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Intramyelinic Conversion of Cerebrosides into Acylgalactosylceramides

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Acylgalactosylceramide (AGC) synthesis was measured in vivo, and in a cell free system. 24 hours post-injection of [^{3}H]palmitic acid into rat brain, more than 60% of the AGC radioactivity was associated with an ester linkage. Isolated rat myelin was incubated in the presence of [^{14}C]palmitic acid, 2mM ATP, 50 μM CoA and 10 mM $MgCl_2$ and acylation of myelin cerebrosides occurred at a linear rate for at least 60 min. Incubation of isolated myelin under standard conditions with [^{3}H] cerebrosides and [^{14}C]palmitic acid produced double labeled AGC. Labeling of AGC was maximum at pH 7.5 and 37°C and appeared to be enzyme mediated inasmuch as it was reduced by myelin incubation with trypsin and drastically reduced by preheating the myelin for 5 min at 80°C. Omission of ATP, CoA, $MgCl_2$ or all three did not reduce fatty acid incorporation into AGC when compared to the values in the complete system. Addition of Triton X100 or Sodium Dodecyl Sulfate had little or no effect on the acylation of cerebrosides. Pulse chase experiments indicated that the reaction involved the net addition of fatty acid to the cerebrosides, rather than a rapid fatty acid exchange.

KEY WORDS: Acylgalactosylceramides; cerebrosides; myelin.

INTRODUCTION

The presence of cerebroside-esters in mammalian brain, spleen epidermis and stomach has been widely reported (1-10). In a previous study (10) we have demonstrated that cerebroside-esters from rat or mouse brains could be resolved into three fractions AGC I, II and III according to their decreasing Rf values. Structural analysis by GC-MS indicated that AGC II was a complex mixture of O-acylgalactosylceramides while the more polar AGC III were mainly galactosyl-N-ω-o-acyl ceramides. AGCs were shown to be components of myelin membranes and were probably involved in the formation and maintenance of myelin sheaths (11). The main feature underlined by the previous structural analysis is the highly specific nature of acylation with respect to the esterifying fatty acid and the cerebroside acceptor. The ester linked fatty acids of both o-acylgalactosylceramides and galactosyl N-ω-o-acylceramides are mainly palmitic and stearic acid. On the other hand, amide linked fatty acids are normally shorter than their counterparts in brain cerebrosides. In O-acylgalactosylceramides, N-acylation predominantly involves palmitic or stearic acid while in galactosyl N-ω-o-acyl ceramides dihydroxy stearic acid (2,18-OH C18:0) is also encountered (10).

Clearly, the fact that only cerebrosides containing C18 fatty acids (in distinction to C24 fatty acids) become acylated in brain, suggests the existence of acyltransferases which exhibit marked differences in specificity for acyl chain length of the cerebroside substrate (10). However to date, the synthesis of AGC has not been precisely delineated in any tissue.

The general pathways of glycosphingolipid metab-

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olism (12) point to two routes for AGC synthesis. One might begin by the acylation of ceramides prior to transfer of galactose, leading to the production of acylceramides and galactosyl N-ω-o-acylceramides. The other might proceed by the specific acylation of cerebroside precursors leading to the synthesis of both O-acylgalactosylceramides and galactosyl N-ω-o-acylceramides. Inhibition of the evolution of cerebroside fatty acid patterns in quaking mutants is consistent with the existence of two pools of cerebroside distinguishable on the basis of their content of C24 fatty acids (13) and supports the concept of this second pathway.

As a first step towards understanding the intracellular synthesis of AGC, we present evidence for a cerebral acylating activity and describe its enzymatic properties and cofactor requirements in myelin membranes.

EXPERIMENTAL PROCEDURE

Materials. Coenzyme A (CoA), ethylene glycol tetraacetic acid (EGTA), adenosine triphosphate (ATP), magnesium chloride ($MgCl_2$) sodium dodecyl sulfate (SDS) and galactocerebrosides from bovine brain hydroxylated or unhydroxylated were obtained from Sigma. Silica gel 60 HPTLC plates were obtained from Merck and U-[^{14}-C]palmitic acid (400-800 mCi/mmol) and [9,10 (n) ^{3}H]palmitic acid (40-60 Ci/mmole) were purchased from Amersham. All organic solvent were of analytical grade unless otherwise specified. Lipofluor and vials were obtained from Backer.

Myelin isolation. Twenty day old rats were killed by decapitation and the cerebrum homogenized in 0.32 M sucrose at 4°C. Purified myelin was obtained from the whole brain homogenate by the method of Norton et al (14) and stored in water at −20°C in small aliquots. All preparations were controlled for 2′3′ cyclic nucleotide 3′ phosphohydrolase activity (15) and analyzed by sodium dodecyl sulphate polyacrylamide gel to attest the purity of the sample (16).

SDS Polyacrylamide Gel Electrophoresis of Myelin Proteins. Prior to electrophoresis, labelled myelin proteins were further delipidated with ethanol/ether, 2/3 v/v (17), dried under nitrogen and dissolved in 100μl of 50 mM Tris-HCl pH 6.8 containing 2% SDS and 1% mercaptoethanol. Total protein (100 μg) was analyzed by electrophoresis on 10-20% gels using the discontinuous buffer system of Laemmli, (18). After electrophoresis, gels were stained with Coomassie Brilliant Blue R250 (19). For the determination of proteolipid protein radioactivity, gel corresponding to Proteolipid protein (PLP) was excised, placed into vials containing 0.3 ml of 30% H_2O_2 and incubated overnight at 50°C. Scintillation liquid (5ml) was then added and the radioactivity measured.

In vivo labeling Experiments. Twenty day old rats of either sex were used throughout. Animals were injected intracranially with 0.5mCi of [^{3}H]-fatty acid suspended by sonication in 8-20 μl of 150 mM NaCl, 5mM Tris-HCl buffer, (pH 7.5) containing 1% (w/v) fatty acid free bovine serum albumin. After 24 hr the animals were killed by decapitation, the cerebrum removed and homogenized in $CHCl_3/CH_3OH$ (2/1, v/v) for lipid analysis.

In Vitro Incubation Conditions. The standard procedure described by Bizzozero (20,21) was employed for in vitro experiments. Purified myelin (500 μg of total protein) was incubated in 0,4 ml of 20 mM Tris-HCl (pH 7.5), containing 2 mM ATP, 50 μM CoA and 10 mM $MgCl_2$. The reaction was started by the addition of the radioactive fatty acid after temperature equilibration. Incubation was carried out for 45 minutes at 37°C.

To stop the reaction, 2ml of $CHCl_3/CH_3OH$ (2/1, v/v) was added, and the mixture processed for lipid analysis.

Lipid Analysis. The lipids were extracted according to Folch's procedure (22) and chromatographed on TLC plates with $CHCl_3/CH_3OH/H_2O$ (40/10/1, v/v/v) as solvent mixture (7,8,10).

Spots were located with iodine vapor, scraped and transfered into vials containing 5 ml of counting solution (lipofluor). Radioactivity was assayed in a LKB Wallac 1214 rack beta liquid scintillation counter.

Saponification of AGC was performed in $CHCl_3$-CH_3OH-10M NaOH (2:7:1 ; v:v:v) (7). After neutralization with HCl, chloroform and water were added to give final concentrations of $CHCl_3$-CH_3OH-H_2O of 8:4:3 by vol. After partition, the lower phase was subjected to TLC analysis and radioactivity counting.

Preparation of [^{3}H]Cerebrosides. Twenty-day old rats were injected intracranially with 0.5 mCi of [^{3}H] palmitate as described for in vivo labeling experiments. Total lipids were extracted with $CHCl_3$-CH_3OH (2:1) and separated on HPTLC plates developed in $CHCl_3$-CH_3OH-H_2O (40:10:1 v:v:v). Lipids were identified by exposure to iodine vapor. Cerebrosides were extracted with $CHCl_3$-CH_3OH (2:1, v:v), dried under nitrogen, and suspended by sonication in 50μl of 20mM Tris HCl buffer (pH 7.5).

For double labeled experiments the ratio of [^{3}H]cerebrosides to [^{14}C]-fatty acids was always 10 to 1 to ensure good efficiency of counting.

RESULTS

In vivo Incorporation of [^{3}H]palmitate into Brain Lipids. Twenty four hours after intracranial injection of [^{3}H]palmitic acid, all brain lipids were labeled. Forty-seven percent of lipid radioactivity was found in brain phospholipids (fractions 2 and 3), and approximately 1,1% of the brain lipid radioactivity appeared in the AGC fraction. After AGC saponification, nearly 60% of the AGC radioactivity was recovered as free fatty acids suggesting that [^{3}H]palmitate was attached to the cerebroside via an ester linkage, and the remaining radioactivity was recovered in the cerebroside itself.

When compared to cerebrosides, which represent 25% of brain lipids, AGC (4 to 5% of total glycolipid) were clearly labeled, suggesting that acylation step of AGC precursors was very active. The ratio of radioactivity to lipid percentage was 0.056 for cerebroside and 0.56 for AGC.

Cell Free Acylation of Myelin Cerebrosides. Incubation of isolated myelin with [^{14}C]palmitic acid for 45 min in the presence of 2 mM ATP, 50 μM CoA and 10 mM $MgCl_2$, labeled all the lipids. Although phospholipids and cerebrosides were the major species that incorporated the [^{14}C] fatty acid, AGC was also radioactive.

The profile of labeling was similar to that obtained in vivo.

To ensure that labeling of AGC was due to fatty acylation of endogenous precursors, purified AGC were submitted to mild alkaline hydrolysis. More than 60% of the radioactivity was again extractable with hexane, suggesting that the [^{14}C]fatty acids were incorporated into the AGC via an ester linkage. Moreover, after incubation of isolated myelin (500μg protein) with [^{14}C]palmitic acid (0.05 μCi) and [^{3}H] cerebrosides (0.1 - 0.5 μCi) under the standard conditions described in methods, the AGC were double labeled (Figure 1). The presence of [^{3}H, ^{14}C]AGC in the myelin lipid extract strongly suggested that esterification of exogenous as well as endogenous cerebrosides was occurring naturally in myelin membranes, inasmuch coincubation of [^{14}C]palmitic acid and [^{3}H]cerebrosides in the absence of myelin did not generate double labeled AGC.

Requirements of Cofactors for Cerebroside Acylation. Under the standard conditions (2mM ATP, 50μM CoA, 10mM $MgCl_2$) the pH optimum for acylation of cerebrosides and AGC synthesis was 7.5 (Figure 2a) and the temperature optimum was 37°C (Figure 2b). Under these optimal conditions, acylation of cerebrosides was linear for at least 60 minutes (Figure 2c).

Incubation at 4°C, decreased the labeling of AGC to 10% of control values, whereas preheating the myelin for 5 min at 80°C completely inhibited the acylation reaction. Moreover when myelin membranes were preincubated for 20 minutes at 37°C in the presence of 0.2% trypsin (w/v), the incorporation of [^{14}C]palmitate into phospholipids, cerebrosides and AGC was lowered and represented ony 24% of the control value. These results strongly suggest that acylating enzymes and the pool of endogenous AGC precursors available for acylation are present in myelin membranes.

Omission of CoA, ATP, Mg^{2+} or all three cofactors reduced fatty acid incorporation into the phospholipid rich fraction to 32%, 5%, 45 and 5%; and into cerebrosides to 42%, 50%, 84%, and 30% respectively, but did not affect the acylation of cerebrosides into AGC (Figure 3). Fatty acid activation thus did not appear to be an absolute requirement for the in vitro acylation of cerebrosides and their metabolic conversion into AGC.

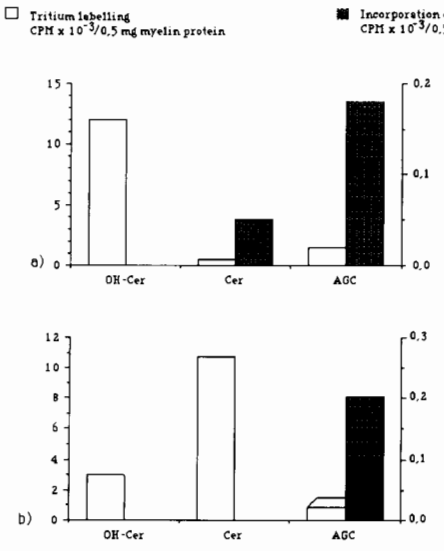

Fig. 1. In vitro synthesis of double labeled Acylgalactosylceramide. Incubation of myelin (500 μg, protein) in the presence of a) [^{3}H]hydroxylated cerebrosides (OH-Cer) or b) [^{3}H]cerebrosides (Cer) and [^{14}C]palmitic acid was performed under the standard conditions (2 mM ATP, 50 mM CoA, 10 mM $MgCl_2$ in 0.4 ml of 20 mM Tris-HCl). The results are expressed as the mean of five experiments (SD < 10%).

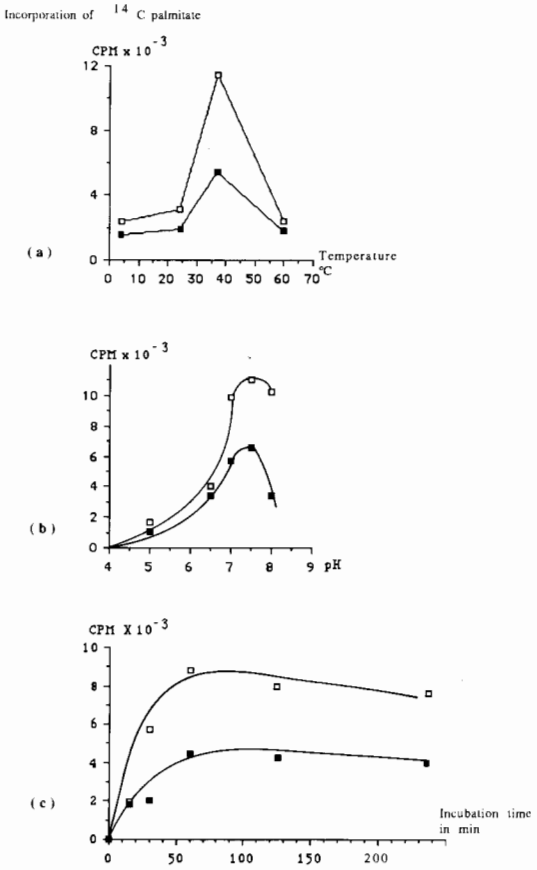

Fig. 2. Influence of temperature (a), pH (b) and incubation time (c) on the incorporation of [^{14}C] palmitate in cerebrosides (□) and acylgalactosylceramides (■). The incubation of myelin were performed under standard conditions as described in the experimental procedure. The results are expressed as the mean of five experiments (SD < 10%).

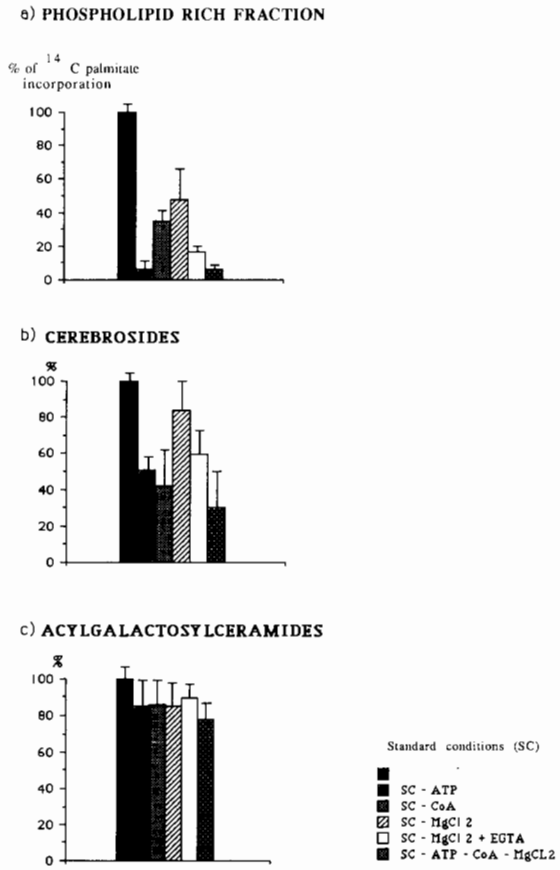

Fig. 3. Cofactor requirements for the incorporation of [^{14}C] palmitate in myelin lipids in a cell-free system. a) Phospholipid rich fraction, b) cerebrosides, c) acylgalactosylceramides. Results are expressed as mean ± SD (<10%) of five experiments.

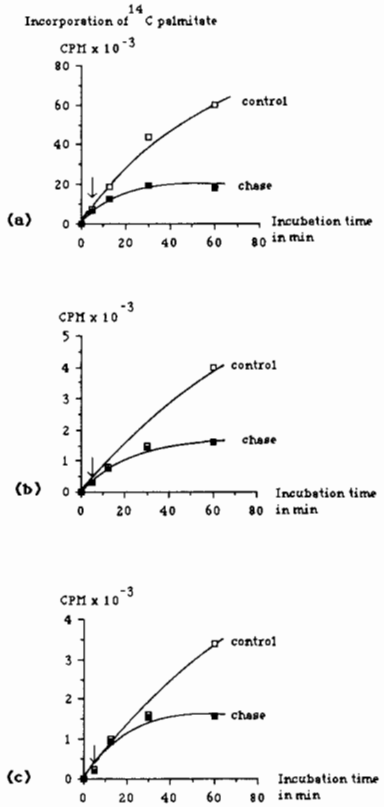

Fig. 4. Effect of chase with unlabeled palmitic acid on the labeling of (a) phospholipid rich fraction, (b) cerebrosides and (c) acylgalactosylceramides. Purified myelin was incubated with 4 mM [^{14}C] palmitate as described under experimental procedure. After 5 minutes, unlabeled palmitic acid (20 mM) was added (arrows) and incubation continued for 55 minutes. The results are expressed as the mean of three experiments (SD <10%).

Pulse Chase Experiment. To determine the stability of the label, pulse chase experiments were carried out. Purified myelin was incubated with [^{14}C]palmitic acid under the standard conditions for 5 min. Excess of unlabeled palmitic acid was then added, and the incubation was continued for another 35 min (Figure 4). Addition of unlabeled palmitic acid stopped almost immediately the incorporation of the radioactivity into the myelin phospholipid rich fraction and into cerebrosides. The arrest of fatty acid incorporation in AGC was delayed, indicating that fatty acid-CoA is not the unique donor for acylation. These results are in good agreement with those above suggesting that AGC labeling is not dependent on fatty acid activation.

The label remained essentially stable after the chase, demonstrating that rapid fatty acid exchange was not occurring under these conditions.

Kinetic Properties. Isolated myelin (500μg protein) was incubated under standard conditions at 37°C for 45 min in the presence of increasing amounts of [^{14}C]palmitic acid. After 45 min the relationship between the rate of radioactivity incorporation and the concentration of labeled fatty acid was sigmoid (Figure 5).

Fatty Acid Donors. The effect of different detergents on the acylation of cerebrosides is shown in Table I. In general, the specific transfer of fatty acid to cerebrosides was not modified by any detergent.

In contrast, addition of SDS in the incubation medium drastically reduced the labeling of proteolipid proteins and the phospholipid rich fraction but had little or no effect on the labeling of myelin AGC. Moreover, the presence of 0.1% Triton X 100, known to markedly stimulate the specific transfer of palmitic acid from acyl-CoA to myelin PLP (21) exhibited only a slight inhibitory effect on labeling of the phospholipid rich fraction and had no effect on AGC labeling.

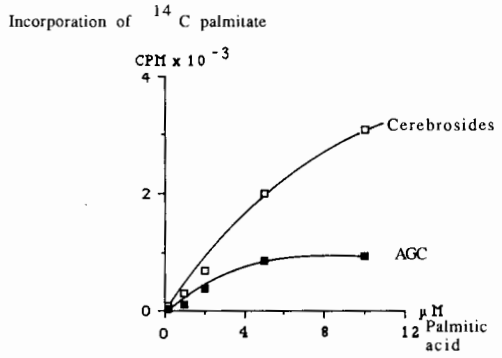

Fig. 5. Effect of concentration of [^{14}C] palmitate on the labeling of cerebrosides and acylgalactosylceramides in the standard conditions. Results are expressed as the mean of three experiments (SD <10%).

Table I. Effect of Triton X100 and SDS on the Incorporation of [^{14}C]Palmitate into Brain lipids and PLP

	AGC	Cb	PL	PLP
Standard conditions	100	100	100	100
+Triton 0,1%	93	91	64	-
+SDS 0,1%	91	97	50	-
+SDS 0,5%	100	100	6	15
+SDS 2%	100	100	4	9

Values represent the average of three experiments (SD <8%).

DISCUSSION

As AGC was shown to be myelin constituents and early markers for differentiation of myelin producing cells (11), the specific goal of this work was to examine their metabolism in myelin membranes. Although the structure of AGC has been extensively described, no studies on their synthesis have been reported. The present investigation suggests that myelin membranes are responsible for the conversion of cerebrosides into AGC through an acylation step.

Injection of radiolabeled [^{3}H]palmitic acid into the brain resulted in the labeling of AGC. The [^{3}H]AGC could be deacylated by mild alkali, and hexane extraction showed that the released acid was labeled suggesting that the additional fatty acid had been incorporated in the AGC via an ester linkage.

Purified myelin membranes were also able to acylate endogenous cerebrosides in a cell free system in the presence of ATP, CoA and $MgCl_2$. The profile of labeling was similar to that obtained in vivo. Double-labeled AGC were also synthesized in the cell free system upon incubation of purified myelin in the presence of [^{14}C]palmitic acid and [^{3}H]cerebrosides, indicating that AGC synthesis pathway proceeds at least in part by specific acylation of myelin cerebrosides.

Labeling of AGC was maximal at pH 7.35 and 37°C and appeared to be enzyme mediated as it was drastically reduced by preheating the myelin preparations for 5 min at 80°C or by proteolysis with trypsin. Incubations performed at 4°C resulted in complete inhibition of the labeling reaction.

In contrast to the dependence of phospholipid and cerebroside labeling on fatty acid activation, palmitate attachment to cerebrosides was relatively insensitive to the complete omission of ATP, CoA, $MgCl_2$ or all three cofactors in the incubation medium. Fatty acid CoA synthetase which is highly purified from rat myelin (23) has an absolute requirement for ATP or $MgCl_2$. It is therefore unlikely to be involved in the AGC labeling.

Cerebroside acylation in myelin could be enzyme mediated without requiring cofactors involved in fatty acid activation. Several lipid metabolizing enzymes have been shown to transfer acyl moities via an autoacylation process. In this regard, two different cholesterol esterifying enzymes have been described; one with optimum activity at pH 7.4 required cofactors for fatty acid activation (24), while the other one with optimum activity at pH 5.2 had no dependence on ATP or CoA (25). The involvement of such fatty acid activation independent enzymes in AGC synthesis is likely.

Since phospholipids and glycolipids were the major species that incorporated the radioactivity, we must consider the possibility that acylation was performed via these intermediates. The inability of phospholipids or glycolipids to serve as direct donors was confirmed by the fact that fatty acid-CoA is the immediate acyl chain donor for acylation of these lipids. In the absence of ATP, CoA and Mg^{2+} in the incubation medium reduced drastically the labeling of phospholipids, but not of AGC. Moreover incubation of myelin in the presence of 0.5 % SDS strongly inhibited phospholipid labeling without affecting AGC.

PLP could also act as acyl chain donor. Autocatalytic regulation of PLP has been shown to take place close to or within the myelin membranes (20,26) and was observed only in the presence of cofactors required for fatty acid acylation. Omission of ATP, CoA, Mg^{2+} or all three reduced fatty acid incorporation into PLP to 27, 44, 8, and 4% respectively (21,27). Moreover, PLP acylation was strongly inhibited by SDS. The presence of 0.5% SDS in the incubation medium strongly reduced acylation of PLP to less than 15% of normal while AGC labeling remained unmodified. The data from this experiment did not permit a definitive conclusion regarding

the nature of the acyl donor for AGC synthesis. Since myelin proteolipid proteins constitute about 50% of the total protein content of myelin and contain 2-4% by weight of fatty acid (28), they cannot be ruled out as the acyl donor. The 15% residual labeling of PLP encountered during SDS incubation represented fatty acid amounts sufficient for AGC synthesis.

Although the precise nature of the fatty acid donor is still unclear, our data point to myelin membranes as being responsible for the enzymatic acylation of cerebrosides and AGC synthesis. The precise role of cerebroside acylation and AGC in myelin is unknown, but as suggested previously (7,10,11), they could contribute to the stabilization of the multilamellar organization of myelin sheaths.

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