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Transport competence of plasma membrane vesicles from cultured human fibroblasts *

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We obtained plasma membranes from cultured human skin fibroblasts. The preparation was enriched 10-fold with about 40 percent yield. There was minimal contamination with other cell membranes. Various observations indicated vesicular conformation of a portion of the plasma membranes, notably by electron microscopy and from the effect of osmotic pressure on the distribution of solutes between mass and medium at equilibrium. Other studies indicated that these fibroblast plasma membrane vesicles retained mediated transport processes for a variety of substrates. The evidence included: stereospecific and temperature-dependent uptake of glucose; dependence of L-alanine uptake on sodium ion and an inward-directed transmembrane Na^+ gradient; stimulation of L-alanine uptake, with overshoot, by enhancement of the interior-negative transmembrane potential; concentration dependent uptake of methotrexate with apparent competitive inhibition by folinic acid; stimulation of L-lysine uptake by *trans*-L-arginine. These findings indicate that human fibroblast plasma membrane vesicles could be used to study membrane transport processes and, perhaps, expression of mutant genes that cause inborn errors of transport.

Introduction

Human skin fibroblasts have been widely used to study normal and mutant cellular phenotypes [1], but rarely to investigate the human Mendelian variation that affects transmembrane transport processes [2]. The membrane vesicle preparation separates the transport process from intracellular events that might influence net uptake of the transported substrate; it also allows manipulation of the events at outer and inner membrane surfaces that drive transport. The process has already been well studied in plasma membrane vesicles prepared from various mammalian tissue culture lines [3,4]. Moreover, the isolation of plasma membranes from human skin fibroblasts has been reported by others [5–7] but there has been no detailed study yet of the transport competence of cultured human fibroblast plasma membrane vesicles.

We show here that the human preparation is useful to study membrane transport of low molecular weight organic solutes. We measured the relative purification of plasma membranes, confirmed their vesicular nature and demonstrated their com-

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Abbreviations: PBS, phosphate-buffered saline: 274 mM NaCl/54 mM KCl/16 mM Na_2HPO_4/1.2 mM KH_2PO_4/11 mM glucose/0.04% chloroform (v/v); MTX, methotrexate (4-amino-10-methylpteroylglutamic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

petence to transport various organic substrates. We used methotrexate (MTX), an analogue of folic acid, because a putative Mendelian disorder of folate transport has been reported [8]; we also studied representative Na^+-dependent cotransports for comparison with findings in mouse fibroblast membrane vesicles [4], preparatory to our study of a specific inborn error of amino acid transport.

Materials and Methods

Cell culture. Human fibroblast cultures were obtained from the Repository for Mutant Human Cell Strains (Montreal Children's Hospital). We used four control strains, derived from infant foreskin. Cultures were screened for mycoplasma contamination [9] before release from the Repository. Fibroblasts were maintained in glass roller bottles 690 cm^2 (Wheaton) in an atmosphere of 5% CO_2/95% air, fed twice weekly with culture medium containing Eagle's modified minimal essential medium supplemented with 10% fetal calf serum (GIBCO or Flow Laboratories), and released for subculture with trypsin (DIFCO) 0.25% w/v in PBS.

Plasma membrane isolation. Fibroblasts were subcultured into polystyrene roller bottles 850 cm^2 (Corning) and grown to confluence. Plasma membranes were isolated according to the method of Kartner et al. [5] with minor modifications. Culture medium was removed from eight roller bottles and these were washed four times with phosphate-buffered saline (see Abbreviations for composition). Cells were lysed in hypotonic medium (20 ml, 1 mM $NaHCO_3$ buffer, pH 8) added to each bottle, then rotated in an ice bath for 1 min. Buffer was removed and the procedure repeated twice. Buffer (20 ml) was then added, the bottles shaken vigorously to release lysed cells, and Na_2EDTA added to pooled lysates to a final concentration of 0.5 mM, followed by centrifugation at $27000 \times g$ for 20 min at 4°C in a type Ti 60 rotor (Beckman) in a Beckman ultracentrifuge (Model L5-75). The pellets were pooled, resuspended in 10% sucrose (w/v, final volume, 10 ml), layered on a discontinuous gradient comprising layers of 60%, 48%, and 30% sucrose, and centrifuged in a SW-27 rotor at $76000 \times g$ for 2 h at 4°C. Bands at each interface were removed, diluted, centrifuged at $65000 \times g$ for 25 min at 4°C, and resuspended, in sodium bicarbonate buffer (as above) or in the appropriate preincubation buffer. The fraction at the 10%–30% interface was further treated by passing it repeatedly through a 27 gauge needle fitted to a 1 ml syringe. This preparation was used for transport experiments after storage overnight on ice.

Enzyme markers. 5'-Nucleotidase activity was measured by the method of Ipata [10] on aliquots of the cell lysate taken before addition of Na_2EDTA. Activity was stable to freezing and thawing or storage on ice. Succinate-cytochrome *c* reductase and NADPH-cytochrome reductase were assayed according to Sottocasa et al. [11]; β-glucosidase by the method of Choy and Davidson [12]; galactosyltransferase activity by the method of Kaplan and Hechtman [13]; hexosaminidase according to Leaback and Walker [14]. Assays were performed on freshly prepared samples unless otherwise indicated. Protein was determined by the method of Lowry et al. [15] with bovine serum albumin as standard.

Uptake assays. Plasma membrane vesicles were preincubated and used for transport assays in one of three buffers: Buffer A, 20 mM Hepes/3 mM $MgCl_2$/225 mM sucrose (adjusted to pH 7.4 with KOH); Buffer B, 10 mM Tris/5 mM $MgCl_2$/225 mM sucrose (adjusted to pH 7.4) with H_3PO_4); Buffer C, 50 mM Hepes/1 mM $MgSO_4$/100 mM K_2SO_4 or Na_2SO_4 (as indicated) (adjusted to pH 7.5 with Tris); with added antibiotics (streptomycin, 0.33 g/l; and penicillin G, 10^5 units/l). When other salts were added to the buffers, the osmotic equivalent of sucrose was removed. 'Stop solutions' were the corresponding buffer in which 0.8 M NaCl replaced sucrose or other salts as described by Lever (cited in Ref. 4) for plasma membrane vesicles. In experiments with methotrexate, unlabelled substrate (0.1 mM) was also added. All solutions were filtered immediately before use through nitrocellulose filters (0.22 or 0.45 μm, Millipore).

Aliquots (10 μl) of preincubated vesicles containing 20–70 μg of protein were equilibrated at incubation temperature and then diluted at zero time with 90 μl of uptake buffer containing radioactive substrate. Uptake was stopped by dilution

with 3 ml ice-cold 'stop solution' and filtered rapidly over vacuum (40–50 cmHg) using 0.45 μm nitrocellulose filters (Millipore) on a Millipore filter manifold, followed by three additional washes (3 ml) with 'stop solution' as proposed by others [4]. Filters were placed in 'minivials' (Fisher) with 5 ml Aquasol (New England Nuclear) and radioactivity measured in an LKB Wallac liquid scintillation counter with quench correction. Correction for background radioactivity was made using samples processed as above but with 'stop solution' added before uptake buffer.

Radiochemicals and pharmaceutical reagents. [^{3}]MTX ([3′,5′,7-^{3}H]methotrexate, sodium salt, Amersham, 10–20 Ci/mmol) was purified before use as follows: [^{3}H]methotrexate was dissolved in 50 mM ammonium bicarbonate (eluting buffer) and applied to a column of Sephadex G-15 (Pharmacia). Eluted fractions were assessed for radioactivity and peak fractions were pooled, lyophilized, resuspended in 10% ethanol and stored at −20°C. Other radiochemicals were used without further purification: D-[^{14}C]glucose (glucose, D-[^{14}C(U)]-, 345 Ci/mol, New England Nuclear), L-[^{3}H]glucose (glucose, L-[1-^{3}H(N)]-, 10.7 Ci/mmol, New England Nuclear), L-[^{3}H]lysine (L-[4,5-^{3}H]lysine monohydrochloride, 77 Ci/mmol, Amersham), L-[^{3}H]alanine (alanine, L-[3-^{3}H]-, 82.7 Ci/mmol, New England Nuclear). Methotrexate (Lederle) was purified as for tritiated methotrexate with peak fractions monitored by absorbance at 280 nm. D,L-folinic acid (Lederle) was used without

TABLE I

MARKER ENZYME ACTIVITY IN FRACTIONS OBTAINED DURING PLASMA MEMBRANE ISOLATION

Units: 5′-nucleotidase, succinate-cytochrome *c* reductase, hexosaminidase, nmol·min^{-1}·(mg protein)$^{-1}$; NADPH-cytochrome *c* reductase, μmol·min^{-1}·(mg protein)$^{-1}$; β-glucosidase, galactosyltransferase, nmol·h^{-1}·(mg protein)$^{-1}$. The cell fractionation procedure is described under Materials and Methods. 'Lysate' refers to the starting material of lysed cells. 'Supernatant' and 'pellet' refer to fractions from the first 27000 × *g* centrifugation. '10/30', '30/48' and '48/60' refer to interface fractions from the sucrose gradient. Abbreviations: S.A., specific activity; -fold, S.A. in fraction/S.A. in lysate; %, 100 × (total activity in fraction/total activity in lysate). Numbers in brackets indicate the number of preparations in which replicate assays were performed for a given fraction.

		Lysate	Supernatant	Pellet	Sucrose gradient fractions		
					10/30	30/48	48/60
Protein	mg	(35) 58.1	(35) 26.3	(24) 22.1	(30) 2.3	(30) 3.1	(23) 2.4
	%	100	46.2	38.1	4.1	5.5	4.3
Plasma membrane	S.A.	(20 98	(15) 14	(20) 273	(21) 944	(19) 329	(15) 166
5′-Nucleotidase	fold	1	0.1	2.9	10.0	3.4	2.0
	%	100	6.0	109.1	38.8	20.8	6.8
Endoplasmic reticulum	S.A.	(2) 0.25	(2) 0.10	(2) 0.38	(2) 0.38	(20) 0.41	(2) 0.20
NADPH-cytochrome *c*	-fold	1	0.4	1.7	1.6	1.7	0.9
reductase	%	100	19.2	59.1	5.1	7.2	1.7
Mitochondria	S.A.	(5) 8.1	(5) 1.0	(9) 18.0	(9) 1.7	(9) 48.6	(8) 7.2
Succinate-cytochrome	-fold	1	0.1	3.0	0.4	6.8	1.5
c reductase	%	100	6.0	131.6	1.9	66.3	4.5
Lysosomal membrane	S.A.	(3) 160	(3) 31	(4) 301	(3) 799	(3) 182	(3) 67
β-Glucosidase	-fold	1	0.2	2.0	5.0	1.4	0.5
	%	100	9.5	80.7	18.0	5.9	0.8
Lysosomes	S.A.	(1) 56.1	(1) 91.5	(1) 21.5	(1) 58.4	(10) 23.5	(10) 13.9
Hexosaminidase	-fold	1	16.	0.4	1.0	0.4	0.3
	%	100	77.0	14.0	2.5	1.7	0.4
Golgi	S.A.	(2) 5.0	(2) 2.1	(1) 4.2	(2) 1.6	(2) 2.1	(1) 1.0
Galactosyl-transferase	-fold	1	0.4	1.1	0.4	0.5	0.2
	%	100	10.5	49.0	2.0	3.5	0.3

further purification. Valinomycin (Sigma) was dissolved in 99% ethanol before addition to aqueous buffers. Equivalent amounts of ethanol (not exceeding 1% v/v) were added to control samples.

Results

Plasma membrane purification

Data for markers of subcellular fractions are summarized in Table I. The fraction at the 10%–30% sucrose gradient interface was enriched 10-fold in 5′-nucleotidase activity, with 39% recovery of the starting activity. Markers for endoplasmic reticulum, mitochondria, and Golgi were not significantly enriched in this fraction. Most hexosaminidase activity was recovered in the supernatant from the first centrifugation, suggesting that lysosomes were disrupted during cell lysis. β-Glucosidase, a membrane-bound lysosomal enzyme, was enriched 5-fold in the 10%–30% interface fraction indicating some contamination of the plasma membrane fraction with lysosomal membranes.

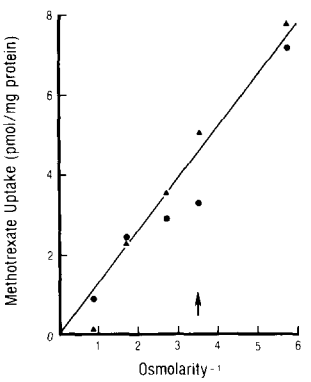

Fig. 1. Effect of varying medium osmolarity (mosmoles) on accumulation of methotrexate. Vesicles were preincubated in Buffer A. Reactions were started by dilution with uptake buffers containing 2 μM [^{3}H]methotrexate + Buffer A containing various concentrations of sucrose (0.125–1 M). The abscissa indicates the inverse of total osmolarity for each medium, with the arrow indicating isotonic buffer. Incubations were for 10 min at 30°C. Each point represents the mean of five determinations with symbols indicating two separate experiments. The best-fitting linear regression was calculated for all data by the method of least squares.

Evidence for a vesicular conformation of plasma membranes

(a) Electron microscopic images. About half of the membrane images were vesiculated with an average diameter of 1 μm [16].

(b) Osmotic sensitivity of uptake. Net uptake of methotrexate at equilibrium decreased when intravesicular volume was diminished by increasing the extravesicular osmolarity (Fig. 1). The best-fitting linear regression of uptake values intersected with the origin indicating that methotrexate did not bind to plasma membranes and was contained intravesicularly. On the other hand 10–20% of L-alanine accumulation under equilibrium conditions could be attributed to binding; uptake of L-lysine also included a binding component (data not shown).

Transport competence of vesicles

(a) Stereospecificity and temperature dependence of uptake. D- and L-glucose were taken up at equivalent low rates by vesicles at 0°C, presumably by a diffusional process (Fig. 2). At 37°C L-glucose uptake was only slightly enhanced whereas D-glucose uptake was strikingly increased. Stereospecificity and temperature dependence of uptake are consistent with carrier-mediated transport of the monosaccharide.

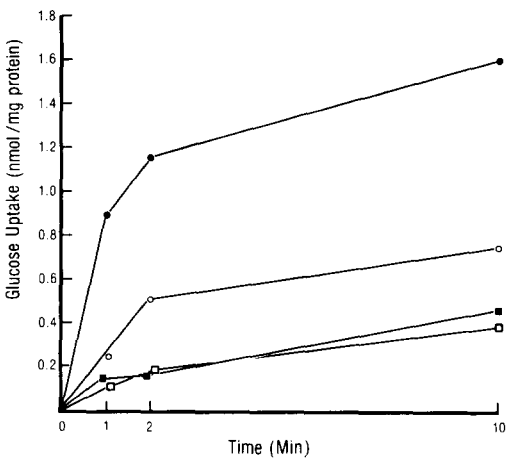

Fig. 2. Stereospecificity and temperature dependence of glucose uptake. Vesicles were preincubated in Buffer A. Reactions were started by dilution with uptake buffers containing Buffer A + 1 mM D-[^{14}C]glucose (●, □) or + 1 mM L-[^{3}H]glucose (○, □). Incubations were at 0°C (□, ■) or 37°C (○, ●). Points represent means of three determinations.

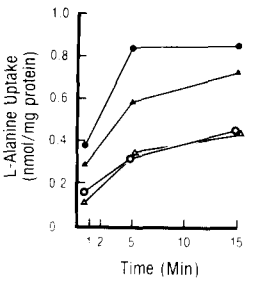

Fig. 3. Effect of a sodium chloride or sodium thiocyanate gradient on L-alanine uptake. Vesicles were preincubated in Buffer B. Reactions were started by dilution with uptake buffers containing Buffer B + 0.2 mM L-[^{3}H]alanine, + 50 mM NaSCN (●), or NaCl (▲), or KSCN (○) or KCl (△) replacing 100 mM sucrose. Incubations were at 37°C. Points represent means of three determinations.

(b) Sodium stimulation of uptake and response to a membrane potential. Uptake of L-alanine was stimulated by an inward-directed Na^+ gradient across the membrane relative to uptake in the presence of a K^+ gradient (Figs. 3 and 4). Na^+-dependent uptake of L-alanine was stimulated by generating an increased interior-negative membrane potential, for example when SCN^- anion was substituted for Cl^- (Fig. 3). The uptake rate also increased in vesicles filled with KCl when valinomycin was added (Fig. 4).

(c) Concentration dependence and competitive inhibition. Temperature-dependent uptake of

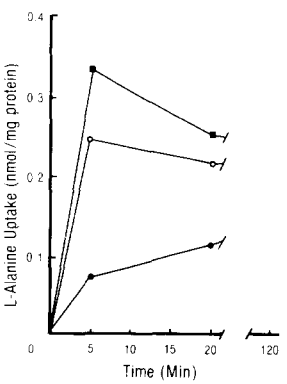

Fig. 4. Effect of a sodium gradient on L-alanine uptake, with or without a potassium diffusion potential + valinomycin. Vesicles were preincubated in Buffer C (K_2SO_4). Reactions were started by dilution with uptake buffers containing 50 μM L-[^{3}H]alanine, + Buffer C (K_2SO_4) (●), or Buffer C (Na_2SO_4) (○) or Buffer C (Na_2SO_4) + 10 μg/ml valinomycin (■). Points represent means of three determinations.

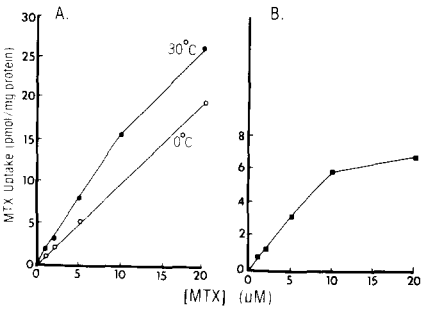

Fig. 5. Concentration dependence of temperature-dependent methotrexate uptake. Vesicles were preincubated in Buffer A. (A) Reactions were started by dilution with uptake buffers containing Buffer A + 1 μm [^{3}H]methotrexate + various concentrations of unlabelled methotrexate to yield final concentrations as indicated. Reactions were stopped after 10 min incubation. (B) Points represent the difference between means of four determinations at 30°C and at 0°C.

methotrexate (uptake at 37°C minus uptake at 0°C) increased asymptotically with increasing concentration of the substrate (Fig. 5); the rate of uptake, at 5 μM methotrexate decreased as the concentration of folinic acid was increased (Fig. 6). Both observations are consistent with a finite number of sites for methotrexate transport in the membrane.

(d) Exchange stimulation. L-Lysine uptake was stimulated when vesicles were filled with L-arginine at ten times the external lysine concentration, relative to uptake into unfilled vesicles (Fig. 7). *Trans*-stimulation of uptake is a test for mediated transport.

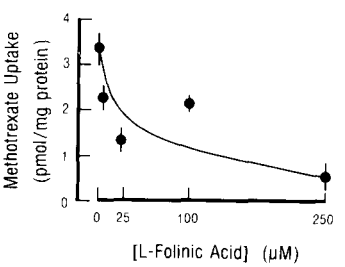

Fig. 6. Effect of L-folinic acid, a competitive inhibitor of methotrexate transport, on the temperature-dependent uptake of methotrexate. Vesicles were preincubated in Buffer A, then diluted with uptake buffer (Buffer A + 5 μM [^{3}H]methotrexate + various concentrations of L-folinic acid as indicated). Uptake was stopped after 10 min incubation. Points represent difference between values obtained at 37°C and (total uptake) at 0°C (diffusional uptake), mean of four measurements.

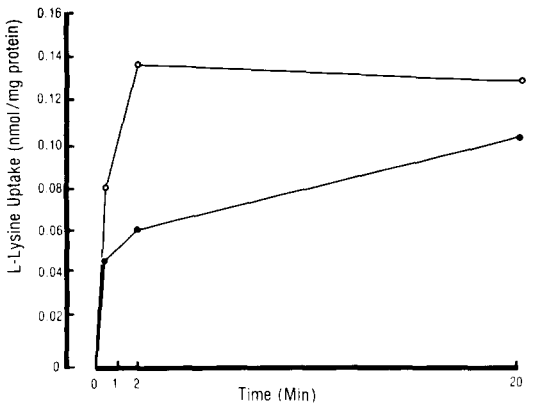

Fig. 7. Effect of intravesicular L-arginine on uptake of L-lysine in vesicles. Vesicles were preincubated in Buffer C (K_2SO_4) (●), or Buffer C (K_2SO_4)+5 mM L-arginine (○). Reactions were started by dilution with uptake buffers containing Buffer C (K_2SO_4)+50 μM L-[^{3}H]lysine+10 μg/ml valinomycin+50 μM L-arginine to control for the extravesicular arginine after dilution. Incubations were at 37°C. Points represent means of three determinations.

Discussion

We used marker enzymes to confirm that the fractionation procedure isolated plasma membranes. Specific activity of 5′-nucleotidase in our plasma membrane fraction, was enriched 10-fold. This value is lower than the apparent enrichment reported by Kartner et al. [5]; however, their homogenate contained EDTA which inactivates the enzyme [6]. Specific activity of the nucleotidase in our membrane fraction was similar to that reported by others [5,6].

Mitochondria were recovered in the 30/48 density gradient fraction, as indicated by the distribution of succinate-cytochrome *c* reductase; mitochondria were obtained in the same fraction by Kartner et al. [5]. Galactosyltransferase specific activity was not enriched in the 10/30 density gradient fraction in our preparations; Kartner et al. [5] reported 2-fold enrichment in their plasma membrane fractions. The only contaminant of the plasma membrane preparation was lysosomal membranes, indicated by the increased specific activity of lysosomal membrane-bound, β-glucosidase. This is not an important contamination since the lysosomal membrane mass in fibroblasts is small compared to the plasma membrane mass. Hexosaminidase, a more readily released enzyme, was recovered in the first supernatant fraction, suggesting that lysosomes were partially ruptured during the stage of hypotonic disruption. Karter et al. [5] did not measure lysosomal markers.

We obtained evidence for a vesicular conformation of some portion of the plasma membranes. First, from electron micrographs, we observed vesicles. Second, an increase in external osmotic pressure reduced the equilibrium volumes for both methotrexate and L-alanine. However, we could not define rigorously the major orientation of the vesicles or the relative proportions of intact and leaky vesicles and sheets in the membrane preparation. Preliminary attempts (Buchanan, J.A., unpublished data) suggest that about half of the membrane mass was vesiculated, and orientation of the vesicles was random.

The osmotic experiments also showed that there was little non-specific binding of methotrexate or L-alanine to the membranes. On the other hand, the cationic amino acid, L-lysine, was both bound and transported, a finding reported by others for renal brush-border membrane vesicles [17].

We performed various studies to demonstrate that our human fibroblast plasma membrane vesicle preparation would support mediated transport of solutes. The findings included evidence for: stereospecific and temperature-dependent uptake of glucose; dependence of L-alanine uptake on sodium ion and an inward-directed transmembrane Na^+ gradient; stimulation of L-alanine uptake with overshoot (uptake against a chemical gradient) by enhancement of the interior-negative transmembrane potential; concentration-dependent uptake of methotrexate; apparent competitive inhibition by folinic acid of methotrexate uptake; stimulation of L-lysine uptake by *trans*-L-arginine. These findings are compatible with the observations summarized by Lever [18] for entry of D-glucose into intact mammalian non-epithelial cells; by Lever [19] and Gazzolla et al. [20] for entry of L-alanine into intact mouse and human fibroblasts, respectively; by Goldman [21] and Huennekens et al. [22] for entry of methotrexate into various mammalian cells; and by White et al. [23] and White and Christensen [24] for entry of L-lysine into human fibroblasts. Accordingly, we conclude that plasma membrane vesicles prepared

from cultured human skin fibroblasts can be used to study the transport processes unmodified by intracellular events, such as incorporation into macromolecules and metabolic conversion, that might influence net uptake into whole cells [25].

Our purpose in undertaking this work was to develop a preparation that would allow investigation of transport processes in human fibroblast plasma membranes and in mutant phenotypes which may affect such processes. We propose that mutations which apparently have their effect at the basal-lateral membrane of renal or intestinal epithelia or in plasma membranes of circulating blood cells, for example, might be expressed in the corresponding plasma membranes of skin fibroblasts. However, we have found no evidence, so far, that a Mendelian phenotype which apparently impairs lymphocyte transport of folate is expressed in the fibroblast plasma membrane [16]; nor did we obtain evidence, in preliminary studies [26], that the lysinuric protein intolerance mutation, which involves the basal-lateral membranes of renal and small-bowel epithelial [27], is expressed in the fibroblast plasma membrane. These negative findings do not invalidate the value of the preparation for studies of transport processes, their ontogeny and adaptation to variant conditions, or their function in mutant phenotypes.

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