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ACYLATION OF MONOACYLGLYCEROPHOSPHOETHANOLAMINE IN THE INNER AND OUTER MEMBRANES OF THE ENVELOPE OF AN *ESCHERICHIA COLI* K12 STRAIN AND ITS PHOSPHOLIPASE A-DEFICIENT MUTANT

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Summary

The site of the *Escherichia coli* envelope of the conversion of 1-acylglycero-3-phosphoethanolamine to diacylglycerophosphoethanolamine was explored, using two K12 strains with a wild-type phospholipid-degradative apparatus and a K12 mutant lacking detectable phospholipase A_1 and A_2 activity.

Experiments with various radioactively labeled substrates show that acylation by crude envelope preparations as well as isolated inner and outer membranes of parent and mutant strains involves neither exogenous fatty acids nor a transacylation reaction with added monoacylglycerophosphoethanolamine. Furthermore, acylation exhibits no absolute requirement for added ATP and coenzyme A.

Specific activity of acylating activity is the same in inner membrane preparations of parent and mutant strain and in outer membrane preparations of the mutant deficient in phospholipase A. Although clearly evident, net diacylglycerophosphoethanolamine formation by outer membranes of the parent strain, however, was about 6-fold less. This lower conversion may be attributed to activation during incubation of phospholipases A within the outer membrane, resulting in breakdown of the diacylcompound formed.

Reacylation of lysophospholipids formed in the *E. coli* envelope by the action of endogenous or exogenous phospholipases A provides the organism with the potential of biochemically inexpensive repair and modification of the envelope phospholipids. Moreover, major phospholipids hydrolyzed in the outer membrane of *E. coli* can be resynthesized in the same location, without need for the transport of the products of hydrolysis to the lipid biosynthetic apparatus associated with the cytoplasmic membrane.

Introduction

The enzymes concerned with the de novo synthesis of phospholipids in *Escherichia coli* and other gram negative bacteria appear to be mainly confined to the cytoplasmic membrane of the bacterial envelope [1—3]. By contrast, phospholipid degradative enzymes are prominently associated with the outer membrane [1,4]. It has been suggested that the anatomical separation of enzymes concerned with the synthesis and the degradation of phospholipids may serve a regulatory function [1].

In the presence of agents and conditions unfavorable to *E. coli*, turnover of phospholipids is increased, apparently because hydrolysis of (outer) membrane phospholipids is activated [5—9]. The increased incorporation of added labeled lipid precursors that often accompanies the increased degradation perhaps reflects the postulated regulation of bacterial phospholipid content and composition. Increased synthesis of phospholipid from substrate added to the medium or resynthesis from degradation products derived from endogenous lipids would require the migration of the precursor from or through the outer membrane to the site of biosynthesis in the cytoplasmic membrane in a manner akin to the translocation of the synthesized phospholipids back to the outer membrane.

In addition to the phosphatidic acid pathway, *E. coli* possesses monoacylglycerophosphoethanolamine acylase activity [10]. Phospholipid turnover in *E. coli* may, therefore, also involve a monoacyl-diacyl phospholipid cycle. It is further conceivable that the acylase activity, unlike most of the enzymes of the phosphatidic acid pathway [3], is associated with the outer membrane, i.e., if the cofactor requirements for acylation can be met in this location. If this were so *E. coli* would be capable of repair and remodeling of its phospholipids at the site of degradation by endogenous [5,69] and/or exogenous [8,9] phospholipases. For example, the killing of *E. coli* by highly purified bactericidal proteins, isolated from rabbit and human granulocytes, is accompanied by two practically immediate envelope changes, namely, discretely increased permeability (to actinomycin D) and net hydrolysis of phospholipids [8—13]. These alterations, apparently involving primarily the outer membrane, can be reversed by addition of Mg^{2+}, Ca^{2+} or trypsin [8—9], causing impermeability to actinomycin D to be restored and phospholipids to be resynthesized from the products of hydrolysis. At least part of the resynthesis of phospholipid appears to take place by reacylation of lysophospholipids.

In the present study we have investigated the localization and some of the properties of lysophospholipid acylase activity in the envelope of *E. coli*. The results indicate that acylation of monoacylglycerophosphoethanolamine occurs in the outer as well as in the inner membrane of the *E. coli* envelope.

Materials and Methods

Preparation of E. coli envelope, inner and outer membrane

Three strains of *E. coli* were used in this study: strain PC 0221, a wild type *E. coli* K12; strain *E. coli* K12 - N 802 (Leu^-, Thi^-, Lac^-, Mal^-)-S15, described henceforth as S15; and a phospholipase A-deficient derivative of this strain,

denoted as S17. (The two latter strains were kindly donated by Dr. S. Nojima, National Institute of Health, Tokyo). Cells were grown in yeast broth, harvested in the logarithmic phase of growth (absorbance, 0.4 at 660 nm) and washed in 0.8% NaCl. For the preparation of crude envelopes the bacteria were suspended in 0.05 M Tris · HCl buffer, pH 8.5, containing 2 mM EDTA and disrupted by sonication. Intact cells were removed by centrifugation at 3000 rev./min for 20 min. A crude cell envelope fraction, obtained by centrifugation for 1 h at 100000 $\times g$, was suspended in 2 mM Tris · HCl buffer, pH 7.4, at a concentration of 1 mg protein/ml. For the preparation of inner and outer membrane fractions the cells were suspended in 10 mM Tris · HCl buffer, pH 7.8, containing 0.75 M sucrose and 0.2 mM dithiothreitol. Conversion into spheroplasts, lysis and separation of inner and outer membrane was performed by following exactly the procedure of Osborn et al. [14].

Preparation of substrates

For preparation of ^{32}P-labeled 1-acyl-glycerol-3-phosphoethanolamine ([^{32}P]-lysophosphatidylethanolamine), *E. coli* was grown at 37°C in yeast broth containing 500 μCi [^{32}P]H_3PO_4 per liter. After harvesting the cells by centrifugation, lipids were extracted according to Bligh and Dyer [15]. Diacylglycerophosphoethanolamine was isolated by preparative thin layer chromatography on silica gel H with chloroform/methanol/acetic acid/water (65/25/1/4, by vol.) as developing system. Lipid species were visualized by exposure of the thin-layer plates to iodine vapor and identified by comparison of R_F to that of authentic lipid standards. The lysophosphatidylethanolamine area was scraped from the plate into a small column plugged with glass wool. Phosphatidylethanolamine was eluted from the silica gel with chloroform/methanol (20/80, v/v). The [^{32}P]lysophosphatidylethanolamine was prepared by hydrolysis of the labeled diacyl-compound in 1 ml diethylether and 1 ml 0.1 M Tris · HCl buffer, pH 7.8, to which had been added 5 mM $CaCl_2$ and 10 mg crude Naja Naja venom phospholipase A_2, during vigorous shaking for 4 h at room temperature.

The lyso derivative was obtained by extraction and preparative thin layer chromatography as described above. The specific radioactivity exceeded 100000 cpm per nmol phosphorus.

[1-^{14}C]Palmitoyl-glycerol-3-phosphoethanolamine ([^{14}C]lysophosphatidylethanolamine) was prepared in exactly the same way using 50 μCi [1-^{14}C]palmitic acid (The Radiochemical Centre, Amersham, U.K.) complexed with 40 mg fatty acid-poor bovine serum albumin per 200 ml medium.

Enzymatic assays

To measure the enzymatic acylation of lysophosphatidylethanolamine the procedure was followed described by Proulx and van Deenen [10]. Radioactive lysophosphatidylethanolamine was mixed with unlabeled carrier (prepared as described above) and oleic acid. The lipid mixture was dried by evaporation and dispersed in water by sonication. Of the lipid suspension 50 μl, representing 22 nmol of the lyso-compound (5000 cpm), and 16 nmol of oleic acid, were incubated with 0.335 mg ATP (Sigma Chem. Co., Missouri)/0.016 mg coenzyme A (Boehringer, Mannheim)/4 μmol $MgCl_2$/5 μmol Tris · HCl buffer, pH 7.4,/10—50 μg membrane protein, in a total volume of 250 μl. Incubations

were carried out at 37°C and terminated by the addition of 3 ml chloroform/methanol (1/2, v/v). Lipids were extracted and separated as described above. Compounds were visualized with iodine. The silica gel was scraped off the plates into counting vials and counted in a liquid scintillation counter, using as scintillation mixture toluene (containing 0.5% 2.5 diphenyloxazole and 0.03% 1.4-bis 2-(5-phenyloxazolyl)-benzene)/Triton X-100/water (2/1/0.2, by vol.).

NADH oxidase was assayed according to Mavis et al. [16] and CTP:phosphatidic acid cytidyltransferase was measured as described by Bell et al. [1], under optimal conditions. The presence of phospholipase activity in membrane fractions was assayed by determination of hydrolysis of endogenous [1-^{14}C]palmitate-labeled phospholipids [8,9] legend Fig. 4). Protein was determined by the method of Lowry [17] and phosphorus according to Chen et al. [18].

Results

Conversion of lysophosphatidylethanolamine to phosphatidylethanolamine by E. coli envelopes

Incubation of a crude envelope preparation of *E. coli* with [^{14}C]lysophosphatidylethanolamine results in the formation of two radioactive products. Fig. 1 shows the time course of accumulation of [^{14}C]phosphatidylethanolamine and ^{14}C-labeled fatty acids. Similar results were obtained with envelope preparations from all three *E. coli* strains studied (see Materials and Methods).

In preliminary experiments the optimal conditions for phosphatidylethanolamine synthesis were established with respect to lysophosphatidylethanolamine concentration (see Materials and Methods) and pH. Assays were carried out at pH 7.4, even though phosphatidylethanolamine formation was somewhat greater at higher pH. The reason for choosing this pH was the markedly enhanced deacylation of the lysophosphatidylethanolamine substrate at pH higher than 7.5. The cofactor requirements of phosphatidylethanolamine formation are shown in Table I. Optimal formation of phosphatidylethanolamine

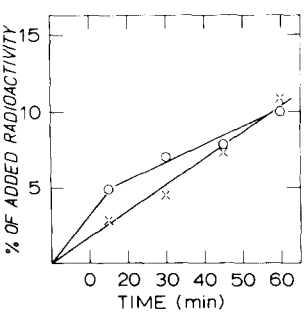

Fig. 1. Time course of formation of phosphatidylethanolamine and fatty acids from lysophosphatidylethanolamine by *E. coli* envelopes. Incubations were carried out with 100 μg of envelope proteins, prepared from strain K12 PC 0221 as described in Methods, in the presence of [^{14}C]lysophosphatidylethanolamine, ATP, coenzyme A and Mg^{2+}. The amounts of free fatty acids (X——X) and phosphatidylethanolamine (o——o) formed are expressed as percent of the amount of ^{14}C-labeled lyso compound present in the incubation mixture.

TABLE I

EFFECTS OF ADDED FATTY ACIDS, ATP AND COENZYME A ON THE REACYLATION OF LYSOPHOSPHATIDYLETHANOLAMINE (LPE) BY AN *E. COLI* ENVELOPE PREPARATION

The complete incubation mixture contained [^{14}C]lysophosphatidylethanolamine, oleic acid, ATP, coenzyme A, Mg^{2+} and 50 μg envelope protein prepared from *E. coli* K12 PC 0221. Incubations were carried out at 37°C for 60 min. The double label experiments (*) were also carried out in the absence of ATP and coenzyme A with results identical to those shown in the table.

Incubation mixture	Phosphatidylethanolamine formed (nmol)	Radioactive label recovered in phosphatidylethanolamine
Complete ([^{14}C]LPE)	34	^{14}C
Complete (boiled membrane preparation)	2	—
Mixture without ATP, CoA	28	^{14}C
Mixture without oleic acid	33	^{14}C
Complete + [^{3}H]oleic acid *	36	^{14}C
Complete + [^{32}P]LPE * ^{14}C/^{32}P = 3.3	32	^{14}C/^{32}P = 3.3

was obtained in the presence of ATP, coenzyme A and Mg^{2+}. However, omission of ATP and coenzyme A from the incubation mixture reduced phosphatidylethanolamine synthesis by 10—20% only. Preincubation of the envelope preparation for 1 or 2 h, in an attempt to deplete substrate and cofactor stores that might be trapped in the preparation, did not markedly increase the dependence of acylation on added ATP and coenzyme A. Thus these envelope preparations may still contain or produce near optimal amounts of cofactors, or the reaction does not require them. It is also apparent from the results in Table I, that addition of oleic acid (cosonicated with lysophosphatidylethanolamine as described by Proulx and van Deenen [10]) has no effect on formation of phosphatidylethanolamine. Moreover, no detectable [^{3}H]oleic acid is incorporated either in the absence or presence of added ATP and coenzyme A. Since exogenous fatty acid is apparently not used in the acylation reaction, the fatty acid might derive from other acyl positions. Such transacylation has been demonstrated for lysophosphatidylcholine (2-lysophosphatidylcholine → phosphatidylcholine + glycerylphosphorylcholine) [19], but so far no evidence has been presented for acyl transfer from one molecule of lysophosphatidylethanolamine to another [20]. Nevertheless, the possibility that in *E. coli* this reaction does occur was investigated using lysophosphatidylethanolamine labeled biosynthetically with both ^{32}P and [1-^{14}C]palmitic acid. The ^{14}C/^{32}P ratio in the newly formed lipid and in the starting material was identical, excluding, therefore, appreciable transacylation of the exogenous lyso derivative. Again, results were the same in the absence and presence of ATP and coenzyme A.

Localization of lysophosphatidylethanolamine acylation activity within the E. coli envelope

Inner and outer membranes were isolated from 2 strains of *E. coli*, a K12 strain (S15) with a phospholipid-degradative apparatus typical of wild type *E. coli*, and a mutant strain (S17) (derived from strain S15) that lacks demonstrable phospholipase A_1 and A_2 activity [21]. The completeness of separation

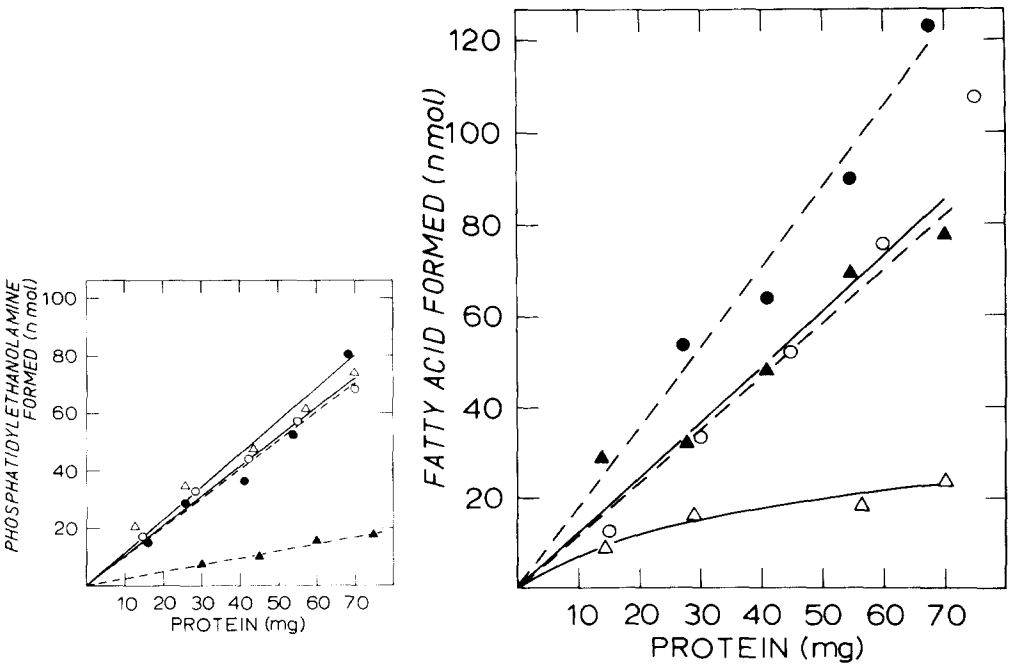

Fig. 2. Phosphatidylethanolamine formation by inner and outer membranes of *E. coli* S15 and S17. [^{14}C]Lysophosphatidylethanolamine was incubated together with ATP, coenzyme A, Mg^{2+} and oleic acid, during 1 h at 37°C with various amounts of inner (●) and outer (▲) membrane preparations of *E. coli* S15 and inner (○) and outer (△) membrane preparations of strain S17. The amounts of phosphatidylethanolamine and fatty acids (see Fig. 3) formed were determined as described in Materials and Methods.

Fig. 3. Free fatty acid formation by inner and outer membrane of *E. coli* S15 and S17. The release of fatty acids from [^{14}C]lysophosphatidylethanolamine was determined in the same incubation procedure as described in the legend to Fig. 2.

of inner and outer membrane fractions was assessed by determining the specific activities of two inner membrane marker enzymes, NADH oxidase and CTP: phosphatidic acid cytidyltransferase. The specific activity of NADH oxidase in different cytoplasmic membrane preparations from the two *E. coli* strains was 30—40-fold greater, and of CTP:phosphatidic acid cytidyltransferase approx. 10-fold higher, than in the corresponding outer membrane preparations. These results are in agreement with published data [1,2,22]. Cytoplasmic membrane preparations were almost devoid of phospholipase A activity towards endogenous substrate (Fig. 4), indicating little contamination with outer membrane material [1].

Fig. 2 shows that acylation of lysophosphatidylethanolamine is carried out to approx. the same extent by inner membrane fractions of *E. coli* S15 and S17 and by outer membrane preparations of S17, and increases in close to linear fashion with increasing protein concentration. Acylation by outer membrane preparations of the parent strain is from 5—8-fold less than by the other membrane preparations. These findings suggest that acylating activity is located in the outer membrane as well as in the cytoplasmic membrane of *E. coli*, but that less [^{14}C]phosphatidylethanolamine accumulates in the outer membrane of the

parent strain because the product is hydrolyzed by the phospholipase A, which is present in this membrane fraction. We have previously shown that formation of *E. coli* spheroplasts is associated with activation of phospholipase A_1, resulting in extensive hydrolysis of phospholipid [7]. To examine further the possibility that [^{14}C]phosphatidylethanolamine accumulation is limited by its breakdown by phospholipase A in outer membrane but not in the cytoplasmic membrane of S15, the envelope phospholipids of *E. coli* S15 were labeled with 1-[^{14}C]palmitic acid before preparation of membranes. As an index of phospholipase activity and distribution within the *E. coli* envelope, hydrolysis was measured of these labeled lipids during incubation of the membrane preparations at 37°C (Fig. 3). The labeled phospholipids of outer membrane fractions undergo substantial degradation, which is enhanced by addition of 10 mM Ca^{2+}, whereas the cytoplasmic membrane preparation is almost devoid of phospholipase activity towards endogenous substrate. Because 1-[^{14}C]palmitic acid is incorporated for more than 90% into the 1-acyl position of *E. coli* phospholipids [9], the accumulation of appreciable amounts of radioactive lyso compounds suggests that the outer membrane of *E. coli* S15 contains phospholipase A_2 in addition to phospholipase A_1 [21].

Although phospholipase A-deficient *E. coli* manifest practically no phospholipid degradation under conditions that cause wild type *E. coli* and *E. coli* S15 to degrade their own lipids extensively [9], the S17 strain does possess lysophospholipase activity [23]. This is also indicated by the release of ^{14}C-labeled fatty acid released from [^{14}C]palmitate labeled lysophosphatidylethanolamine (Fig. 4). Fatty acid release by cytoplasmic and outer membrane of S15 and by cytoplasmic membrane of preparations of S17 is roughly similar, but deacylation of [^{14}C]lysophosphatidylethanolamine by outer membrane preparations of S17 is much less. It is probable that some of the [^{14}C]fatty acids released by outer membrane preparations from S15 are the product of hydrolysis of phosphatidylethanolamine synthesized from [^{14}C]lysophosphatidylethanolamine.

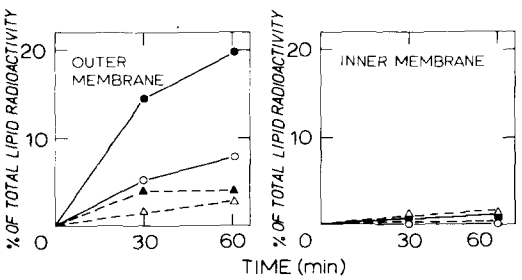

Fig. 4. Degradation of 1-[^{14}C]palmitate-labeled phospholipids of inner and outer membrane fractions. Inner and outer membranes of *E. coli* S15 were incubated in a mixture that contained the same ingredients as employed for the acylation reaction: 22 nmol lysophosphatidylethanolamine/16 nmol oleate/5 μmol Tris · HCl buffer (pH 7.4)/0.335 mg ATP/0.016 mg coenzyme A/4 μmol $MgCl_2$/50 μg membrane protein, in a total volume of 0.3 ml. Final concentration of added Ca^{2+} was 10 mM (●, ▲). The reaction was stopped by addition of 3 ml of chloroform/methanol (1/2, v/v). Lyso compounds (▲, △) and fatty acids (●, ○) were separated from phospholipids by thin layer chromatography in a chloroform/methanol/water/glacial acetic acid (65/25/4/1, v/v) solvent mixture. Remaining parts of the assay were as described in Materials and Methods. Open symbols represent incubation in the absence of added Ca^{2+}.

Discussion

The biosynthesis of the constituents of the outer membrane, including lipopolysaccharides, proteins, lipoproteins and lipids [1,2,24,25], generally appears to take place in close association with the cytoplasmic membrane. The concept has arisen therefore that no biosynthesis takes place in the outer membrane and that its assembly requires the transport of the products of biosynthesis from the cytoplasmic to the outer membrane, probably through zones of adhesion between both membranes [24]. Machtiger and Fox have pointed out however that certain enzymes of the Weiss-Kennedy pathway are not restricted to the cytoplasmic membrane, but appear to be more widely distributed among *E. coli* cell fractions, including the outer membrane and non-particulate fractions [3]. It is not clear to what extent these observations reflect true heterogeneity in native distribution of enzymes of lipid biosynthesis, and to what extent some of these enzymes are prone to be redistributed artefactually during the fractionation procedure. The finding that the distribution of those enzymes that were not restricted to the cytoplasmic membrane varied with the homogenization technique used [3], suggests that some cytoplasmic membrane enzymes are less tightly bound than others and, when released during fractionation, reassociate with other membrane fractions.

In the present study the acylase activity was about equally divided between inner and outer membrane of *E. coli* S17, both in terms of total and specific activity. Essentially no acylase activity was found in non-particulate fractions (not shown), suggesting a tight membrane association. This renders it less likely that artefactual redistribution during fractionation accounts for association of the acylase with the outer membrane of *E. coli* S17 and leads us to propose that the acylase is in fact a constituent of outer as well as inner membrane.

The much lower conversion of lysophosphatidylethanolamine to its diacyl derivative by the outer membrane of *E. coli* S15, reflects, we believe, the presence of phospholipase A in this fraction. In *E. coli* with a complete phospholipid degradative apparatus, net phospholipid breakdown becomes manifest upon surface perturbation [6,8,9] and when the envelope is disrupted, for example during spheroplast formation [7] and in isolated outer membranes as demonstrated in Fig. 4. Hence, phosphatidylethanolamine synthesis is readily apparent in the outer membrane of a phospholipase A-deficient mutant, and in the cytoplasmic membrane which lacks appreciable phospholipase A activity in both strains; but is underestimated in outer membrane of S15 because the product degraded by activated phospholipases A.

The existence in the outer membrane of a biosynthetic pathway for resynthesis of major outer membrane phospholipids from the products of hydrolysis, generated by deacylation in the same location, provides *E. coli* with a biochemically inexpensive mechanism for repair of the outer membrane phospholipids. The Mg^{2+}- (Ca^{2+})- or trypsin-induced resynthesis of outer membrane phospholipids, previously hydrolyzed during the action of membrane-active bactericidal leukocyte proteins [8,9,13] may well represent an example of this biosynthetic potential.

A deacylation-reacylation cycle in the outer membrane of *E. coli* may further facilitate in situ remodeling of the outer membrane phospholipids in

response to environmental stimuli, thereby modifying the physicochemical properties of the external envelope layer. These properties determine in large measure the sensitivity of gram negative bacteria to noxious factors such as antibiotics and humoral and cellular host defense systems. It is also conceivable that the balance of phospholipid deacylation and reacylation within the outer membrane contributes importantly to regulation of envelope growth.

The origin of the fatty acid used in the conversion of lysophosphatidylethanolamine to phosphatidylethanolamine has not been established. Identification of the fatty acid source will be an important part of the further elucidation of the role of lysophospholipid acylation in the lipid biochemical pathways within the outer membrane of the gram negative bacterial envelope.

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