Newly biosynthesized cytochrome *P*-450 associated with the golgi apparatus from livers of rats induced with phenobarbital

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1. Cytochrome *P*-450 has been detected in preparations of golgi apparatus from the livers of phenobarbital-induced rats. 2. Newly biosynthesized cytochrome *P*-450 is also present in preparations of golgi apparatus. By using three different techniques to fractionate the golgi into vesicle contents and membrane components it was found that newly biosynthesized cytochrome *P*-450 is associated solely with the membrane fraction. 3. By investigating the susceptibility of cytochrome *P*-450, present in the golgi apparatus, to the action of trypsin it was found that the majority was orientated on the cytosolic face of the membrane.

It is well established that the golgi apparatus is involved in the modification and export of proteins subsequent to their synthesis on membrane bound polysomes (Jamieson & Palade, 1977; Palade, 1975). Modifications such as glycosylation and proteolytic cleavage may also occur during passage of newly biosynthesized proteins from their site of membrane translocation, through the lumen of the smooth reticulum to the golgi apparatus (Schacter *et al.*, 1970; Palade, 1975; Campbell & Blobel, 1976). The enzymes catalysing the addition of the terminal sugar residues to glycoproteins are thought, however, to be located solely in the membranes of the golgi apparatus (Fleischer *et al.*, 1969; Morré *et al.*, 1970).

The synthesis of cytochrome *P*-450, which is located in the membranes of the endoplasmic reticulum and is reported to be a glycoprotein (Haugen & Coon, 1976), has been shown to occur on membrane bound polysomes (Cooper *et al.*, 1979; Craft *et al.*, 1975, 1978; Harano & Omura, 1977; Negishi *et al.*, 1976). It has been suggested that this protein is inserted directly, in the course of biosynthesis, into the membrane of the rough reticulum and that the protein may diffuse subsequently in the lateral plane to its ultimate destination, where this differs from the site of insertion (Fujii-Kuriyama *et al.*, 1979; Omura & Kuriyama,

1971). However, it has been demonstrated that a portion of newly biosynthesized cytochrome *P*-450 can be detected in soluble form in the lumen of vesicles derived from both rough and smooth endoplasmic reticulum and, furthermore, can also be detected in preparations of golgi apparatus (Craft *et al.*, 1979*a,b*). We have shown also that cytochrome *P*-450 is distributed on both sides of the phospholipid bilayer in vesicles derived from the smooth endoplasmic reticulum (Cooper *et al.*, 1980), in contrast to glucose 6-phosphatase which is found only on the luminal face. Newly biosynthesized cytochrome *P*-450 found in soluble form inside vesicles derived from membranes of the endoplasmic reticulum may be a variant destined to be inserted into the luminal face of the membrane. An alternative possibility is that the intravesicular protein undergoes modification in the lumen of reticular membranes and/or the golgi apparatus before insertion into the cytosolic face of the membranes.

In the present study we present evidence that newly biosynthesized cytochrome *P*-450 is associated intrinsically with elements of the golgi apparatus in rats induced with phenobarbital. We show that the newly biosynthesized cytochrome *P*-450 is associated with the membrane and is absent from the vesicle contents. We have also examined the distribution of cytochrome *P*-450 in the transverse plane of the membranes of the golgi apparatus and present evidence that the bulk of the newly biosynthesized *P*-450 is orientated on the cytosolic face of these membranes.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Methods

Male Sprague–Dawley rats of body weight 200–220 g were used. Each animal received one intra-peritoneal injection of phenobarbital (40 mg/kg body wt., in 0.15 M-NaCl) on each of five successive days before being killed. The animals were permitted food and water *ad libitum*.

Perfusion of livers in situ

Perfusion of the livers *in situ* was carried out by the method of Hems *et al.* (1966), with the modifications described elsewhere (Craft *et al.*, 1979a,b). To radiolabel the proteins of the reticular membranes, the livers were each perfused for 30 min with a medium containing 20 μ Ci (0.74 MBq) of [14 C]leucine.

Preparation of endoplasmic reticulum membranes

Golgi apparatus was isolated by the method of Morré (1971). Four perfused livers were used for each preparation. The final material was characterized morphologically and enzymically. Vesicles derived from rough and smooth endoplasmic reticulum were isolated as described previously (Blyth *et al.*, 1971).

Subfractionation of golgi apparatus into vesicle contents and residual membranes

French-press method (Ehrenreich *et al.*, 1973). Pelleted golgi material was resuspended to approx. 4 mg of protein/ml in a solution containing sodium veronal (0.04 M) adjusted to pH 8.5 with HCl. The suspension was then passed through a French press (American Instrument Co., U.S.A.) at 6.9×10^4 kPa internal pressure. The resultant material was left on ice for 30 min, and the particulate fraction then separated from the vesicle contents by centrifugation at 105 000 g for 1 h at 4°C. The supernatant, containing the vesicle contents, was aspirated from the pellet of membrane material which was then resuspended to a protein concentration of 15 mg/ml in a solution containing Na₃PO₄ (0.1 M)/EDTA (0.01 M)/sucrose (0.25 M)/glycerol (20%, v/v), adjusted to pH 7.5 at 20°C with NaOH. The trichloroacetic acid-insoluble 14 C-labelled material in the subfractions was determined as described previously (Craft *et al.*, 1978). For analysis by immunoprecipitation the membrane fraction was solubilized by the addition of sodium cholate (3 mg/mg of protein) and sonicated for 30 s at 0°C. Non-solubilized material was sedimented by centrifugation at 105 000 g, at 4°C, for 1 h. The recovery of trichloroacetic acid-insoluble 14 C-labelled material at this stage was within the range 87–95%.

Sonication treatment. Pelleted golgi material was resuspended in 1.5 ml of 0.15 M-NaCl, and 3.5 ml of NaHCO₃, pH 7.8, added. The resultant suspension

was sonicated at 0°C for 30 s. Released material was separated from the particulate fraction by centrifugation as described in the previous section.

Treatment with deoxycholate. The use of low concentrations of deoxycholate (0.05%) to release the contents of vesicles from rough and smooth endoplasmic reticulum, without solubilization of intrinsic membrane proteins, has been documented (Kreibech *et al.*, 1973). Pelleted golgi material was resuspended in a solution containing KCl (0.05 M)/MgCl₂ (0.005 M)/Tris (0.05 M), adjusted to pH 7.5 at 20°C with HCl, to a concn. of 1.8 mg/ml of protein. A solution of deoxycholate (1%, w/v) was added with constant mixing to a final concentration of 0.05%. The suspension was incubated on ice for 30 min and the released vesicle contents and residual membrane proteins separated as in the French-press method section.

Trypsin treatment of intact golgi vesicles

Golgi preparations were incubated at 30°C in the presence of trypsin (50 μ g/mg of golgi protein) for 45 min. The reaction was stopped by the addition of soya bean trypsin inhibitor (2 mg/mg of trypsin) and the membrane recovered by centrifugation at 105 000 g for 1 h at 4°C. Control incubations were carried out in the absence of trypsin. The resultant membranous material was assayed for the presence of spectrally detectable cytochrome P-450 and solubilized for analysis by immunoprecipitation.

Immunoprecipitations

Subfractions of golgi apparatus were assayed for their content of 14 C-labelled serum proteins and cytochrome P-450 by quantitative immunoprecipitation from three separate experiments. The purification of cytochrome P-450, and the specificity of the antibody raised to it, has been fully described (Craft *et al.*, 1979a,b).

The vesicle contents or solubilized residual membrane fractions were dialysed for 15 h at 4°C against a solution containing Na₃PO₄ (0.1 M)/EDTA (0.01 M)/sucrose (0.25 M)/glycerol (20%, v/v)/sodium cholate (3%, w/v), adjusted to pH 7.7 at 20°C with NaOH. The immunoreaction was carried out as described previously (Craft *et al.*, 1978). Each incubation contained not less than 1000 c.p.m. of 14 C-labelled trichloroacetic acid-insoluble material. When necessary, sufficient unlabelled carrier antigen was included to ensure formation of an immunoprecipitate. The data were corrected for non-specific precipitation by running control incubations in which specific antibody was replaced by serum prepared from non-immune rabbits. Antibody to rabbit IgG, raised in a goat, was then added and radioactivity associated with the resultant immunoprecipitate determined. The correction for

EXPLANATION OF PLATE 1

Electron micrograph of isolated golgi apparatus

Golgi apparatus was prepared as described in the Methods section. Samples were fixed in osmium tetroxide (4%, w/v) and sodium cacodylate (0.2 M, pH 7.0), postfixed in glutaraldehyde (12.5% in cacodylate buffer) and embedded in Spurr resin. The horizontal bar represents 0.5 μm.

non-specific precipitation never exceeded 5% of the radioactivity precipitated by the specific antisera. All incubations were for 1 h at 32°C followed by 15 h at 4°C. Immunoprecipitates obtained were washed three times in a solution containing boric acid (0.05 M)/NaBH₄ (0.001 M)/NaCl (0.15 M) pH 8.4. The washed material was dispersed into 1 ml of water by sonication. Trichloroacetic acid-insoluble material was precipitated by the addition of 0.2 ml of 50% (w/v) trichloroacetic acid. After incubation at 0°C for 30 min the precipitates were collected on glass-fibre discs and the radioactivity present was determined.

Preparation of specific anti-(cytochrome P-450) IgG

The IgG fraction from immune rabbit serum was prepared as described by Campbell *et al.* (1970a). Specific anti-(cytochrome *P*-450) IgG was obtained from the total IgG as described by Campbell *et al.* (1970b).

Preparation of antibody-conjugated polyacrylamide beads

Polyacrylamide beads were conjugated with goat anti-(rabbit IgG) by the method of Ito & Palade (1978). The beads were mixed with excess rabbit anti-(cytochrome *P*-450) IgG and incubated for 30 min at 32°C followed by gentle stirring for 15 h at 4°C. The reacted beads were recovered by centrifugation, washed twice with a solution containing NaCl (0.15 M) and Tris (0.01 M) pH 7.4, and finally resuspended in that buffer. The affinity of the beads for membrane-bound cytochrome *P*-450 was determined by titration against known amounts of vesicles containing cytochrome *P*-450. Non-specific absorption of protein by the beads was determined by titration against bovine serum albumin and found to be negligible. Control beads were prepared by the same procedure except that specific anti-(cytochrome *P*-450) IgG was replaced by rabbit IgG from non-immune animals.

Other methods

Trichloroacetic acid-insoluble radioactivity collected on glass-fibre discs was determined in a scintillation fluid containing PPO (2,5-diphenyloxazole) (5 g/l) in toluene. Measurements were made with a Packard type 3375 scintillation counter, at constant counting efficiency as determined by automatic external standard. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. RNA was measured by the method of Fleck & Begg (1965). Phospholipid was extracted by the method of Folch *et al.* (1957), and its phosphorus content determined as described by Chen *et al.* (1956). SDS/polyacrylamide-gel electrophoresis was by the method of Laemmli (1970). Enzyme assays were

carried out on freshly isolated golgi preparations by the following methods: glucose 6-phosphatase (EC 3.1.3.9), Leskes *et al.* (1971); 5'-nucleotidase (EC 3.1.3.5) and UDP-galactose-glycoprotein galactosyltransferase (EC 2.4.1.38), Morré (1971); succinic dehydrogenase (EC 1.3.99.1), Pennington (1971); cytochrome *P*-450, Omura & Sato (1964). When mentioned in the text, results show the means $\pm$ S.D. from four separate experiments.

Materials

Bovine serum albumin (fraction V) and rabbit IgG were purchased from Sigma Chemical Co., U.K., rabbit anti-(rat serum protein) antibody was from ICN Biochemicals, U.S.A., and goat anti-(rabbit IgG) from Miles, U.K. [¹⁴C]Leucine and UDP-*N*-acetyl-[¹⁴C]-galactosamine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Polyacrylamide beads (Affi-Gel 701) were obtained from Bio-Rad Laboratories, U.S.A.

Results

Purity of the golgi preparation

The isolated golgi fraction was examined by electron microscopy and assayed for the presence of specific enzymes which are regarded as markers for various subcellular organelles.

Plate 1 shows a positively stained sample of golgi preparation isolated as described. Dictyosomes are clearly visible and there are also numerous small secretory vesicles. Some contaminating vesicles from rough endoplasmic reticulum are present in small amounts. However, the general morphology of the preparation is similar to previously reported results (Morré, 1971). Contamination with material derived from other cellular organelles was further assessed by assays for the presence of the following marker enzymes: 5'-nucleotidase (plasma membrane), succinic dehydrogenase (mitochondria), glucose 6-phosphatase (endoplasmic reticulum). UDP-galactose-glycoprotein galactosyltransferase was assayed as a marker enzyme of the golgi apparatus. The results of these analyses are shown in Table 1. Protein, RNA and phospholipid content of the preparation were also determined. The ratio of RNA to protein present in the preparation was found to be 0.18 ± 0.06 (mg of RNA/mg of protein) and that of phospholipid to protein 18 ± 2 (μ g of lipid phosphorus/mg of protein). Some contamination of the preparation with plasma membrane can be inferred; however, the general level of contamination appears to be small and the sample is certainly greatly enriched in the marker enzyme specific for the golgi apparatus. The findings that labile forms of enzymes, including glucose 6-phosphatase, can be detected in preparations of golgi apparatus (Howell *et al.*, 1978;

Table 1. *Enzymic characterization and chemical composition of isolated golgi apparatus from liver of male rats induced with phenobarbital*

Enzyme activities were assayed as described in the Methods section; specific activities are expressed as μmol of product formed $\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ of protein, other than succinic dehydrogenase that is μmol of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-tetrazolium reduced $\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ of protein and UDP-galactose-glycoprotein galactosyltransferase that is pmol of galactose transferred to *N*-acetylglucosamine $\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ of protein. N.D., Not detectable.

Enzyme	Subcellular fraction	Sp. act.	Sp. act. of fraction/ sp. act. of homogenate	Homogenate activity recovered in reticular fraction (%)
Glucose 6-phosphatase	Golgi	0.026	0.7	8
	Rough vesicles	0.15	4.7	24
	Smooth vesicles	0.17	5.1	32
5'-Nucleotidase	Golgi	0.028	2.02	<5
	Rough vesicles	N.D.	—	—
	Smooth vesicles	N.D.	—	—
Succinic dehydrogenase	Golgi	0.004	0.1	<5
	Rough vesicles	0.038	1.3	7.6
	Smooth vesicles	0.038	1.3	7.6
UDP-Gal-glycoprotein galactosyltransferase	Golgi	3.26	82.0	64
	Rough vesicles	N.D.	—	—
	Smooth vesicles	0.06	1.8	<4

Palade, 1975) will tend to exaggerate the assessment of the levels of contaminating organelles. Differences are also seen between the chemical composition of the golgi preparation and vesicles derived from rough and smooth endoplasmic reticulum. From the results we conclude that the preparation consists largely of vesicles from the golgi apparatus.

Presence of cytochrome P-450 in golgi apparatus

Cytochrome *P*-450 has been reported to be present in preparations of golgi apparatus from the livers of phenobarbital-induced rabbits (Ichikawa & Yamano, 1970), and from rat liver (Jarasch *et al.*, 1979). Under conditions of phenobarbital induction the cytochrome can be detected spectrally in the preparation of golgi apparatus at levels approx. 4-fold those observed in control animals ($0.31 \pm 0.10 \text{ nmol/mg}$ of protein in control animals and $1.23 \pm 0.30 \text{ nmol/mg}$ of protein in phenobarbital-induced animals). The levels of cytochrome *P*-450 are approx. one-third of those found in smooth or degranulated rough membrane vesicles. The reported presence of ferrihaemoprotein *P*-450 reductase (EC 1.6.2.4) in preparations of golgi apparatus suggest that the cytochrome *P*-450 may have a catalytic role in this organelle (Ito & Palade, 1978). However, it is possible that these proteins are present in the golgi apparatus in the process of translocation to the ultimate membrane locus.

To test whether cytochrome *P*-450 is intrinsically associated with elements of the golgi apparatus, or whether its presence is in fact due to contamination, experiments were conducted by using immunochemical techniques. A sample of golgi

preparation, containing known amounts of both cytochrome *P*-450 and UDP-galactose-glycoprotein galactosyltransferase activity, was incubated with an excess amount of polyacrylamide beads with anti-(cytochrome *P*-450) IgG attached. A similar amount of golgi-derived material was mixed with control beads with rabbit IgG, containing no antibody to cytochrome *P*-450, attached. After incubation at 32°C for 45 min the samples were layered over 5 ml of 1.25 M-sucrose and the tubes centrifuged at 600 g at 4°C for 5 min. This caused the beads to sediment to the interface of the sucrose solution. The supernatant above the beads was aspirated from the tubes and the content of the cytochrome *P*-450 and UDP-galactose-glycoprotein galactosyltransferase activity determined; when the golgi preparation was incubated with control beads the major part of both the activities are recovered ($82 \pm 5\%$ and $82 \pm 10\%$ respectively) together with the majority of the protein ($87 \pm 4\%$). However, when beads having antibody to cytochrome *P*-450 attached were used, neither activity could be detected and only $10 \pm 5\%$ of the protein. This strongly suggests that the cytochrome *P*-450 is associated with membranes that also possess UDP-galactose-glycoprotein galactosyltransferase activity. If the cytochrome *P*-450 was present wholly as a microsomal contaminant the transferase activity should have been recovered as found in the control.

Fractionation of the golgi preparation into membrane components and vesicle contents

Livers were labelled by perfusion *in situ* with [^{14}C]leucine for 30 min, as described in the Methods section. The golgi apparatus was isolated and then

fractionated into vesicle contents and residual membrane components by the techniques described in the Methods section.

The distribution of proteins between the two fractions was examined by SDS/polyacrylamide-gel electrophoresis. As shown in Fig. 1 major differences can be observed between the membrane-bound proteins and those present in the released fractions. In particular a protein of approx. 70 000 mol.wt. is present only in the released fraction, and has a similar electrophoretic mobility as albumin. In the membrane-associated material a preponderance of proteins of approx. 50 000 mol.wt. are present and are largely absent from the released fraction. Fractionation of the golgi preparation by either the detergent treatment or by the use of the French-press can be seen to give identical segregation of the golgi proteins into vesicle contents and membrane-associated components. When the preparation from the golgi apparatus is fractionated by either of the three methods described, the distribution of trichloroacetic acid-insoluble ^{14}C is essentially the same: $60 \pm 4\%$ of the trichloroacetic acid-insoluble ^{14}C is present in the membrane-associated material, whereas the remainder is present in the vesicle content.

The nature of the ^{14}C -labelled proteins in the membrane-associated and released fractions, prepared by the three techniques described, was investigated by immunoprecipitation with a specific antibody to cytochrome P-450 and antiserum to rat serum proteins. More than 95% (97 ± 0.5) of the cytochrome P-450 was contained in the residual membrane fraction, whereas the majority ($82 \pm 4\%$) of the serum proteins were present in the released fractions.

Distribution of cytochrome P-450 in the transverse plane of the phospholipid bilayer of golgi membranes

The use of denaturing agents, incapable of penetrating the phospholipid bilayer of intracellular membranes, to establish the orientation of membrane bound enzymes is well documented (Cooper *et al.*, 1980; Nilsson *et al.*, 1978). Proteins attached to the cytosolic face of the membrane can be inactivated by the action of trypsin, whereas those on the luminal face are protected from proteolysis.

Golgi-derived material was incubated with trypsin as described in the Methods section. Of the spectrally detectable cytochrome P-450 $81 \pm 5\%$ is susceptible to the action of trypsin. Prolonged incubation of the golgi material with trypsin for 45 min gave no further decrease in the level of spectrally detectable cytochrome P-450, neither was this level affected by increasing the amount of trypsin present in the incubation 2-fold (data not shown). The proteolytic degradation of cytosolically orientated cytochrome P-450 present in prepara-

Fig. 1. Electrophoretic separation of proteins remaining associated with the membrane and those released from the vesicles after subfractionation of isolated golgi apparatus

SDS/polyacrylamide-gel electrophoresis was carried out with a 3% stacking gel (pH 6.8) and 10% separation gel (pH 8.8). Samples ($30 \mu\text{g}$) were electrophoresed at 30 mA/gel for approx. 4 h, as described in the Methods section. Channel A shows the separation of molecular-weight marker proteins: phosphorylase A (mol.wt. 94 000), bovine serum albumin (66 000), pyruvate kinase (57 000), ovalbumin (43 000), alcohol dehydrogenase (41 000). Channel B shows the electrophoretic mobilities of membrane-associated proteins, and C those released from golgi vesicles by the French-press method (see the Methods section). Channel D shows the mobilities of membrane-associated proteins and E those released from golgi vesicles by the use of deoxycholate as described in the Methods section.

tions of Golgi apparatus appears to be identical with that observed when smooth membranes from livers of phenobarbital-induced rats are exposed to the action of trypsin under the same conditions (Cooper *et al.*, 1980). When golgi preparations from livers perfused with [^{14}C]leucine are similarly treated with trypsin the majority ($80 \pm 5\%$) of the newly biosynthesized cytochrome *P*-450 is susceptible to its action. This is in contrast to $90 \pm 5\%$ of newly biosynthesized serum proteins that are protected from proteolysis.

Discussion

In the present study we have demonstrated that cytochrome *P*-450 could be detected in preparations of golgi apparatus from livers of animals that were induced with phenobarbital. The cytochrome *P*-450 appears to be associated with elements of the golgi apparatus that contain UDP-galactose-glycoprotein galactosyltransferase, which is an asserted marker enzyme for golgi membranes (Fleischer *et al.*, 1969; Morré *et al.*, 1970). The possibility that the cytochrome *P*-450 is not associated with the golgi apparatus but is present as a contaminant has been virtually eliminated. Moreover, treatment with detergent of the golgi preparation causes no release of newly biosynthesized cytochrome *P*-450, in sharp contrast to similar treatment of vesicles derived from rough and smooth reticular membranes (Craft *et al.*, 1978, 1979a,b). This strongly supports the contention that the cytochrome *P*-450 present in our golgi preparations was not an artifact of contamination by other reticular membrane fractions. Other workers have also reported the presence of cytochrome *P*-450 as a constituent protein of the golgi apparatus (Ichikawa & Yamano, 1970; Jarasch *et al.*, 1979), furthermore it now seems certain that ferrihaemoprotein *P*-450 reductase is also present in preparations of golgi apparatus (Ito & Palade, 1978; Howell *et al.*, 1978). In contrast, other workers using immunoelectron microscopy have failed to demonstrate the existence of significant amounts of cytochrome *P*-450 in the golgi apparatus of non-phenobarbital-induced rats (Matsuura *et al.*, 1978). Fleischer *et al.* (1971) were able to detect cytochrome *P*-450 in preparations of golgi apparatus from non-induced rats at levels 23% of that detectable in smooth membrane vesicles. However, they attributed this presence to microsomal contamination. The report that marker enzymes, previously thought to be specific for the endoplasmic reticulum, could be detected in preparations from golgi apparatus is not consistent with this view (Howell *et al.*, 1978).

Cytochrome *P*-450 may be present in the golgi apparatus as a precursor that is then inserted into other intracellular membranes. Evidence exists for such a role for the golgi apparatus in membrane

protein biosynthesis (Autuori *et al.*, 1975; Bennett & Leblond, 1971; Bennett *et al.*, 1974; Elhammer *et al.*, 1975; Fambrough & Devroetes, 1978). Alternatively, the cytochrome *P*-450 may be a constituent protein of the golgi membranes and its pathway of biosynthesis may involve translocation through the rough and smooth endoplasmic reticulum, possible as a soluble, intracisternal form (Craft *et al.*, 1975, 1979a,b), before insertion into the golgi membranes.

The cytochrome *P*-450 associated with the golgi membranes is distributed on both sides of the phospholipid bilayer of the membrane. Approx. 80% of the cytochrome appears to be cytosolically orientated in preparations from phenobarbital-induced animals. This is in agreement with the finding that the majority of cytochrome *P*-450 induced by phenobarbital in vesicles from the smooth endoplasmic reticulum, is similarly distributed (Cooper *et al.*, 1980). This possibly suggests that cytochrome *P*-450 is a normal component of the golgi apparatus and has a definite metabolic role in these membranes.

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