Acyl-CoA Synthetase Activity in Liver Microsomes from Calcium-Deficient Rats

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ABSTRACT: A study on the kinetic properties of the nonspecific acyl-coenzyme A (CoA) synthetase activity in liver microsomal vesicles from both normal and calcium-deficient Wistar rats was carried out. After a 65-d treatment, the calcium-deficient diet reflected a 75% increase in the synthetase activity with respect to control animals. The apparent Vm was significantly enhanced, while the Km remained unchanged. We also provided experimental evidence about various fatty acids of different carbon length and unsaturation which depressed the biosynthesis of palmitoyl-CoA following different behaviors in control or calcium-deprived liver microsomes. In addition, we studied in detail the inhibition reflected by stearic, α-linolenic, or arachidonic acids, in the biosynthesis of palmitoyl-CoA in microsomal suspensions either from control or hypocalcemic rats. In control microsomes, stearic acid produced a pure competitive effect, while the other fatty acids followed a mixed-type inhibition. The competitive effect of stearic acid was not observed in calcium-deprived microsomes. At the same time, a mixed-type inhibition produced by either α-linolenic or arachidonic acid was diminished in deprived microsomes due to an increase in the noncompetitive component (αKi). These changes observed in apparent kinetic constants (Km, Vm, Ki, and αKi), as determined by Lineweaver-Burks and Dixon plots, were attributed to the important alterations in the physicochemical properties of the endoplasmic reticulum membranes induced by the calcium-deficient diet. The solubilization of the enzyme activity from both types of microsomes demonstrated that the kinetic behavior of the enzyme depends on the microenvironment in the membrane, and that the calcium ion plays a crucial role in determining the alterations observed.

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The importance of long-chain acyl-coenzyme A (CoA) derivatives as intermediates and regulators of lipid metabolism has prompted a number of investigators to study the long-chain acyl-CoA-synthetase (ACS) AMP-forming (EC 6.2.1.3), which was first demonstrated by Kornberg and Pricer (1) in particulate preparations from guinea pig liver. Subsequent works extended research to different sources such as rat tissues (2–9), yeasts (10,11), infected erythrocytes (12), murine T lymphocytes (13), and various human cells and tissues (14–16). In rat livers, ACS is located in microsomes (1,17–19), outer mitochondrial membranes (20,21), and peroxisomal membranes (22,23). Purified ACS from these three different organelles are identical regarding all molecular and catalytic properties (2,24). The identity of the enzymes was also confirmed immunologically (25). Wilson *et al.* (14) and Laposata *et al*. (16) demonstrated the occurrence of an arachidonic acid-specific enzyme activity, and they suggested its potential role in regulating free arachidonic acid within the cell. Another enzyme activity requiring GTP instead of ATP was reported to occur in the mitochondrial fraction (26), though the significance of this enzyme reaction is controversial (27,28). More recently, Sugiura *et al.* (29) have suggested that certain types of fatty acyl chains of membrane lipids are continuously being converted transiently into acyl-CoA esters by an ATP-independent acyl-CoA synthetase. In any case, it is clear that the long chain acyl-CoA synthetase activity existing in liver microsomes is attributed to a well-characterized protein different from other enzyme activities (12,13,16,30). This enzyme, called nonspecific ACS, is very important because it catalyzes the initial reaction for the overall fatty acid metabolism, and it further regulates the rate of fatty acid biosynthesis (31,32). It has been recently demonstrated that acyl-CoA is necessary for the thyroid hormone receptor function (33), the budding of transport vesicles from Golgi cisternae (34), and the modulation of several enzyme activities such as Na^+,K^+-ATPase (35), Ca^{2+}-ATPase (36), and protein kinase C (37). In addition, three recent reports have indicated that acyl-CoA esters are involved in the intracellular handling of Ca^{2+} (38–40). We should also bear in mind that acyl-CoA have substantial, complex, and differing effects on Ca^{2+} movements in rat liver microsomes. These facts led us to study the relationship between calcium availability and the ACS activity in liver microsomal vesicles from both normal and calcium-deficient Wistar rats. In subsequent experiments, the catalytic properties of this enzyme were also examined under a calcium-deprived diet and the results obtained were discussed in terms of their kinetic significance.

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Abbreviations: ACS, acyl-CoA synthetase; CD, calcium-deficient; S, standard (control); SEM, standard error of the mean.

MATERIALS AND METHODS

Fatty acids and other chemicals. All the unlabeled fatty acids were obtained from Nu-Chek-Prep. (Elysian, MN). ATP (disodium salt), dithiothreitol, coenzyme A (lithium salt), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Palmitic (58.0 mCi/mmol, 99% radiochemically pure) and [1-^{14}C]stearic acids (56.5 mCi/mmol, 98% radiochemically pure) were supplied by Amersham International (Buckinghamshire, United Kingdom). Other chemicals used were of analytical or chromatographic grade, and they were provided by commercial sources. The concentration and purity degrees of fatty acids were routinely checked by liquid-scintillation counting and gas–liquid chromatography of fatty acid methyl esters prepared in the presence of internal standards. Fatty acids were dissolved in ethanol and stored in the dark at −20°C under an atmosphere of N_2 until they were used.

Animal treatment. Female Wistar rats from Comisión Nacional de Energía Atómica (Buenos Aires, Argentina) weighing 170 ± 10 g were bred and maintained on a control diet (Cargill type "C", Rosario, Argentina) throughout gestation and lactation. The dams were housed in plastic cages (one animal per cage) in a vivarium kept at 22 ± 1°C with a 12-h light/dark cycle and a relative humidity of 60 ± 10%. After weaning, 24 female pups (weighing 47 ± 4 g/animal) were randomly divided into two groups of 12 animals each, and fed *ad libitum* either on a calcium-deficient diet (group CD), or on a balanced diet (group S). The composition of the calcium-deficient diet prepared in our laboratory is shown in Table 1. The Ca^{2+} content (0.5 g/Kg) was determined by a Shimadzu Atomic Absorption Spectrophotometer AA-630-12 (Kyoto, Japan) following the mineralization procedure described elsewhere (41). Control animals were fed on a standard balanced diet supplemented with 5.0 g/Kg calcium in order to supply the mineral at a level equivalent to that recommended by The American Institute of Nutrition (AIN)-93 Purified Diets for Laboratory Rodents (42). The content of Ca^{2+} in drinking water (given *ad libitum*) was determined either by atomic absorption or by calcium-selective electrode Orion model 93-20, Orion Research Inc. (Cambridge, MA), and it was generally below 5 ppm. During the feeding period, body weights, water consumption, and food intakes were determined every day. Samples of blood were collected in order to determine plasma calcium levels. The food intake and water consumption relative to body weights were not significantly different between both groups of animals at the time the rats were killed, after 60 d of feeding. Calcium-deficient animals grew at a rate similar to the control group during the initial 20 d of feeding, then they grew at a reduced rate until day 59 when differences between groups became significant. In order to avoid individual differences among animals that might have resulted from *ad-libitum* feeding, on day 59 all the rats were fasted for 24 h, refed with the corresponding diet for 2 h, and then killed by decapitation without prior anesthesia 12 h after the refeeding period.

TABLE 1
Composition of Calcium-Deficient Diet

Ingredients	g/kg
Casein, high protein (free of calcium)	200.0
Sucrose	505.9
Corn starch	150.0
Cellulose	50.0
Corn oil[a]	50.0
Mineral mixture (calcium-deficient)[b]	13.4
Sodium phosphate, monobasic	8.9
Potassium phosphate, monobasic	8.8
Vitamin mixture[c]	10.0
DL-Methionine	3.0

[a]Contained 11.5% 16:0, 2.1% 18:0, 26.6% 18:1, 57.3% 18:2n-6, 1.4% 18:3n-3, 0.2% 20:3n-6, 0.2% 20:4n-6, 0.2% 20:1, and other fatty acids as trace amounts (<0.2%).
[b]Contained (g/kg mix): NaCl, 183.7; potassium citrate, 576.0; K_2SO_4, 136.1; MgO, 62.8; $MnCO_3$, 9.2; ferric citrate, 16.7; $ZnCO_3$, 4.2; $CuCO_3$, 0.80; KIO_4, 0.03; $Na_2SeO_3 \cdot 5H_2O$, 0.03; $CrK(SO_4) \cdot 12H_2O$, 1.40.
[c]Contained (g/kg mix): choline dihydrogen citrate, 349.7; ascorbic acid, 101.7; vitamin E acetate, 24.2; p-aminobenzoic acid, 11.0; inositol, 11.0; niacin, 9.9; panthothenate, 6.6; menadione, 5.0; vitamin A palmitate, 4.0; vitamin B_{12}, 3.0; pyridoxine HCL, 2.2; riboflavin, 2.2, thiamine, 2.2; vitamin D_3, 0.44; folic acid, 0.2; biotin, 0.04; and corn starch, 466.7.

All the diet components used were purchased from Carlo Erba (Milan, Italy) or Mallinckrodt Chem. Works (NY). The casein was deprived of calcium by EGTA treatment and then defatted with boiling acetone. The calcium content in the extracted casein was negligible. Animal maintenance and handling were in accordance with the National Institutes of Health guide for the care and use of laboratory animals (43).

Preparation of liver microsomal suspensions. Livers were rapidly excised and immediately placed in an ice-cold homogenizing medium (44). The homogenate from each liver was processed individually at temperatures varying from 0° through 2°C, and the microsomal fractions were separated by differential centrifugation at 110,000 × *g*, as described previously (44). Microsomal pellets were resuspended in a cold homogenizing solution up to a final protein concentration of 30–40 mg/mL.

Assay of long-chain fatty acyl-CoA synthetase activity. The enzyme activity was radiochemically determined basically following the method of Tanaka *et al.* (2), consisting of the enzymatic conversion of heptane-extractable radioactively labeled fatty acid into water-soluble acyl-CoA esters. The standard reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.0), 5 mM dithiothreitol, 0.15 M KCl, 15 mM $MgCl_2$, 10 mM ATP(Na_2), 1 mM CoA (lithium salt), and the substrate (1 mM palmitic acid, in a final volume of 0.2 mL.). To prepare the palmitic acid solution, labeled and unlabeled acids were dissolved in ethanol and mixed to obtain 200,000 dpm/incubation tube. The solvent was evaporated to dryness under N_2 and the fatty acids resuspended by sonication in 375 mM Tris-HCl buffer, pH 8.0, containing 1% polyethyleneglycol. The reaction was started by adding 0.04 mg liver microsomal protein in a solution of 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA. After 3 min of incubation, the reaction was stopped by adding 2.25 mL of a mixture of isopropanol/heptane/2 M-sulfuric acid (40:10:1, vol/vol). Then 1.5 mL heptane and 1 mL distilled water were added and the

upper layer was discarded. The lower layer was washed 3 times with 2 mL heptane containing 1 mg/mL of carrier palmitic acid to remove the unreacted radioactive fatty acid. The radioactivity of the aqueous phase was counted in a Wallac 1214 Rackbeta Liquid Scintillation Counter (Turku, Finland). In some experiments, different amounts of unlabeled fatty acids (tested as enzyme inhibitors) were added to the incubation mixture at the concentration indicated in the figures, while in other experiments palmitic acid was replaced by stearic acid under the incubation conditions described above. Blanks were routinely run in all incubations. These blanks consisted of tubes in which boiled microsomes (10 min at 100°C) were added. In preliminary experiments using different experimental conditions, the recovery of radioactivity from the aqueous phase (labeled acyl-CoA) of the blanks did not exceed 1 to 2% of the added radioactivity. The aqueous extract from the reaction mixture was reacted with neutral hydroxylamine, and the hydroxamate derivative was chromatographed on silica gel G with chloroform/methanol/water (95:5:0.5, vol/vol), following the procedure described elsewhere (10). The reaction products were identified by means of authentic standards. Ninety percent of the radioactivity in the reaction product was found in palmitoyl-CoA and *ca.* 3% in free palmitic acid; the remaining 7%, which migrated below the acyl-CoA ester, may be in oxidation products (5).

Solubilization of enzyme activity. The ACS activity was solubilized from the microsomal membrane of control or calcium-deficient rat livers as described by Nagamatsu *et al.* (30) with minor modifications. Briefly, microsomes were diluted in Tris-HCl buffer (50 mM, pH 8.0) containing 5 mM dithiothreitol (instead of 2-mercaptoethanol as indicated in the original method) and 1 mM EGTA, so that the protein concentration was 4.0 mg/mL. A solution of Triton X-100 was added to make the final detergent concentration 5 mM (various detergent concentrations were previously tested). The mixture was allowed to stand at 0–2°C for 60 min and centrifuged at 105,000 × *g* for 1 h. The enzyme activity was partially purified by ammonium sulfate precipitation as described elsewhere (2). The excess of Triton X-100 was removed by washing the pellet three times with 10 vol of the ammonium sulfate solution. The washed pellet was dialyzed overnight at 2°C against 0.02 M sodium phosphate buffer (pH 7.0), 130 mM NaCl, and 1 mM EGTA. This enzyme preparation had a specific activity of 177 ± 6 nmol of palmitoyl-CoA synthesized per milligram of protein per minute, which was similar to that reported by other authors (45) in the presence of residual amounts of Triton X-100. Solubilization of the ACS was also performed by means of the modified method of Juarez *et al.* (46). The extraction was carried out using a high ionic strength solution of KCl. The liver microsomal pellet was resuspended in a buffer containing 0.05 M K_2HPO_4 (pH 7.00), 1.50 M KCl, 2 mM EDTA, and 0.5 mM dithiothreitol. The suspension (4.0 mg protein/mL) was incubated with stirring at 2°C for 30 min in the presence of 10% glycerol. At the end of this period, the solubilized enzyme was separated from the pellet by centrifugation in the cold at 110,000 × *g* for 60 min.

Diphenylhexatriene labeling of microsomes and steady-state fluorescence anisotropy determinations. The 110,000 × *g* microsomal pellet from either control or calcium-deficient diets was washed according to the method of Glaumann *et al.* (47) with minor modifications (48). Fluorescence anisotropy was determined in these washed microsomal suspensions, as previously described (49). All measurements were performed at 37°C in an Aminco-Bowman spectrofluorometer equipped with two glan prism polarizers. Both methodology and calculations were described in a previous paper (48).

Other analytical determinations and statistical treatment of the data. The fatty acid composition of lipids from microsomal suspensions was determined as described previously (48). Total lipids were extracted from microsomes according to the procedure of Folch *et al.* (50), and determined gravimetrically after evaporating aliquots to constant weight (48). The cholesterol content was measured by the method of Allain *et al.* (51). Phospholipid and neutral lipid fractions were isolated from the total lipid extract by silicic acid microchromatography (Bio-Rad Lab., Richmond, CA) according to the method of Hanakan *et al.* (52). Phosphorus analysis was performed following the method of Chen *et al.* (53). Protein content was determined by the micromethod of Lowry *et al.* (54) with crystalline bovine albumin as standard. Samples for gas–liquid chromatography analysis were transesterified as previously described (48), and they were analyzed in a Hewlett-Packard 5840-A gas–liquid chromatography apparatus (Avondale, PA) equipped with a 5840-A terminal computer integrator system and with a 6-ft glass column packed with 10% SP-2330 on a 100–200 mesh Chromosorb WAW-DMCS (Supelco Inc., Bellefonte, PA). Eicosa-11-monoenoic acid (1 µg/tube) was used as an internal standard.

All values represented the mean of 3 to 6 individual determinations (assayed in triplicate) ± 1 standard error of the mean (SEM). In order to test the statistical significance of numerical differences in results, data were analyzed by either the Student's *t*-test or by ANOVA (analysis of variance), with the aid of the GB-STAT Professional Statistics Program (version 4.0) from Dynamic Microsystems, Inc. (Silver Springs, MD). Data were plotted using Sigma Plot Scientific Graphing Software (version 2.0) from Sigma Chem. Co., (St. Louis, MO).

RESULTS

In the present experiments, the ACS activity was studied in liver microsomal suspensions from both normal and calcium-deprived rats. At weaning, CD animals were fed on a diet whose calcium content was 10-fold lower than that recommended for animal breeding (42). During the feeding experimental period, calcium levels in the serum of the CD-group progressively decreased up to 1.8 times lower than those of the S-group (1.44 ± 0.10 vs. 2.60 ± 0.10 mmol/L, respectively). Using this kind of microsomal suspension, we optimized the assay design with respect to protein concentration and incubation time. Previous studies with ACS from rat liver microsomes had shown that the interpretation of experimen-

TABLE 2
ACS Activity for Palmitic Acid from Control (S) or Calcium-Deficient (CD) Rat Liver Microsomes[a]

Microsomes	Specific activity (nmol/min·mg)
S	150.1 ± 3.1
CD	261.4 ± 4.0*

[a]Results were obtained as described in the Materials and Methods section and they are given as nmoles of product formed per min per mg of microsomal protein. Each value represents the mean ± 1 SEM of six animals assayed in triplicate.
*Significantly different from S-group, $P < 0.001$.

tal results could become complicated by a high ratio of endogenous fatty acid to enzyme protein, or to an excessively lengthy incubation period (55). Thus, following the procedure of Tanaka *et al.* (2), we verified that the enzyme activity with palmitic acid as substrate (1.0 mM concentration) was completely linear as a function of a microsomal protein up to 0.40 mg/tube or 2 mg/mL (constant incubation time 10 min), and to time for up to 15 min (constant protein concentration of 0.50 mg/mL). Under the experimental conditions stated in the Materials and Methods section, the activity of nonspecific ACS was measured at initial velocity conditions using palmitic acid as substrate. Therefore, the resulting rate of formation of the product was directly proportional to the enzyme activity. As indicated in Table 2, the activity of nonspecific ACS increased *ca.* 75% over control values in animals fed on the calcium-deficient diet.

Then we examined the effect of the calcium-deficient diet on the apparent kinetic parameters by determining the values of Vm and Km for palmitic acid through double reciprocal plots according to the method of Lineweaver and Burk (56). Figure 1 shows that the apparent Vm for the calcium-deficient control or calcium-deficient microsomes, evoked by the presence of varying amounts of different fatty acids, labeled palmitate was used as substrate—at a saturated concentration of 1 mM—while another fatty acid was simultaneously added as an alternative substrate within the range 0.05 to 0.40 mM. The results obtained are illustrated in Figure 2A for control microsomes and Figure 2B for calcium-deficient ones. In control microsomes, the sole saturated fatty acid tested as alternative substrate (stearic acid) evidenced a weak inhibitory effect only at concentrations greater than 0.3 mM. Unsaturated fatty acids derived from linoleate or α-linolenate showed a greater inhibition capacity of nonspecific ACS activity than that observed for fatty acids of the n-9 family. In calcium-deficient microsomes the pattern of the inhibitory effects displayed by the tested fatty acids was less noticeable than that observed in control microsomes. Moreover, in the case of the

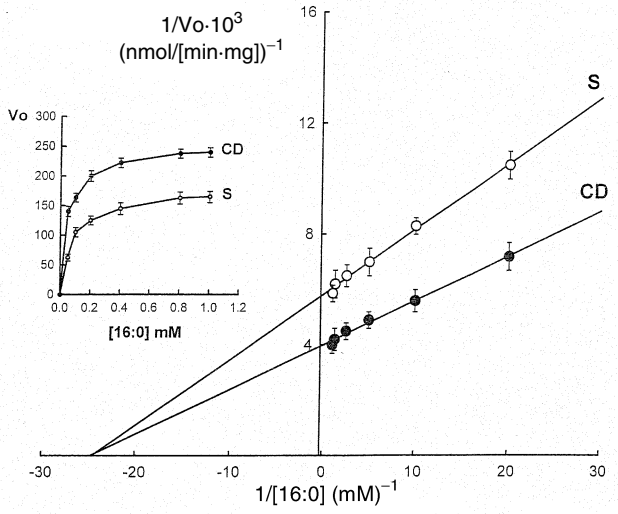

FIG. 1. Linear regressions for the Lineweaver-Burk plots of acyl-coenzyme A-synthetase ACS activity for palmitic acid from control or calcium-deficient rat liver microsomes. Enzyme activity was assayed as described in the Materials and Methods section. Typical Michaelis-Menten plots are shown in the insert. Each point represents the mean ± 1 standard error of the mean (SEM) of three different incubations assayed in triplicate.

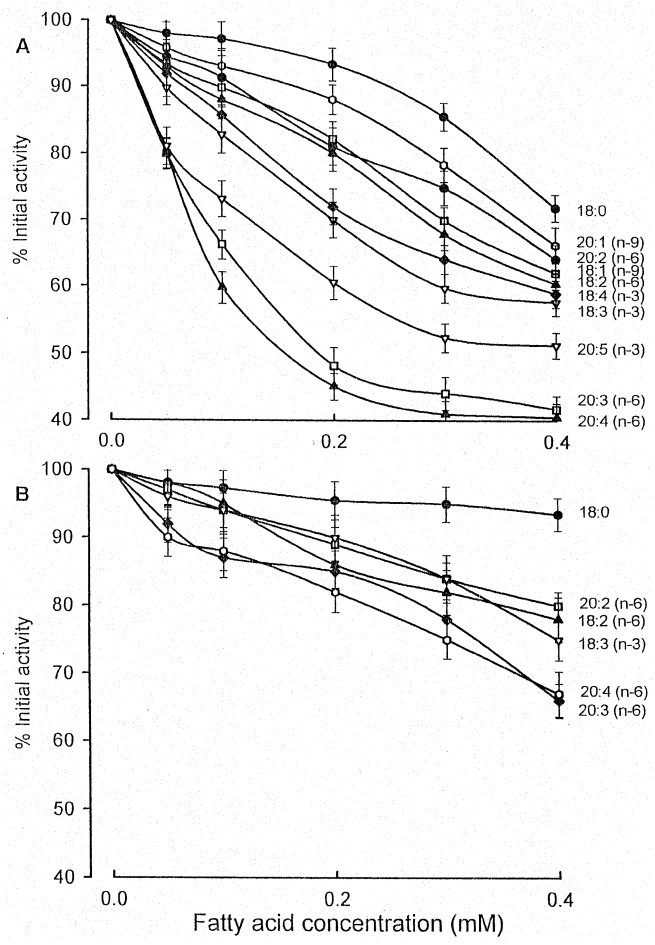

FIG. 2. Inhibition of nonspecific ACS activity for palmitic acid from control (A) or calcium-deficient (B) microsomes in the presence of various fatty acids. The enzyme was assayed as described in the Materials and Methods section. The reaction mixture contained saturated amounts of labeled palmitic acid as the main substrate and varying amounts of unlabeled inhibitor fatty acids at the concentrations indicated. Basal activity was 143.5 ± 4.0 and 271.1 ± 4.4 nmol·min^{-1}(mg protein)$^{-1}$ in control and calcium-deficient microsomes, respectively. Each point represents the mean ± 1 SEM of three different incubations using microsomes from three animals. For abbreviations, see Figure 1.

saturated fatty acid (stearic), there was no inhibitory effect within the range of concentrations assayed. In spite of the quantitative differences observed, the relative capacity shown by the alternative substrates to reduce palmitate activation was the same in both types of microsomal preparations.

In order to further investigate the type of inhibition provoked by the alternative substrates and the calcium-induced modifications in the lipid composition of the endoplasmic reticulum membranes, we carried out a series of experiments using palmitic acid as substrate with stearic, α-linolenic, and arachidonic acids, as weak, medium, or strong inhibitors, respectively, of the nonspecific ACS activity. In these incubations, for each concentration of palmitic acid studied (0.05 to 1.00 mM), various concentrations of inhibitors were tested (stearic acid: 0 to 0.36 mM or 0 to 0.40 mM in either control or calcium-deficient microsomes; α-linolenic acid: 0 to 0.12 mM or 0 to 0.20 mM; and arachidonic acid: 0 to 0.08 mM or 0 to 0.20 mM). In all instances initial velocities were measured and plotted according to the method of Lineweaver and Burk (56) (Figs. 3 and 4 for control or calcium-deficient microsomes, respectively). The analysis of the double reciprocal plots shown in Figure 3 indicates that the stearic acid displayed a competitive behavior only at relatively high fatty acid concentrations, whereas α-linolenic and arachidonic acids evidenced a mixed-type inhibition consisting of uncompetitive and competitive components (57). In calcium-deficient microsomes (Fig. 4) the stearic acid was not effective as a competitive inhibitor even at higher concentrations tested. Although α-linolenic and arachidonic acids showed a mixed-type inhibition pattern, the extent of their effects was significantly lower than that observed in control preparations. To estimate quantitatively the magnitude of these inhibitory effects on nonspecific ACS activity, data from Figures 3 and 4 were re-plotted as the inverse of the initial velocities vs. inhibitor concentrations (Dixon plots). Dixon plots are frequently used to identify the type of inhibition and to determine Ki values. The velocity equation for competitive inhibition may be converted into a linear form in which the varied ligand is [i]. The straight lines obtained at different substrate concentrations have positive slopes. Drawing a horizontal line at a height of 1/Vm, these lines can be intercepted at the −[i] value representing the kinetic parameter "αKi". This parameter considers the noncompetitive component of a linear-mixed-type inhibition (57). Figures 5 and 6 show the results obtained for control or calcium-deficient microsomes, respectively. The slopes of these plots are given by Km/−[S].Vm.Ki. Since Km [S] and Vm are constants, the slope is inversely proportional to Ki. Comparing the plots obtained for both types of microsomal preparations, relative Ki and αKi values were obtained. These data were consistent with the apparent kinetic parameters obtained from the Lineweaver-Burk plots shown in Figures 3 and 4. All these kinetic data are summarized in Table 3. These results clearly indicate that the calcium-deficient diet produced a significant decrease in the inhibitory effects presented by all the tested fatty acids. In the case of the stearic acid, Vm was increased *ca.* 80% over control values, while

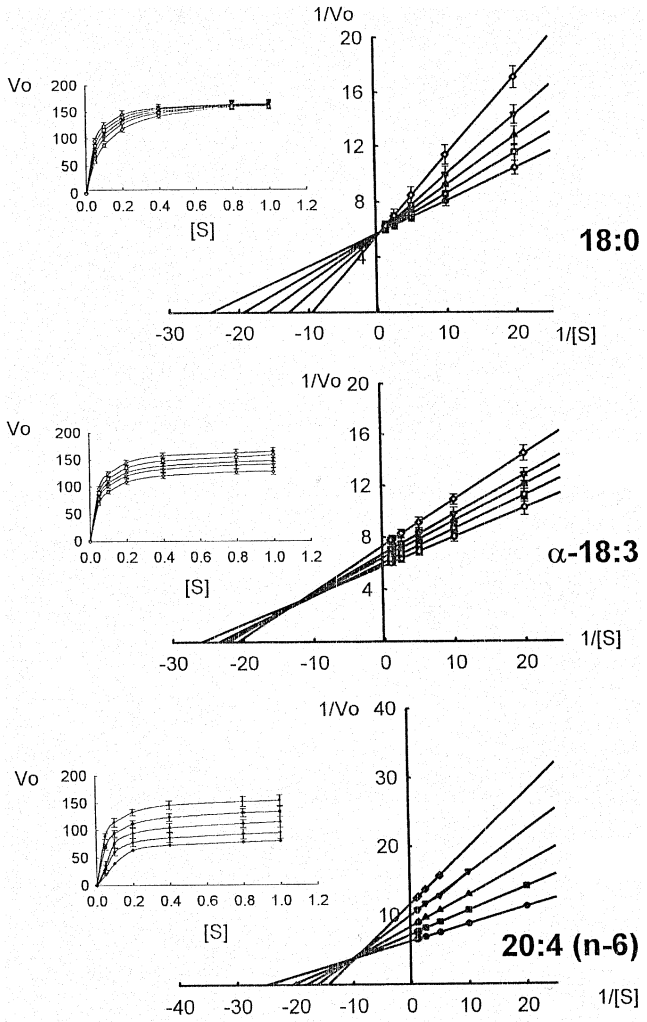

FIG. 3. Linear regressions for Lineweaver-Burk plots of ACS activity for palmitic acid from control microsomes in the presence of various concentrations of inhibitory fatty acids. The enzyme was assayed as described in the Materials and Methods section. The reaction mixture contained saturated amounts of labeled palmitic acid and varying amounts of the unlabeled inhibitory fatty acid as indicated. Typical Michaelis-Menten plots are given in the inserts. 1/Vo was expressed as $[nmol/(min \cdot mg)]^{-1} \cdot 10^3$ and 1/[S] was given as $(mM)^{-1}$. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

its competitive effect disappeared at the concentrations assayed. The increase in Vm values observed with hepatic microsomes of the CD-group, even in the presence of the α-linolenic or arachidonic acids as inhibitors, was produced through a decrease in the competitive component. Although this finding was markedly evident in the case of arachidonic acid, Ca deficiency significantly raised both Ki and αKi in all the studied fatty acids.

We also studied the linear regressions for Lineweaver-Burk plots of nonspecific ACS activity using either palmitic or stearic acid as substrate (Fig. 7). It was observed that the Km value for stearic acid (a pure competitive inhibitor) increased in calcium-deficient microsomes, compared to controls, while Km for palmitic acid remained unchanged in both

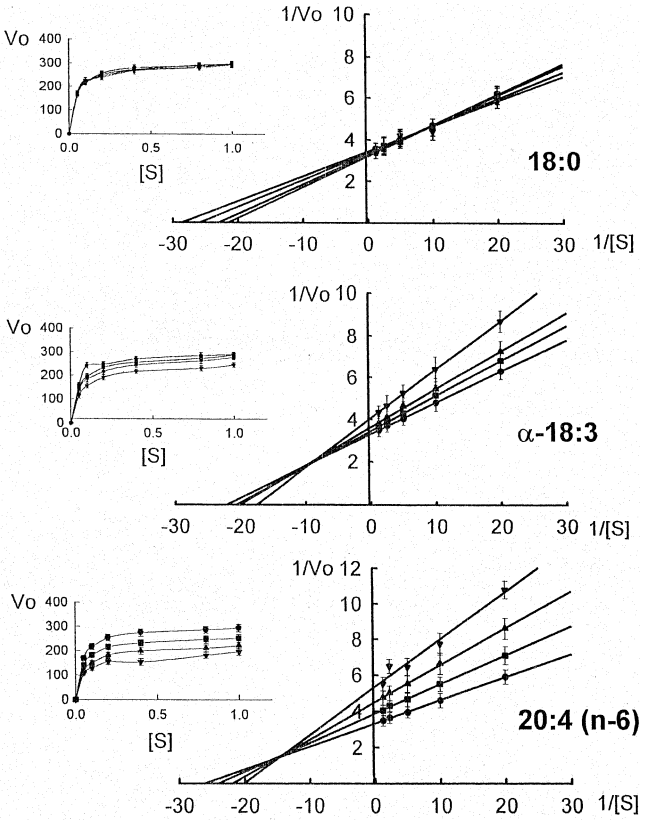

FIG. 4. Linear regressions for Lineweaver-Burk plots of nonspecific ACS activity for palmitic acid from calcium-deficient microsomes in the presence of various concentrations of inhibitory fatty acids. The enzyme was assayed as described in the Materials and Methods section. The reaction mixture contained saturated amounts of labeled palmitic acid and varying amounts of the unlabeled inhibitory fatty acid as indicated. Typical Michaelis-Menten plots are given in the inserts. 1/Vo was expressed as $[nmol/(min \cdot mg)]^{-1} \cdot 10^3$ and 1/[S] was given as $(mM)^{-1}$. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

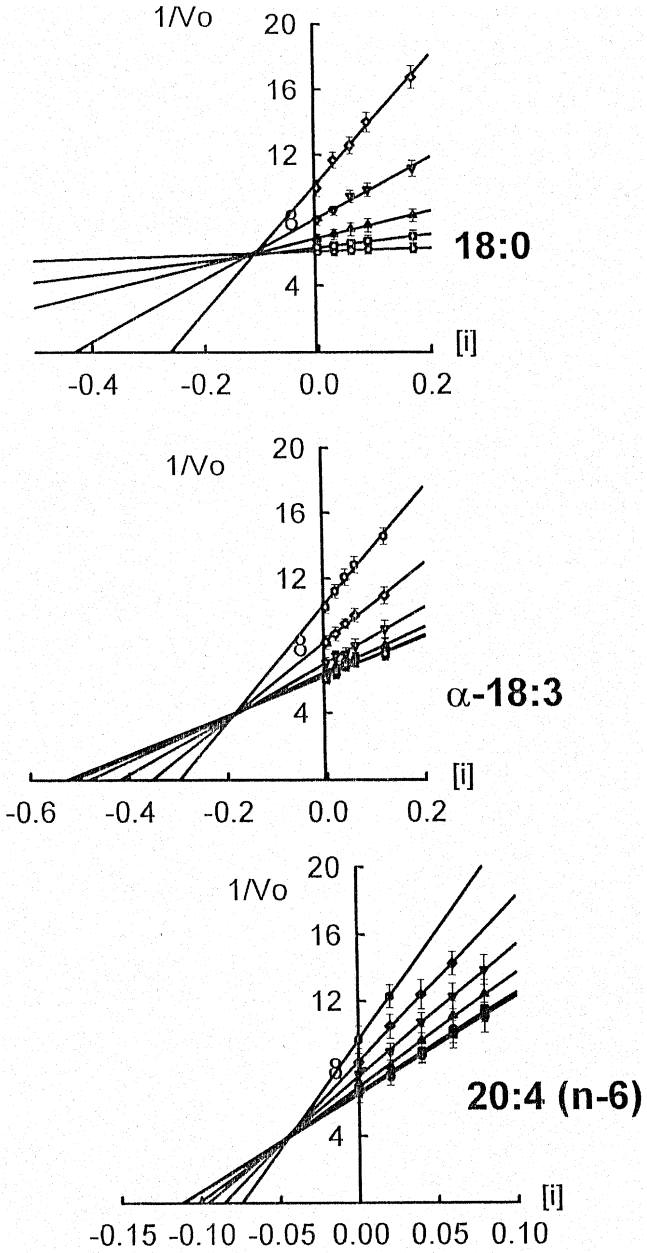

FIG. 5. Linear regressions of Dixon plots from control microsomes. Data were taken from the reciprocal plots shown in Figure 3. 1/Vo was expressed as $[nmol/(min \cdot mg)]^{-1} \cdot 10^3$ and [i] was given as mM. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

kinds of microsomes.

Table 4 shows the interlipid relationships and fluorescence anisotropy of liver microsomal membranes from either control or calcium-deficient rats. The results clearly indicate that in fact the lipid bilayer of the endoplasmic reticulum membrane was significantly modified by calcium deficiency. The relative content of lipids was decreased at the time that the neutral lipid was significantly raised. The proportion of cholesterol and phospholipids was diminished by calcium deficiency. On the other hand, Table 5 shows that the fatty acid composition of the different lipid fractions was significantly modified by calcium deprivation. The amounts of saturated fatty acids significantly increase in both triglyceride and phospholipid fractions, while a concomitant decrease was observed in the content of phospholipid polyunsaturated fatty acids. The decrease produced in the unsaturation index suggested that the quality and/or quantity of the fatty acid chains acylated to the lipid structures were substantially modified in the microsomes of the CD-group. These modifications in the physicochemical state of the lipid bilayer, induced by calcium deficiency, could be closely associated with the difference found in the nonspecific ACS activity, and with the altered response of this enzyme to the fatty acid tested as inhibitors. Taking into account these considerations, we investigated the ACS activity deprived of its microenvironment in the biomembrane. The linear regressions for Lineweaver-Burk plots of the solubilized enzyme from control or calcium-deficient microsomes are shown in Figure 8. It is evident that the kinetic behavior exhibited by nonspecific ACS between control and calcium-deficient microsomal preparations was the same after solubilization procedures either with Triton X-100 or

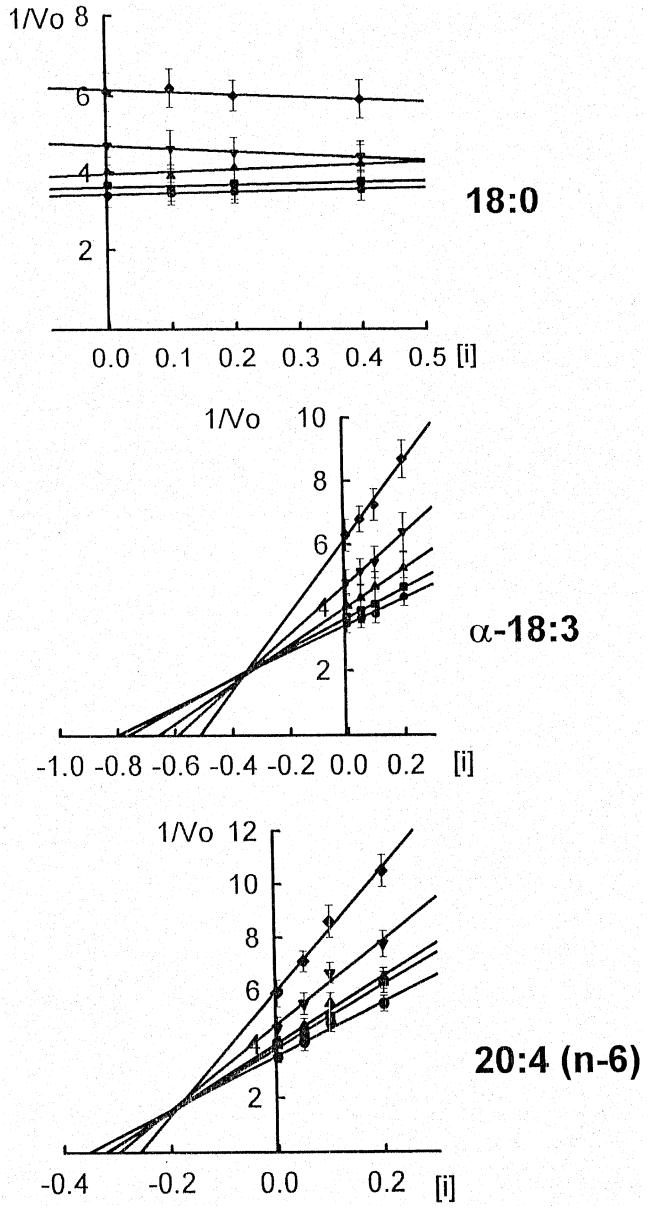

FIG. 6. Linear regressions of Dixon plots from calcium-deficient microsomes. Data were taken from the reciprocal plots shown in Figure 4. 1/Vo was expressed as $[nmol/(min \cdot mg)]^{-1} \cdot 10^3$ and [i] was given as mM. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

KCl. These apparent kinetic parameters calculated from Figure 8 are summarized in Table 6.

DISCUSSION

An examination of the nonspecific ACS activity in liver microsomal suspensions from both normal and calcium-deprived rats using palmitic acid as substrate revealed that calcium deprivation promotes a significant increase in this enzymatic activity measured at initial velocity conditions. To our knowledge, this is the first experimental evidence of the regulatory effect caused by calcium deprivation on the palmitoyl-CoA synthesis in liver microsomes. Previous studies by other authors clearly indicate that the formation of palmitoyl-CoA causes a significant increase in the release of Ca^{2+} from microsomal vesicles (38–40). Based on these findings, it is reasonable to speculate that in calcium-deficient rats the stimulation observed in the biosynthesis of palmitoyl-CoA would preserve calcium levels within the cells.

From the results shown in Figure 1, we can assume that, under calcium-deficient conditions, the increased Vm resulted from an increment in the specific activity of the enzyme, whereas the substrate affinity remained unchanged. Previous studies from other laboratories showed that ACS seems to be largely dependent on the extent of substrate unsaturation and the carbon chain length (2–5,8,12). On the other hand, studies on the fatty acid composition carried out in platelets (4), lymphocytes (13), rat brain microsomes (5), and plasmodium-infected erythrocytes (12) demonstrated that the activation of palmitic acid is markedly decreased in the presence of unlabeled unsaturated fatty acids.

The interpretation of the competition studies shown in Figures 2–4 is rather difficult because of unknown factors, such as fatty acid solubility, critical micellar concentration of the different fatty acids, and the form in which fatty acids are present in the incubation medium. However, it was shown that in calcium-deficient microsomes the pattern of the inhibitory effects displayed by the tested fatty acids was similar but less noticeable than the one observed in control microsomes (Fig. 2A,B). The highest inhibitory capacity of unsaturated fatty acids provoked in the activation of palmitic acid (especially the polyunsaturated fatty acids of the n-6 or n-3 series), compared with the other fatty acids studied, may be due to their relatively easy accessibility and higher affinity for the membrane-bound enzyme. This explanation was first suggested by Reddy *et al*. (5) to justify the fatty acid specificity of long chain ACS from rat brain microsomes and mitochondria. Thus the minor response obtained for each fatty acid in calcium-deficient microsomes would be the consequence of some alterations in the physical and/or chemical state of these membranes.

The different kinetic behavior induced by calcium deficiency seems to be the consequence of a change in the concentration range at which the inhibitory effects are produced. In this regard, the kinetic pattern in both types of microsomes (Figs. 3 and 4) was similar, having a minor response in the CD-microsomal suspensions despite the highest concentrations of the fatty acids used as inhibitors. Taking into account that the three fatty acids studied exhibited special variations in their inhibition mechanisms, we may presume that there exist at least two binding sites in the nonspecific liver ACS. α-Linolenate, which behaves like arachidonate, would be activated at the same site and would have a better affinity for polyunsaturated species, whereas palmitate and stearate would be activated at the other site. The results summarized in Table 3 would support this hypothesis because of the differential effect on the palmitoyl-CoA synthesis caused by the unsaturated inhibitors, compared to stearic acid. On the other

TABLE 3
Apparent Kinetic Parameters of ACS for Palmitic Acid from Control or Calcium-Deficient Microsomes[a]

	Control			Calcium-deficient			Change (%)		
	18:0	α-18:3	20:4	18:0	α-18:3	20:4	18:0	α-18:3	20:4
Km	41 ±3	39 ±4	43 ±2	39 ±4	43 ±5	37 ±5	−5	10	−14
Vm	176 ±11	170 ±8	163 ±9	306* ±18	308* ±16	312* ±21	74	81	92
Ki	117 ±9	178 ±13	47 ±3	∞*	292* ±17	189* ±11	∞	64	302
αKi	—	537 ±22	113 ±7	—	789* ±53	347* ±29	—	48	207

[a]Apparent kinetic parameters are expressed as mean ± 1 SEM of six different incubations assayed in duplicate using at least five different substrate concentrations and four inhibitor concentrations (see Figures 3 and 4 for control or calcium-deficient diet, respectively). Km, Ki , and αKi are given in μM concentrations. Vm was expressed as nmoles/min.mg.protein. The inhibition constants (Ki for the competitive and αKi for the noncompetitive component) of the linear-mixed-type inhibition were calculated as described in the text. Percent changes were calculated with respect to the corresponding value of the control group. —, no inhibition observed; ∞, too high to be calculated; *significantly different with respect to control ($P < 0.01$).

hand, if saturated fatty acids had been activated at the same molecular site, the Km value of stearic acid as substrate would have had a value similar to its Ki in inhibiting the activation of palmitate. As shown in Figure 7, the Km for stearic acid, a pure competitive inhibitor, was 52.0 ± 4.1 and 101.3 ± 6.5 μM for control and CD-microsomes, respectively, whereas the Km for palmitic acid in both kinds of microsomes remained unchanged (41.6 ± 3.3 vs. 40.9 ± 4.1 μM). This finding indicates that, under calcium-deficient conditions, the affinity for stearic acid significantly declines; consequently, this acid works as a bad competitor for other substrates. It is worth noticing that the abnormal changes introduced by calcium deficiency only imply a significant increase in the Vm for palmitic acid, with no substantial modifications in Km values. On the contrary, in the case of stearic acid, Vm modification was less important than that observed in Km val-

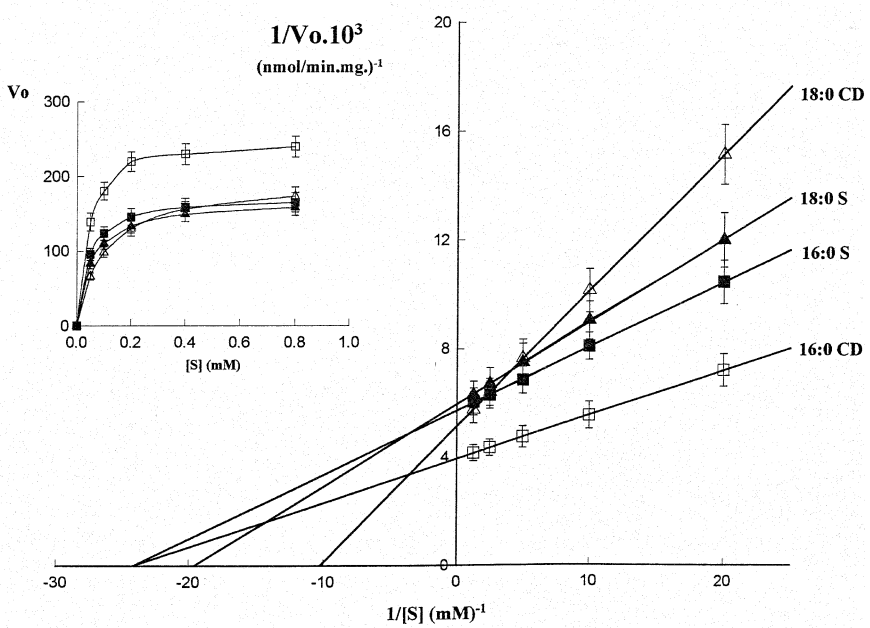

FIG. 7. Linear regressions for Lineweaver-Burk plots of ACS activity using palmitic acid (squares) or stearic acid (triangles) as substrates, from control (S) (solid symbols) or calcium-deficient (CD) (open symbols) microsomes. Enzyme activities were assayed as described in the Materials and Methods section. Typical Michaelis-Menten plots are shown in the insert. Each point represents the mean ± 1 SEM of three different incubations assayed in triplicate. For abbreviations, see Figure 1.

TABLE 4
Interlipid Relationships and Fluorescence Anisotropy of Liver Microsomal Membranes from Control or Calcium-Deficient Rats[a]

	Control	Calcium-deficient	*n*	*P* <
Cholesterol/phospholipid (μmol/μmol)	0.31 ± 0.02	0.23 ± 0.01	3	0.02
Total lipid/phospholipid (mg/μmol)	0.79 ± 0.03	0.98 ± 0.04	3	0.02
Total lipid/cholesterol (mg/μmol)	2.48 ± 0.02	3.00 ± 0.03	4	0.001
Phospholipid/protein (μmol/mg)	0.45 ± 0.03	0.30 ± 0.01	3	0.01
Neutral lipid/protein (μg/μg)	0.06 ± 0.003	0.11 ± 0.01	3	0.01
Neutral lipid/polar lipid (μg/μg)	0.13 ± 0.01	0.36 ± 0.02	5	0.01
Fluorescence anisotropy (r_s)	0.1405 ± 0.0010	0.1112 ± 0.0008	6	0.01
Unsaturation index	3.80 ± 0.05	2.90 ± 0.1	4	0.001

[a]Results were expressed as the mean ± 1 SEM of the experiments indicated as "*n*". For details see the Materials and Methods section. Unsaturation index was calculated as the total double bonds in total unsaturated fatty acids/mol saturated fatty acids. *P* <, indicates the value at which calcium-deficient results are significantly different from the respective control value.

ues (Fig. 7). Whether changes in affinity and/or in the number of active enzyme molecules are the consequence or the cause of calcium-induced alterations in the lipid bilayer awaits further clarification.

Previous studies have demonstrated that the lipid domain fluidity is determined by the cholesterol/phospholipid molar ratio, lipid/protein ratio, and degree of unsaturation of lipid acyl chains (58,59). Such changes were reflected in our experimental system through a modification in the diphenylhexatriene steady-state fluorescence anisotropy (r_s) (Table 4), as detected by an increase in the rotational mobility of the probe within the membrane lipid phase when compared to those values observed in the microsomal membranes from control animals. As previously reported, this parameter depends on the overall membrane lipid mobility (60) and earlier studies with diphenylhexatriene have demonstrated that this probe is a useful tool to estimate membrane fluidity (61). Storch and Schachter (62) reported that an increase in calcium ion evokes a decrease in lipid fluidity of isolated rat hepatocyte plasma membranes by the activation of phospholipase A_2, demonstrating that the cation decreased the arachidonic acid content and the overall double bond index of membrane lipids. The effect of calcium deficiency on the physicochemical state of the microsomal membrane has not been shown until now, and further investigation on the precise role of this ion seems to be needed.

Previous studies carried out with long-chain ACS from rat liver microsomes showed that Triton X-100 enhanced fourfold the Vm value without affecting Km (5). Taking this fact into account, it seemed vital to investigate the ACS activity deprived of its microenvironment in the biomembrane.

Our results demonstrated that the enzyme preparation obtained after a solubilization with Triton X-100 exhibited a similar specific activity as compared with the one shown by a fraction solubilized with KCl. In accordance with data previously reported by other authors (45,63), the results presented

TABLE 5
Fatty Acid Composition (μg/mg microsomal protein) of Triglycerides and Phospholipids in Liver Microsomes from Control (S) or Calcium-Deficient (CD) Rats[a]

	Triglycerides		Phospholipids	
Fatty acids	S	CD	S	CD
14:0	0.6 ± 0.1	0.6 ± 0.1	0.1 ± 0.01	2.1 ± 0.1*
16:0	38.9 ± 1.2	44.9 ± 1.5*	16.0 ± 0.6	25.6 ± 1.0*
16:1n-7	3.3 ± 0.1	4.2 ± 0.2	0.9 ± 0.1	1.0 ± 0.1
18:0	7.8 ± 0.2	11.6 ± 0.9*	19.4 ± 0.2	27.1 ± 0.4*
18:1n-9	36.2 ± 2.0	34.5 ± 1.8	5.8 ± 0.1	5.6 ± 0.2
18:1n-7	3.0 ± 0.1	4.0 ± 0.2	4.1 ± 0.2	5.1 ± 0.2
18:2n-6	6.9 ± 0.3	13.6 ± 0.7*	11.3 ± 0.2	15.6 ± 0.4*
18:3n-3	0.1 ± 0.0	traces	0.2 ± 0.0	0.3 ± 0.02
20:3n-6	0.3 ± 0.1	1.1 ± 0.1*	2.3 ± 0.1	2.6 ± 0.1
20:4n-6	0.4 ± 0.1	traces	25.6 ± 0.7	15.7 ± 0.5*
20:5n-3	0.1 ± 0.01	traces	0.4 ± 0.1	0.6 ± 0.1
22:5n-3	traces	traces	1.0 ± 0.2	0.3 ± 0.1*
22:6n-3	traces	traces	9.6 ± 0.1	4.4 ± 0.2*

[a]Results are expressed as the mean ± 1 SEM from six animals assayed in duplicate. Asterisks indicate significantly different ($P < 0.01$) with respect to the corresponding control value. GLC analyses were performed as described in the Material and Methods section. Only relevant fatty acids were included.

TABLE 6
Apparent Kinetic Parameters of Solubilized ACS Activity for Palmitic Acid, from Control (S) or Calcium-Deficient (CD) Microsomes[a]

	Treatment			
	KCl		Triton X-100	
	S	CD	S	CD
Km (μM)	42.6 ± 4.1	39.2 ± 5.2	56.8 ± 4.3	60.4 ± 5.5
Vm (nmol/min·mg)	177.1 ± 8.7	176.9 ± 6.6	167.3 ± 9.8	158.6 ± 5.5

[a]Results were obtained from the linear regressions shown in Figure 8. Data are given as the mean ± 1 SEM of four different incubations assayed in triplicate.

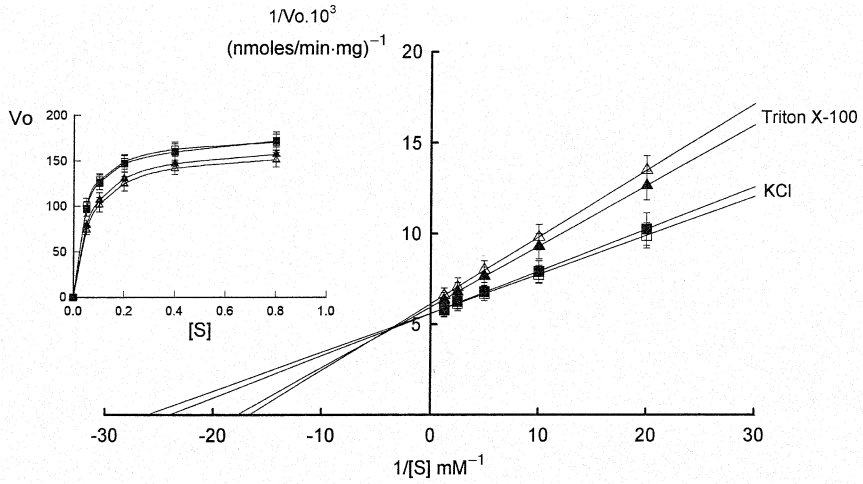

FIG. 8. Linear regressions for Lineweaver-Burk plots of solubilized ACS activity for palmitic acid, from control (open symbols) or calcium-deficient (solid symbols) microsomes. Both types of microsomes were treated with Triton X-100 (triangles) or a buffer with KCl (squares). After partial purification, the activity was assayed as described in the Materials and Methods section. Michaelis-Menten plots are shown in the insert. Each point represents the mean ± 1 SEM of four different incubations assayed in triplicate. For abbreviations, see Figure 1.

in this paper also show that residual amounts of Triton X-100 have no significant effect on the palmitoyl-CoA synthetase activity, but they promote an increase in Km values without affecting Vm (Table 5).

Considering that membrane enzymes such as ACS interact with surrounding lipids and proteins in the membrane, it is conceivable that the configuration of the molecule would be altered if its microenvironment changed. It is important to remark that the different behavior exhibited by ACS between S- and CD-microsomal preparations was eliminated after solubilization procedures. From the results shown in Figure 8 and Table 5, we may assume that both modifications in the specific activity of the enzyme and the differential response observed in competition experiments were the consequence of the alterations in the reticulum membranes induced by the calcium-deficient diet.

In conclusion, this work shows experimental evidence of the role of calcium ions in relation to fatty acid activation. These studies would indicate an alteration in the physicochemical state of the lipid bilayer, evoked by calcium deficiency, that indirectly modifies the kinetic properties of the enzyme. The results presented here would be of interest when examining the intracellular regulation of acyl-CoA synthesis, from the lipid metabolism as well as cell biology and physiology points of view.

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