Assay of acyl-CoA:monoglyceride acyltransferase from rat small intestine using continuous recording spectrophotometry

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ABSTRACT Acyl-CoA:monoglyceride acyltransferase in microsomal preparations from the small intestine of the rat has been measured by means of continuous recording spectrophotometry. The reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with CoA has been employed for this assay and optimal conditions for the reaction have been defined. One of the substrates, palmitoyl-CoA, inhibits the reaction even in modest concentrations. This inhibition is largely prevented by the addition of bovine serum albumin to the incubation medium. The reliability of the assay method was confirmed by comparison with the more cumbersome assay method that uses radioactive substrate.

SUPPLEMENTARY KEY WORDS small bowel mucosa · microsomal preparation · substrate requirements · substrate inhibition · bovine serum albumin · nonionic detergent

ACYL-COA:MONOGLYCERIDE acyltransferase is an enzyme present in the small bowel mucosa concerned with recombining the absorbed products (fatty acid and monoglyceride) of dietary fat digestion (1). Once free fatty acid has been activated to fatty acyl-CoA, reesterification may proceed either by the glycerol 3-phosphate pathway (2, 3) or by the monoglyceride pathway (4–6). It has been demonstrated that the latter pathway is quantitatively the more important in man and laboratory animals (1, 7). The enzyme required for direct acylation of monoglyceride has been found to have its highest activity in the microsomal fraction of the small bowel mucosa (4, 8).

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

In various diseases of the small intestine associated with steatorrhea (such as nontropical sprue), in which esterification of fatty acids is abnormal (9, 10), it is likely that the activity of acyl-CoA:monoglyceride acyltransferase and possibly other enzymatic activities is reduced. Assay of the enzyme using radioactive substrate demands much time and effort.

Recently, a spectrophotometric method employing 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, was developed for the assay of acyl-CoA:phospholipid acyltransferase (11). DTNB reacts with the sulfhydryl group of liberated CoA; it was recently used to measure acyl-CoA:monoglyceride acyltransferase in the small bowel of adrenalectomized rats (12).

The present study, employing continuous spectrophotometric monitoring, was undertaken to study the kinetics of the reaction and to determine optimal substrate requirements. One of the substrates, palmitoyl-CoA, was found to inhibit the reaction even at moderate concentrations; the inhibition was largely overcome by the addition of bovine serum albumin, which greatly facilitates the measurement of acyl-CoA:monoglyceride acyltransferase activity.

EXPERIMENTAL PROCEDURES

Microsomal preparations from the mucosa of the upper half of the small intestine of fasting male Sprague-Dawley rats were made as previously described (12). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (13). The enzyme was assayed within 6–10 hr after the rats were killed.

Monoolein, mainly the 1-isomer, was purchased from Calbiochem, Los Angeles, Calif., and purified by silicic acid column chromatography. The 2-isomer of mono-

olein was a generous gift of Dr. F. H. Mattson, Procter and Gamble Company, Cincinnati, Ohio. Palmitoyl-CoA was purchased from Nutritional Biochemicals, Cleveland, Ohio. When used, it was dissolved in 0.01 M KH_2PO_4, pH 5.0. Bovine albumin, fraction V, was purchased from Pentex, Inc., Kankakee, Ill. DTNB was purchased from Aldrich Chemical Company, Milwaukee, Wis. Palmitoyl-1-^{14}C CoA was purchased from Tracerlab, Waltham, Mass. Scintillation material, Pre Mix "M," was purchased from Packard Instrument Company, Inc., Downers Grove, Ill., and dissolved in toluene to make a 0.55% solution.

The incubation medium contained 0.2 ml of 0.1 M Tris–HCl buffer, pH 8.0, 0.03 ml of 0.01 M DTNB in 0.1 M Na_2HPO_4, pH 7.0, and 0.02 ml of 10% bovine albumin solution. To this were added 25 mμmoles of monoolein in 0.01 ml of 95% ethanol, 50 mμmoles of palmitoyl-CoA in 0.02 ml of 0.01 M KH_2PO_4, pH 5.0, and 0.01 mg of microsomal protein. The final volume in the microcuvette was 0.3 ml. The temperature in the cuvette chamber of the Gilford spectrophotometer was kept constant at 30°C by water circulating through the chamber jacket. Changes in optical density at 412 nm were recorded continuously for at least 4 min. The molar absorptivity of CoA, 13,600 M^{-1} cm^{-1}, was used to convert change in optical density to mμmoles of CoA released. CoA was released by one of two processes:

1. Deacylation: palmitoyl-CoA → palmitic acid + CoA.

2. Acylation: monoglyceride + palmitoyl-CoA → diglyceride + CoA

diglyceride + palmitoyl-CoA → triglyceride + CoA.

To determine the relative rates of deacylation and acylation occurring during the incubation period, an incubation was carried out in two cuvettes simultaneously. One had the complete incubation system and recorded the CoA released by both acylation and deacylation. The other cuvette contained the entire system minus monoolein. The same amount of 95% ethanol was added to this cuvette to keep conditions otherwise the same. This cuvette measured the CoA released by deacylation alone. The change in optical density recorded in the "deacylase" cuvette was subtracted from the change in optical density recorded in the other cuvette to obtain the rate of acylation.

When the "radioactive substrate" method of assaying the enzyme was used, the incubation was carried out in the same manner except that 50 mμmoles of palmitoyl-^{14}C CoA, specific activity 910 cpm/mμmole, was used. The specific activity of this material was determined by dissolving 50 mμmoles in 13 ml of scintillation liquid containing 2 ml of ethanol. The ethanol was added to help dissolve palmitoyl-CoA in toluene. After the rate of acylation had been determined spectrophotometrically, the contents of the cuvettes were transferred to separatory funnels containing 20 ml of chloroform–methanol 2:1 (v/v) and lipids were extracted by the method of Folch, Lees, and Sloane Stanley (14). The chloroform layer was separated and measured and a 1 ml aliquot was put in 15 ml of scintillation liquid for determination of total radioactivity per sample. The remainder was taken to dryness, redissolved in 0.5 ml of chloroform, and subjected to thin-layer chromatography on Silica Gel H in hexane–ether–acetic acid 40:10:1. The fractions, triglyceride, diglyceride, monoglyceride–phospholipid, and free fatty acid, were made visible by exposure of the plate to iodine vapor and each zone was placed in a microcolumn and eluted with 20 ml of chloroform into a counting vial. The chloroform was removed by evaporation and 15 ml of scintillation liquid was added. The amount of radioactivity in each fraction was calculated by multiplying the percentage of radioactivity found in this fraction by the total radioactivity in the original sample. Quenching was determined by means of an internal standard. Samples were counted in a Nuclear Mark I liquid scintillation spectrometer.

The rate of deacylation of palmitoyl-^{14}C CoA was further studied by the Dole (15) procedure as modified by Fredrickson, Ono, and Davis (16) for extraction of free fatty acid. After the spectrophotometric determination, the entire sample was transferred to a tube containing 5 ml of isopropanol–isooctane–1 N H_2SO_4 40:10:1 to which was added 1.5% (w/v) polyoxyethylene dodecyl alcohol. The tubes were vigorously agitated and then 5 ml of heptane was added and thoroughly mixed. After separation the heptane layer was taken off, transferred to scintillation vials, and taken to dryness. Then 15 ml of scintillation liquid was added and radioactivity was determined.

The effect of a nonionic detergent, polyoxyethylene sorbitan monolaurate (Tween 20), on acyl-CoA:monoglyceride acyltransferase activity was also determined. 0.01 ml of a 10% solution of the detergent was added to the incubation medium and the rates of deacylation and acylation were determined in the usual way.

The microsomal preparations were also preincubated with small amounts of palmitoyl-CoA to determine if this substrate had direct toxic effects on the system. Microsomal protein, 0.01 mg, and 12.5 mμmoles of palmitoyl-CoA in 0.01 ml of 0.01 M KH_2PO_4, pH 5.0, were added to 0.2 ml of 0.1 M Tris–HCl buffer, pH 8.0, and 0.03 ml of 0.01 M DTNB and incubated at 30°C for 14 min. Then 0.02 ml of 10% bovine serum albumin, 25 mμmoles of monoolein in 0.02 ml of 95% ethanol, and 37.5 mμmoles of palmitoyl-CoA in 0.015 ml of KH_2PO_4 buffer were added. The final volume of the incubation

medium was 0.32 ml. The mixture was kept at 30°C for another 4 min. The rates of deacylation and acylation measured during this period were compared to those obtained from microsomal enzyme that had not been preincubated. Microsomal protein, 0.01 mg, was also preincubated for the same length of time in Tris–HCl buffer and DTNB alone to determine what effect preincubation at 30°C had on the enzyme.

In an investigation of factors contributing to the change in optical density in the "deacylase" cuvette, another study was performed omitting palmitoyl-CoA and substituting 50 mμmoles of sodium dodecyl sulfate. Tris–HCl buffer, DTNB, bovine serum albumin, 95% ethanol, and microsomal protein were added in the usual amounts and a 4 min incubation at 30°C was performed.

On certain microsomal preparations fatty acid:CoA ligase was also assayed by the method of Senior and Isselbacher (17). The method was modified by the use of 8% bovine serum albumin to suspend the palmitic acid substrate in the aqueous incubation medium and only 0.1 mg of microsomal protein was utilized. Incubation was for 15 min at 37°C.

RESULTS

Variation of Substrate Concentration

Preliminary studies indicated that one of the substances, palmitoyl-CoA, inhibited acyl-CoA:monoglyceride acyltransferase activity even in modest concentrations. With very low concentrations of palmitoyl-CoA, acylation occurred but the rate quickly decreased as the limited amount of substrate was utilized. Increasing palmitoyl-CoA concentration greatly increased the rate of deacylation but acylase activity declined. If the concentration of this substrate was further increased, acylase activity was completely abolished. This inhibitory effect of palmitoyl-CoA was altered by the addition of albumin to the incubation medium. Albumin inhibited deacylation, and acylation was now observed to proceed at the maximal rate for at least 4 min (Fig. 1).

The requirement for albumin increased with higher concentrations of palmitoyl-CoA, although for any given amount of palmitoyl-CoA it was possible to exceed the optimal requirement for albumin. With excess albumin, acylation as well as deacylation was inhibited. The concentrations of both palmitoyl-CoA and bovine albumin were varied and their optimum concentrations determined. When the concentration of monoolein was kept constant at 25 mμmoles per 0.3 ml of incubation medium (Fig. 2), maximal acyl-CoA:monoglyceride acyltransferase activity was observed with either 50 mμmoles of palmitoyl-CoA and 2 mg of albumin or 100 mμmoles of palmitoyl-CoA and 4 mg of albumin.

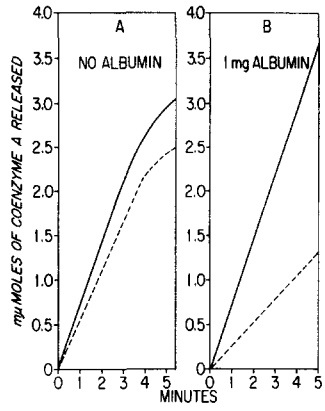

FIG. 1. Effect of bovine albumin on acyl-CoA:monoglyceride acyltransferase activity. The medium contained 0.2 ml of 0.1 M Tris–HCl buffer, pH 8.0, 0.03 ml of 0.01 M DTNB in 0.1 M Na_2HPO_4, pH 7.0, 12.5 mμmoles of palmitoyl-CoA in 0.02 ml of 0.01 M KH_2PO_4, pH 5.0, 25 mμmoles of monoolein in 0.01 ml of 95% ethanol, and 0.01 mg of microsomal protein. The pH of this mixture was 7.82. The final volume was 0.3 ml and incubation was done at 30°C. - -, mμmoles of CoA released in "deacylase" cuvette; —, mμmoles of CoA released in "deacylase plus acylase" cuvette. *A*: without albumin, net acylation (difference between curves) after 5 min was 0.57 mμmole/0.01 mg of microsomal protein. *B*: 1 mg of albumin added; net acylation after 5 min was 2.36 mμmoles/0.01 mg of microsomal protein.

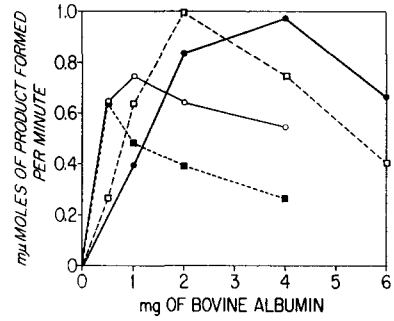

FIG. 2. Effect of concentration of palmitoyl-CoA and bovine albumin on acyl-CoA:monoglyceride acyltransferase activity. Incubation medium contained 0.2 ml of 0.1 M Tris HCl buffer, pH 8.0, 0.03 ml of 0.01 M DTNB, 25 mμmoles of monoolein, and 0.01 mg of microsomal protein. Amounts of albumin shown on the abscissa. Enzyme activity on the ordinate is expressed as mμmoles of product formed/min per 0.01 mg of microsomal protein. Amounts of palmitoyl-CoA: ■—■ 12.5 mμmoles; ○—○ 25 mμmoles; □—□ 50 mμmoles; ●—● 100 mμmoles. Final volume of the incubation medium was 0.3 ml and incubation was for 4 min at 30°C.

With the amount of palmitoyl-CoA and albumin now held constant, the effect of monoolein concentration was studied (Table 1). Results were similar for the 1- and 2-isomers: maximal activity with 25–50 mμmoles of 1-monoolein or 12.5–25 mμmoles of 2-monoolein in the incubation mixture (0.3 ml). Higher concentrations of either isomer produced slight inhibition of the reaction.

Effect of pH

Maximal activity was observed over a pH range of 7.0–8.5 (Table 2). In an attempt to study this reaction above

TABLE 1 EFFECTS OF CONCENTRATION OF 1- OR 2-MONOOLEIN ON ACYL-CoA:MONOGLYCERIDE ACYLTRANSFERASE

Concentration of Monoolein	Activity	
	1-Monoolein	2-Monoolein
mμmoles/0.3 ml	*mμmoles product/min/mg protein*	
6.25	53	
12.5	82	134
25	137	147
25	126	102
100	95	

Incubation medium contained 0.2 ml of 0.1 M Tris–HCl buffer, pH 8.0, 0.03 ml of 0.01 M DTNB in 0.1 M Na_2HPO_4, pH 7.0, 0.02 ml of 10% bovine albumin, 50 mμmoles of palmitoyl-CoA in 0.02 ml of 0.01 M KH_2PO_4, pH 5.0, and 0.01 mg of microsomal protein. Monoolein in the amounts indicated was added in 0.01 ml of 95% ethanol. The final volume was 0.3 ml and incubation was for 4 min at 30°C.

TABLE 2 EFFECT OF pH ON ACYL-CoA:MONOGLYCERIDE ACYLTRANSFERASE ACTIVITY

	pH						
	5	6	7	7.5	8	8.5	9
	mμmoles product/min/mg protein						
Activity	13	55	126	133	137	117	104

Incubation medium contained 0.03 ml of 0.01 M DTNB in 0.1 M Na_2HPO_4, pH 7.0, 0.02 ml of 10% bovine albumin, 25 mμmoles of monoolein in 0.01 ml of 95% ethanol, 50 mμmoles of palmitoyl-CoA in 0.02 ml of 0.01 M KH_2PO_4, pH 5.0, and 0.01 mg of microsomal protein. 0.1 M sodium acetate–acetic acid buffer was used at pH 5, 0.1 M sodium phosphate buffer at pH 6 and 7, and 0.1 M Tris–HCl buffer at pH 7.5–9. 0.2 ml of buffer was used in each study. The final volume was 0.3 ml and incubation was for 4 min at 30°C.

pH 9.0, spontaneous changes occurred in optical density without any addition of palmitoyl-CoA.

Linearity with Enzyme Concentration

Various proportions of active and heat-inactivated microsomal preparations were employed to determine if there was a linear relationship between enzyme activity and the amount of active enzyme present. Total amount of both active and heat-treated microsomal protein added to each incubation was always 0.01 mg. The relationship between the amount of active microsomal protein and enzyme activity was linear up to 0.01 mg of protein (specific activity, 1.5 mμmoles of CoA released by acylation reaction per minute per 0.01 mg of protein).

Comparison of Spectrophotometric Method with Assay Using Palmitoyl-^{14}C CoA

Two rats were used for this study and the amount of glyceride formed was determined in intestinal tissue from each after two separate incubations, one for 4 and the other for 9 min (Fig. 3). The results of the two methods

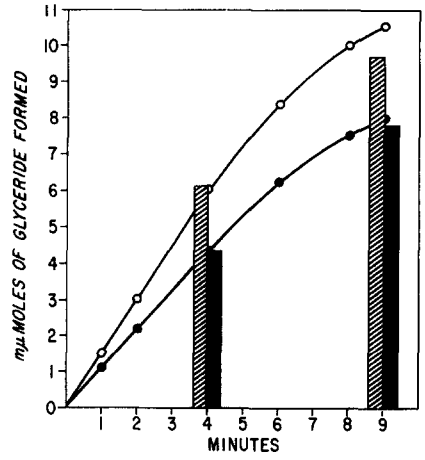

FIG. 3. Comparison of spectrophotometric and radioactive methods of assay. Incubation medium contained 0.2 ml of Tris–HCl, pH 8.0, 0.03 ml of 0.01 M DTNB, 0.02 ml of 10% bovine albumin, 25 mμmoles of monoolein, 50 mμmoles of palmitoyl-CoA, and 0.01 mg of microsomal protein. The final volume was 0.3 ml and incubation was at 30°C. The curves represent the amount of product formed per 0.01 mg of microsomal protein as determined by continuous recording spectrophotometry; ○—○ rat A, ●—● rat B. The bars represent the amount of ^{14}C-glycerides formed at the end of 4 and 9 min of incubation; hatched column rat A, solid column rat B.

for acylase activity compared very well, with a difference of less than 10%. The rate of deacylation as determined by these two methods did not correlate well, however; the spectrophotometric assay indicated a higher deacylase activity. Since the incubation medium contained significant quantities of protein and since the amount of palmitoyl-CoA added was rather small, it is possible that a significant amount of the ^{14}C-palmitic acid liberated by deacylation became bound to protein, remained in the aqueous layer, and was therefore not determined.

The rate of deacylation was reexamined using the Dole (15) method of extraction to determine if more ^{14}C-free fatty acid could be recovered in the lipid phase. The amount of albumin added to the incubation medium was varied to produce changes in the deacylase rate. As the amount of albumin added increased, the rate of deacylation decreased as determined by each method but the values were still greater for the spectrophotometric method (Table 3).

The relative amounts of ^{14}C-di- and triglycerides formed using palmitoyl-^{14}C CoA as substrate depended upon whether 1- or 2-monoolein was used. With 1-monoolein, 88% of the ^{14}C-glyceride formed after 10 min was in the diglyceride and 12% in the triglyceride fraction. When 2-monoolein was used, 48% was found in the diglyceride and 52% in the triglyceride fraction.

Effect of Tween 20

When the nonionic detergent Tween 20 (0.01 ml of a 10% solution) was added to the incubation medium

TABLE 3 COMPARISON OF RATES OF DEACYLATION WITH VARIATION OF ALBUMIN CONCENTRATION BY SPECTROPHOTOMETRIC AND RADIOACTIVE METHODS

Albumin	Amount of Deacylation	
	Spectrophotometric Method*	Radioactive Method†
mg/0.3 ml	*mμmoles/5 min/0.01 mg protein*	
0	7.55	1.74
1	5.30	1.58
2	2.38	0.87

Incubation medium contained 0.2 ml of 0.1 M Tris–HCl buffer, pH 8.0, 0.03 ml of 0.01 M DTNB in 0.1 M Na_2HPO_4, pH 7.0, 50 mμmoles of palmitoyl-CoA in 0.02 ml of 0.01 M KH_2PO_4, pH 5.0, 0.01 ml of 95% ethanol, and 0.01 mg of microsomal protein. Albumin was added in amounts indicated above. Final volume was 0.3 ml and incubation was for 5 min at 30°C.

* CoA released measured spectrophotometrically.

† Fatty acid released measured by extraction (15) and radioactivity determination.

(0.3 ml), the rate of acylation declined from 150 to 54 mμmoles/min per mg of protein.

Effects of Preincubation of Microsomal Enzyme With Palmitoyl-CoA

After preincubation of 0.01 mg of microsomal protein with 12.5 mμmoles of palmitoyl-CoA for 14 min at 30°C, acylase activity could no longer be detected when monoolein, albumin, and palmitoyl-CoA were added in the usual amounts and the mixture was reincubated. Deacylase activity, however, was unchanged. Preincubation of 0.01 mg of microsomal protein without the addition of palmitoyl-CoA resulted in 45% inhibition of acylase activity when compared with values obtained from microsomal protein not subjected to any form of preincubation.

Are Changes in Optical Density due to Factors Other than Deacylation of Palmitoyl-CoA?

When optical density was studied in the "deacylase" cuvette with palmitoyl-CoA omitted, no change in optical density was observed. Substitution of palmitoyl-CoA with 50 mμmoles of sodium dodecyl sulfate (which might mimic the detergent effect of palmitoyl-CoA and expose microsomal membrane -SH groups to the action of DTNB) also failed to produce any change in optical density during a 4 min incubation.

Comparison of the Rates of Acyl-CoA:Monoglyceride Acyltransferase and Fatty Acid:CoA Ligase in the Fasted State

With an incubation system containing 25 mμmoles of 1-monoolein, 50 mμmoles of palmitoyl-CoA, and 2 mg of albumin at pH 8.0, the rate of acyl-CoA:monoglyceride acyltransferase in intestinal microsomes from six fasted rats was 115 ± 16.6 (SD) mμmoles/min per mg of protein. Fatty acid:CoA ligase determined independently on the same samples was 97 ± 16.5 (SD) mμmoles/min per mg of protein.

DISCUSSION

The rate of acyl-CoA:monoglyceride acyltransferase in the rat small intestine can be determined rapidly by the spectrophotometric method. Rao and Johnston, however, recently attempted to use the same spectrophotometric method employing DTNB to assay the activity of this enzyme in hamster intestine (18). They found little correlation between the DTNB and radioactive methods and concluded that this spectrophotometric technique was unsatisfactory for assaying acyl-CoA:monoglyceride acyltransferase because the liberated CoA became tightly bound to the microsomal membrane and was not available to react with DTNB.

This was not so for rat intestine, as demonstrated by the close correlation between the spectrophotometric and radioactive assays (Fig. 3). The amount of ^{14}C-glycerides formed at the end of incubation was similar to the amount predicted by the spectrophotometric method. Species variation might explain the observed difference. Also, Rao and Johnston employed a partially purified form of the enzyme for their assay as opposed to the microsomal preparation used in this study. The latter type of enzyme source may not bind liberated CoA as strongly as the former, so that it may be more available to react with DTNB. The addition of albumin to the incubation medium in the present study also may have interfered with the binding of CoA to the enzyme.

The rates of deacylation of palmitoyl-CoA determined by the DTNB and radioactive methods did not correlate well; the DTNB method gave higher values. Use of the Dole (15) procedure for extraction of ^{14}C-palmitic acid did not appreciably improve the agreement. Both methods did show increasing rates of deacylation with decreasing amounts of albumin in the system. The amount of ^{14}C-palmitate liberated during these studies was quite small. A possible explanation for the difference is that the extraction procedure did not fully extract all of the liberated ^{14}C-palmitic acid from a strong binding to albumin and enzyme protein.

During this study it has been assumed that the entire change in optical density observed in the "deacylase" cuvette was produced from hydrolysis of palmitoyl-CoA. When palmitoyl-CoA was omitted from the reaction no change in optical density was observed. The assumption, therefore, appears valid. However, palmitoyl-CoA has detergent properties and if during incubation it altered the microsomal membrane, it is possible that membrane sulfhydryl groups would become ex-

posed and also react with DTNB, thus contributing to change in optical density. This could also explain the difference in deacylase activity recorded by the spectrophotometric and radioactive methods. The explanation seems unlikely, however, as when an ionic detergent, sodium dodecyl sulfate, replaced palmitoyl-CoA in the "deacylase" cuvette there was no change in optical density upon incubation.

The specific activities of acyl-CoA:monoglyceride acyltransferase and fatty acid:CoA ligase were similar in microsomal preparations from the intestines of fasting rats. Rao and Johnston found that these two enzymes were so closely associated with each other in the microsomal fraction of hamster intestinal mucosa as to be impossible to separate by methods currently available (18). It is not surprising, therefore, to find that these two enzymes have similar values for maximal activity.

The inhibition of acyl-CoA:monoglyceride acyltransferase by palmitoyl-CoA is not unexpected, as this compound has been shown to inhibit a number of reactions at high concentrations (11, 19–22). The addition of albumin to the incubation medium markedly stimulated acylase activity. The stimulating effect of albumin on other varieties of acyltransferase enzymes has been shown previously (11, 21, 22). The explanation for this is not known, but most probably the albumin lowers the effective concentration of palmitoyl-CoA by binding with this substrate. Deacylase activity seems directly related to the amount of substrate present over a wide range of concentration and with a reduction in "free" palmitoyl-CoA that apparently occurs with the addition of albumin, deacylase decreases. Acylation, however, occurs only at low substrate concentration and would be expected to work maximally when palmitoyl-CoA is present in reduced amounts.

Palmitoyl-CoA has detergent properties. In this study it was shown that addition of small amounts of nonionic detergent, Tween 20, inhibited the reaction. Dorsey and Porter demonstrated that inhibition of pigeon liver fatty acid synthetase by palmitoyl-CoA could be duplicated by replacing palmitoyl-CoA with an ionic detergent, sodium dodecyl sulfate (23). The complete inhibition of acylase activity observed in the present study when microsomal protein was preincubated with palmitoyl-CoA may be the result of the detergent effects of this substrate. The detergent properties of this compound may also be contributing to the inhibition observed when high concentrations of "free" palmitoyl-CoA are present during regular incubation.

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