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LIPOLYTIC CLEAVAGE OF DOLICHYL OLEATE CATALYZED BY CALF BRAIN MEMBRANES

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SUMMARY: Calf brain membranes catalyze the lipolytic cleavage of dolichyl [^{14}C]oleate added as an aqueous dispersion in Triton X-100. The enzymatic release of [^{14}C]oleate from the dolichyl ester is not affected by divalent cations or EDTA, but the lipase activity is inhibited by iodoacetamide and pHMB. The amount of [^{14}C]oleate released is dependent on the time of incubation, the amount of membrane protein added and the concentration of the radiolabeled lipid substrate. Dolichyl ester hydrolase activity exhibits a pH optimum of 7.5, distinguishing this lipase activity from cholesteryl ester hydrolase (5.0-5.5) and triolein hydrolase (5.0) activity associated with the same membrane preparations. The enzymatic hydrolysis of dolichyl [^{14}C]oleate is also partially inhibited by oleate and free dolichol, possibly by end-product inhibition.

The function of dolichyl monophosphate as a eukaryotic glycosyl carrier lipid in the biosynthesis of asparagine-linked oligosaccharides has attracted considerable attention from researchers during the past decade (1). In addition to the phosphomonoester, dolichol is also present in animal tissues as the free polyisoprenol and the fatty acyl ester (2,3). While there is good evidence for the biosynthesis of the fatty acyl esters occurring by a transesterification reaction with phosphatidylcholine acting as the acyl donor (4), there is, as yet, no direct evidence for the enzymatic deacylation of dolichyl esters.

This paper presents the first enzymatic studies indicating that membranes from central nervous tissue are capable of catalyzing the lipolytic cleavage of dolichyl oleate. Some properties of the calf brain lipase activity have been examined. Although further work will be required to conclusively define the exact specificity and function of the enzyme hydrolyzing dolichyl oleate in these *in vitro* studies, the release of oleate from the polyisoprenyl ester does not appear to be due to the non-specific action of cholesteryl ester hydrolase or triacylglycerol hydrolase activities associated with the same membrane preparations.

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If dolichyl esters represent a reserve pool of dolichol that can be converted to the "activated" form of the glycosyl carrier lipid, the activation process would require a lipase, like the activity described here, to regenerate the preformed polyisoprenol to be phosphorylated by the CTP-mediated kinase (5,6). Enzymatic evidence for a change in the level of dolichyl monophosphate available to mannosylphosphoryldolichol synthase in developing brain was presented in earlier papers (7,8). It will be interesting to see if de novo biosynthesis of dolichyl monophosphate can be augmented during developmental changes in the central nervous system by the utilization of dolichyl esters as a metabolic source of preformed dolichol.

MATERIALS AND METHODS

Materials: Dolichyl [^{14}C]oleate was chemically synthesized according to the procedure described below, and a sample of dolichyl [^{14}C]oleate, prepared similarly, was a generous gift from Dr. L.S. Wolfe, McGill University. Cholesteryl [^{14}C]oleate (51 μCi/μmole), [^{14}C]triolein (95 μCi/μmol) and [^{14}C]oleic acid (57 μCi/μmol) were purchased from New England Nuclear. Bovine brain phosphatidylcholine was obtained from Serdary Research Laboratories, Inc. Precoated thin layer sheets of Silica Gel 60 F-254, produced by EM Laboratories, Inc., were from VWR Scientific. Dolichol (Grade I), other unlabeled lipids and bovine serum albumin were obtained from Sigma Chemical Co. National Diagnostics was the supplier of Liquiscint.

Preparation of calf brain membranes: A crude microsomal fraction from whole calf brain was obtained by the procedure described for pig brain (9). For comparison of the microsomal and myelin fractions, calf brain white matter was used. The 39,000 xg (microsomal) pellet was further fractionated by centrifugation through 0.9 M sucrose to remove myelin (10). Purified myelin was prepared according to the procedure of Norton (11). All membrane fractions were suspended in 0.1 M Tris-HCl, pH 7.1, 0.25 M sucrose, 1.0 mM EDTA. The protein concentration of the suspensions was determined by the method of Lowry et al. (12).

Synthesis of dolichyl oleate: [^{14}C]oleic acid (32.4 μmoles, 4 μCi/μmol) was converted to the acid chloride during a 2 h reaction with oxalyl chloride (97.2 μmoles) in benzene solution (13). Excess oxalyl chloride was removed with N_2 and the addition of fresh oxalyl chloride was repeated. After 2 h the reaction mixture was dried with N_2, and dolichol (10.8 μmoles), dissolved in 1.0 ml of dry benzene, and pyridine (86.9 nmoles) were added for the esterification reaction. After 2 h at room temperature the sample was dried with N_2 and benzene and water were added. The lipids in the organic phase were purified by chromatography on thin layer plates of Silica Gel H developed with solvent A) hexane-diethyl ether (80:20). The ester, located by autoradiography was eluted with $CHCl_3$. Only a single radioactive compound with the mobility of unlabeled dolichyl oleate (prepared in the same way) was detected when the purified lipid was analyzed by thin layer chromatography with solvent systems B) petroleum ether-diethyl ether (90:1), C) CCl_4, and D) toluene. An anisaldehyde spray reagent was used to detect the unlabeled polyisoprenoid compounds (14).

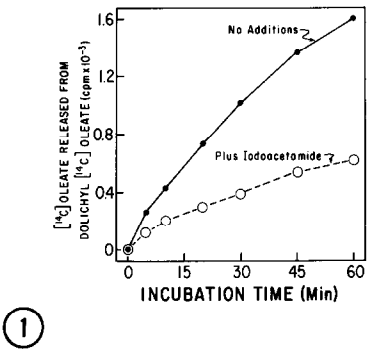
Assay for the release of [^{14}C]oleate from exogenous dolichyl [^{14}C]oleate, cholesteryl [^{14}C]oleate and [^{14}C]triolein: Dolichyl ester lipase was assayed by following the enzymatic release of [^{14}C]oleate from exogenous dolichyl [^{14}C]oleate using a

modification of the procedure described by Khoo et al. (15) for lipase activity. Enzyme reactions were terminated by the addition of 2.5 ml of $CHCl_3$–CH_3OH–benzene (2:2.4:1) containing 0.1 mM oleic acid. Carrier oleic acid was omitted when the reaction product was prepared for chromatographic analysis. The suspension was agitated with a vortex mixer and the membrane residue was sedimented by centrifugation (1,500 rpm, 7 min) in an IEC HN-S centrifuge. The lipid extract was saved and the membranous residue was washed with 0.5 ml of the organic solvent mixture. The lipid extracts were pooled and chilled on ice for 5 min. After chilling, 0.3 ml of either 0.033 or 0.33 N NaOH was added. The samples were vigorously mixed and a two phase system was produced by centrifugation (1,500 rpm, 1 min). The upper phase was saved and the lower phase was washed twice with 1.6 ml of synthetic upper phase. The pooled upper phases were transferred into a scintillation vial and dried by a stream of air in a water bath (40–50°). The radioactivity recovered in the upper phases was measured after the addition of 0.5 ml of 1.0% SDS and 10 ml of Liquiscint. The enzymatic release of [^{14}C]oleate from exogenous cholesteryl [^{14}C]oleate and [^{14}C]triolein was assayed by the same procedure. The radiolabeled product recovered in the upper phase cochromatographed with authentic oleic acid when chromatographed on thin layer sheets of Silica Gel 60 F-254 developed with solvent mixtures A) and E) hexane–diethyl ether–glacial acetic acid (80:20:1). The labeled product was located by autoradiography using Kodak No-Screen X-ray film. Unlabeled oleic acid was detected by spraying with a 0.001% solution of Rhodamine 6G followed by UV irradiation.

Determination of radioactivity: The release of [^{14}C]oleic acid was monitored by counting the samples in 10 ml of Liquiscint. All data were average values obtained by counting each sample for 10 min in a Packard Tri-Carb Scintillation Spectrometer.

RESULTS AND DISCUSSION: When calf brain microsomes were incubated with exogenous dolichyl [^{14}C]oleate, added in an aqueous dispersion with Triton X-100 (final concentration = 0.04%), there was a time-dependent release of [^{14}C]oleate for 60 min (Figure 1). The enzymatic release of labeled fatty acid was inhibited by Triton X-100 and sodium taurocholate when the detergent concentration exceeded 0.06%. The calf brain lipase activity was also inhibited 61% by 63 mM iodoacetamide (Figure 1) and 54% by 2 mM pHMB. The dolichyl ester hydrolase activity was dependent on the amount of membrane protein and the concentration of exogenous dolichyl [^{14}C]oleate added to the incubation mixture. An accurate determination of an apparent Km for the polyisoprenyl ester was not possible due to the difficulty encountered in preparing aqueous dispersions of dolichyl oleate in concentrations high enough to saturate the calf brain lipase. The dolichyl oleate hydrolase activity was virtually unaffected by $MgCl_2$, $MnCl_2$, $CaCl_2$ or EDTA added at 20 mM. Dolichyl oleate hydrolysis was optimal at pH 7.5. This result indicates that the enzyme(s) catalyzing the deacylation of dolichyl oleate is probably not a lysosomal hydrolase.

A crucial objective for future studies will be to establish the specificity of the neutral lipase activity hydrolyzing exogenous dolichyl oleate under these *in vitro* condi-



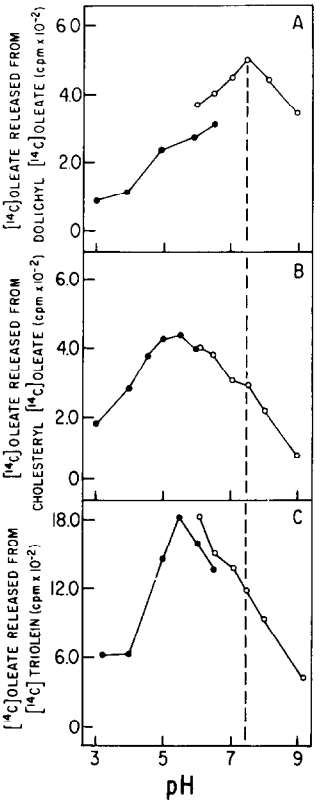


Figure 1. Time course for the enzymatic release of [^{14}C]oleate from dolichyl [^{14}C]oleate catalyzed by calf brain membranes in the presence and absence of iodoacetamide. The reaction mixtures consisted of calf brain membranes (14 mg membrane protein); 50 mM Tris-HCl (pH 7.1); 125 mM sucrose; 0.5 mM EDTA; 20 mM $MgCl_2$; 4 mg bovine serum albumin and 0.073 mM dolichyl [^{14}C]oleate (3,579 cpm/nmol) dispersed in Triton X-100 (final concentration = 0.04%) in a total volume of 0.8 ml. The reaction mixtures were incubated in the presence (O—O) or absence (●—●) of 63 mM iodoacetamide at 37°. At the indicated period of time 0.1 ml samples were withdrawn and transferred into 2.5 ml of $CHCl_3$-CH_3OH-benzene (2:2.4:1), containing 0.1 mM oleic acid. The amount of [^{14}C]oleate released at each time point was measured as described in MATERIALS AND METHODS.

Figure 2. Effect of varying the pH on the enzymatic hydrolysis of dolichyl [^{14}C]oleate (Panel A), cholesteryl [^{14}C]oleate (Panel B) and [^{14}C]triolein (Panel C) catalyzed by calf brain membranes. The reaction mixtures contained calf brain membranes (2.5-2.7 mg membrane protein); 50 mM Tris-HCl (O—O) or 50 mM sodium acetate (●—●) at the indicated pH; 125 mM sucrose; 0.5 mM EDTA; 20 mM $MgCl_2$; 1 mg bovine serum albumin and either 0.014 mM dolichyl [^{14}C]oleate (8,100 cpm/nmol), 0.011 mM cholesteryl [^{14}C]oleate (8,100 cpm/nmol) or 0.009 mM [^{14}C]triolein (8,565 cpm/ nmol) and 0.04% Triton X-100 in a total volume of 0.2 ml. After a 20 min incubation at 37° the amount of [^{14}C]oleate released from each lipid substrate was measured as described in MATERIALS AND METHODS.

tions. Since cholesteryl ester hydrolase (16,17) and glyceride lipase activities (18,19) have been previously reported for brain membranes, a preliminary attempt was made to exclude the possibility that the dolichyl ester was being enzymatically deacylated by the non-specific action of one of these lipases. From the results depicted in Figure 2 it can be seen that the lipolytic activity cleaving dolichyl oleate can be distinguished from cholesteryl ester hydrolase (5.0-5.5) and triglyceride lipase (5.0) activity associated with the same membrane preparations on the basis of their pH optima. Moreover, although the lipases are similarly sensitive to detergents, the dolichyl ester hydrolase activity detected in these studies is apparently distinct from the brain lipase that hydrolyzes dioleoylglyceride optimally at pH 4.8, and the alkaline lipase that cleaves monooleoylglyceride maximally at pH 8.0-8.6 (18). It is also unlikely that dolichyl oleate is being hydrolyzed indiscriminately by the calf brain phospholipase A_1 described by Gatt (20) because that enzyme is optimally active at pH 4.0 in detergent concentrations that inhibit dolichyl ester hydrolysis.

It was also of interest to test the effect of other lipids on the enzymatic hydrolysis of dolichyl [^{14}C]oleate. A limited survey (TABLE I) revealed that addition of free dolichol (4.3-fold) and oleic acid (12.9-fold) in excess of the lipid substrate produced 49% and 76% inhibition, respectively, of lipase activity. This result may be due to end-product inhibition. Cholesteryl oleate (76%) and triolein (69%) also caused partial inhibition when added in a 4.3-fold excess of the radiolabeled substrate. It is possible that these results could be partly accounted for by the production of oleic acid during the incubation period. Consistent with this conjecture, the inhibitory effects were reduced by the addition of bovine serum albumin to the incubation mixtures. Monoolein, cholesterol and phosphatidylcholine did not appreciably affect the lipolytic reaction when included in the assay mixtures at a 4.3-fold excess.

Since one form of cholesteryl ester hydrolase has been found associated with myelin (17), the level of dolichyl ester lipase activity in myelin was compared with the activity bound to the microsomal fraction. However, myelin prepared from bovine white matter by the procedure of Norton (11) was found to contain a very low level of dolichyl ester lipase as compared to activity associated with the microsomal fraction (TABLE

TABLE I. Effect of various lipids on the enzymatic hydrolysis of dolichyl [^{14}C]oleate.

lipid added	[^{14}C]oleate release
(0.1 mM)	(% of control)
none	100
dolichol	49
oleic acid (0.3 mM)	76
cholesteryl oleate	76
cholesterol	100
triolein	69
monoolein	88
phosphatidylcholine	90

Reaction mixtures contained calf brain membranes (3.5 mg membrane protein); 50 mM Tris-HCl (pH 7.1); 125 mM sucrose; 0.5 mM EDTA; 20 mM $MgCl_2$; 0.023 mM dolichyl [^{14}C]oleate (3,579 cpm/nmol) and the indicated lipid in 0.04% Triton X-100 in a total volume of 0.2 ml. Following a 15 min incubation at 37°, the reactions were terminated as described in Figure 1 and the extent of dolichyl [^{14}C]oleate hydrolysis was measured by the procedure in MATERIALS AND METHODS.

II). A more detailed study on the subcellular distribution of this activity will be important for ultimately determining its function and possible relationship to glycoprotein biosynthesis.

Efforts are now underway to solubilize and purify the calf brain enzyme catalyzing the lipolytic cleavage of dolichyl oleate to conclusively define its properties and specificity. If the lipase reported here proves to be specific for fatty acyl esters of dolichol, one function may be to provide free, preformed dolichol that could be converted to the glycosyl carrier lipid by dolichol kinase in brain, and perhaps extra-neural tissues.

TABLE II. Comparison of dolichyl ester lipase activity in myelin and microsomal fractions from calf brain white matter.

subcellular fraction	[^{14}C]oleate released
	(pmol/mg membrane protein)
crude microsomal	110
myelin	18

Reaction mixtures contained white matter membranes (1.2-1.4 mg membrane protein); 60 mM Tris-HCl (pH 7.1); 188 mM sucrose; 0.6 mM EDTA; 20 mM $MgCl_2$; 1 mg bovine serum albumin; 0.04% Triton X-100 and 0.040 mM dolichyl [^{14}C]oleate (3,579 cpm/nmol) in a total volume of 0.2 ml. After a 15 min incubation at 37°, the amount of [^{14}C]oleate released was measured as in Figure 1.

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