by layering a more concentrated solution over a less concentrated solution, with both solutions maintained above the CMC. For the sedimentation coefficient, the effective concentrations plotted on the abscissa are $(C_1 V_1 + C_2 V_2)/(V_1 + V_2)$ where $C_1$ and $V_1$ are the concentration and volume of the liponucleotide solution placed in the cell, and $C_2$ and $V_2$ are the concentration and volume of the overlying solution (22). For the diffusion coefficient, the plotted concentrations are $(C_1 + C_2)/2$. The rotor was an AD-N operated at 50,740 rpm for sedimentation and 4,098 rpm for diffusion.](image)
The calculated anhydrous mass of a sphere having a sedimentation coefficient of 4.2 S, a partial specific volume of 0.70 ml g⁻¹ (6), and a hydration of 0.30 g H₂O g⁻¹ of micelle (37) is in fact 40,000 daltons. The anhydrous radius of the sphere is 22 Å, and its hydrated radius is 25 Å. If the usual analysis of the hydrodynamic data is carried out using the sedimentation and diffusion coefficients and a partial specific volume of 0.70 ml g⁻¹, the value obtained for f/f₀ is 1.3. This fractional ratio, taken together with a hydration of 0.3, is compatible with a prolate ellipsoid having an axial ratio of 3.6 or an oblate ellipsoid with an axial ratio of 4.0 (37). For the prolate ellipsoid the major and minor axes would be 113 and 31 Å; for the oblate ellipsoid they would be 76 and 19 Å.

For an ordinary detergent such as SDS or sodium palmitate, the molecules are usually considered to be fully or nearly fully extended radially in the micelles, and the dimensions obtained from hydrodynamic data are consistent with such a structure. In these cases such a model is reasonable, since the hydrocarbon chain accounts for most of the molecular length, the polar head extends radially in the micelles, and the dimensions obtained from the CMC are in fact due to the gradual appearance of micelles rather than a discontinuity at the CMC. That is, the extended palmitoyl-CoA molecule, which is about 50 Å (11, 38), cannot be accommodated within the calculated dimensions. The molecules might be accommodated, however, if the CoA portions were folded at the micelle surface, and this possibility will be discussed later.

**DISCUSSION**

In the physical studies reported here, surface tension, conductivity, and fluorescence were employed to characterize the micelles formed by long-chain fatty acyl-CoAs. CMC values obtained under various conditions, as well as size determinations of the micelles, are inconsistent with earlier reported values (10) but in good agreement with more recent studies (6).

Surface tension and conductivity are direct methods, where interference from added probes can be avoided, and they are commonly used with many detergents. Surface tension is very sensitive to traces of surface-active impurities (24), giving a minimum in the surface tension-concentration plot, and unless a highly purified surfactant is employed an approximate value of the CMC is obtained. Conductivity, on the other hand, is less sensitive to impurities, and determinations can be carried out in a short period of time. It is a method of choice for ionic detergents but becomes unsatisfactory at high salt concentrations where the increments in conductance are occluded by the elevated background conductance (21). In such cases fluorescence can be used as an alternative method. Fluorescent dyes are widely employed as indicators for the micellization of surfactants, but for ionic detergents unless the dye and detergent are carefully selected with respect to charge, the dye can perturb the system and introduce serious artifacts. Dyes and surfactants having opposite charges can form a micelle-soluble salt at concentrations much below the CMC (12, 13), but such premicellar association can be avoided if the dye and surfactant have like charges (13, 15).

Unfortunately, cationic dyes, particularly pinacyanol chloride, have been the most commonly used indicators for fatty acyl-CoA micellization (10). Under these conditions a salt can be formed between pinacyanol chloride and fatty acyl-CoA, as Fig. 3 shows, at concentrations well below the CMC. Similar salt formation has been reported earlier between pinacyanol chloride and SDS (12). However, ANS and TNS, both negatively charged dyes, have been successfully used to measure the CMC of SDS (14, 15); the values obtained are in agreement with those measured by conductance and surface tension and are considerably higher than those obtained by using cationic dyes. A similar effect is seen when TNS is substituted for pinacyanol chloride in solutions of the fatty acyl-CoAs. Measured either in a phosphate buffer used earlier with pinacyanol chloride (10) or in a Tris buffer where the formation of a salt with pinacyanol chloride has been demonstrated (Fig. 3), the CMC values obtained with TNS were approximately an order of magnitude higher (Table 1) than those recorded with the cationic dye.

The results obtained with palmitoyl-CoA and TNS, together with the following observations from the present and other studies, argue that pinacyanol chloride might cause an earlier event to occur which otherwise would not occur during micelle formation by long-chain fatty acyl-CoA. First, in a recent study (7) where both pinacyanol chloride and the more direct methods of ultracentrifugation and ultrafiltration were employed to measure the CMC of palmitoyl-CoA, CMC values obtained with pinacyanol chloride were consistently lower (4 μM) than those obtained by the other methods (15-30 μM). Second, pinacyanol chloride was used for the CMC determination of palmitoyl-CoA, increasing the CMC of the medium had either no effect on the measured CMC (39) or produced a considerably smaller effect (7) than the one observed here with ANS and TNS (Fig. 6) and predicted by theory (28, 31, 34). Finally, when measured with pinacyanol chloride (7), the CMCs of oleoyl-CoA (6.8 μM), palmitoyl-CoA (4.5 μM), and stearoyl-CoA (1.0 μM) followed the order CMC oleoyl-CoA > CMC palmitoyl-CoA > CMC stearoyl-CoA. This order, however, is not predicted from theory (28) and is in variance with the one observed in the present studies, where CMC palmitoyl-CoA > CMC oleoyl-CoA > CMC stearoyl-CoA (70 > 33 > 12 μM), an order which is parallel with the CMC order of the corresponding free fatty acids (30) and which is predicted from theory (28, 29).

A general feature of the fluorimetric titrations obtained with TNS or ANS is that the fluorescence enhancement above the CMC is strongly dependent upon the ionic strength of the buffer; the higher the ionic strength, the greater the enhancement. This effect can be explained in terms of increased partitioning of the dye into the micelles because of a lowered surface potential on the micelle, possibly combined with a tendency for the apolar portions of the dye molecules to salt out. In fact, efforts to determine the CMC of palmitoyl-CoA in glass-distilled water or in 0.01 M Tris, pH 8.3, failed because of insufficient fluorescence enhancement above the CMC to allow a well-defined intersection and hence a precise value of the CMC. Increasing the dye concentration of both ANS and TNS increases fluorescence (Fig. 4), but not in a proportional fashion. This behavior may reflect a real property of the system but could equally well be an instrumental artifact; it was not pursued because it does not affect the CMC measurement. The possibility that an early event occurs during micelle formation by long-chain fatty acyl-CoAs but is not detected by conductance or fluorescence seems unlikely, since the line below the observed intersection extrapolates to zero palmitoyl-CoA concentration.

Both fluorescence and conductance indicate a smooth transition in the CMC region rather than a discontinuity at the CMC, in agreement with solubility and conductivity data with SDS (21). It has been shown that this curvature in the region of the CMC is in fact due to the gradual appearance of micelles and not to an experimental artifact (13, 21). Therefore, when
data points were fitted to straight lines both below and above the CMC, points in the immediate vicinity of the CMC were ignored. Conventionally, the CMC is defined as the intersection of these straight lines. The CMC obtained in this way is equivalent to the concentration at which micelle formation would begin if suddenly full-size micelles were formed. An estimate of the concentration range over which micellization takes place can be done by careful inspection of the fluorescent plots (Fig. 4) or the more precise conductance plots (Fig. 2). In glass-distilled water (Fig. 2) or 0.050 M KPF, pH 7.4 (Fig. 4), where the CMCs are relatively high, the concentration range over which the micellization of palmitoyl-CoA takes place is between 10-20 μM.

The CMC of ionic surfactants is highly dependent upon the ionic strength of the medium (28, 31) and approximately halves for every doubling of salt concentration (Fig. 6). Linearity between log CMC and log C, implies that ΔGo is independent of salt concentration (31). Many enzymatic studies employing fatty acyl-CoAs as substrates have ignored the dependence of CMC upon conditions, especially salt concentration. In 0.1 M Tris-HCl containing 0.4 M KCl at pH 8.5, the CMC was found to be approximately 10 times lower than in 0.1 M Tris-HCl, pH 8.5, alone (Table 1). The former buffer is used with reconstituted acyltransferase systems (27), while the latter gives optimal acyltransferase activity in cell-free systems with bacterial enzymes (26). Although high salt concentration seems to stabilize the enzyme (27), it can clearly also directly affect the physical state of the substrate. For example, the low CMC value for palmitoyl-CoA recently determined by low-angle light scattering photometry (40), in a rather complex acyltransferase assay medium containing high salt, can be explained primarily as a salt effect. Plots of log CMC-log C, (Fig. 6) can be useful in predicting the CMC of fatty acyl-CoAs in buffers of various ionic strengths used in enzymatic assays.

In agreement with response of other detergents (29, 32, 34), the effect of temperature on the CMC of palmitoyl-CoA is smaller than the salt effect. The thermodynamic parameters of micellization of palmitoyl-CoA in 0.01 M Tris, pH 8.3, fall within the range encountered with other ionic surfactants. For ionic surfactants, unlike nonionic surfactants, the standard free energy of micellization includes the free energy involved in transferring the free molecules in their standard state to the micelle in its standard state and the transfer of α ions/molecule to the micelle from their standard state in solution. The ionic contribution can be the greater of the two as is likely to be the case for palmitoyl-CoA. Since ΔHi is not large, the major contribution to ΔGo is the entropy term, and the effect of ionic binding is reflected in ΔSo.

A detailed analysis of hydrodynamic data for highly charged particles such as fatty acyl-CoA micelles should be viewed with some skepticism because of electrokinetic effects. Linear extrapolation, for example, may not be warranted even though it is commonly used to obtain s0 and D0 for globular proteins (38). Nevertheless, the extrapolated value of 4.6 S agrees reasonably well with the S0 recently obtained from sedimentation velocity measurements carried out at low concentrations of palmitoyl-CoA by ultraviolet optics (6), and the anhydrous mass of 50,000 daltons is in the range determined by sedimentation equilibrium (6). Furthermore, extensive studies with SDS, a common detergent which forms charged micelles, have shown good agreement of diffusion coefficients measured by boundary spreading (22) and quasi-elastic light scattering (41, 42).

It was pointed out earlier that the hydrodynamic data yields dimensions for the micelles which cannot accommodate fully extended molecules. Conversely, the sedimentation and diffusion coefficients calculated for a spherical particle with extended molecules are grossly incompatible with the experimentally determined hydrodynamic parameters. For a sphere having a radius of 50 Å, which is the length of an extended palmitoyl-CoA molecule (11, 38), and a partial specific volume of 0.70 ml g⁻¹, the calculated anhydrous molecular weight is 450,000 daltons. This mass corresponds to an aggregation number of about 450, and if a reasonable hydration is assumed (0.3) gives a calculated sedimentation coefficient of 21 S and a diffusion coefficient of 3.8 × 10⁻⁷ cm²s⁻¹. Thus, for the real particle to be a conventional micelle would require an error of several hundred per cent in the hydrodynamic parameters. Such a micelle can also be ruled out by considering the geometry of packing without reference to the observed hydrodynamic parameters. The mass of each hydrocarbon portion of palmitoyl-CoA is 211 daltons, and the mass of the hydrocarbon core of a micelle having a molecular weight of 450,000 would be 95,000 daltons. Taking the partial specific volume of the hydrocarbon as 1.2 ml g⁻¹ gives a radius of 36 Å for the core, considerably greater than the 20 Å length of a fully extended hydrocarbon chain (3). A similar argument (3) can be used to place an upper limit of 84 on the aggregation number of a detergent having extended chains consisting of 15 carbons. Even if some error in the hydrodynamic parameter is allowed and this upper limit of 84 is assumed in fact to be the aggregation number of palmitoyl-CoA micelles, their anhydro radius would be 29 Å. Once again, the core would have a radius of 20 Å, and only about 9 Å would remain outside the core to accommodate the CoA portion of the molecules.

Thus, serious difficulties arise no matter what approach is used to devise a model for the palmitoyl-CoA micelle if the model is based upon fully extended lipid molecule molecules. Both the experimental data and geometrical considerations yield particle dimensions which are too small for the molecular length. It is likely, and in fact thermodynamically preferable, for the apolar portions of the molecules to reside within the cores of the micelles, but a consistent model apparently must also permit folding. A precedent for folding of simpler CoA esters has been demonstrated (43-45), and in fact a proper model for the palmitoyl-CoA micelle should incorporate this feature.

The work reported here underscores three of the important characteristics of the critical micelle concentration of the fatty acyl-CoAs in solution: the CMCs can frequently be far higher than they are often assumed to be, they are strongly dependent upon salt concentration, and they vary markedly with the nature of the fatty acid chain. These findings emphasize the necessity of coupling enzyme characterization with proper measurements of the CMC if a correlation is to be made between the behavior of the enzyme and the properties of the substrate. Although the published data is not extensive, comparison of the CMC values found in this investigation with reported concentrations of fatty acyl-CoAs required for enzyme inhibition suggests that the inhibitory concentrations may be unrelated to the CMC. A similar suggestion has been made by Powell et al. (6), who found inhibition of citrate synthase at 4 μM palmitoyl-CoA but a CMC in the neighborhood of 50 μM.

However, great caution should be exercised when the results from the present and other physical studies are extrapolated to the conditions used in many enzyme assays. Although CMCs have been recently determined under assay conditions (7, 40), their interpretation is not straightforward since assay mixtures are often more complex than those encountered here. Membranes are always present and bovine
serum albumin is frequently added, and both can bind fatty acyl-CoA substrates with high affinity (38, 46-48). The lipid-polymer might be possibly bound in solution under assay conditions, and as a result the concentration of free molecules in assays containing membranes and/or bovine serum albumin is likely to be well below the CMC. Additional complications could arise from the presence of Mg²⁺, a cofactor needed for maximum acyltransferase activity (49, 50), where solubility of the fatty acyl-CoA can be a limitation. Insolubility of the substrate could introduce serious artifacts when attempts are made to measure the CMC of fatty acyl-CoAs in the presence of Mg²⁺ (7). A more thorough definition of how CMCs affect the role that the fatty acyl-CoA as enzyme substrates requires further investigation. The present studies, together with others cited in this report, may provide a useful approach toward a better understanding of the properties of this important class of amphiphiles.

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