Retinol esterification by mammary gland microsomes from the lactating rat

A. Catharine Ross

Department of Physiology and Biochemistry and Department of Pediatrics, The Medical College of Pennsylvania, Philadelphia, PA 19129

Abstract Because vitamin A in milk is largely present as esterified retinol while blood plasma predominantly contains unesterified retinol, experiments were conducted to determine whether membranes from the lactating mammary gland are able to synthesize retinyl esters in vitro. When microsomes from rats lactating for 7 to 14 days were incubated with [^{3}H]retinol dispersed in dimethylsulfoxide, some [^{3}H]retinol esterification was observed (147 pmol/5 min per 0.5 mg protein). However, 3- to 7-fold increases in retinyl ester synthesis could be achieved by supplying either a fatty acyl CoA-generating system or preformed fatty acyl CoA thioesters; thus, the major activity in vitro has the characteristics of a fatty acyl CoA: retinol acyltransferase. Both long-chain and medium-chain fatty acyl CoA esters stimulated [^{3}H]-labeled retinyl ester synthesis in vitro. Concordantly, analysis of the retinyl ester pattern of rat milk demonstrated the presence of eight different esters of retinol ranging in fatty acyl chain length from 8 to 18 carbons. Retinol esterification by microsomes was maximal at neutral pH (7.1) in the presence of approximately 50 μM palmitoyl CoA, and was linear with time of incubation for at least 5 min. Retinyl ester synthesis increased with the apparent concentration of [^{3}H]retinol to approximately 200 nmol/ml, but was also dependent on the ratio of retinol relative to total microsomal protein in the incubation mixture. These experiments demonstrate for the first time retinol esterification by mammary gland membranes and point to the hypothesis that free retinol from plasma is esterified in this organ before secretion of retinyl esters in milk.—**Ross, A. C.**, Retinol esterification by mammary gland microsomes from the lactating rat. *J. Lipid Res.* 1982. **23:** 133–144

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During lactation, vitamin A is secreted from the mammary gland as one component of milk fat. In 1947, Parrish, Wise, and Hughes (1) and Ganguly, Kon, and Thompson (2) analyzed the carotene and vitamin A contents of milk and reported that nearly all (94–100%) of vitamin A is in the form of retinyl ester. Little is known about the origin of this esterified retinol. It is known, however, that approximately 95% of vitamin A in fasting blood circulates as the alcohol, retinol, bound specifically to serum retinol-binding protein (3, 4). A smaller amount of vitamin A in plasma is also found in the form of esterified retinol as a constituent of the chylomicron (5–7) or chylomicron remnant (7) which is largely recognized and catabolized by liver (8–10), thus delivering most newly absorbed vitamin A from the intestine to liver, the major storage organ for this nutrient. Either retinol bound to retinol-binding protein or retinyl ester in the chylomicron might be delivered to vitamin A's target organs such as the mammary gland during lactation. In one study of the lactating rhesus monkey, Vahlquist and Nilsson (11) have presented evidence that retinol-binding protein is the major carrier of vitamin A to the mammary gland. If retinol is delivered to this organ, or if chylomicron retinyl esters are taken up by processes involving ester bond hydrolysis, then it is necessary to postulate that esterification of retinol occurs in the mammary gland during transport of vitamin A from blood to milk.

This paper reports experiments conducted in vitro to determine whether membrane fractions prepared from the lactating mammary gland of the rat have enzymatic activity capable of esterifying retinol. Attention has been focused on the microsomal fraction due to its known role in milk triglyceride (12, 13) and phospholipid biosynthesis (14), and because this fraction has previously been reported to catalyze retinyl ester synthesis in other organs, namely liver (15) and the retina of the eye (16, 17). Initially, we asked whether mammary gland microsomes can catalyze retinol esterification and, having observed activity (18), have explored the nature of the esterification reaction and some of the characteristics of this microsomal enzyme in vitro.

MATERIALS AND METHODS

Isotopes and chemicals

[1-^{3}H(N)]Vitamin A_1 (all-*trans*) was purchased from New England Nuclear and was mixed with all-*trans*-

Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; p-CMB, sodium p-chloromercuribenzoate; DTT, dithiothreitol; EDTA, disodium ethylenediaminetetraacetate; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonylfluoride.

retinol (Sigma Chemical Co., type X) to a specific radioactivity of approximately 6 μCi/μmol and a concentration of 3 mM in ethanol. This stock solution was kept under argon at −20°C in the dark; its purity was checked by aluminum oxide column chromatography (below). The substrate was repurified on alumina when more than 0.3% of ^{3}H eluted in the retinyl ester fraction. [1-^{14}C]Palmitoyl coenzyme A (New England Nuclear) was mixed with palmitoyl CoA to 2 μCi/μmol and 1 mM in 0.001 M sodium acetate buffer, pH 6.0, and stored in the same manner. Coenzyme A (lithium salt) and its fatty acid esters were obtained from P–L Biochemicals Inc. Other reagents included ATP and sodium taurocholate from Calbiochem-Behring Corp., bovine serum albumin (BSA, essentially fatty acid-free), sodium palmitate, dithiothreitol (DTT), butylated hydroxytoluene (BHT), phenylmethylsulfonylfluoride (PMSF), hydroxylamine hydrochloride, and disodium ethylenediaminetetraacetate (EDTA) from Sigma Chemical Co., sodium p-chloromercuribenzoate (p-CMB) from Mann Research Laboratories, Inc., and Triton X-100 from Rohm and Haas Co.

Preparation of mammary gland fractions

Lactating Sprague-Dawley rats with litters, purchased from Charles River Breeding Labs, were housed in our animal facility with free access to pelleted rat diet (Wayne Lab-Blox) and water until 7 to 14 days after parturition. Rats weighing 220–345 g were anesthetized with sodium pentobarbital (130 mg/kg, intraperitoneally) and both inguinal-abdominal and pectoral mammary glands were removed with attached skin and immediately placed on ice. All subsequent procedures were performed at 4°C. The mammary glands were dissected free of skin and sheets of connective tissue, then minced in 0.25 M sucrose–0.15 M potassium phosphate buffer, pH 7.1, 4 ml/g tissue. The suspension was homogenized in 15-ml portions with a Polytron homogenizer with PT10/ST probe generator at setting 7 and filtered through gauze to produce the whole homogenate. Microsomes were prepared routinely by three successive centrifugation steps under the following conditions: *1*) 725 *g* for 10 min in 50-ml tubes in a swinging bucket rotor of an International PR-2 centrifuge; *2*) 37,000 *g* for 13 min in 50-ml tubes in the SS-34 rotor of a Sorvall RC2-B centrifuge; *3*) 6.7×10^6 *g*·min in the 60 Ti rotor of a Beckman ultracentrifuge. After each step, floating fat was skimmed from each supernatant before centrifuging at higher force. The final supernatant (cytosol) was decanted and the microsomal pellet was suspended in 0.15 M potassium phosphate buffer, pH 7.1, with 1 mM DTT except where noted, to a concentration of 6–14 mg protein/ml. One-half- to 1-ml portions were frozen in capped glass vials in dry ice/acetone before storage at −70°C. The stability of the esterifying activity was tested after 10 weeks and 6 months of storage under these conditions. Compared to the same microsomes assayed when fresh, frozen microsomes retained 90% or more of retinol esterifying activity after 10 weeks and 57% after 6 months at −70°C.

In two experiments, mammary gland microsomes were treated with 0.5 M hydroxylamine hydrochloride, adjusted to pH 7.4 with NH_4OH (19, 20), or with 0.15 M potassium phosphate buffer, pH 7.4. Thawed microsomes, 2 mg protein/ml, were incubated with hydroxylamine or buffer for 20 or 30 min at 37°C in a shaking waterbath. After dilution to 0.3 mg/ml with cold buffer, microsomes were collected by centrifugation at 105,000 *g* for 30 min at 4°C. The pellets were washed with 2 ml of buffer to remove residual hydroxylamine, then hand-homogenized in buffer at a concentration of 0.5 mg protein/ml. The suspension was centrifuged again under the same conditions and the final pellets were suspended in a small volume of potassium phosphate buffer, pH 7.1, for incubation with retinol. Protein recoveries equaled 25.4 to 31.3% and 29.1 to 32.4% after treatment with hydroxylamine and buffer, respectively.

Protein was measured by the dye-binding method of Bradford (21) using reagent from Bio-Rad Laboratories, Inc. Protein values were determined with bovine immunoglobulin G as standard.

Milk was expressed manually from three lactating rats approximately 10 min after an intramuscular injection of 0.1 unit oxytocin. The samples were frozen at −20°C before extraction with ethanol and hexane, below, and analysis by high performance liquid chromatography (HPLC).

Incubations with retinol

Mammary gland microsomes or other tissue fractions were incubated with [^{3}H]retinol in the dark in an air atmosphere. The [^{3}H]retinol substrate was prepared just before use by evaporating ethanol from the stock solution under argon and redissolving [^{3}H]retinol in dimethylsulfoxide. Each tube containing microsomes was transferred from ice to a shaking water bath (120 rev/min, 37°C) to warm for 50 sec. At that time, any additions such as cofactors were added so that the final volume was 0.25 ml. Ten seconds later the reaction was begun by adding 5 μl of substrate solution and vortexing the tube for 3 sec. Each experimental condition was conducted in duplicate; a third tube containing boiled microsomes served as a blank in each case. In some experiments, controls containing no protein were included; these gave the same blank values as boiled microsomes (0.1–0.3% of added [^{3}H]retinol). Reactions were stopped by adding 1 ml of ethanol containing 100 μg of BHT to each tube, with vortexing.

Extraction and chromatography

Neutral lipids were quantitatively extracted from the ethanol-precipitated microsomes into hexane (22). To each tube, 4 ml of hexane with BHT (100 μg/ml) was added and, after shaking, 1 ml of water was added. Tubes were centrifuged briefly (1,500 rpm, 10 min) to produce clear hexane upper phases. A 0.5-ml portion of each hexane extract was counted in liquid scintillation fluid (ScintiLene, Fisher Scientific Co.) to determine recovery of ^{3}H; routinely, recoveries equaled 95–98% compared to the [^{3}H]retinol substrate which was counted directly in ScintiLene. A 3-ml portion of each hexane extract was taken to dryness under nitrogen at 37–40°C, redissolved in 1 ml of hexane with BHT, and chromatographed on columns of 1.5 g neutral aluminum oxide deactivated with water (5 ml/100 g). The sample was applied in hexane and rinsed with 2 ml of hexane. ^{3}H-labeled retinyl esters were eluted by addition of 12 ml of hexane–diethyl ether 97:3 (v/v). In some cases, [^{3}H]retinol was eluted subsequently with 20 ml of hexane–diethyl ether 50:50 (v/v). Since retinyl esters and cholesteryl esters coelute in this chromatographic system, as do unesterified retinol and cholesterol (7), we tested, in some experiments, for any leakage of [^{3}H]retinol into the column fraction containing ^{3}H-labeled retinyl esters by adding an internal standard of [^{14}C]cholesterol to the ethanol-precipitated microsomes. The absence of ^{14}C in the retinyl ester fraction after alumina column chromatography assured that no [^{3}H]retinol contaminated the ^{3}H-labeled retinyl esters. Eluates from alumina columns were dried under air and dissolved in ScintiLene for determination of radioactivity by liquid scintillation counting; efficiencies were determined using calibrated standards of [^{3}H]toluene or [^{14}C]toluene. Data are expressed as pmol of retinol esterified after subtraction of values for incubations with boiled microsomes or blank incubations without microsomes.

In one experiment, noted below, alumina column chromatography with the solvent system of Bhat, et al. (23) was used in comparison with our standard chromatographic procedure. After chromatography, the solvents were evaporated under nitrogen, lipids were dissolved in hexane, and absorption spectra were recorded on a Beckman Acta II spectrophotometer.

Analysis of rat milk retinyl esters by HPLC

Rat milk lipids were extracted by the ethanol–hexane procedure, above, and chromatographed on alumina to isolate retinyl esters from triglycerides. Solvents did not contain BHT and care was taken to keep extracts under argon. The fraction eluting with hexane–ether 97:3, was dried under nitrogen and dissolved in 100 μl of acetonitrile. Retinyl esters were separated by HPLC on a 5 μM Supelcosil LC-8 column, 25 cm × 4.6 mm ID, using mobile phases of acetonitrile–water, 88:12 and 98:2 (24). Retinyl esters were detected with an absorbance meter having a 340 nm filter (Waters Associates, Inc.). The eluate from a blank extract was chromatographed and its absorbance was subtracted. Milk retinyl esters were identified by comparison to retinyl ester standards which were synthesized from retinol and either fatty acyl chlorides (5) or fatty acid anhydrides (25).

RESULTS

Esterification of retinol by microsomes

Our initial objective was to determine whether tissue fractions from the mammary gland of the lactating rat are able to esterify retinol. Microsomes were chosen for investigation because reports had shown esterifying activity in this fraction from both liver (15) and retina (16, 17), and because other lipid ester synthases are bound to membranes of the microsomal fraction (12, 14). ^{3}H-labeled retinyl ester synthesis was consistently observed when mammary gland microsomes were incubated with [^{3}H]retinol only. As shown in **Table 1,** activity under these conditions, referred to hereafter as the basal reaction, equaled 147 pmol of ^{3}H-labeled retinyl ester per 5 min per 0.5 mg of protein.

We then asked whether various additions constituting a fatty acyl CoA-generating system (26) could increase the esterification of [^{3}H]retinol. Microsomes incubated with a combination of DTT, ATP, coenzyme A, $MgSO_4$, and BSA produced 977 pmol of ^{3}H-labeled retinyl ester, a 6.65-fold increase over the basal reaction (Table 1). These data suggested that this combination of cofactors provided substrates to microsomal fatty acid: CoA ligase, an active enzyme in the lactating mammary gland (27), and that an activated fatty acid was subsequently used as substrate for ^{3}H-labeled retinyl ester synthesis. In a second experiment, we asked whether supplying exogenous fatty acid to the microsomal preparation would produce a further increase in retinol esterification. As shown in Table 1, the addition of 20 μM or 50 μM palmitic acid did not increase retinyl ester yield over the amount esterified in the presence of cofactors but without added free fatty acid. Apparently, sufficient endogenous fatty acid is present in the microsomal membrane preparation. Higher concentrations of exogenous palmitic acid decreased retinol esterification by mammary gland microsomes. A third set of incubations was conducted to determine whether preformed fatty acyl CoA (in the form of palmitoyl CoA) could also increase [^{3}H]retinol esterification as compared to the basal reaction. Palmitoyl CoA (20 μM) alone increased synthesis 3.3-fold; when

TABLE 1. Esterification of [^{3}H]retinol by mammary gland microsomes in the presence of various additions

	Retinyl Ester Synthesis, pmol[a]	Percent of Control
Control, microsomes with buffer only	147 (±1.7%)	100
Experiment I:		
Dithiothreitol (DTT), 5 mM	142 (±5.8%)	97
DTT + ATP, 10 mM	245 (±2.5%)	167
DTT + coenzyme A, 1.25 mM	402 (±2.7%)	274
DTT + ATP + coenzyme A	666 (±0.1%)	454
DTT + ATP + coenzyme A + $MgSO_4$, 4 mM	750 (±0.5%)	511
DTT + ATP + coenzyme A + $MgSO_4$ + BSA, 20 μM	977 (±0.1%)	665
		Percent of activity without added fatty acid
Experiment II:		
Cofactors[b]	1,007 (±7.1%)	100
Cofactors + sodium palmitate, 20 μM	1,034 (±1.4%)	103
Cofactors + sodium palmitate, 50 μM	971 (±0.6%)	96
Cofactors + sodium palmitate, 200 μM	820 (±0.5%)	81
Cofactors + sodium palmitate, 500 μM	588 (±4.5%)	58
Cofactors + sodium palmitate, 1 mM	391 (±1.8%)	39
		Percent of control
Experiment III:		
Palmitoyl CoA, 20 μM	488 (±0.4%)	332
Palmitoyl CoA + BSA, 20 μM + DTT, 5 mM	623 (±2.2%)	423

[a] Incubations were conducted for 5 min at 37°C using 0.5 mg of microsomal protein in 0.25 ml of 0.15 M potassium phosphate buffer, pH 7.1. [^{3}H]Retinol, 45 nmol, was added in 5 μl of dimethylsulfoxide. Each value reported is the average of duplicate incubations; the percent deviation of each duplicate from the mean is reported in parentheses.

[b] 'Cofactors' denotes the presence of ATP (10 mM), reduced coenzyme A (1.25 mM), DTT (5 mM), $MgSO_4$ (4 mM) and BSA (20 μM).

BSA (20 μM) and DTT (5 mM) were also present, esterification was 4.2-fold greater than when microsomes were incubated only with buffer (Table 1). Since this experiment strongly suggested that retinyl ester synthesis by rat mammary gland microsomes can proceed by a fatty acyl CoA: retinol acyltransferase-catalyzed reaction, incubations were performed where [1-^{14}C]palmitoyl CoA was employed as a putative substrate and the aluminum oxide column fraction containing fatty acyl esters of retinol (and cholesterol) was then resolved into individual retinyl esters by HPLC. Of the ^{14}C radioactivity recovered from the HPLC column, 69% co-migrated with a standard of retinyl palmitate, confirming that exogenous palmitoyl CoA can be used as one of the substrates for retinyl ester synthesis.

Effect of hydroxylamine treatment on the basal reaction

Hydroxylamine at neutral pH reacts with fatty acyl coenzyme A thioesters to form fatty acyl hydroxamates (19). To determine whether the basal esterification reaction might be due to the presence of endogenous fatty acyl CoA in the microsomal preparation, we incubated microsomes with hydroxylamine at pH 7.4, or with buffer only, and then washed the membranes by repeated ultracentrifugation. Incubations with [^{3}H]retinol were conducted under basal conditions, with added palmitoyl CoA, or with added cofactors. Retinol esterification under basal conditions was reduced by 70% (**Table 2**) to 79% after treatment with hydroxylamine. Washing with buffer alone either had no effect on the basal reaction (Table 2) or produced a much smaller effect than hydroxylamine. After hydroxylamine treatment, microsomes were still fully able to respond to exogenous palmitoyl CoA or cofactors. Compared to the basal reaction, esterification of [^{3}H]retinol in the presence of palmitoyl CoA was 27-fold (Table 2) to 39-fold greater, while addition of cofactors resulted in a 34-fold (Table 2) to 65-fold increase in esterified retinol. Microsomes washed with buffer only responded similarly to untreated mammary gland microsomes. These experiments provide indirect evidence that endogenous fatty acyl CoA ester bound to or trapped within mammary gland microsomes can provide substrate for [^{3}H]retinyl ester synthesis and that this pool is depleted during incubated with hydroxylamine. The reason for the increased response of mi-

TABLE 2. Retinol esterification by microsomes treated with hydroxylamine

Treatment Before Assay	Additions during Incubation with [^{3}H]Retinol[b]		
	Buffer Only	Palmitoyl CoA Mix	ATP + Coenzyme A Mix
	Esterified retinol, pmol/5 min/mg protein		
None	154 (±2.5%)	525 (±0.9%)	576 (±3.4%)
Hydroxylamine, 0.5 M[a]	47 (±17.8%)	1,276 (±1.9%)	1,608 (±2.4%)
Buffer[a]	159 (±0.5%)	481 (±0.6%)	511 (±1.1%)

[a] Mammary gland microsomes were incubated at 37°C for 20 min with either 0.5 M hydroxylamine hydrochloride, pH 7.4, or 0.15 M potassium phosphate buffer, pH 7.4, then washed twice by ultracentrifugation (see Methods). Recovery of protein equaled 31.3% and 32.4% for microsomes treated with hydroxylamine and buffer, respectively.

[b] All incubation conditions included 0.15 M potassium phosphate buffer, pH 7.1, and 45 nmol [^{3}H]retinol. The palmitoyl CoA mix included palmitoyl CoA (50 μM), DTT (5 mM), and BSA (20 μM). The ATP + coenzyme A mix included ATP (10 mM), Coenzyme A (1.25 mM), DTT (5 mM), BSA (20 μM), and $MgSO_4$ (4 mM).

crosomes to exogenous palmitoyl CoA and to cofactors after hydroxylamine treatment is not yet known.

Effects of varying reaction conditions

The esterification reaction in the presence of palmitoyl CoA was studied further to determine the pH optimum for retinyl ester synthesis, the effects of varying substrate concentration, and the reaction's characteristics with regard to incubation time and the amount of microsomal protein incubated. ^{3}H-labeled retinyl ester synthesis was maximal at neutral pH (**Fig. 1,** panel A), and all subsequent experiments were conducted with potassium phosphate buffer, pH 7.1. Esterification increased as the concentration of palmitoyl CoA was increased from zero to 50–60 μM; however, at even higher concentrations we observed a decrease in retinyl ester yield instead of a plateau indicative of substrate saturation. This type of dependency on fatty acyl CoA concentration has been reported for other acyltransferases and is thought to be due to the detergent properties of this amphiphile at high concentrations (28–31).

When the concentration of [^{3}H]retinol in the incubation mixture was increased from 17 to 191 nmol/ml, microsomal ^{3}H-labeled retinyl ester synthesis also increased both under basal conditions and in the presence of 50 μM palmitoyl CoA. However, a hyperbolic curve was observed only in the presence of palmitoyl CoA (panel C). With palmitoyl CoA present, half-maximal velocity was obtained with 36 nmol [^{3}H]retinol per ml (panel D). In most subsequent studies, microsomes were incubated with [^{3}H]retinol dispersed in dimethylsulfoxide at 180 nmol/ml and, under these conditions, only a small fraction (less than 5%) of [^{3}H]retinol was esterified.

Incubations of microsomes with cofactors were also performed at various temperatures. Esterification was greater at 37°C than at either 30°C or 45°C, and all other incubations were conducted at 37°C.

The effects of incubation time and of amount of microsomal protein on ^{3}H-labeled retinyl ester synthesis were also studied. Product formation was proportional to incubation time for 5 min (**Fig. 2,** panel A). However,

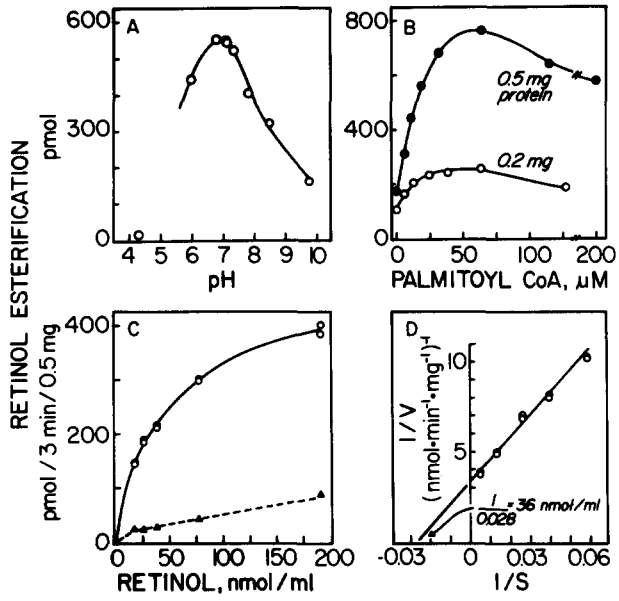

Fig. 1. Characteristics of retinyl ester synthesis by microsomes from the lactating rat mammary gland. Panel A: Determination of pH optimum. At each pH, 5-min incubations were carried out with 0.5 mg of protein and 45 nmol of [^{3}H]retinol in a volume of 0.25 ml containing 50 μM palmitoyl CoA, 5 mM DTT, and 20 μM BSA. Buffers included 0.15 M concentrations of potassium phosphate for pH 4.3, 6.0, 6.8, 7.2, and 7.4, and Tris–HCl for pH 7.8, and 8.5, and glycine–NaOH for pH 9.8. The pH's of mock incubation mixtures containing all incubation components except [^{3}H]retinol were measured at 37°C. Points are means of duplicate incubations. Panel B: Effect of increasing concentrations of palmitoyl CoA on retinyl ester yield. All 5-min incubations contained 45 nmol [^{3}H]retinol, 5 mM DTT, and 20 μM BSA in 0.25 ml of 0.15 M potassium phosphate buffer, pH 7.1. Points are means of duplicate incubations. Panel C: Effect of [^{3}H]retinol concentration. Microsomes, 0.5 mg protein, were incubated at 37°C for 3 min in the presence of 50 μM palmitoyl CoA, 5 mM DTT, and 20 μM BSA (○), or under basal conditions (with buffer only, ▲). Panel D: Double reciprocal plot of data in panel C with palmitoyl CoA showing half-maximal velocity with 36 nmol of [^{3}H]retinol per ml incubation medium.

esterification catalyzed by 0.2 mg of microsomal protein was only 21% of that catalyzed by 0.5 mg of protein instead of the expected value of 40%. Subsequently, the synthesis of ^{3}H-labeled retinyl ester by increasing amounts of microsomal protein was explored further using a standard incubation period of 5 min, [^{3}H]retinol at 180 nmol/ml, and incubations with either palmitoyl CoA or the cofactor mixture or buffer alone. As illustrated in panel B of Fig. 2, although esterification was linear with protein *between* 0.2 mg and 0.5 mg with either palmitoyl CoA or cofactors present, this portion of each curve would extrapolate to zero esterifying activity with approximately 0.1 mg of microsomal protein. Two possible causes for the observed low activities of small amounts of microsomal protein were considered: *1*) dilution of microsomes might in itself have reduced synthesis if interaction of microsomal vesicles is required for esterification, or *2*) esterification of retinol by small amounts of microsomal protein might have been disproportionately reduced as the *ratio* of retinol to membrane increased to an inhibitory level. A dilution experiment was conducted (**Table 3**) which showed that a 2.5-fold increase in incubation volume alone did not reduce ^{3}H-labeled retinyl ester synthesis when either 0.2 or 0.5 mg of microsomal protein was included, nor was esterification reduced when the amount of palmitoyl CoA was increased 2.5-fold, back to a concentration of 50 μM. However, when both the incubation volume and the amount of [^{3}H]retinol were increased 2.5-fold, thereby maintaining retinol at 180 nmol/ml but increasing the retinol:microsomal protein ratio 2.5-fold, esterification was reduced by 39%. This experiment demonstrates that the amount of retinyl ester synthesized under these in vitro conditions is a function of the *ratio* of retinol to microsomal membrane, and not strictly a function of the apparent concentration of retinol.

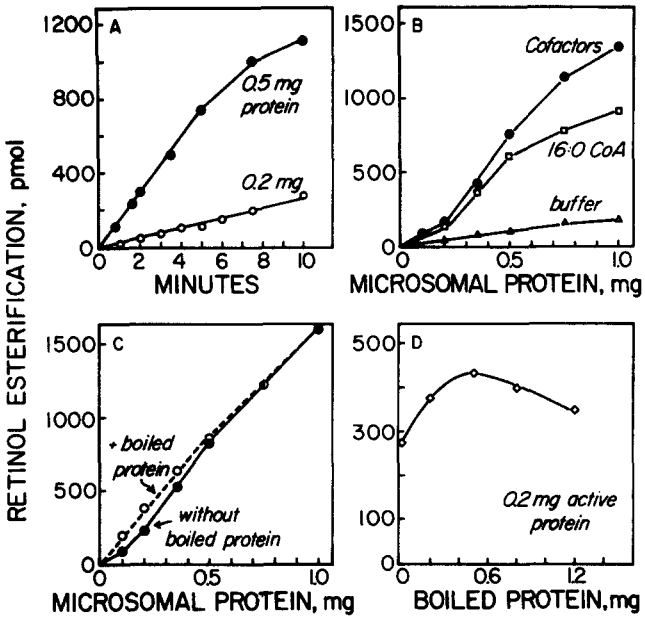

Fig. 2. Effects of incubation time and amount of mammary gland protein on ^{3}H-labeled retinyl ester synthesis. All points are means of duplicate determinations. Panel A: Esterification as a function of time of incubation. Microsomes were incubated at 37°C with 45 nmol of [^{3}H]retinol, palmitoyl CoA (50 μM), DTT (5 mM), and BSA (20 μM). Panel B: Different amounts of microsomal protein and 45 nmol of [^{3}H]retinol were incubated for 5 min at 37°C with buffer alone (△); with 50 μM palmitoyl CoA, 5 mM DTT, and 20 μM BSA (□); or with a mixture of cofactors (●; 10 mM ATP, 1.25 mM coenzyme A, 5 mM DTT, 20 μM BSA, and 4 mM $MgSO_4$). Panel C: Comparison of ^{3}H-labeled retinyl ester yield in the presence of cofactors when the amount of active microsomal protein indicated on the abscissa was incubated alone (●) or was mixed with boiled microsomal protein so that all incubations contained 1 mg total protein per 0.25 ml (○). Panel D: Effect of varying amount of boiled microsomal protein on ^{3}H-labeled retinyl ester synthesis catalyzed by 0.2 mg of active protein in the presence of 45 nmol of [^{3}H]retinol and cofactors.

It became possible to demonstrate proportionality between ^{3}H-labeled retinyl ester synthesis and the amount of active microsomal enzyme under standard incubation conditions when heat-inactivated microsomes were included in the incubation mixture (Fig. 2, panel C). Retinyl ester synthesis by 0.2 mg of active microsomal protein was maximal when approximately 0.5 mg of heat-inactivated protein was also included, resulting in a ratio of retinol to *total* protein of 45 nmol/0.7 mg (64 nmol/mg; Fig. 2, panel D).

Effects of potential enzyme inhibitors and detergents

Esterification of [^{3}H]retinol by mammary gland microsomes was measured in the presence of a sulfhydryl blocking reagent, an inhibitor of serine-dependent enzymes, and a chelator of divalent cations. Inhibition was observed only with p-chloromercuribenzoate (**Table 4**), indicating that reduced sulfhydryl groups are essential, either on the enzyme or its thioester substrate. Inhibition was essentially complete in the presence of 5 mM p-CMB.

The addition of either bile salt or non-ionic detergent to the incubation medium resulted in a marked inhibition of ^{3}H-labeled retinyl ester synthesis by mammary gland microsomes (**Fig. 3**). Esterification in the presence of 50 μM palmitoyl CoA, 20 μM BSA, and 5 mM DTT was reduced 43% by 0.05% (0.93 mM) sodium taurocholate, and 78% in the presence of only 0.02% Triton X-100.

Stimulation of retinyl ester synthesis by various fatty acyl CoA esters

It was of interest to ask whether fatty acyl CoA esters other than palmitoyl CoA also could increase the esterification of [^{3}H]retinol, and to determine whether they were able to do so below their critical micellar concentrations. Five coenzyme A esters with medium or long fatty acyl chains were incubated with mammary gland microsomes at concentrations of 5 μM and 50 μM; ad-

TABLE 3. Effect of retinol concentration versus retinol:microsomal protein ratio on the synthesis of ^{3}H-labeled retinyl esters

	Conditions[a]				Ratio, nmol [^{3}H]Retinol: mg Protein	pmol ^{3}H-Labeled Retinyl Ester Synthesized	% of Standard Incubation
	Volume,	Palmitoyl CoA,	[^{3}H]Retinol,	[^{3}H]Retinol,			
	ml	*nmol*	*nmol*	*nmol/ml*			
With 0.5 mg protein:							
A Standard incubation	0.25	12.5	45	180	90	753 (±0.0%)	100
B Increased volume	0.625	12.5	45	72	90	780 (±1.7%)	104
C Increased palmitoyl CoA	0.625	31.25	45	72	90	815 (±1.5%)	108
D Increased [^{3}H]retinol	0.625	31.25	112.5	180	225	457 (±1.8%)	61
With 0.2 mg protein:							
A Standard incubation	0.25	12.5	45	180	225	230 (±1.8%)	100
B Increased volume	0.625	12.5	45	72	225	252 (±11.6%)	110
C Increased palmitoyl CoA	0.625	31.25	45	72	225	268 (±0.4%)	117
D Increased [^{3}H]retinol	0.625	31.25	112.5	180	562.5	141 (±2.8%)	61

[a] All incubations were conducted in 0.15 M potassium buffer, pH 7.1, at 37°C for 5 min.
Incubations *A* included 20 μM BSA, 5 mM DTT, and 50 μM palmitoyl CoA, as noted. Incubations *B* contained amounts of additions equal to incubation *A* in a total volume of 0.625 ml. Incubations *C* and *D* contained 20 μM BSA, 5 mM DTT, and 50 μM palmitoyl CoA, with either 45 nmol or 112.5 nmol of [^{3}H]retinol. The concentration of dimethylsulfoxide was maintained at 2% of volume in all incubations.

ditionally, palmitoyl and oleoyl CoA were added at 10μM and at 1 μM which is below the reported critical micellar concentration of 4 to 5 μM for these coenzyme A thioesters in low ionic strength buffers at netural pH (30, 31). **Table 5** shows that 50 μM concentrations of medium or long chain fatty acyl CoA esters increased retinol esterification by 2.2 to 4.0-fold. When added at only 1 μM, palmitoyl CoA and oleoyl CoA each produced statistically significant increases in retinyl ester synthesis, suggesting that monomeric fatty acyl CoA esters were effective substrates in this esterification reaction.

Pattern of retinyl esters in rat milk

Although it has been known that vitamin A in milk is largely esterified, the composition of its retinyl esters has not been reported. Here, the distribution of rat milk retinyl esters has been determined by HPLC (24). **Fig. 4** shows a tracing from a rat milk extract (panel A) compared to a mixture of various retinyl ester standards (panel B). In milk, eight different esters of retinol could be detected, ranging in fatty acyl chain length from eight to eighteen carbons. The predominant retinyl ester was retinyl palmitate (34% of retinyl ester mass), followed by retinyl stearate (18%), retinyl oleate (10.3%), retinyl linoleate (10.0%), retinyl decanoate (9.7%), retinyl myristate (7.1%), retinyl laurate (6.9%), and retinyl octanoate (4.0%). Synthesis and esterification of medium chain fatty acids is a property of the mammary gland during lactation (32, 33), and it is of some interest that medium chain fatty acid esters of retinol are clearly detectable in milk. These esters have not been found during comparable analyses of rat lymph chylomicrons or postprandial human plasma (24).

Comparison of effects of cytosol and cofactors on retinyl ester synthesis by microsomes

It has been reported that retinyl ester synthesis by microsomes from liver (15) and retina (18) increased

TABLE 4. Effects of some enzyme inhibitors on retinol esterification by mammary gland microsomes from the lactating rat

p-Chloromercuribenzoate (p-CMB)			Phenylmethylsulfonylfluoride (PMSF)			Ethylenediaminetetraacetate (EDTA)		
	Retinol Esterified, pmol	Percent of Control		Retinol Esterified, pmol	Percent of Control		Retinol Esterified, pmol	Percent of Control
Control[a,b]	476	100	Control[a,c]	488	100	Control[a,d]	534	100
p-CMB, 2 mM	65	13.7	PMSF, 1 μM	462	94.7	EDTA, 2 mM	529	99.1
p-CMB, 5 mM	6	1.3	PMSF, 10 μM	459	94.0			
			PMSF, 100 μM	482	98.8			

[a] All incubations included 0.5 mg of microsomal protein in 0.25 ml of 0.15 M potassium phosphate buffer, pH 7.1, containing palmitoyl CoA (50 μM), BSA (20 μM), and [^{3}H]retinol (180 nmol/ml); incubations to test PMSF and EDTA also included DTT (5 mM). Reaction times were 5 min; values reported are the means of duplicate incubations.
[b] Microsomes were incubated with buffer, with or without 2 mM or 5 mM p-CMB, for 10 min at 37°C prior to addition of palmitoyl CoA, BSA, and [^{3}H]retinol. During the 5-min incubation with substrate, p-CMB concentrations were 1 and 2.5 mM.
[c] Microsomes, 5 mg protein/ml, were incubated for 10 min at 37°C with various concentrations of PMSF added in 5 μl dimethylsulfoxide before addition of substrate. Control incubations included dimethylsulfoxide.
[d] EDTA was added to microsomes immediately before the 5-min incubation period with [^{3}H]retinol.

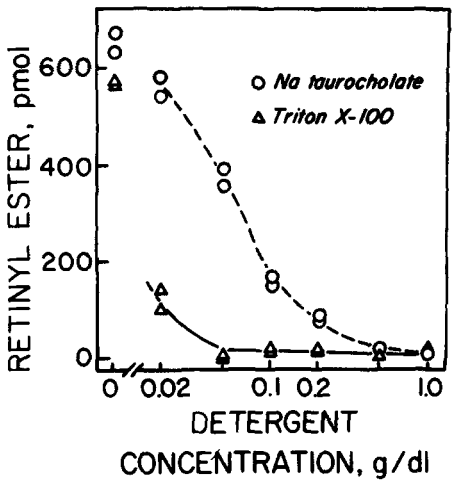

Fig. 3. Inhibition of retinyl ester synthesis by bile salt and non-ionic detergents. All mixtures included 0.5 mg of microsomal protein, 45 nmol of [^{3}H]retinol, 50 μM palmitoyl CoA, 5 mM DTT, and 20 μM BSA and either sodium taurocholate cr Triton X-100 in 0.15 M potassium phosphate buffer, pH 7.1. Detergents were added to microsomes 10 sec before the addition of retinol for the 5-min incubation.

when cytosol fractions were included during incubation with retinol. Therefore, an experiment was designed in which mammary gland microsomes were incubated with buffer alone, with cofactors (ATP, coenzyme A, $MgSO_4$, BSA, and DTT), with mammary gland or rat liver cytosol, or with both cytosol and cofactors. Whereas inclusion of the cofactors increased [^{3}H]retinol esterification 6.78-fold over that observed with buffer only, inclusion of either rat mammary gland or rat liver cytosol (0.5 mg protein) had no large or consistent effect on retinyl ester synthesis (**Table 6**). However, the cofactors remained nearly equally effective when added to microsomes in the presence of the cytosol fractions. Thus, under our incubation conditions, no evidence was found to suggest that cytosol has a stimulatory effect of retinyl ester synthesis by microsomes, nor did it decrease esterification when added in the presence of a fatty acyl CoA-generating system.

Test for other reactions during incubation with retinol

While we were conducting these experiments, Bhat et al. (23) reported that [^{14}C]retinol is dehydrated to [^{14}C]anhydroretinol during incubation with microsomes from mouse fibroblasts. Anhydroretinol, a hydrocarbon, elutes from aluminum oxide with pure hexane, but could have contaminated the retinyl ester fraction in some of our studies. To test for anhydroretinol synthesis by rat mammary gland microsomes, incubations were conducted with [^{3}H]retinol on a larger scale (5 mg of protein in 1.5-ml incubation volume for 10 min) with buffer only or with cofactors. Aluminum oxide columns were developed either with the solvent system described by Bhat and coworkers (23) or in the usual manner. Absorption spectra were recorded for column fractions eluting with pure hexane and possibly containing anhydroretinol, and for more polar fractions thought to contain only retinyl esters or retinol; portions were also taken to measure the tritium content of each fraction. We found essentially no absorbance, and no tritium, in the hexane eluates, indicating that [^{3}H]retinol had not been dehydrated to

TABLE 5. Synthesis of retinyl esters in the presence of medium or long chain fatty acid esters of coenzyme A

	1 μM		5 μM		10 μM		50 μM	
Fatty acyl CoA ester	pmol[a]	% of Control[b]	pmol[a]	% of Control[b]	pmol[a]	% of Control[b]	pmol[a]	% of Control[b]
Octanoyl CoA							416 (±2.5%)	226
Lauroyl CoA			183 (±9.5%)	99			478 (±2.9%)	259
Palmitoyl CoA	201 (±0.4%)	119	353 (±1.7%)	191	460 (±1.2%)	274	663 (±3.4%)	360
Stearoyl CoA			330 (±0.7%)	179			681 (±1.0%)	369
Oleoyl CoA	203 (±1.1%)	121	528 (±0.1%)	286	488 (±1.2%)	290	744 (±2.5%)	403

[a] Values are the means of duplicate incubations; ± the deviation from the mean expressed as a percentage.

[b] All incubations included 0.5 mg of microsomal protein in 0.25 ml of potassium phosphate buffer, pH 7.1, containing 5 mM DTT, 20 μM BSA, 45 nmol [^{3}H]retinol, and fatty acyl CoA esters, as indicated; control incubations contained no added fatty acyl CoA. When 5 μM and 50 μM concentrations of fatty acyl CoA's were tested, the control value equaled 184 ± 5 pmol of retinyl ester per 5 min (mean ± SD, n = 3); incubations without BSA or DTT produced 133 pmol of retinyl ester per 5 min in this experiment. In a separate experiment to test 1 μM and 10 μM concentrations of palmitoyl CoA and oleoyl CoA, the control value, with BSA and DTT, was 168 ± 4 pmol per 5 min (n = 3).

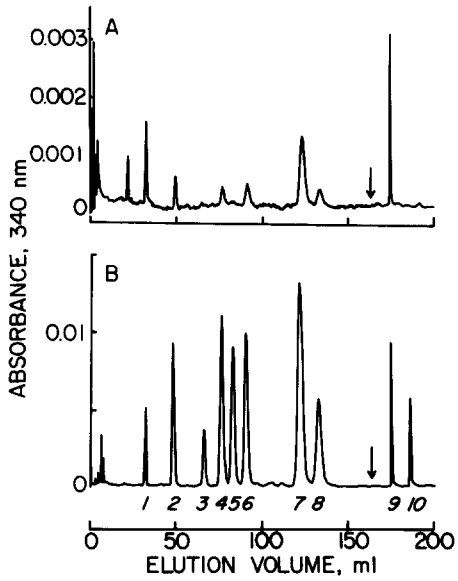

Fig. 4. Composition of rat milk retinyl ester fraction. Panel A: Milk retinyl esters separated by HPLC on an octyldimethylsilyl bonded phase column with acetonitrile–water 88:12 (v/v) as mobile phase for the first 170 ml and acetonitrile–water 98:2 for rest of chromatography (change noted by ↓). Flow rate: 3 ml/min; sensitivity: 0.005 absorbance units full scale. Panel B: Synthetic retinyl ester standards chromatographed under identical conditions immediately after the sample in panel A. Sensitivity: 0.02 absorbance units full scale. Retinyl ester standards include: *1*) retinyl decanoate, *2*) retinyl laurate, *3*) retinyl γ-linolenate, *4*) retinyl myristate, *5*) retinyl palmitoleate, *6*) retinyl linoleate, *7*) retinyl palmitate, *8*) retinyl oleate, *9*) retinyl stearate, and *10*) retinyl arachidonate. Retinyl octanoate, which immediately precedes retinyl decanoate in panel A, was identified by extrapolating a plot of relative elution volume (relative to retinyl palmitate) versus fatty acyl chain length for saturated fatty acid esters of retinol (24).

[^{3}H]anhydroretinol. On the other hand, those eluates that were expected to contain only fatty acid esters of retinol had the correct ultraviolet absorption spectra showing a single absorption maximum at 325–326 nm. Thus, by chromatographic and spectral criteria, esterified retinol appears to be the only product that we have measured by radioassay after incubation of microsomes with [^{3}H]retinol.

An experiment was also performed to determine whether [^{3}H]retinyl palmitate can be hydrolyzed under the incubation conditions we developed to measure retinyl ester synthesis. Mammary gland microsomes (0.5 mg of protein) were incubated with 50 μM palmitoyl CoA, 5 mM DTT, 20 μM BSA, and dispersions of [^{3}H]retinyl palmitate at either 1.8 or 18 nmol/ml plus unlabeled retinol so that the total vitamin A concentration equaled 180 nmol/ml. After incubation for 5 or 20 min, microsomal extracts were chromatographed to separate and collect [^{3}H]retinyl palmitate and any [^{3}H]retinol. Under these conditions, no enzymatic hydrolysis of retinyl palmitate was observed. It is still possible, of course, that retinyl esters newly synthesized by microsomes are in a different environment and, therefore, are available for subsequent hydrolysis by microsomal enzymes.

DISCUSSION

Microsomal vesicles prepared from mammary glands of the lactating rat were able to synthesize retinyl esters when incubated with retinol alone, but esterification was consistently increased 6- to 7-fold in the presence of cofactors constituting a fatty acyl CoA-generating system, and 3- to 6-fold by preformed palmitoyl CoA. Thus, the retinyl ester synthase activity in these membranes appears to qualify as a fatty acyl CoA:retinol acyltransferase. When microsomes were treated before assay with

TABLE 6. Comparison of effects of cytosol and cofactors on retinyl ester synthesis by mammary gland microsomes

	Retinol Esterification, pmol (± Percent Deviation)	Percent of Control
Control, microsomes with buffer[a]	137 (±21%)[b]	100
Additions:		
Cofactors[c]	926	678
Mammary gland cytosol, 0.5 mg protein	182 (±0.3%)	
(minus mammary gland cytosol, alone[d]) (−)	68	
Effect of mammary gland cytosol:	114	86
Cofactors + mammary gland cytosol	843 (±3.6%)	617
Rat liver cytosol, 0.5 mg protein	179 (±1.0%)	
(minus liver cytosol, alone[d]) (−)	8	
Effect of liver cytosol:	171	125
Cofactors + rat liver cytosol	979 (±0.8%)	717

[a] Microsomal protein, 0.5 mg, and 45 nmol of [^{3}H]retinol were incubated together for 5 min at 37°C.
[b] ± Percent deviation from the mean of duplicate incubations.
[c] 'Cofactors' denotes the presence of ATP (10 mM), reduced coenzyme A (1.25 mM), DTT (5 mM), $MgSO_4$ (4 mM), and BSA (20 μM).
[d] Values for incubations containing cytosol but no microsomal protein.

neutral hydroxylamine, a reagent that "traps" activated fatty acids by forming hydroxamates (19), [^{3}H]retinol esterification was nearly totally dependent on exogenous palmitoyl CoA (Table 2). Thus, the basal reaction we observed rountinely in the absence of added fatty acyl CoA is most likely due to endogenous fatty acyl CoA thioesters bound to (31) or within the microsomal vesicles. Washing with buffer alone did not reduce this basal reaction. At present, the concentrations of endogenous activated fatty acids in the lactating mammary gland and their affinities for these membranes are not known.

The properties of retinyl ester synthesis in the presence of exogenous fatty acyl CoA were studied most extensively using palmitoyl CoA as fatty acyl donor because retinyl palmitate is the predominant ester in liver (15), intestinal chylomicrons (5, 6), retina (34), and, as our own analysis later showed, milk. In addition to palmitoyl CoA, four other medium or long chain fatty acyl CoA esters that we tested also increased ^{3}H-labeled retinyl ester synthesis; the greater effects were observed with the longer chain esters of coenzyme A. It appears, then, that the enzyme or enzymes responsible for retinyl ester synthesis display a rather broad specificity towards activated fatty acids in vitro. When we determined the pattern of retinyl esters in rat milk, we found that it differed from that of other tissues by including several medium chain (C_8–C_{12}) esters of retinol that composed approximately 20% of the total vitamin A esters in milk. It seems likely that the composition of milk retinyl esters is determined in part by the relative rates of activation of various fatty acids by the lactating mammary gland and, hence, by the availability of these CoA esters to the membrane-bound esterifying enzyme. It has been estimated that a large portion, approximately 80%, of total fatty acids in rat milk is derived from blood, whereas 20% is synthesized within the mammary gland (35). An interesting possibility is that retinyl ester distribution in rat milk can be modified somewhat by varying the fatty acid composition of the diet and, thereby, the types of fatty acids delivered to this organ.

These experiments in vitro have revealed certain similarities between retinyl ester synthesis catalyzed by mammary gland microsomes and the acylation of other lipids such as cholesterol by microsomal membranes from a variety of organs. The activity of acyl CoA:cholesterol acyltransferase (ACAT) has been studied over a number of years either in incubations where labeled cholesterol was added in solvent to membranes or, more recently, by employing radiolabeled faty acyl CoA as one substrate and endogenous microsomal cholesterol as the other (29). In this second assay condition, ACAT and one of its substrates, cholesterol, are covariates and thus the ratio of cholesterol to enzyme remains constant when different amounts of microsomal membrane are assayed. Whether the measured activity of ACAT is limited by the enzyme's catalytic activity or the pool size of endogenous cholesterol has been investigated and discussed (29, 36–39); it seems that both factors are important. In the present experiments on retinol esterification, it was our plan to add [^{3}H]retinol dispersed in dimethylsulfoxide to microsomes in an attempt to saturate the enzyme with substrate and thus to produce conditions where initial enzyme velocities might be measured in vitro. However, because our experiments revealed complex relationships between the amounts of retinol and microsomal membrane included in the incubation mixture, we have not determined kinetic constants for the reaction. Clearly, the yield of ^{3}H-labeled retinyl ester was increased when increasing amounts of [^{3}H]retinol were added to a constant amount of microsomal membrane (Fig. 1C); nevertheless, other experiments pointed to the importance of the *ratio* of retinol to membrane rather than apparent concentrations as a determinant of the yield of [^{3}H]retinyl ester (Fig. 2D and Table 3). Retinol has previously been shown to be membrane-seeking and membranolytic (40, 41), thus its adsorption to or penetration into the microsomal membrane is not surprising. Our data indicate, however, that at a sufficiently high ratio of retinol to microsomes, this interaction can inhibit the synthesis of ^{3}H-labeled retinyl ester. In a similar way, palmitoyl CoA also had two effects: acting as substrate at all concentrations tested and, at sufficiently high level, acting as both substrate and inhibitor. The detergent properties of fatty acyl CoA have been studied and discussed previously by Cleland and coworkers (28, 30, 31) who observed substrate inhibition of 1-palmitoylglycerol-3-phosphate synthesis by palmitoyl CoA in micellar form. The extent of inhibition in their studies was also a function of the ratio of palmitoyl CoA to microsomal membrane (28). Retinyl ester synthesis by mammary gland microsomes was also inhibited by bile salt and non-ionic detergents (Fig. 3). Taken together, these results point toward the hypothesis that retinol esterification is catalyzed by a membrane-bound enzyme consisting of one or more integral proteins whose activity is highly sensitive to changes in physical environment.

The present experiments were begun to ask whether any enzymatic activity is present in mammary gland tissue that could esterify retinol prior to its secretion in milk. Retinol for this reaction could be obtained by the mammary gland either by uptake of unesterified retinol from plasma (11) or by uptake and hydrolysis of retinyl esters contained in the chylomicron or the chylomicron remnant which is generated at the mammary gland endothelial surface during lactation (35). Data from our present study make it unlikely that retinyl esters from

plasma are incorporated into the milk fat droplet directly, i.e., without hydrolysis and re-esterification in the lactating cell. The composition of milk retinyl esters differed considerably from the distribution of retinyl ester mass in intestinal chylomicrons which we have found to consist of 58% retinyl palmitate, 21% retinyl stearate, 12% retinyl oleate, and 7% retinyl linoleate when rats were fed olive oil (24). Although shorter chain fatty acid esters of retinol were not detected in rat lymph chylomicrons, they were clearly present in rat milk. Important questions still remain concerning the physiological delivery of vitamin A to its target organs including the lactating mammary gland, and the mechanisms of its transport across the mammary gland epithelium.

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