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STUDIES ON MILK FAT GLOBULE MEMBRANES

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SUMMARY

The fat globules in milk are surrounded by a membranous envelope about 90 Å thick. Ghosts can be formed by freeze–thawing or sonication. Membrane preparations were found to contain alkaline phosphomonoesterase, acid phosphomonoesterase, phosphodiesterase, glucose-6-phosphatase, Mg^{2+}-activated ATPase, (Na^+-K^+-Mg^{2+})-activated ATPase, true cholinesterase, xanthine oxidase and aldolase. In the main, the enzyme activities found in the fat globule membranes are found in the fraction of other tissues thought to contain plasma membranes. Enzymes characteristic of other tissue fractions were generally absent from the fat globule membranes. Antisera to fat globule membranes agglutinated and hemolyzed bovine erythrocytes. The fat globules appear to be freely permeable to potassium. The fat globule membrane appears to be a derivative of the cell membrane of the mammary cells.

INTRODUCTION

ANTONI VAN LEEUWENHOEK[1], an indefatigable and omnivorous observer, was the first to report that the butterfat in milk was dispersed as globules approx. 2 μ in diameter. He likened the microscopic appearance of a film of milk to that of blood. Since ASCHERSON[2] suggested that the fat globules are surrounded by an emulsion-stabilizing protein envelope, and STORCH[3] demonstrated it by direct staining, the fat globule membrane has been the subject of numerous studies. In this communication, observations are presented which support the view that the milk fat globule membrane is a true biological membrane generically related to cell membranes.

While it has been suggested by some[4,5] that the fat globule membrane merely consists of skim milk proteins adsorbed on the fat globules, a number of findings are not consistent with this view. The isolated proteins appear to have unique amino acid composition and physical properties[6,7] and differ immunologically from all but a

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minor protein component of skim milk[8,9]. In contrast to the butteroil which is composed essentially of triglycerides, the lipids of the fat globule membrane are principally phospholipids[10,11].

Several milk enzymes appear to be associated with the cream. These include xanthine oxidase[12], alkaline phosphomonoesterase[13] and aldolase[14]. BAILIE AND MORTON[15] found these enzymes and NADH–cytochrome *c* reductase in both the protein of cream and the microsomes of mammary gland cells and suggested that the fat globules are covered by an adsorbed layer of cytoplasmic protein to which a concentrated layer of microsomes is attached. Recently, phosphodiesterase has also been found in cream protein[16].

The fat globule envelope appears to arise during the process of milk secretion. It has been shown, first by JEFFERS[17] using only light microscopy, and recently by the elegant electron microscope studies of BARGMANN and co-workers[18,19], that fat secretion takes place by a sort of "reverse" pinocytosis. The fat globules are synthesized in the acinar cells of the mammary gland and, upon reaching a critical size, project into the duct, pushing the acinar cell membrane out around them. Eventually, the cell membrane pinches off behind and completely encases the free fat globule. Very little cytoplasm appears to be carried along. If, indeed, the fat globule membrane is a derivative of the mammary cell membrane, it should be an useful system for study for investigators interested in the structure and composition of cell membranes.

EXPERIMENTAL

Isolation of fat globule membranes

Cream, obtained by the immediate separation from fresh, uncooled milk using a De Laval cream separator, was used for all experiments. The fresh, cream-free skim milk was retained for some experiments. The cream was washed four times at 35° with 3 vol. of 0.25 M sucrose solution containing $2 \cdot 10^{-3}$ M $MgCl_2$. It was found that higher enzyme activity was obtained when the cream was washed with 0.25 M sucrose than when distilled water or isotonic saline was used. The washed cream was adjusted to 35% fat with 0.25 M sucrose, cooled to 12–15° and churned. Occasionally, membranes were prepared by freeze–thawing. After straining through cheese-cloth, most of the remaining butterfat was removed by centrifugation at 3000 × *g* for 10 min. The preparation was carried out as quickly as possible and usually was completed within 2.5 h of milking. Some membrane preparations were centrifuged at 100 000 × *g* for 1 h. A dense brown pellet covered by a fluffy pale yellow precipitate and a clear supernatant fraction were obtained.

Electron microscopy

Droplets of diluted washed cream were placed on carbon-coated copper grids and shadowed with chromium. In some cases a grid was momentarily frozen and thawed prior to shadowing. Some preparations of washed cream, or washed cream which had been frozen and thawed, were fixed for 2 h in 2 vol. of 2.5% glutaraldehyde and 2.0% acrolein in 0.1 M acetate–veronal buffer (pH 7.2). After thorough washing in ice-cold buffer, the material was suspended in a 2% osmium tetroxide solution for 1 h. After thorough washing in buffer, the material was dehydrated in acetone and embedded in Araldite 502. Silver to gray sections were collected on carbon-coated

grids, stained with uranyl acetate and lead, and examined in an R.C.A. EMU 3 microscope.

Immune studies

Samples of membrane preparations were adjusted to contain 0.5% protein with isotonic saline, and emulsions were prepared with equal volumes of complete Freund's adjuvant. Rabbits were immunized by subcutaneous injection of 0.5 ml emulsion followed by a similar injection a week later with an emulsion prepared using incomplete Freund's adjuvant. After a 30-day period the rabbits were exsanguinated and the serum obtained after clotting was divided into aliquots and stored at −20° without a preservative.

Agglutinating antibodies were detected by mixing 0.2 ml of a 2% washed erythrocyte suspension in isotonic saline with 0.2 ml serum and incubating at 37° for 1 h with gentle mixing. If no agglutination was observed, the cells were washed twice with isotonic saline and 0.2 ml anti-rabbit horse serum or anti-human rabbit serum added (depending on whether rabbit or human serum was used initially). The mixture was incubated at 37° for 15 min and then centrifuged and examined. Titers were estimated by serial dilutions.

Enzyme determinations

The following enzymes were assayed essentially as described in the references cited: acid phosphomonoesterase (EC 3.1.3.2) and alkaline phosphomonoesterase (EC 3.1.3.1) using *p*-nitrophenylphosphate as substrate[20], phosphodiesterase (EC 3.1.4.1) using bis(*p*-nitrophenyl)phosphate as substrate[20], NADH–cytochrome *c* reductase (EC 1.6.2.1)[21], succinate–cytochrome *c* reductase (EC 1.3.99.1)[22], cytochrome *c* oxidase (EC 1.9.3.1)[23], acetylcholinesterase (EC 3.1.1.7) using acetyl-β-methylcholine as substrate[24], β-glucuronidase (EC 3.2.1.31)[25], aldolase (EC 4.2.1.7)[26], aspartate–oxoglutarate aminotransferase (EC 2.6.1.1)[27], lipase (EC 3.1.1.3)[28], lactate dehydrogenase (EC 1.1.1.27)[29] and glucose-6-phosphatase (EC 3.1.3.9)[30].

Assays of Mg^{2+}-activated ATPase (EC 3.6.1.4) were performed in an incubation medium consisting of 3 mM Tris–ATP (Na^+-free), 6 mM $MgCl_2$, 3 mM cysteine and 30 mM Tris (pH 7.4). The (Na^+-K^+-Mg^{2+})-activated ATPase was assayed in a medium containing 80 mM NaCl and 40 mM KCl in addition to the forementioned components. The inhibition of (Na^+-K^+-Mg^{2+})-activated ATPase was assayed by adding ouabain, final concentration 0.1 mM, to the incubation mixture.

Total protein was determined on dried aliquots after extraction of lipid with ether by the biuret method using standard samples calibrated by Kjeldahl nitrogen determinations.

Potassium uptake by fat globules

The uptake and turnover of potassium by the fat globules was determined essentially as described by KAHN AND ACHESON[31]. Tracer amounts of ^{42}K were added to washed, fresh cream in Locke–Ringers solution adjusted to contain 25–30% butterfat, and mixed thoroughly. Aliquots were removed at 5-min intervals and the cream layer and plasma were assayed for radioactivity.

RESULTS

Electron microscopy

Ghosts of fat globule membranes were obtained by use of ultrasonic vibrations or freeze–thawing; shadowed specimens were examined with the electron microscope (Fig. 1). They appeared to be empty sacs, surrounded by tiny fat droplets when prepared directly on a grid. No ultrafine structure was observed. Sections of washed cream, particularly after fat extraction, showed a membrane approx. 90 Å thick (Fig. 2). A triple layer could be clearly distinguished, but only in places where the section was cut at right angles to the membrane. In places where the section was cut at an angle, a single line or a wider smeared band was observed. Dense particles, 30–110 Å in diameter, appeared to adhere to the outer surface of the membranes.

The average yield of eight membrane preparations was 0.494 g per l fresh milk. Using a measured density for the membranes of 1.16 g/cm^3 and assuming a fat globule surface area of 0.55 $\times$ 10^6 cm^2 per l milk[32], a thickness of 78 Å is calculated assuming all of the membrane preparation is from the surface of the fat globules. Because of

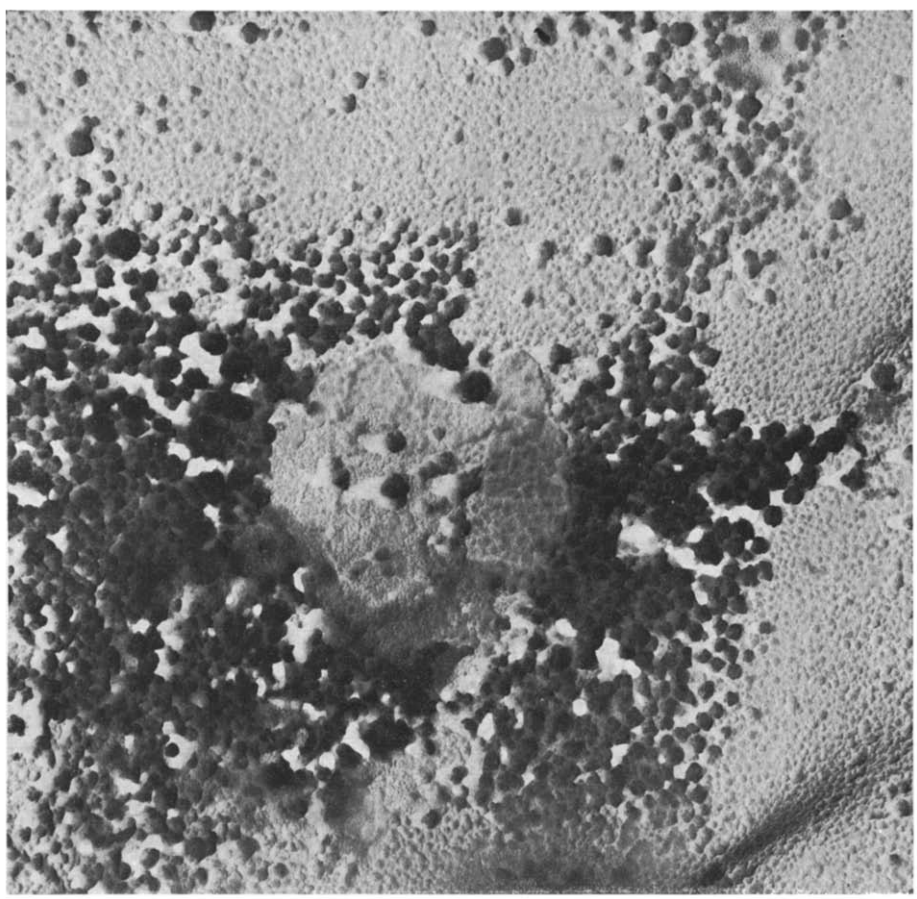

Fig. 1. An electron photomicrograph of a fat globule ghost surrounded by droplets of butterfat.

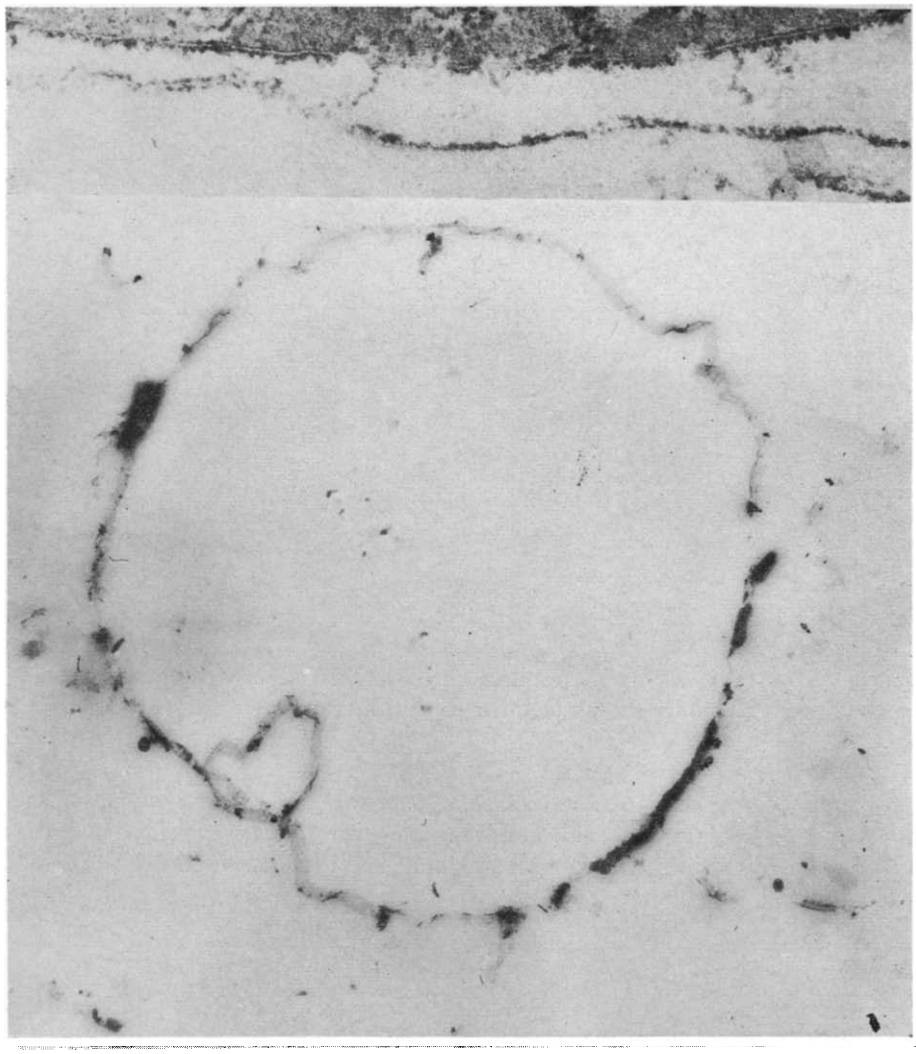

Fig. 2. An electron photomicrograph of a section through a fixed and stained fat globule.

losses in the preparation of the membranes, the figure calculated for thickness probably represents a lower limit.

Immune studies

Rabbit antisera prepared using fat globule membranes as antigen agglutinated bovine but not rabbit nor human erythrocytes (Table I). Hemolysis was observed when fresh plasma was added to the incubation mixture. Non-immune rabbit serum did not agglutinate bovine erythrocytes. Nine of twelve randomly obtained human sera agglutinated bovine erythrocytes; the highest titer (approx. 1:2048) was obtained from a young girl with multiple allergies, including a profound milk allergy. Sera from

TABLE I

INTERACTION OF VARIOUS SERA AND ERYTHROCYTES

Upper result agglutination upon mixing at room temperature.
Lower result hemolysis upon incubation at 37° for 2 h

Sera	*Erythrocytes*		
	Bovine	*Rabbit*	*Human*
Rabbit antimembrane	++++ / ++++	O / O	+ / O
Rabbit control	O / O	O / O	O / O
Mixed bovine	++++ / ++	+ / +	+ / O
Chicken	O / O	O / O	O / O
Mixed human	O to ++++ / O to ++++	O / O	++++ / ++++

four children afflicted with phenylketonuria who had received only casein hydrolysate but no cows' milk all showed weak agglutinating titers for bovine erythrocytes.

Enzyme studies

No succinate–cytochrome *c* reductase, cytochrome *c* oxidase, β-glucuronidase, lipase, aspartate–oxoglutarate aminotransferase nor lactate dehydrogenase activities could be demonstrated in the membrane preparations. Enzymes found to be present

TABLE II

Enzyme	*Number of determinations*	*Average (range)*
Alkaline phosphomonoesterase	8	0.63 (0.21–0.92)*
Acid phosphomonoesterase	8	0.087 (0.061–0.14)*
Phosphodiesterase (pH 8.9)	6	0.134 (0.092–0.161)*
Phosphodiesterase (pH 5.0)	6	0.008 (0.004–0.016)*
NADH–cytochrome *c* reductase	6	0.0068 (0.0031–0.0106)**
Cholinesterase	4	0.171 (0.139–0.227)***
Mg^{2+}-activated ATPase	8	0.74 (0.29–0.94)*
$(Na^+$-K^+-$Mg^{2+})$-activated ATPase	8	0.91 (0.54–1.18)*
Mg^{2+}-activated ATPase + ouabain	8	0.68 (0.29–0.91)*
$(Na^+$-K^+-$Mg^{2+})$-activated ATPase + ouabain	8	0.71 (0.32–0.92)*
Glucose-6-phosphatase	3	0.064 (0.056–0.074)*
Xanthine oxidase	4	1.304 (1.008–1.656)†
Aldolase	4	0.016 (0.012–0.024)††

* μmoles P_i liberated/mg protein per h.
** μmoles cytochrome *c* reduced/mg protein per h.
*** μmoles acetyl-β-methylcholine hydrolyzed/mg protein per h.
† μg xanthine oxidized/mg protein per h.
†† μmoles fructose diphosphate split/mg protein per h.

in the membrane preparations included alkaline phosphomonoesterase, acid phosphomonoesterase, phosphodiesterase, NADH–cytochrome *c* reductase, Mg^{2+}-activated ATPase, a (Na^+-K^+-Mg^{2+})-activated ATPase which is inhibited by ouabain, acetylcholinesterase, xanthine oxidase, aldolase and glucose-6-phosphatase. The activities found for the various enzymes are summarized in Table II. The activity of NADH–cytochrome *c* reductase and (Na^+-K^+-Mg^{2+})-activated ATPase were very labile and were largely absent from the membrane preparation 12–18 h after milking.

Upon centrifugation of the membrane preparation at 100 000 × *g* for 1 h, approx. 22.0% of the protein was found in the supernatant fraction, 38.3% in the pellet and 39.7% in the fluffy precipitate. The distribution of several enzymes in these fractions is summarized in Table III. The distribution of alkaline phosphomonoesterase and xanthine oxidase were similar to those obtained previously by ZITTLE *et al.*[33].

TABLE III

DISTRIBUTION OF MILK MEMBRANE ENZYMES

Enzyme	*Membrane fraction*		
	Pellet	*Fluff*	*Supernatant*
Alkaline phosphomonoesterase*	0.362	0.496	0.163
Alkaline phosphodiesterase*	0.157	0.129	0.307
Mg^{2+}-activated ATPase*	0.023	0.124	0.138
(Na^+-K^+-Mg^{2+})-activated ATPase*	0.048	0.148	0.016
Xanthine oxidase**	1.670	1.526	0.087
Aldolase***	0.0000	0.0043	0.067
Total protein	38.3%	39.7%	22.0%

* μmoles P_i/mg protein per h.
** μg xanthine oxidized/mg protein per min.
*** μmoles fructose diphosphate split/mg protein per h.

Potassium uptake

Upon the addition of ^{42}K to washed cream suspended in Locke–Ringers solution, equilibrium between the cream and plasma was reached before the first samples were obtained 5 min after mixing. The time for half exchange of ^{42}K by erythrocytes, in contrast, is about 35 h (ref. 47). The fat globule membrane does not appear to present a significant barrier to the diffusion of ions such as K^+.

DISCUSSION

A number of views of the fat globule interfacial protein have been put forward to explain its unique protein composition, the preponderance of phospholipids in its lipids, and the presence of enzymes (alkaline phosphomonoesterase, xanthine oxidase and NADH–cytochrome *c* reductase) characteristic of mammary gland cell microsomes. Of the modern hypotheses, KING[4,34] proposed that the fat globules are coated with a layer of phospholipids which strongly adsorb specific proteins. MORTON[15,35] suggested that a layer of cytoplasmic protein surrounds the fat globules to which a dense layer of microsomes is attached. We favor the view that the fat globule inter-

facial protein is a true biological membrane. From their electron microscopic studies of fat globules in various stages of formation and secretion in the mammary gland epithelium, BARGMANN *et al.*[17,18] concluded that the fat globules are secreted by projecting into the lumen of the duct surrounded by acinar cell membrane. Finally, the fat globule is amputated from the secretory cell in an envelope of cell membrane containing perhaps a very small amount of cytoplasm. In contrast, the protein and carbohydrate components of milk accumulate in vacuoles which move to the cell surface and open, discharging their contents into the duct.

We have observed many similarities of fat globules to erythrocytes. The fat globules are lysed by freeze–thawing, by sonication or by sapogenins. They are not lysed by hypotonic media presumably because they are freely permeable to water and low-molecular-weight solutes. If any cytoplasm were trapped within the fat globule, high permeability of the membrane would lead to loss of free water and low-molecular-weight solutes. Upon rupture the butterfat is released, leaving a ghost behind. SCHWARZ AND FISCHER[36], the first research workers to study milk fat globules by electron microscopy, proposed that the interfacial protein was about 100 Å thick and consisted of a "sandwich" of phospholipid between two layers of protein. KNOOP[37], however, did not find an unit membrane in his electron photomicrographs of fresh milk fat globules. He suggested that a morphological rearrangement of the unit membrane occurs shortly after the fat is secreted. The fat globules are surrounded by a membrane; in our own electron photomicrographs a definite unit membrane can be seen in places. In addition, there are small granules associated with the membrane. Although they are smaller in size, these granules may be identical with those described by MORTON.

The agglutination and hemolysis of bovine erythrocytes by antisera prepared by immunization with fat globule membranes is further evidence in support of the view that they are a cell-membrane derivative.

The presence of xanthine oxidase, alkaline phosphomonoesterase, phosphodiesterase and NADH–cytochrome *c* reductase, previously reported in cream[12,13,15,16,35], has also been found in these studies. In addition, we found Mg^{2+}-activated ATPase, (Na^{+}-K^{+}-Mg^{2+})-activated ATPase, glucose-6-phosphatase and true cholinesterase activity in the fat globule membranes*. These enzymes are all characteristically found upon differential centrifugation of homogenized tissue in the fraction which contains membranous material, the microsomal fraction, used as an operational term (see SIEKEVITZ[38] for a pertinent review). Aldolase, an enzyme found in the high-speed supernatant, was present in the membrane preparations, but lactate dehydrogenase and aspartate–oxoglutarate aminotransferase, other high-speed supernatant enzymes, were not present. Acid phosphomonoesterase, which is present in large quantities in skim milk[39], was found in the membrane preparations. Lipase and β-glucuronidase, two other hydrolytic enzymes, were not found. Lipase, which is reported to be present in large quantities in skim milk, may preferentially adsorb on cream when it is chilled[40]. The aldolase in the fat globule membranes appears to be loosely bound, as may be deduced from the relatively large fraction found in the clear supernatant fraction when the membranes were centrifuged at 100 000 $\times$ *g* for 1 h. Succinate–cytochrome

* Professor L. E. HOKIN found appreciable quantities of phosphatidic acid phosphatase and small amounts of diglyceride kinase activities in these fat globule membrane preparations.

c reductase and cytochrome *c* oxidase, enzymes which are characteristically associated with the mitochondrial fraction, were not found in the fat globule membranes.

The microsomal fraction contains fragments of the endoplasmic reticulum, an intracellular membranous system which resembles the cell membrane and probably is continuous with it[41,42]. On the basis of antigenicity and localization of (Na^+-K^+-Mg^{2+})-activated ATPase activity, WALLACH AND KAMAT[43] concluded that the cell-membrane fragments of Ehrlich ascites tumor cells sediment with the microsomal fraction. EMMELOT *et al.*[44] found these same "microsomal" enzymes (except cholinesterase) in their preparations of plasma membranes from rat-liver cells. Cholinesterase, alkaline phosphomonoesterase and (Na^+-K^+-Mg^{2+})-activated ATPase are present in erythrocyte ghosts[45,46]. The presence of "microsomal" enzymes in the fat globule membrane, therefore, lends support to the concept that it is derived from a true cell membrane. The presence of a (Na^+-K^+-Mg^{2+})-activated ATPase in the fat globule membranes is particularly noteworthy since this enzyme has been isolated from a variety of tissues which show selective permeability to cations and it is believed to be involved in "active transport" across the cell membrane[46].

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