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# Evidence for Triacylglycerol Synthesis in the Lumen of Microsomes via a Lipolysis-Esterification Pathway Involving Carnitine Acyltransferases*

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**In this study a pathway for the synthesis of triacylglycerol (TAG) within the lumen of the endoplasmic reticulum has been identified, using microsomes that had been preconditioned by depleting their endogenous substrates and then fusing them with biotinylated phosphatidylserine liposomes containing CoASH and $Mg^{2+}$. Incubating these fused microsomes with tri[$^3$H]oleoylglycerol and [$^{14}$C]oleoyl-CoA yielded microsome-associated triacylglycerol, which resisted extensive washing and had a [$^3$H]:[$^{14}$C] ratio close to 2:1. The data suggest that the precursor tri[$^3$H]oleoylglycerol was hydrolyzed by microsomal lipase to membrane-bound di[$^3$H]oleoylglycerol and subsequently re-esterified with luminal [$^{14}$C]oleoyl-CoA. The accumulation of TAG within the microsomes, even when overt diacylglycerol acyltransferase (DGAT I) was inactive, is consistent with the existence of a latent diacylglycerol acyltransferase (DGAT II) within the microsomal lumen. Moreover, because luminal synthesis of TAG was carnitine-dependent and markedly reduced by glybenclamide, a potent carnitine acyltransferase inhibitor, microsomal carnitine acyltransferase appears to be essential for trafficking the [$^{14}$C]oleoyl-CoA into the microsomal lumen for subsequent incorporation into newly synthesized TAG. This study thus provides the first direct demonstration of an enzymatic process leading to the synthesis of luminal triacylglycerol, which is a major component of very low density lipoproteins.**

Overt and latent forms of carnitine acyltransferase (CAT I[1] and CAT II, respectively) have been shown to exist in mitochondria, peroxisomes, and microsomes (1), suggesting a parallel occurrence of the two isozymic forms of this enzyme in these organelles. Although these two enzymes are known to facilitate the uptake of acyl-CoAs (via interconversion with acylcarnitine) into mitochondria and peroxisomes for β-oxidation (2, 3), their physiological function in microsomes is yet to be established. It has been suggested that microsomal CAT is likewise part of a system that enables delivery of acyl moieties across the membrane of the endoplasmic reticulum (4, 5), which is considered to be an integral step in the synthesis of VLDL triacylglycerol (TAG). This is supported by the fact that the sulfonylurea drug tolbutamide inhibits microsomal CAT I (4) and suppresses VLDL TAG secretion by hepatocytes to the same extent (6).

If acyl-CoA generated within the lumen of the microsomes is employed in the acylation of DAG to form VLDL TAG, the existence of a luminal (latent) diacylglycerol acyltransferase (DGAT II), as distinct from the overt enzyme (DGAT I), is suggested (4, 5). However, currently only one DGAT gene has been identified in mice (7). Nevertheless, Cases *et al.* (7) raised the possibility of a second DGAT mRNA to explain the fact that in Northern blots more than one band hybridizes with the DGAT probe (7). The present investigation provides novel metabolic evidence supporting the existence of DGAT II within the microsomal lumen. It is proposed that this enzyme catalyzes the formation of intraluminal TAG from membrane-bound diacylglycerol (DAG) and luminal acyl-CoA, which are generated by microsomal lipase and a carnitine-dependent process, respectively.

## EXPERIMENTAL PROCEDURES

*An Outline of the Methodology*—I. Because carnitine can be unidirectionally transported (8), microsomes were first incubated with carnitine (8) to ensure that their intraluminal carnitine concentration was sufficient to facilitate oleoylcarnitine uptake through an exchange reaction. Simultaneously, they were exposed to excess CDP-choline (9) to convert endogenous diacylglycerol to phosphatidylcholine, thus eliminating endogenous DAG. The microsomes were then centrifuged through dibutyl phthalate, an oil immiscible with water, to remove the unreacted reagents.

II. The microsomes were resuspended and incubated with tri[$^3$H]oleoylglycerol (tri[$^3$H]TAG) to generate membrane-bound di[$^3$H]oleoylglycerol (di[$^3$H]DAG) through the action of microsomal lipase, followed by a further centrifugation of the microsomes through dibutyl phthalate to remove the unreacted tri[$^3$H]TAG and liberated [$^3$H]oleic acid.

III. Because CoASH is depleted from microsomes during their isolation (10) and cannot be taken up by simple diffusion (8, 11), the CoASH was replenished by fusing them with biotinylated phosphatidylserine liposomes encapsulating CoASH and $Mg^{2+}$.

IV. Microsomes were then incubated with [$^{14}$C]oleoyl-CoA in the presence and absence of carnitine to prove that the pathway of internal, but not external, TAG synthesis from the membrane-bound DAG is carnitine-dependent. Both pools of newly synthesized TAG were subsequently quantified. TAG was extracted and purified by thin-layer chromatography before determining the relative amounts of $^3$H and $^{14}$C incorporated into the TAG.

*Isolation of Microsomes*—Rough microsomes were isolated from the livers of 10-week old male Wistar rats using a previously described method (1, 12) but with the inclusion of 50 mM EDTA to remove ribosomes (13). Removal of ribosomes exposes the highly positively charged N-terminal membrane-anchoring domain (pI = 11) (14), thus

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[1] The abbreviations used are: CAT I and II, carnitine acyltransferase I (overt) and II (latent); VLDL, very low density lipoproteins; DAG, diacylglycerol; TAG, triacylglycerol; DGAT I and II, diacylglycerol acyltransferase I (overt) and II (latent); BSA, bovine serum albumin; PS, phosphatidylserine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MES, 2-(*N*-morpholino)-ethanesulfonic acid.



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providing greater charge attraction for the negatively charged liposomes in the fusion step. Microsomes were essentially "outside-out" vesicles with the same orientation of the membrane as in the endoplasmic reticulum. This was confirmed by the latency of the enzyme glucose-6-phosphatase, which is bound to the inner face of the microsomal membrane and was assayed using mannose-6-phosphate as a substrate, as described previously (15, 16).

*Preconditioning of Microsomes and the Verification of Microsomal Lipase Activity*—Microsomal-bound di[$^3$H]oleoylglycerol was generated by allowing microsomal lipase to hydrolyze exogenously added tri[$^3$H]oleoylglycerol (900 mCi/mmol), under conditions slightly modified from that previously described (17). A higher concentration of tri[$^3$H]TAG (50 $\mu$M) was used to compete with the newly synthesized di[$^3$H]DAG, thus preventing further hydrolysis of di[$^3$H]DAG by DAG lipase. The assay was carried out in a final volume of 1 ml containing ~150 $\mu$g of microsomal protein, 175 mM morpholinoethanosulfonic acid (pH 6.0), 400 $\mu$g of BSA, and 50 $\mu$g of a 1:1 (w/w) mixture of phosphatidylcholine and phosphatidylserine, previously sonicated for 2 min in 10 mM Tris-HCl (pH 7.0) containing 10 mM $MnCl_2$. The reaction was started by the addition of 50 $\mu$M tri[$^3$H]oleoylglycerol in 25 $\mu$l of acetone (final concentration in the assay mixture was 2.5%). Note that the assay components employed above have previously been shown to have no effect on the latency of glucose-6-phosphatase (17). After 40 min of incubation at 37 °C, 3 ml of cold homogenization buffer was added to rapidly cool the reaction mixture, which was then put on ice. The ice-cold mixture was layered over 3 ml of dibutyl phthalate oil (top) and 1 ml of 50 mM triethanolamine, pH 7.3, containing 0.5 M sucrose and 1 mM dithiothreitol (bottom) in a 10-ml polycarbonate tube and centrifuged at 115,000 × *g* at 4 °C for 60 min. The aqueous solution above the oil layer was carefully aspirated with a Pasteur pipette, and the wall of the tube was gently washed with water and wiped with a tissue before removing the oil and the buffer underneath. For the determination of microsomal-bound di[$^3$H]DAG, 1.5 ml of isopropanol:heptane:water, 80:20:2 (v/v/v), was added to the microsomal pellet followed by vigorous mixing. After mixing, 1 ml of heptane and 0.5 ml of water were added. The tube was then capped and vortexed vigorously and centrifuged (700 × *g* for 10 min). After phase separation, an aliquot (400 $\mu$l) was taken from the upper heptane phase (total volume approximately 1.3 ml) and evaporated to dryness under nitrogen. The dried material was solubilized in ≤50 $\mu$l of diethylether and applied to a thin-layer chromatography plate (250 $\mu$m silica gel LK6DF), which was developed using hexane:diethylether:acetic acid (80:20:1) as the solvent. The regions corresponding to TAG and DAG were identified by comparison with known standards and scraped into scintillation vials to which 3 ml of Optiphase scintillation fluid was added and the radioactivity measured in a Beckman LS 3802 liquid scintillation $\beta$-counter (Beckman Instruments). Greater than 98% of TAG and 94% of DAG was extracted and recovered as judged by the application of standard amounts of radiolabeled TAG and DAG. The yield of di[$^3$H]DAG synthesized is described under "Results and Discussion."

*Preparation of Liposomes and Their Subsequent Fusion with Microsomes*—Biotinylated phosphatidylserine (PS) was prepared from phosphatidylserine according to a previously described method (18). The generation of biotinylated PS liposomes (encapsulating a buffer containing 0.32 M sucrose, 2 mM HEPES (pH 7.0), 2 mM dithiothreitol, 2 mM NaCl, with and without 16 mM CoASH and 2 mM $MgCl_2$) and their subsequent fusion with microsomes were carried out as described previously (19, 20). Fused microsomes, which as a consequence of fusion had biotinylated PS on their surface, were separated from unfused microsomes by allowing them to bind to streptavidin-coated Dynabeads, a step which exploits the remarkably high affinity of streptavidin for biotin ($K_d$ = $10^{-15}$ M) (21). The fused microsomes bound to the Dynabeads were then isolated using a Magnetic Particle Concentrator. Dynabeads were applied to only one-tenth of the mixture of fused and unfused microsomes after the removal of unfused liposomes, which would otherwise have interfered with the fused microsomes binding to the Dynabeads. Unfused liposomes were removed by layering an aliquot of the microsome/liposome mixture (5 ml) over 3 ml of dibutyl phthalate (top) and 1 ml of 50 mM triethanolamine, pH 7.3, containing 0.5 M sucrose and 1 mM dithiothreitol (bottom) in a 10-ml polycarbonate tube and centrifuging at 115,000 × *g* for 60 min.

*Initiation of TAG Synthesis*—The synthesis of TAG from the membrane-bound di[$^3$H]DAG precursor was facilitated by providing the optimum conditions for CAT I and DGAT. Because DGAT (22, 23) and CAT I (24) are both active at neutral pH when phosphate is present, potassium phosphate was included as described previously (25). Additions were made to the microsomal-liposomal suspension to give final concentrations of 220 mM sucrose, 40 mM KCl, 1 mM EGTA, 4 mM $MgCl_2$, 4 mM ATP, 100 mM potassium phosphate, and 40 mM Tris-HCl (pH 7.4), with or without 0.5 mM carnitine. The reaction was initiated by the addition of [$^{14}$C]oleoyl-CoA (5 mCi/mmol) at concentrations of 0.8, 1, 2, 5, 25, and 50 $\mu$M, and included fatty acid-free bovine serum albumin (BSA) at an oleoyl-CoA:BSA molar ratio of 9:1. The role of BSA as a binder and delivery system for acyl-CoA was partially substituted for by the presence of liposomes in the system (26, 27). The mixtures were incubated at 37 °C for 40 min before being rapidly cooled on ice. The microsomes were then washed by centrifugation through dibutyl phthalate. Acyl-CoA hydrolysis during the incubation period was minimized by using microsomes that had been frozen overnight, a procedure that inactivates deacylase activity (28). In addition, ATP was included in the assay as recommended by McGarry *et al.* (29) to facilitate acyl-CoA synthetase activity and thus offset any hydrolysis of acyl-CoA. Moreover, alkaline hydrolysis of acyl-CoA only occurs at a considerable rate at pH >9.0 (30), whereas the pH at which our assay was performed was 7.4.

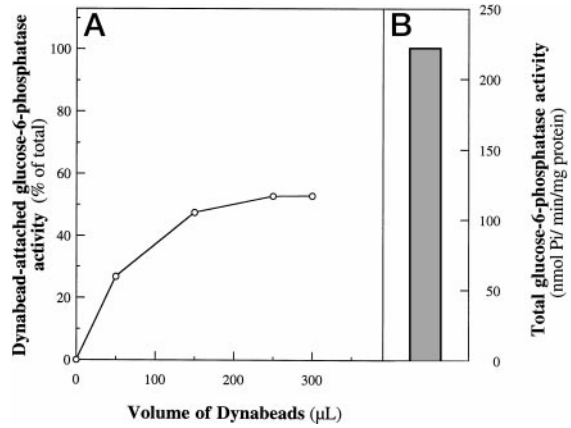


FIG. 1. **Extent of fusion of rat liver microsomes with biotinylated PS liposomes.** Rat liver microsomes (about 128 $\mu$g of protein) were incubated with biotinylated PS liposomes (~0.6 $\mu$mol of biotinylated PS) encapsulating 16 mM CoASH and 2 mM $Mg^{2+}$ in a total volume of 420 $\mu$l. Microsomal fusogenic protein-mediated fusion was triggered at pH 5.0, which maintains the biotinylated PS carboxylate group in a negatively charged state (57). The buffer was 20 mM MES, pH 5.0, containing 0.32 M sucrose. The glucose-6-phosphatase activity was determined in fused microsomes that bound to the indicated volume (0–300 $\mu$l) of a 10 mg/ml suspension of streptavidin-coated Dynabeads (*A*) and in the mixture of fused and unfused (total) microsomes (*B*). The extent of fusion was calculated from the enzyme activity (nmol $P_i$/min) that bound to a saturating volume of Dynabeads, expressed as a percentage of the total activity.

TAG synthesized externally (in the supernatant) and within the microsomal lumen, as well as membrane-bound DAG, was determined after thin-layer chromatography purification, as described above. In one experiment, conducted in triplicate, 220 $\mu$M glybenclamide (1-{4[2-(5-chloro-2-methoxy benzamido)-ethyl]benzene-sulfonyl}-3-cyclohexylurea) was added to the assay in dimethylformamide (final concentration 0.5%) at the times indicated in the legend to Fig. 5.

*Miscellaneous Procedures*—Recombinant acyl-CoA-binding protein was isolated from *Escherichia coli* as described previously (31). Protein was measured using a modification of the Lowry procedure to facilitate quantitation of protein in the presence of high concentrations of lipids (32).

## RESULTS AND DISCUSSION

*Fusion Experiments*—In this study, microsomes were fused with liposomes encapsulating various components to investigate the proposed involvement of latent activities of DGAT and CAT in the luminal synthesis of TAG. Fused microsomes were magnetically separated from the unfused microsomes by being bound to streptavidin-coated Dynabeads. The extent of fusion was 54.2% ± 5.0, as determined by expressing the glucose-6-phosphatase activity in microsomes bound to a saturating amount (300 $\mu$l) of Dynabeads (Fig. 1*A*) as a percentage of the total activity of the enzyme in the original mixture of fused and unfused microsomes (Fig. 1*B*). This extent of fusion was higher than the 39% recorded by Pistolesi *et al.* (19) using PS lipo-

somes, confirming our assumption that biotinylation of the PS would enhance subsequent fusion by increasing the electronegativity of the liposomes (18) and thus their attraction for the positively charged microsomes (33).

*Production of Microsomal-bound Di[$^3$H]Oleoyl Glycerol by Microsomal Lipase*—When microsomes were incubated with 50 $\mu$M tri[$^3$H]TAG and washed free of unreacted tri[$^3$H]TAG by centrifuging the microsomes through dibutyl phthalate, 14.6 ± 2.9 nmol of di[$^3$H]DAG/mg of microsomal protein was shown to be bound to the microsomal membrane. Microsomal lipase has been shown to hydrolyze TAG to DAG and 2-monoacylglycerols (34). However, under our assay conditions the concentration of TAG was vastly in excess of newly formed DAG and would thus have prevented further breakdown of DAG to 2-monoacylglycerol by preferentially binding to the active site of the lipase (35).

*Distinction between TAG Produced External and Internal to the Microsomal Membrane*—When microsomes containing membrane-bound di[$^3$H]DAG and loaded with CoASH were further incubated with [$^{14}$C]oleoyl-CoA in the absence of carnitine, 98% of the resultant radiolabeled TAG could be removed by centrifuging the microsomes through dibutyl phthalate. Thus only 0.08 ± 0.01 nmol of TAG/mg of microsomal protein remained associated with the microsomes, implying that the TAG synthesized in the absence of carnitine must have been predominantly external to the microsomes. In contrast, if microsomes were incubated with [$^{14}$C]oleoyl-CoA in the presence of carnitine, the amount of TAG remaining associated with the washed microsomes was 11.58 ± 2.36 nmol of TAG/mg of fused microsomal protein, corresponding to a ~140-fold increase. Because dibutyl phthalate was very effective in removing the externally synthesized TAG, it is concluded that the majority of TAG generated in the presence of carnitine, and remaining associated with the microsomes after passage through dibutyl phthalate, must have been synthesized inside the microsomes. Because only trace amounts (~2%) of externally synthesized TAG remain associated with dibutyl phthalate-washed microsomes, the TAG synthesized by fused microsomes in the presence of carnitine must be predominantly free in the lumen and/or bound to the inner surface of the microsomal membrane.

The $^3$H:$^{14}$C molar ratios in the external and internal pools of TAG were 2.02 ± 0.03 and 2.13 ± 0.04, respectively, confirming that, for both pools of TAG, the tri[$^3$H]TAG was initially hydrolyzed to di[$^3$H]DAG, which is membrane-diffusible (36), and that this DAG was subsequently re-esterified with the introduced [$^{14}$C]oleoyl-CoA. It has been previously shown that DGAT has a stereospecific preference for *sn*-1,2-DAG over *sn*-2,3-DAG or *sn*-1,3-DAG (23, 37–39), suggesting that the acylation of the DAG is likely to have occurred in the *sn*-3 position. This demonstration of the existence of a lipolysis/esterification pathway is consistent with the observation of Yang *et al.* (40) that the fatty acid composition of the TAG of liver and of VLDLs were homologous in the *sn*-1 and *sn*-2 positions but distinctly different in the *sn*-3 position. The fact that the $^3$H:$^{14}$C molar ratio in the TAG produced within the microsomal lumen was slightly higher than the expected ratio of 2.0 suggests that there may have been some endogenous acyl-CoA within the lumen, which would have competed with the [$^{14}$C]oleoyl-CoA during the re-esterification of DAG. Furthermore, if the newly synthesized TAG had been derived from monoacylglycerol then the $^3$H:$^{14}$C ratio would be expected to be 1:2. Clearly this was not the case.

*Dependence of DGAT I and DGAT II Activities on Acyl-CoA*—The extent of external and internal synthesis of TAG by the carnitine-independent and carnitine-dependent pathways, respectively, at varying concentrations of oleoyl-CoA and at a fixed amount of membrane-bound DAG, is compared in Fig. 2. When these data were analyzed using double reciprocal plots, the amounts of extraluminal and internal TAG that were synthesized at a saturating concentration of [$^{14}$C]oleoyl-CoA, were calculated to be 5.6 ± 0.9 and 10.9 ± 2.9 nmol of TAG/mg of microsomal protein, respectively. That is, the extent of synthesis of internal TAG (the carnitine-dependent pathway) was 1.95-fold higher than that of external TAG (the carnitine-independent pathway) shown in this study. The total TAG synthesized at this saturating concentration of oleoyl-CoA was 16.5 ± 3.8 nmol of TAG/mg of microsomal protein, which is not significantly different ($p > 0.1$) from the original amount of DAG bound to the microsomal membrane (see above). This study used microsomes with an essentially outside-out orientation, *i.e.* having a similar sidedness to intact endoplasmic reticulum, as judged by the latency of glucose-6-phosphatase (data not shown). Thus, TAG synthesized within the lumen and in the extraluminal space correspond to the distinct microsomal and cytoplasmic pools of TAG, respectively.

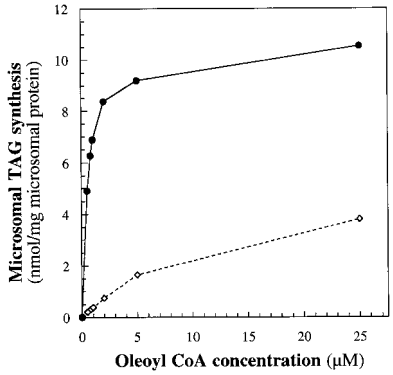


FIG. 2. **Internal and external synthesis of TAG in fused microsomes.** The amounts of internal (●) and external TAG (◇) that were synthesized by rat liver microsomes, from membrane-bound 1,2-di[$^3$H]oleoylglycerol (14.6 ± 2.9 nmol/mg of microsomal protein) and the indicated concentrations of [$^{14}$C]oleoyl-CoA, were determined. Microsomes were preconditioned for luminal TAG synthesis by loading them with carnitine and subsequently fusing them with liposomes encapsulating CoASH and $Mg^{2+}$. The assay was performed at pH 7.4 as described under "Experimental Procedures." Greater than 98% of the TAG was recovered after thin-layer chromatography on 250 $\mu$m silica gel LK6DF plates. The data presented were corrected for the extent of fusion and correspond to the mean values from three separate experiments.

The luminal synthesis of TAG within a mixture of fused and unfused microsomes (6.21 ± 0.96 nmol/mg of total microsomal protein) was shown to be predominantly associated with those microsomes that had fused with biotinylated PS liposomes, previously loaded with 16 mM CoASH and 2 mM $Mg^{2+}$ (Fig. 3). This is evident from the fact that only the fused microsomes, *i.e.* those bound to streptavidin-coated Dynabeads, had appreciable amounts of internal radiolabeled TAG (11.58 ± 2.36 nmol/mg of fused microsomal protein). Unfused microsomes, *i.e.* those not bound to the streptavidin-coated Dynabeads, contained negligible amounts of internal radiolabeled TAG. There was also negligible TAG produced within the lumen of microsomes that had been fused with either empty liposomes (data not shown) or with liposomes containing $Mg^{2+}$ but no CoASH (Fig. 4). Therefore, it is concluded that it was not simply the act of fusion but rather was the consequence of CoASH being delivered to the microsomal lumen, thereby facilitating intraluminal acyl-CoA synthesis, that promoted the production of luminal TAG.

*DGAT II Activity When DGAT I Is Specifically Inactivated*—The conclusion proposed earlier that the TAG generated in the presence of carnitine, which survived washing through dibutyl

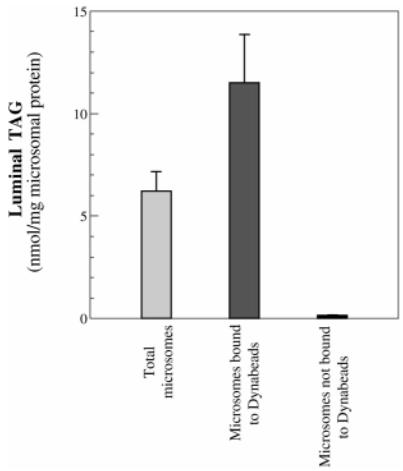


FIG. 3. **Internal synthesis of TAG in fused and unfused microsomes.** The amount of newly synthesized luminal TAG was determined for total (fused + unfused) microsomes (□), fused microsomes (▩), f), *i.e.* those bound to streptavidin-coated Dynabeads, and unfused microsomes (■), *i.e.* those not bound to Dynabeads. The data represent the means ± S.E. of three separate experiments.

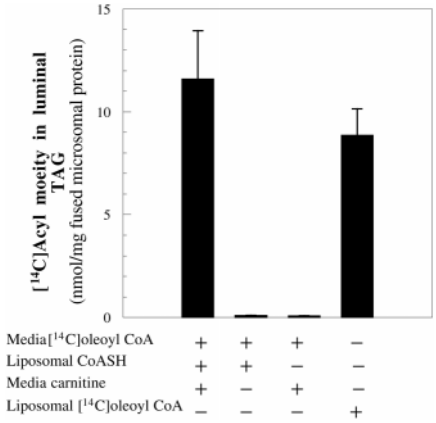


FIG. 4. **Incorporation of the acyl moiety of exogenously added [$^{14}$C]oleoyl-CoA into newly formed luminal TAG.** The amount of exogenous [$^{14}$C]oleoyl-CoA incorporated into newly synthesized luminal TAG was determined under different experimental conditions. In a typical complete assay, DAG-bound microsomes were loaded with CoASH by fusing them with liposomes encapsulating 16 mM CoASH. These microsomes were incubated with 50 μM [$^{14}$C]oleoyl-CoA and 0.5 mM carnitine. The extent of incorporation of the [$^{14}$C]oleoyl moiety into luminal TAG was quantitated in the absence (−) or presence (+) of CoASH in the microsomal lumen (via liposomes) and carnitine in the incubation media. When both CoASH and carnitine were absent, [$^{14}$C] oleoyl-CoA was directly loaded into microsomes by fusing them with liposomes encapsulating 50 μM of the labeled acyl-CoA. The data represent the means ± S.E. of triplicate experiments.

phthalate, was synthesized inside the microsomes, rather than resulting from the internalization of externally synthesized TAG, was confirmed by an additional experiment. Microsomes were fused with liposomes encapsulating 50 μM [$^{14}$C]oleoyl-CoA and then subsequently treated with excess (>350 μM) of acyl-CoA-binding protein and washed through dibutyl phthalate. Because acyl-CoA-binding protein binds acyl-CoAs with a very high affinity (nanomolar $K_d$), this procedure not only removes external acyl-CoA but also that bound to the outer leaflet of the microsomal membrane (31, 41, 42). These microsomes failed to acylate exogenously added 1 mM DAG (data not shown), prepared as a sonicated mixture with phosphatidylglycerol (PG) (43). Sonicated DAG-PG mixtures have been shown in this laboratory to be very effective in presenting DAG in an appropriate physical form for microsomal diacylglycerolcholine phosphotransferase activity (44). In addition, this type of preparation of DAG has previously been shown to be suitable for the DGAT I reaction (5). Therefore, the lack of acylation activity in this study indicates that DGAT I was rendered completely inactive by the removal of extraluminal acyl-CoA and was not due to the exogenous DAG being in an inappropriate physical form. This observation also demonstrates that DAG not bound to the membrane (in contrast to membrane-bound DAG (36)) is inaccessible to DGAT II and that the intraluminal [$^{14}$C]oleoyl-CoA is impermeable to the microsomal membrane (45) and therefore inaccessible to DGAT I. When the above microsomes were incubated with tri[$^{3}$H]TAG, under conditions that generate membrane-bound di[$^{3}$H]DAG, a high level of newly synthesized di[$^{3}$H]oleoyl-mono[$^{14}$C]oleoylglycerol (8.84 ± 1.30 nmol/mg of microsomal membrane) was generated, which survived washing through oil (Fig. 4). Because DGAT I was deprived of one of its two substrates, namely acyl-CoA, this TAG most plausibly originated from DGAT II activity.

*Delivery of Acyl Moieties across the Microsomal Membrane to DGAT II*—Distinct overt and latent forms of microsomal CAT have been independently isolated and purified in two different laboratories (1, 46). This suggests that carnitine-mediated transfer of acyl moieties across the microsomal membrane might occur. However, whereas mitochondria and peroxisomes have been shown to possess a carnitine-acylcarnitine translocase system (8, 47), such a system has not been detected in microsomes (47). Nevertheless, acylcarnitine (but not acyl-CoA) transfer across the endoplasmic reticulum membrane does occur, perhaps by a gated channel mechanism (47). In such a system, acyl-CoA (which is impermeable to microsomal membranes (45, 47)) is converted by CAT I into its corresponding acylcarnitine. This crosses the membrane via a gated channel and is re-esterified to acyl-CoA by a CAT II-catalyzed reaction. The experiment represented in Fig. 4 supports the existence of such a system. When all of the assay components were present, the amount of the [$^{14}$C]acyl moiety incorporated into the newly synthesized luminal TAG by DGAT II was 11.58 ± 2.36 nmol/mg of fused microsomal protein. This amount was ~140-fold higher than that produced when carnitine was omitted from the medium and ~236-fold higher than that produced when microsomes were devoid of CoASH (Fig. 4). In the former case, acylcarnitine would not have formed and in the latter case, luminal acylcarnitine would not have been reconverted to acyl-CoA. To prove that the availability of acyl-CoA within the microsomal lumen is critical for the DGAT II reaction, the system was bypassed by directly loading acyl-CoA into the microsomes by fusing them with liposomes encapsulating 50 μM [$^{14}$C]oleoyl-CoA. In these microsomes, despite being devoid of CoASH and deprived of exogenously added carnitine, appreciable amounts of the [$^{14}$C]oleoyl moiety (8.84 ± 1.30 nmol/mg of fused microsomal protein) were incorporated into newly synthesized luminal TAG (Fig. 4). When microsomes were fused with liposomes containing 50 μM [$^{14}$C] oleoyl-CoA and 8 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), there was a high level of inhibition (71.0 ± 8.3%) of luminal synthesis of TAG (data not shown). Because DTNB is unable to penetrate the membrane (48), this suggests that one or more thiol groups are at or sufficiently near to the active center of DGAT II such that the enzyme is inhibited when they are reacted with DTNB. DTNB has been previously shown to strongly inhibit acyl-CoA:cholesterol acyltransferase (49), which is genetically and structurally related to DGAT (50), but to have no effect on microsomal lipase activity (51).

*Effect of Glybenclamide on the Production of Luminal TAG*—When the sulfonylurea drug glybenclamide (220 μM), a potent inhibitor of CAT I (4), was included during the incubation of microsomes with tri[$^{3}$H]TAG but removed by washing the mi-

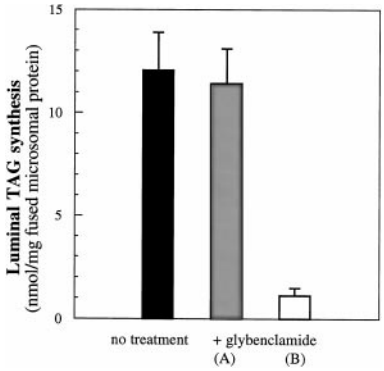


FIG. 5. **Influence of glybenclamide on the luminal synthesis of TAG in fused microsomes.** The amount of newly synthesized luminal TAG was determined in microsomes, previously fused with biotinylated liposomes containing 16 mM CoASH and 2 mM $Mg^{2+}$, in the absence (no treatment) (■) and presence of 0.2 mM glybenclamide. The addition of glybenclamide was made 5 min before either the initiation of TAG hydrolysis to membrane-bound DAG (▨) and then removed at the end of lipase-catalyzed reaction by centrifugation through dibutyl phthalate (*A*) or before the initiation of the CAT I reaction (□) (*B*).

crosomes through dibutyl phthalate prior to the addition of [$^{14}$C]oleoyl-CoA, the production of luminal TAG was only marginally reduced (5.3%; see Fig. 5). This indicates that microsomal lipase, unlike lysosomal lipase (6), is not affected by sulfonylurea drugs. However, if glybenclamide was present during the incubation of the microsomes with [$^{14}$C]oleoyl-CoA, *i.e.* during the CAT I-dependent transfer of [$^{14}$C]oleoyl-CoA into the microsomes, the production of TAG in the lumen was markedly reduced by 90.8% (Fig. 5). This suggests that CAT I is an essential component of the pathway involved in TAG synthesis within the microsomal lumen.

*A Postulated Pathway for TAG Synthesis within the Microsomal Lumen*—The concept of a lipolysis-esterification pathway being involved in the secretion of VLDL TAG was previously suggested to explain the differences in fatty acid composition of TAG in the liver and in VLDLs (40). However, the molecular mechanism and intracellular location of the enzymes responsible for lipolysis and re-esterification have been the subject of conjecture (6) and have remained obscure (34). Microsomal lipase has been suggested as a candidate for the lipolysis of cytosolic TAG (17, 51), which is an integral step in the proposed VLDL-TAG lipolysis-esterification cycle (6). Recently, Lehner *et al.* (34) have ruled out a possible contribution from lysosomal lipase and/or hormone-sensitive lipase in this process and have shown that microsomal lipase is confined to the centrilobular regions of the liver where lipoprotein assembly and secretion would be expected to occur. In addition, cells that are impaired in VLDL assembly/secretion lack microsomal lipase (34). These findings provide strong support for the involvement of microsomal lipase in the VLDL-TAG lipolysis-esterification cycle. This enzyme has been shown to reside on the cytoplasmic side of microsomal vesicles as is evident from its susceptibility to limited proteolysis (17).

The enzymes involved in the proposed pathway for intraluminal TAG synthesis are microsomal lipase and CAT I, which act on the cytosolic side of microsomal membrane, and CAT II and DGAT II, which act on the luminal side of microsomal membrane. Although our study has demonstrated that microsomal transfer protein is not required for intraluminal synthesis of TAG (data not shown) it could be involved in the subsequent utilization of this TAG in the assembly of VLDL. Microsomal transfer protein, which is localized within the microsomal lumen (52), has been shown to be required for the lipidation of apoB (53, 54). The critical role of microsomal transfer protein in the assembly and secretion of VLDL has been supported by detailed studies in liver-specific microsomal transfer protein knockout mice (55, 56).

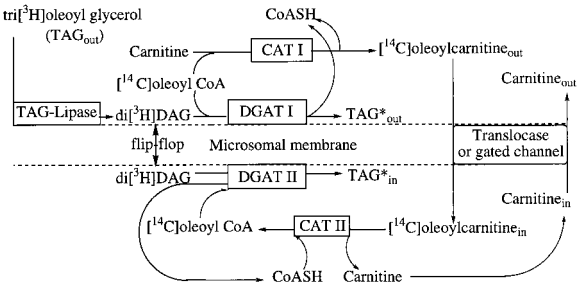


FIG. 6. **Enzyme reactions involved in the synthesis of TAG in the extra- and intraluminal spaces of the microsomes.** The $^3$H isotopic label of the acyl moieties at positions *sn*-1 and *sn*-2 of the tri[$^3$H]oleoyl glycerol were retained and that at the position *sn*-3 was replaced by the [$^{14}$C]oleoyl moiety in the nascent TAG. Symbols are: CAT I and II and DGAT I and II, overt (*I*) and latent (*II*) microsomal carnitine- and diacylglycerol-acyltransferases; *TAG**, 1,2-di[$^3$H]oleoyl-3-[$^{14}$C]-*sn*-oleoylglycerol. Subscripts *in* and *out* refer to the luminal and extraluminal spaces of the microsomes, respectively.

In conclusion, a proposed pathway, by which intraluminal microsomal TAG is derived from cytoplasmic TAG and acyl-CoA, is suggested to be as follows. First, microsomal TAG lipase generates membrane-bound DAG from TAG. Second, CAT I and CAT II work sequentially to generate acyl-CoA inside the microsomes. Third, DGAT II utilizes this intraluminal acyl-CoA to synthesize TAG from the membrane-bound DAG, which is membrane-diffusible and thus exists on both sides of the membrane. The pathway is depicted in Fig. 6, which shows the radiolabeled precursors and products used experimentally to confirm this pathway.

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