CoA- and Non-CoA-dependent Retinol Esterification in Retinal Pigment Epithelium*

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Washed, buffered microsomes from bovine retinal pigment epithelium catalyze retinyl ester synthesis from retinol in the absence of an exogenous acyl donor. A plot of retinyl ester synthesis versus time reaches a plateau at 123 ± 26 nmol of retinyl ester mg⁻¹ microsomal protein, providing a minimum value of the concentration of the endogenous acyl donor. Fatty acyl-CoA analysis by three different methods employing high performance liquid chromatography resulted in the detection of less than 1 nmol mg⁻¹ protein of acyl-CoA, indicating that fatty acyl-CoA is not the endogenous acyl donor.

Stimulation of the rate of retinyl ester synthesis by palmitoyl-CoA or ATP, CoA, and palmitate is observed following its addition at the beginning of the reaction or after the endogenous acyl source has been exhausted by 20 min of reaction with retinol. Palmitate from [¹⁴C]palmitoyl-CoA is incorporated into retinyl ester at a rate similar to that for the incorporation of [¹⁴C]retinol, demonstrating the presence of an apparent acyl-CoA:retinol acyl transferase activity. The acyl group from palmitoyl-CoA can be transferred initially to a component of the microsomes and subsequently to retinol.

The product of retinyl ester synthesis from all-trans-retinol and palmitoyl-CoA is all-trans-retinyl palmitate, indicating that the stereochirnical configuration is retained during esterification. The kinetic parameters for the esterification of 11-cis-retinol and all-trans-retinol are similar.

Retinyl ester synthesis in retinal pigment epithelium (RPE) is one of several reactions that make up the visual cycle in vertebrate retina (Wald, 1968). An unusual feature of the reaction in RPE, noted in several studies (Andrews and Futterman, 1964; Berman et al., 1980; Krinsky, 1958), is the presence of an endogenous fatty acyl donor in microsomal preparations allowing retinyl ester synthesis upon addition of retinol alone. Endogenous fatty acyl-CoAs have been suggested to be responsible for the activity (Berman et al. 1980; Krinsky, 1958), although their presence in RPE microsomes has not been documented. In addition, there are apparently conflicting reports regarding stimulation of retinyl ester synthesis by exogenous palmitoyl-CoA. Krinsky (1958) reported stimulation of the rate of retinol esterification by addition of ATP, palmitate, and CoA, substrates for palmitoyl-CoA generation. However, Berman et al. (1980) with RPE microsomes and Andrews and Futterman (1964) with retinal microsomes did not observe this stimulation. The latter authors also reported that palmitoyl-CoA failed to stimulate retinyl ester synthesis. This communication describes studies that examine the question of the endogenous fatty acyl donor in more detail. The results demonstrate that endogenous fatty acyl-CoAs cannot be detected in bovine RPE microsomes in amounts sufficient to account for retinyl ester synthesis from retinol alone. Exogenous palmitoyl-CoA stimulates retinyl ester synthesis in calf and adult RPE microsomes in a reaction apparently involving intermediate acylation of a microsomal component. The identity of the endogenous fatty acyl donor is unknown but it is likely to be a lipid. A preliminary account of portions of this work has appeared previously (Saari and Bredberg, 1987).

EXPERIMENTAL PROCEDURES

Materials

The sources and purifications of retinoids have been given in a previous communication (Saari et al., 1985). Fatty acyl-CoAs, CoA, and fatty acyl chlorides were purchased from Sigma; radioactive retinol, palmitate, and palmitoyl-CoA were purchased from Du Pont-New England Nuclear. These substrates were purified by HPLC when necessary. Vydac C₁₈ and Ultrasphere ODS columns were purchased from The Separations Group and Rainin, respectively. Activated alumina was obtained from EM Science. All-trans-, 9-cis-, 11-cis-, and 13-cis-retinyl palmitate were synthesized from the corresponding retinols and palmitoyl chloride following the procedure described by Huang and Goodman (1965). All other chemicals and reagents were the highest quality obtainable. Adult and calf bovine eyes were obtained from local meat processing firms.

Preparation of Microsomes

The procedure applies to calf and adult bovine eyes. Bovine eyes were bisected and the anterior portion and vitreous discarded. After removal of the retina, 1 ml of cold 0.25 M sucrose, 25 mM Tris acetate, pH 7, 1 mM DTT was added to the eye cup and the RPE cells dislodged by gentle stroking with a small brush (Krinsky, 1958). The suspension of cells was removed with a Pasteur pipette. After repeating the procedure, the cells were homogenized with 8–10 passes of a motor-driven Teflon-glass homogenizer and centrifuged at 27,000 × g, 5 °C, for 20 min. After decanting, the supernatant was centrifuged at 150,000 × g for 1 h. The microsomal pellet from this spin was resuspended in 10 mM Tris acetate, pH 7, 1 mM DTT (25 μl/calf eye, 200 μl/adult eye) by sonication and stored at –80 °C in 50-μl portions. After thawing, microsomes were kept at 0 °C and used during the day or discarded.
[**][H]All-trans-retinol (in ethanol, 2 mM) was added to 40 mM Tris acetate, pH 8.0, 60 µM BSA (fatty acid-depleted), 1 mM EDTA, at 37 °C, to give a final retinol concentration of 10 µM.  After cooling, portions of the sample were analyzed for CoA using a reversed phase Vydac CI column.  The column was equilibrated with 4% acetonitrile, 85% water, and 1 mM perchloric acid was added to bring the pH to 2.0.  Palmitoyl-CoA (2 mM, in 10 mM phosphate buffer, pH 6.0) was added in some studies to give a final concentration of 20 µM.  The reaction was initiated by the addition of microsomes to give an assay concentration of ~50 µg of microsomal protein ml⁻¹.  Retinol and BSA were incubated at 37 °C for less than 2 min before addition of microsomes.  The concentration of ethanol in the reaction was 0.5% or less.  For routine assays, tubes were flushed with argon and maintained in subdued light at 37 °C.  The assays described in this communication were carried out at 37 °C except for those described in Fig. 1.  The reaction was stopped by the addition of 2 volumes of ice-cold ethanol.  Retinoids were extracted with 5 volumes of petroleum ether.  Following extraction of the petroleum ether with 2 ml of water to remove residual alanin, retinoids were separated on alumina as described by Putterman and Andrews (1964).  Briefly, the washed petroleum ether extract was applied to 0.4 g of 6% water-deactivitated alumina in a Pasteur pipette plugged with glass wool.  One ml of petroleum ether was passed through the column followed by 2 ml of 3% diethyl ether in petroleum ether to elute retinyl esters and 2 ml of 60% diethyl ether in petroleum ether to elute retinoids.  Retinoid fractions were collected in scintillation vials, petroleum ether evaporated with argon, and radioactivity determined by liquid scintillation counting.  Recovery of radioactivity was ~95%.  For experiments in which endogenous acyl donor levels were compared to endogenous fatty acyl-CoA levels (Table 1), separate portions of frozen microsomes were used for each analysis.  When microsomes had been prelabeled with [³²P]palmitoyl-CoA, transfer of [³²P]palmitate to retinol was demonstrated after fatty acyl-CoA analysis had been completed.

**Fatty Acyl-CoA Analysis**

*Precipitation with Perchloric Acid—* The procedure of Tubbs and Garland (1964, 1969) was followed.  An equal volume of ice-cold, 10% perchloric acid was added to a thawed suspension of microsomes (containing ~55nmol of endogenous acyl donor) in a Microfuge tube followed by centrifugation in a Beckman Microfuge for 5 min at 11,640 × g.  After removal of the supernatant, the pellet was washed with 2.5% perchloric acid and dissolved in 300 µl of 0.2 M Tris acetate, pH 7.  Following an additional centrifugation, portions of the supernatant were analyzed for fatty acyl-CoAs by reversed phase HPLC (see below). Palmitoyl-CoA was added to some samples to determine the efficiency of the procedure.

*Chloroform Methanol Extraction—* Five µl of 4% SDS was added to 75 µl of buffered microsomes followed by 20 µl of palmitoyl-CoA (external standard) or buffer to give a final SDS concentration of 0.2%.  The amount of exogenous palmitoyl-CoA added was approximately one-half the calculated concentration of endogenous acyl donor in the microsomal sample.  Following addition of 400 µl of CH₃OH/CH₂Cl₂ 2:1 v/v (cold) to the tube, the tube was vortexed and centrifuged in an Eppendorf Mini-fuge for 5 min (at 8000 × g).  The resulting biphasic mixture consisted of 200 µl of upper phase and 300 µl of lower phase.  One-half of each phase was analyzed for fatty acyl-CoAs by reversed phase HPLC (see below).  The upper phase was injected directly, the lower phase after drying with argon and dissolving in running buffer.  The distribution of authentic palmitoyl-CoA using this procedure was 95% upper phase, 7% lower phase.  HPLC—Fatty acyl-CoAs were separated with a Vydac C₈ column employing isotropic elution with 65% acetonitrile, 15 mM ammonium acetate, pH 5, at a flow rate of 1 ml min⁻¹ with monitoring at 260 nm.  An example of the resolution of this system is shown in Fig. 3.  The minimum detectable amount of palmitoyl-CoA was ~50 pmol.

Hydrolysis to CoA—Fatty acyl-CoAs were hydrolyzed to CoA (Corkey et al., 1981), which was analyzed by reversed phase HPLC.  After addition of 290 µl of 10 mM KOH, pH 11.5, to 10 µl of buffered microsomes, the reaction mixture was heated at 55 °C for 10 min.  After cooling, 20 µl of 0.7% perchloric acid was added to bring the pH to 5.  Following centrifugation in a Beckman Microfuge for 10 min, portions of the sample were analyzed for CoA using a reversed phase Vydac C₈ column.  The column was equilibrated with 4% acetonitrile, 0.2 mM ammonium acetate, pH 5 (buffer A) at a flow rate of 2 ml min⁻¹.  Buffer B was the same solvent with 30% acetonitrile.  After 5 min of pumping buffer A, a 6-min gradient was initiated from 0 to 100% buffer B followed by 6 min of buffer B.

**Assay for Retinyl Ester Synthesis**

The procedure of Purr et al. (1986) was used with a Vydac C₈ column to separate all-trans-retinyl palmitate, oleate, and stearate plus palmitoleate.

**Analysis of Retinyl Esters**

Microsomes (300 µl) were diluted to give a 2-ml suspension in 40 mM Tris acetate, pH 7.0, 60 µM BSA, 1 mM EDTA (final microsomal protein concentration, ~0.9 mg ml⁻¹).  After addition of [³⁵S]palmitoyl-CoA (final concentration 18-40 µM), the mixture was incubated at 37 °C for 10 min, followed by 1 ml ice, diluted to 5 ml with the above buffer, and centrifuged at 150,000 × g for 1 h.  After removal of the supernatant an additional 5 ml of buffer was added, and the microsomes were sonicated and centrifuged at 150,000 × g for 1 h.  Finally, the pellet was resuspended in 300 µl of 10 mM Tris acetate, pH 7.1, 1 mM EDTA by sonication and used immediately or stored frozen until analysis.

**RESULTS**

**Esterification of Retinol in the Absence of Exogenous Acyl Donor**—Other investigators have reported that RPE microsomes are able to synthesize retinyl ester in the absence of an exogenous acyl donor (Andrews and Putterman, 1964; Berman et al., 1980; Krinsky, 1958).  An example of this phenomenon is depicted in Fig. 1 in which the synthesis of retinyl ester from retinol is shown as a function of time.  Of the only external substrate added to the microsomes was all-trans-retinol, the microsomes must provide a pool of endogenous acyl groups as well as enzyme responsible for the synthesis.  The progress curve reaches a maximum of ~1.23 ± 0.26 nmol of retinyl ester mg⁻¹ microsomal protein (n = 7) after approximately 20 min, providing an approximate value for the concentration of the endogenous acyl donor.  In the example shown, ~13% of the added retinol was esterified.  Further additions of retinol at the plateau do not produce additional esterification (see Fig. 4).  Incubation of the microsomes with NH₄OH, as described by Ross (1982a) produces a decrease in the synthesis of retinyl ester; however, addition of palmitoyl-CoA failed to restore the activity, suggesting that the enzyme was inactivated by the treatment.  A similar finding was noted by Ong et al. (1987).

The concentration of the endogenous acyl donor was routinely determined using an assay in which "retinyl ester" is taken to be proportional to the radioactivity that elutes from an alumina column with 3% diethyl ether in petroleum ether (see description under "Experimental Procedures").  The

**FIG. 1.** Time course of the synthesis of retinyl ester from retinol by RPE microsomes at room temperature (21 °C).  The reaction was initiated by the addition of 20 µM all-trans-retinol.  The fatty acyl group was provided by a microsomal component.  Additions of retinol at 60 min did not result in further synthesis of retinyl ester.  The amount of RE synthesis at the plateau is a minimum estimate of the amount of endogenous acyl donor present in the microsomes (125 nmol mg⁻¹ protein in this experiment).
amount of retinyl ester synthesized, determined by this assay, was compared with that determined by direct HPLC analysis and UV absorbance. The agreement of the two determinations is within 18%, indicating the validity of the routine assay procedure. In addition, HPLC analysis of the 3% diethyl ether fraction revealed only retinyl esters.

The fatty acid composition of the retinyl esters resulting from esterification by the endogenous acyl pool was evaluated using a reversed phase HPLC system (Furr et al., 1986). Retinyl palmitate, oleate, and palmitoleate plus stearate are the main products (approximate molar ratios 64:19:17), in reasonable agreement with published data (Futterman and Andrews, 1964). The sensitivity of the method is not sufficient to rule out trace amounts of other retinyl esters.

**Kinetic Parameters of the Reaction**—The \( V_{\text{max}} \) with all-trans-retinol (103 nmol min\(^{-1}\) mg\(^{-1}\)) is ~3000-fold higher than reported for liver microsomes in the absence of exogenous CoA (Ross, 1982). Berman et al. (1980) reported a \( V_{\text{max}} \) of 8.3 nmol min\(^{-1}\) mg\(^{-1}\) with RPE microsomes at 30°C, lower than that observed here. However, their conditions for microsome isolation and assay do not include DTT, a reagent we find to preserve enzymatic activity. The total activity of one calf eye equivalent of RPE (12.5 nmol min\(^{-1}\)) is approximately equal to the activity present in one rat liver indicating the relative abundance of this activity in RPE.

The \( K_m \) values for synthesis of retinyl esters from all-trans- and 11-cis-retinol are similar (1.9 and 2.5 \( \mu \)M, respectively) as are the \( V_{\text{max}} \) values for the two retinoids (103 and 80 nmol min\(^{-1}\) mg\(^{-1}\)), respectively. This is in contrast to a retinyl ester hydrolase of human RPE, which is reported to hydrolyze 11-cis-retinyl ester 20 times faster than all-trans-retinyl ester (Blaner et al., 1987).

To determine if the stereochemistry of the retinol is retained during esterification, all-trans-retinol was incubated with microsomes and the retinyl ester fraction from alumina analyzed by a normal phase HPLC system that resolves 9-, 13-, 11-cis-, and all-trans-retinyl esters (Fig. 2). The product of the reaction was primarily all-trans-retinyl palmitate. The shoulder evident in Fig. 2, trace \( B \), was not identified but is likely to be all-trans-retinyl oleate or stearate. Residual unesterified retinol from this experiment was also analyzed using a normal phase HPLC system that resolves geometrical isomers (Saari et al., 1982). Only small amounts (~5%) of 11- and 13-cis-retinol were found, suggesting that retinol isomerase activity (Bernstein et al., 1987; Bridges and Alvarez, 1987) is not active in our preparations under these conditions.

**Acyl-CoA Analysis of RPE Microsomes**—The estimated endogenous acyl donor content of the RPE microsomes indicated that fatty acyl-CoAs should be present at a concentration of ~800 \( \mu \)M in the microsomal suspension, a relatively high concentration, if they were to account for the endogenous acyl donor activity. Three methods of analysis were employed in an attempt to correlate endogenous fatty acyl-CoA and endogenous acyl donor levels. In each case, solutions of authentic palmitoyl-CoA of known concentration were added to microsome preparations to assess the efficiency of the method. Method 1 utilized perchloric acid to precipitate long chain acyl-CoAs (Tubbs and Garland, 1964, 1969), which were subsequently dissolved and analyzed by reversed phase HPLC. In method 2, microsomes were dissolved initially in 0.2% SDS and then extracted with CHCl\(_3\)-MeOH (Folch et al., 1957). Both upper and lower phases were analyzed for fatty acyl-CoAs by HPLC. An example of the HPLC separation of fatty acyl-CoAs and the results obtained during the analysis of one preparation of microsomes are shown in Fig. 3. Method 3 involved alkaline hydrolysis of either microsomes or the precipitate obtained after addition of perchloric acid and subsequent analysis of CoA by reversed phase HPLC (profile not shown). The results of these analyses, presented in Table I, demonstrate that exogenous palmitoyl-CoA can be recovered in good yield (~80%) with methods 2 and 3, whereas lower recoveries of exogenous standard were obtained with method 1 (~60%). However, virtually no endogenous fatty acyl-CoA
CoA and Non-CoA-dependent Retinol Esterification

TABLE I

| Method                        | % Recovery*                      |
|-------------------------------|----------------------------------|
| Perchloric acid precipitation | 57 ± 12  (n = 4)                 |
| CHCl₃:MeOH extraction         | 81 ± 9  (n = 7)                  |
| Base hydrolysis               | 83 ± 13 (n = 6)                  |

*The results are presented as percent recovery of exogenous palmitoyl-CoA and calculated endogenous acyl donor concentration.

Indirect Acyl Transfer from Palmitoyl-CoA to Retinol—To assess whether palmitoyl-CoA was acting as an alternate substrate for the esterification reaction or was providing palmitate to an endogenous active acyl pool, [¹⁴C]palmitoyl-CoA (40 μM) was added to microsomes in the absence of retinol and incubated for 20 min. The microsomes were then pelleted, washed with BSA, and analyzed. Approximately 47% of the added [¹⁴C] remained associated with the microsomes. After dissolving the microsomes in 0.2% SDS and extraction with CHCl₃:MeOH (fatty acyl-CoA analysis 2, “Experimental Procedures”), ~1% of the [¹⁴C] was detected in the upper phase.

Fig. 4. Stimulation of the rate of retinyl ester synthesis by exogenous palmitoyl-CoA added after depletion of the endogenous acyl donor. Microsomes were incubated with retinol at 37 °C for 30 min, at which time the reaction had essentially stopped. A second addition of retinol at this time produced no stimulation of retinyl ester synthesis (lower curve). Addition of palmitoyl-CoA and retinol produced a stimulation of the rate of retinyl ester synthesis (ascending curve) demonstrating the presence of an apparent acyl-CoA:retinol acyltransferase activity.

can be detected in the microsomal preparations. The small amount of CoA detected by method 3 (Table I) corresponds to 0.1 nmol of CoA mg⁻¹ protein, a value similar to that reported by Garland et al. (1965) for rat liver microsomes. The concordance observed with three independent methods for fatty acyl-CoA analysis, each showing reasonable recovery of exogenous palmitoyl-CoA, indicates that esterification of retinol with microsomes alone is non-CoA-mediated.

Detection of Apparent Acyl-CoA:Retinol Acyltransferase Activity—Addition of palmitoyl-CoA to a mixture of retinol and microsomes after the endogenous acyl donor had apparently been depleted (i.e. at the plateau of the progress curve) produces a considerable stimulation of retinyl ester synthesis (Fig. 4), demonstrating the presence of an apparent acyl-CoA:retinol acyltransferase activity. Retinol or palmitate, added at this point, has no effect. Addition of palmitate, CoA, Mg²⁺, and ATP produced ~15% the stimulation observed with an equivalent amount of palmitoyl-CoA. Addition of [¹⁴C]palmitoyl-CoA and [³H]retinol at the beginning of the reaction demonstrates that the rate of esterification is similar when either retinyl or palmitoyl group incorporation is followed (Fig. 5). Addition of palmitoyl-CoA at the beginning of the reaction also produces a stimulation of the extent of esterification (Fig. 6). Complex kinetics were observed on attempting to determine a Kₘ for palmitoyl-CoA.

Fig. 5. Time course of retinyl ester synthesis at 37 °C from retinol and palmitoyl-CoA indicating that similar results are obtained when either the incorporation of retinol or palmitate is followed. Upper curve, [³H]retinol + palmitoyl-CoA. Lower curve, retinol + [¹⁴C]palmitoyl-CoA.

Fig. 6. Stimulation of extent of retinyl ester formation by exogenous palmitoyl-CoA. The curves depict the amount of retinyl ester formed following the addition of retinol (O) or retinol and palmitoyl-CoA (●).
the phase in which palmitoyl-CoA partitions nearly quantitatively. HPLC analysis of both phases failed to detect palmitoyl-CoA whether assayed by A$_{260}$ or 4°C. Subsequent incubation of the labeled microsomes with [3H]retinol resulted in transfer of 24% of the microsomal 4°C to retinol. Retinyl ester that contained both H and 14C was demonstrated by HPLC isolation. The 14C-specific activity of retinyl palmitate in the recovered ester was approximately one-fourth that of added palmitoyl-CoA. This experiment was performed six times, with comparable results obtained each time. The results demonstrate that an endogenous active acyl pool can be labeled by preincubation of microsomes with [14C]palmitoyl-CoA but leave open the question of whether the palmitoyl group can be transferred directly to retinol as well.

**DISCUSSION**

Enzymatic retinyl ester synthesis has been reported in several tissues including RPE (Berman et al., 1980; Krinsky, 1958), liver (Ross, 1982a), mammary gland (Ross, 1982b), testis (Chaudhary and Nelson, 1987), intestinal epithelium (Helgerud et al., 1982; Mahadevan et al., 1961; Ong et al., 1987), pancreas (Pollard and Bieri, 1960), and retinoblastoma cell line WERI-Rb1 (Saari et al., 1980). Andrews and Puttermann (1964) observed retinyl ester synthesis in retinal microsomes; however, it is likely that the activity they measured was due to contamination with RPE microsomes since Berman et al. (1980) report that the activity in retina is only about 1% that observed in RPE.

Retinyl ester synthesis in RPE is characterized by at least two unusual features. The activity of the enzyme is orders of magnitude higher than the activities of two other visual cycle enzymes in this tissue (e.g., retinyl palmitate hydrolase (Blair et al., 1987) and retinol isomerase (Bernstein et al., 1987)), raising questions regarding the control of retinol metabolism. In addition, the enzyme appears to function with an endogenous, non-acyl-CoA source of fatty acids for synthesis of retinyl esters. This study was designed to examine the second of these features in more detail.

The extent of retinyl ester synthesis from retinol alone, observed at the plateau of a progress curve (Fig. 1), was used to estimate the concentration of endogenous acyl-CoA needed to account for the esterification. The value obtained is undoubtedly a minimum as no correction was made for loss of basis for analysis of endogenous acyl-CoA levels, the validity of the enzyme assay was assessed by comparing the level of activity obtained from the radioactivity in the "ester fraction" from alumina with the amount of retinyl ester determined by direct HPLC analysis of the extracted digest. The good agreement between the two methods (~18% difference) provides a measure of confidence in the expected acyl donor concentration.

Three methods for the determination of fatty acyl-CoA were employed. In methods 1 and 2, palmitoyl-CoA was analyzed directly by HPLC (Fig. 5) following precipitation and extraction, respectively. In method 1, perchloric acid was used to disrupt microsomal components and precipitate long chain fatty acyl-CoAs (Tubbs and Garland, 1969). Samples of microsomes to which palmitoyl-CoA had been added were included to determine the efficiency of the procedure. The recovery of exogenous palmitoyl-CoA was relatively poor with this method (~60%). However, endogenous fatty acyl-CoA could not be detected (Table I). Since it could be argued that exogenous palmitoyl-CoA did not mix with the endogenous fatty acyl-CoA pool and was not dissolved following precipitation, method 2 was employed in which microsomes were dissolved initially in 0.2% SDS to achieve a single phase and then extracted with CHCl₃:MeOH (Folch et al., 1957). Preliminary experiments demonstrated that [14C]palmitoyl-CoA partitioned nearly quantitatively (93%) into the upper, methanol-water phase. Nonetheless, both phases were analyzed for fatty acyl-CoA by HPLC analysis. Approximately 80% of the exogenous palmitoyl-CoA was recovered whereas endogenous fatty acyl-CoA could not be detected (Table I). In method 3 either microsomes or the perchloric acid precipitate from microsomes were hydrolyzed at pH 11.5 (Williamson and Corkey, 1969), and CoA was analyzed in the neutralized hydrolysates by HPLC analysis. The recovery of standard palmitoyl-CoA (as CoA) was ~80% with this method. Little if any endogenous CoA was detected, in agreement with the results of methods 1 and 2. In each of the three sets of analytical experiments, an amount of microsomes was analyzed with a calculated endogenous acyl donor content 2-3 times the amount of exogenous palmitoyl-CoA added, and the minimum detectable amount of fatty acyl-CoA was ~2.5% the amount of added palmitoyl-CoA.

Although it is possible to suggest several reasons why endogenous fatty acyl-CoAs might have escaped detection, no one explanation is entirely tenable. We considered that endogenous fatty acyl-CoAs might be hydrolyzed to palmitate and CoA upon addition of extraction reagents. However, recoveries of exogenous standards are sufficiently high with two of the methods we employed (~80%, see Table I) to rule this unlikely, and method 3 would have detected CoA, one of the hydrolysis products. We also considered that endogenous fatty acyl-CoAs might be sequestered in a compartment that does not mix with the exogenous standards and is not affected by extraction conditions. However, in method 2 an apparent single phase is obtained by the addition of SDS before addition of CHCl₃:MeOH solvents, ensuring equilibration of exogenous standards with endogenous components. It should also be noted that usually separate portions of frozen microsomes were used for the estimation of endogenous acyl donor and fatty acyl-CoA levels. When the same sample was used for both analyses, the extent of esterification with the endogenous acyl donor was measured after the concentration of endogenous fatty acyl-CoA had been determined, thus eliminating the possibility of underestimating the concentration of fatty acyl-CoA relative to endogenous acyl donor. The results, employing different extraction conditions and chemical procedures, convincingly demonstrate that endogenous fatty acyl-CoA cannot account for the amount of retinyl ester synthesis observed in the absence of an exogenous source of acyl groups.

Endogenous fatty acyl-CoA should be susceptible to cleavage by NH₄OH (e.g., Ross, 1982a) and, indeed, treatment of the microsomes with 300 mM NH₄OH at 37°C resulted in a decrease in their activity. However, no restoration of activity was observed with subsequent addition of palmitoyl-CoA, suggesting that the treatment resulted in a general inactivation of the enzyme. Ong et al. (1987) noted a similar finding with intestinal microsomes. Monitoring the rate of esterification is observed if palmitoyl-CoA is added following a preincubation of microsomes with retinol (Fig. 4) that presumably depletes the preparation of endogenous acyl donor. Approximately 15% as much stimulation was observed by addition of ATP, magnesium, palmitate, and CoA as the substrates for palmitoyl-CoA formation, suggesting the presence of a fatty acyl-CoA generating
system. Palmitoyl alone was without effect. Incorporation of palmitoyl from \[^{14}C\]palmitoyl-CoA into retinyl ester could be used to follow the rate of esterification, indicating the presence of an apparent ARAT activity (Fig. 5). An effect on the initial rate of esterification by addition of palmitoyl-CoA was more difficult to document, presumably because the enzyme is already saturated with endogenous acyl donor. An increased amount of retinyl ester synthesis resulting from addition of palmitoyl-CoA at the beginning of the reaction was readily apparent (Fig. 6).

Other investigators, working with RPE microsomes, have not examined directly the question of stimulation of esterification by palmitoyl-CoA. Bridges et al. (1986) reported that 30 \(\mu\)M palmitoyl-CoA did not enhance the esterification of retinol in cultured bovine RPE cells. However, since the cells lose activity in culture this result is difficult to extrapolate to the fresh tissue situation. Berman et al. (1980), working with RPE microsomes, reported no stimulation with added ATP, magnesium, CoA, and palmitoleate but apparently did not add palmitoyl-CoA. Since these substrates for palmitoyl-CoA synthesis were only 15% as effective as palmitoyl-CoA in this study, it is possible that the acyl-CoA synthetase is present in low and/or variable amounts. Andrews and Futterman (1964) reported no stimulation by either ATP, magnesium, CoA, and palmitoyl-CoA or by palmitoyl-CoA; however, retinal microsomes were studied. Krinsky (1988) observed a "marked increase" in the rate of esterification with added ATP, palmitate, cysteine, and CoA, in reasonable agreement with our results. Although calf eyes were used for most of the studies described here, adult bovine RPE microsomes behaved similarly.

Low levels of esterification with retinol alone are characteristic of microsomal preparations from several tissues. This activity has been attributed to endogenous fatty acyl-CoA as treatment with NH\(_2\)OH increases the fatty acyl-CoA dependence of the reaction (Ross, 1982a, 1982b; Helgerud et al., 1982; Chaudhary and Nelson, 1987). Ong et al. (1987) have provided evidence that retinyl ester synthesis with rat intestinal microsomes is non-acyl-CoA-mediated, analogous to the system described here. Further work is needed to ascertain the extent to which these two reactions are related.

Liver microsomes display limited esterification of retinol in the absence of exogenous fatty acyl-CoA (Ross, 1982a), although a comparison with RPE is difficult because the specific activity of the preparations differs by a factor of more than 3000. Since liver is a complex organ it is interesting to speculate that a higher specific activity microsomal preparation, perhaps displaying non-acyl-CoA dependent esterification, might be obtained from one of the several cell types present in liver, e.g. the vitamin A-ester-storing stellate cells (Hendriks et al., 1985; Blomhoff et al., 1984).

Addition of exogenous palmitoyl-CoA could stimulate retinyl ester synthesis after exhaustion of the endogenous acyl donor directly by acting as an alternative substrate for the reaction or indirectly by recharging an endogenous acyl pool. The results of the 2-step transfer experiment indicate that the latter mechanism is operative but do not rule out the former. \[^{14}C\]Palmite from exogenous palmitoyl-CoA was transferred to component(s) of the microsomes and then, after removal of residual palmitoyl-CoA, to retinol. The validity of the incorporation depends on demonstrating the absence of palmitoyl-CoA following preincubation and washing. In the experiment described, it would have been necessary for the microsomes to retain 14% (28 nmol; \(-10^6\) dpm) of the added \[^{14}C\]palmitoyl-CoA to account for the \[^{14}C\]palmitate transferred to retinol, an amount easily detectable. However, extraction of the labeled microsomes with CHCl\(_3\):MeOH showed no radioactivity in the upper phase, and no palmitoyl-CoA was detectable in either phase by HPLC analysis. Thus the \[^{14}C\]palmate transferred to retinol had to originate from an acylated microsomal component and not residual palmitoyl-CoA. A tentative mechanism for the apparent acyl-CoA:retinol acyltransferase activity is shown below.

![Diagram showing reaction pathways involving CoA Palmitoyl, Microsomes, Retinol, and Retinyl palmitate]

Ross (1982a) speculated that such a mechanism could account for the acyl-CoA:retinol acyltransferase activity of liver microsomes but did not test the hypothesis. The ability to label what is likely to be the endogenous acyl donor with \[^{14}C\]palmitate should be helpful in its isolation and identification.

Retinyl ester synthesis in RPE occurs with both all-trans-retinol and 11-cis-retinol, the only geometrical isomers of retinol present in major amounts in retina and RPE. The \(K_m\) values for the two isomers were within experimental error as were the \(V_{max}\) values. It is unknown at present if this is the result of one enzyme or two. Retinyl ester hydrolysis and retinol oxidation in retina involve separable activities, relatively specific for 11-cis- or trans-isomers (Blaner et al., 1987; Lion et al., 1975; Saari and Bredberg, 1982).

Extraction and HPLC analysis of the retinooids generated during incubation with microsomes indicates that the stereoechemical configuration of all-trans-retinol is retained during esterification (Fig. 2). In addition, unesterified retinol remains predominantly in the all-trans configuration although small amounts of 11-cis- and 13-cis-retinol are present. It is not certain whether this is an indication of retinol isomerase activity (Bernstein et al., 1987; Bridges and Alvarez, 1987). Based on the reported activity of this enzyme in bovine RPE (Bridges and Alvarez, 1987) it is unlikely to play a role in 11-cis-retinol generation during a 20 min incubation. In addition, the presence of ethanol in the reaction mixture may have inhibited the reaction (Bernstein et al., 1987).

In summary, less than 1% of the amount of endogenous fatty acyl-CoA required for retinyl ester synthesis can be detected in bovine RPE microsomal preparations. Endogenous esterification of retinol is therefore non-fatty acyl-CoA mediated. The identity of the endogenous donor is unknown but is likely to be a lipid. Labeling of an endogenous acyl donor with \[^{14}C\]palmate is achieved by incubation of microsomes with \[^{14}C\]palmitoyl-CoA. The apparent acyl-CoA:retinol acyltransferase activity of the preparation can be explained by a transfer of palmate from palmitoyl-CoA to a microsomal component and then to retinol.

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