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# Evidence for the translocation of 5′-nucleotidase across hepatic membranes *in vivo*

**(Golgi fractions/membrane protein orientation/cytochemistry/antibody/concanavalin A)**

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**ABSTRACT** Hepatic 5′-nucleotidase (EC 3.1.3.5; 5′-ribonucleotide phosphohydrolase) activity has been studied in cisternal elements of the Golgi complex and in secretion vacuoles, both isolated after ethanol administration to rats *in vivo*. The enzyme in secretion vacuoles was latent, so that a 5-fold increase in activity was observed when incubations were carried out in the presence of detergent; evidence is presented that the latency is caused by the impermeability of the membrane to substrate. Essentially no latency was observed in Golgi cisternae. Confirming the results of Farquhar *et al.* [(1974) *J. Cell Biol.* 60, 8–25], reaction product from 5′-nucleotidase was localized by cytochemical procedures on the inside of secretion vacuoles and on the cytoplasmic side of Golgi cisternae. After solubilization in detergent, the enzyme from both fractions reacted almost identically with both antibody to the purified enzyme and concanavalin A. In contrast, when intact fractions were incubated with an excess of antibody or concanavalin A, only 22–23% of the enzyme was inhibited in secretion vacuoles whereas 51–84% was inhibited in Golgi cisternae. Sonication of secretion vacuoles in the presence of antibody or concanavalin A increased the inhibition 2- to 3-fold. It is suggested that during the formation of secretion vacuoles from the Golgi cisternae, 5′-nucleotidase is translocated from the cytoplasmic side of the membrane to the inside.

Very low density lipoproteins are synthesized and secreted by the liver. The elaboration of these lipoproteins takes place within the membranes of the endoplasmic reticulum and the cisternae of the Golgi complex; they are then transported from the Golgi complex to the plasma membrane within secretion vacuoles, which are apparently derived from the periphery of the Golgi cisternae (1–6). Although the precise function of the different membranes involved in the secretion pathway has yet to be established, it is clear that, in general, the endoplasmic reticulum, Golgi, and plasma membranes contain different enzymes and proteins (7). So far, the only protein whose presence has been clearly established in all of these membranes is the enzyme 5′-nucleotidase (EC 3.1.3.5; 5′-ribonucleotide phosphohydrolase) (8, 9).

When cytochemical procedures, involving the trapping of inorganic phosphate as lead phosphate, are used to localize 5′-nucleotidase activity, the reaction product is localized on the cytoplasmic side of microsomal membranes in isolated cell fractions (8) and on the external side of plasma membranes both in isolated cell fractions and *in situ* (8–11). Farquhar *et al.* (9) have recently shown that reaction product is localized on the cytoplasmic side of cisternal elements of the Golgi complex, and on the inside of secretion vacuoles. The results could suggest that the reversed asymmetry of the site of precipitation of $P_i$ in plasma membranes compared to microsome membranes is the result of a reorientation of the enzyme during the formation of secretion vacuoles from Golgi cisternae.

Abbreviation: DOC, sodium deoxycholate.

We have therefore studied the properties of 5′-nucleotidase in cisternal elements of the Golgi complex and in secretion vacuoles. We show here that the enzyme in secretion vacuoles is latent, and relatively inaccessible to both antibody and concanavalin A, whereas in cisternal elements of the Golgi complex, the enzyme is not latent, and is accessible to both antibody and concanavalin A. In addition, we have confirmed the cytochemical studies of Farquhar *et al.* (9).

## MATERIALS AND METHODS

**Materials.** The sources of materials, the purification of 5′-nucleotidase, and the preparation of antibody have been described elsewhere (12, 13).

**Cell Fractions.** Golgi fractions were isolated either as described by Ehrenreich *et al.* (5) or by Leelavathi *et al.* (14), and plasma membranes and microsomes as described elsewhere (8).

**Assay for 5′-Nucleotidase.** The determination of the specific activity of 5′-nucleotidase has been described in detail (12, 13). Any variation is indicated in the legends to the figures and table. The cytochemical assay was carried out as described elsewhere (8), or as modified by Farquhar *et al.* (9). This technique resulted in an identical localization and an improved morphology of the fractions.

**Inhibition of 5′-Nucleotidase by Antibody or Concanavalin A.** Because a major fraction of the 5′-nucleotidase activity in secretion vacuoles was latent, two methods were used to determine the inhibition by antibody and concanavalin A. In Procedure A, samples were preincubated at 0° in (0.1 ml) 0.25 M sucrose, 0.1 M Tris·HCl (pH 7.5), 10 mM $MgCl_2$ either with no additions (controls), or with purified gamma globulin (15) containing antibody to 5′-nucleotidase, or concanavalin A, both added at twice the concentration (as determined in preliminary assays) required to cause maximum inhibition in fractions solubilized in 1% Triton X-100, 0.5% sodium deoxycholate (DOC). After 30 min, the samples were diluted to 1.0 ml with the same medium containing in addition 11 mM AMP, and the rate of release of $P_i$ was determined at 25°.

In Procedure B, fractions were preincubated as described above, except that the volume was 0.25 ml, and antibody or concanavalin A was added at five times the concentration required for maximum inhibition of the total activity. Samples incubated with antibody were reisolated by flotation from 1.5 M sucrose to a 1.3/0.25 M sucrose interface by centrifugation for 45 min at 50,000 rpm in the Spinco SW50.1 rotor. This reisolation procedure decreased the inhibition by concanavalin A, so that fractions incubated with the lectin were recovered, after dilution with 5 ml of preincubation medium, by centrifugation at 50,000 rpm in the Spinco 50 rotor for 30 min. The specific activity of 5′-nucleotidase was then determined at 37° in samples solubilized in 1% Triton X-100, 0.5% DOC.

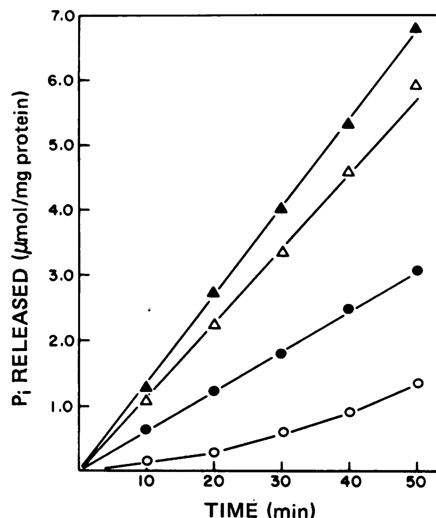
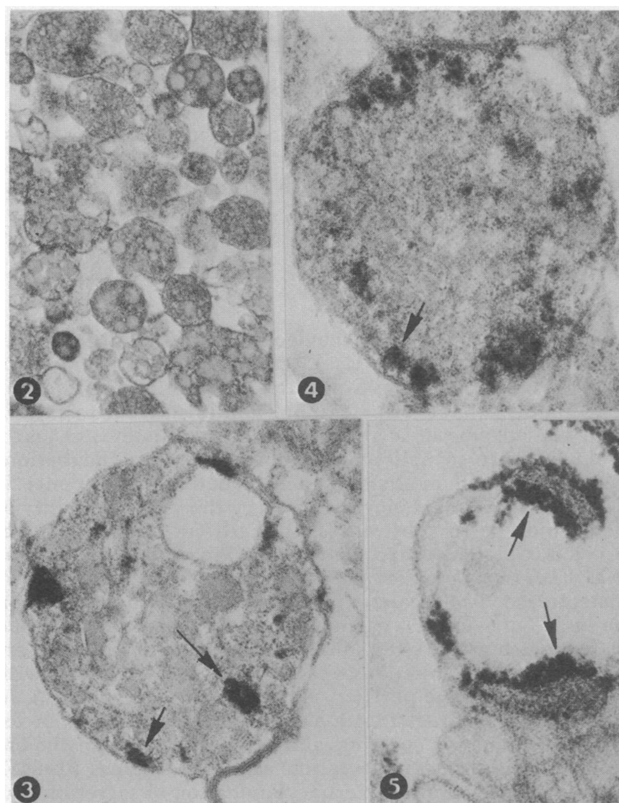



FIG. 1. Hydrolysis of 5′-AMP by 5′-nucleotidase in Golgi fractions. The fractions were isolated by the procedure of Ehrenreich *et al.* (5), and incubations were carried out at 25° in a medium containing 0.1 M Tris·HCl pH 7.5, 10 mM $MgCl_2$, and 10 mM 5′-AMP. $P_i$ was determined as described in the text. *Circles* denote Golgi fraction 1 (secretion vacuoles) and *triangles*, Golgi fraction 3 (cisternae). Incubations were performed in the absence of detergent (open symbols) or with fractions solubilized in 1% Triton X-100, 0.5% DOC (closed symbols).



FIGS. 2–5. Electron micrographs of thin sections of pellets of Golgi fractions. *Fig. 2.* Unincubated secretion vacuoles (5). Representative field from the center of a uniform pellet. Most of the vacuoles appear intact and contain very low density lipoproteins. ×27,500. *Fig. 3.* Secretion vacuoles were incubated in the cytochemical medium for 20 min with 5′-AMP. Typical field from the center of the pellet. The profile represents a buckled cisterna (see ref. 5) with lead precipitate on the inside, both associated with the membrane (short arrow) and in the matrix (long arrow). ×70,000. *Fig. 4.* Secretion vacuoles were incubated in the cytochemical medium without substrate for 30 min and then for 20 min with 5′-AMP. Typical field from the center of the pellet, illustrating a secretion vacuole with lead precipitate on the inside of the membrane (arrow). ×70,000. *Fig. 5.* Golgi cisternae (14) were incubated in the cytochemical medium for 20 min with 5′-AMP. Typical field from the center of the pellet illustrating a cisterna with reaction product concentrated at the rims (arrows). ×110,000.

## RESULTS AND DISCUSSION

### Latency of 5′-nucleotidase in secretion vacuoles

In agreement with the original report (7), the specific activity of 5′-nucleotidase in secretion vacuoles and cisternal elements of the Golgi complex isolated from ethanol-treated rats (5) was less than that of the plasma membrane, but greater than that of rough microsomes. The contamination of the preparations by glucose-6-phosphatase was also very similar to that described in the original report (7), and the properties of Golgi cisternae isolated from untreated rats (14) were essentially the same as in fractions from ethanol-treated animals. In all Golgi fractions, the enzyme was firmly bound to membranes as judged by its insolubility in 1% Triton X-100 (see refs. 12 and 13).

In contrast to other cell fractions, 5′-nucleotidase activity in secretion vacuoles was latent, in that there was a marked increase in activity when detergent was included in the assay. The latency was most evident when assays were performed at 25° and at pH 7.5. Under these conditions, the rate of $P_i$ release was linear with time when Golgi cisternae (or microsomes or plasma membranes) were incubated either with or without Triton X-100 and DOC, and when secretion vacuoles were incubated in the presence of the detergents (Fig. 1).

When secretion vacuoles were assayed in the absence of detergent, the rate of $P_i$ release increased as the incubation progressed (Fig. 1); the initial rate of $P_i$ release was 20%, and the final rate 60%, of that in the presence of Triton X-100. The addition of 0.25 M sucrose apparently stabilized the vacuoles, so that the final rate decreased; the final rate increased when $Mg^{2+}$ was replaced by $K^+$. Although $Pb^{2+}$ inhibited 5′-nucleotidase (see ref. 8), it also released the latency, so that when assays were performed in the cytochemical medium, the final rate of reaction was the same in the presence or absence of detergent. No latency was observed when the vacuoles were sonicated before assay.

It is likely that the latency reflects a change in the $V_{max}$ since the substrate concentration used in these assays was 500 times the $K_m$ of the enzyme for 5′-AMP in microsomes or plasma membranes. This implies the unmasking of previously inactive enzyme molecules, perhaps because the membranes of secretion vacuoles became spontaneously permeable to the substrate during incubation.

### Cytochemical localization of 5′-nucleotidase in Golgi fractions

The morphology of unincubated secretion vacuoles is illustrated in Fig. 2. After incubation for 20 min in the cytochemical medium for 5′-nucleotidase, lead phosphate precipitates were observed entirely on the inside of the vacuoles (Fig. 3); controls incubated with 2′-AMP were free of reaction product (not shown). These results are in complete agreement with the study of Farquhar *et al.* (9).

Secretion vacuoles were preincubated with $Pb^{2+}$ but without substrate at 25° for 30 min, and then incubated for a further 20 min with 5′-AMP; under these conditions essen-

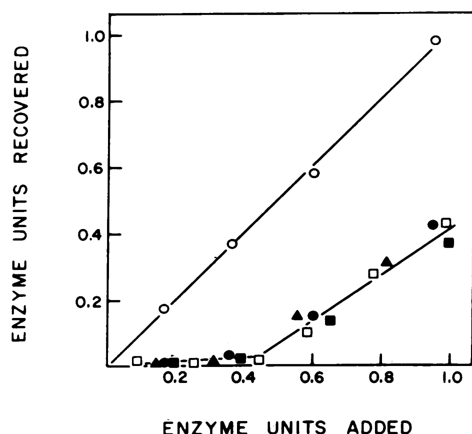



FIG. 6. Inhibition of 5′-nucleotidase by antibody. Golgi fractions (5) were solubilized in 0.1 M Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1% Triton X-100, 0.5% DOC, and samples containing the desired enzyme activity were incubated with 10 μl of serum at 0° overnight. Enzyme activity was determined as in Table 1; 1 unit of enzyme activity released 1 μmol of $P_i$ per min at 37°. Golgi fractions 1 (▲), 2 (●), and 3 (■) and purified 5′-nucleotidase (□) were incubated with serum containing antibody to 5′-nucleotidase, and Golgi fraction 2 (○) was incubated with control serum.

tially all the latent activity was expressed. Reaction product was again localized on the inside of the vacuoles (Fig. 4), but a larger number seemed to contain lead precipitate. This would be expected if the number of vacuoles that were permeable to substrate increased as the incubation progressed. The fraction of stained vacuoles was estimated by counting profiles with and without reaction product in fields selected at random from the top to the bottom of the pellet, and at several different locations within the pellet. In preincubated preparations 65% (1979/2900) of the vacuoles were stained, whereas without preincubation only 37% (1043/2800) were stained.

Golgi cisternae, obtained after ethanol treatment, were incubated cytochemically for 5′-nucleotidase. As described by Farquhar *et al.* (9), reaction product from 5′-nucleotidase was localized on the cytoplasmic side of the membrane (not shown). A Golgi fraction from untreated rats (14) was studied to test the possibility that ethanol treatment might affect the localization of the enzyme. A typical field is shown in Fig. 5. Lead phosphate precipitates were localized at the ends of the cisternae and on the cytoplasmic side of the membrane, exactly as in preparations from ethanol-treated animals (9).

The solubility of the reaction product in EDTA was investigated, since it has been noted (8) that lead phosphate inside microsomal vesicles is insoluble in EDTA whereas lead phosphate localized on the cytoplasmic side of the membrane is soluble in EDTA. As was expected, after cytochemical incubation of cisternal elements of the Golgi complex for 5′-nucleotidase, 98% of the lead phosphate was solubilized by 1 mM EDTA after incubation for 30 min at 0°. This result was obtained with preparations from both ethanol-treated and control animals.

After cytochemical incubation of secretion vacuoles for 5′-nucleotidase, with or without preincubation to release latent activity, at least 95% of the reaction product was solubilized by 1 mM EDTA, even though it was localized on the inside of the membrane. It is unlikely that EDTA itself changed the permeability of the membrane in these experiments, since treatment of secretion vacuoles with 1 mM EDTA at 0° for 30 min had no effect on the latency of the enzyme. This result shows that vacuoles in which 5′-nucleotidase activity was detected cytochemically were also permeable to EDTA, and supports the suggestion that the latency is related to the permeability of the membrane to anions.

Table 1. Inhibition of 5′-nucleotidase in Golgi fractions by antibody and concanavalin A

| Fraction | Experimental procedure* | Specific activity of 5′-nucleotidase (μmol of $P_i$ released/20 min per mg of protein) | | |
|---|---|---|---|---|
| | | Control | + Antibody† | + Concanavalin A† |
| Golgi | A | 1.11 | 0.22 (20) | 0.30 (27) |
| Cisternae‡ | B | 3.27 | 0.39 (12) | 1.99 (61) |
| Golgi | A | 2.88 | 0.51 (18) | 0.95 (33) |
| Cisternae§ | B | 8.48 | 1.36 (16) | 4.19 (49) |
| Secretion | A | 0.36 | 0.24 (67) | 0.28 (78) |
| Vacuoles§ | B | 4.53 | 3.54 (78) | 3.48 (77) |
| | B¶ | 7.16 | 3.10 (43) | 3.41 (48) |

Each value represents the mean of results obtained with two different preparations. Experiments with antibody and concanavalin A were carried out with different preparations, and the results were normalized to the same specific activity for controls.

* The procedures are described in *Materials and Methods*.

† Values in parentheses represent % of activity in controls.

‡ Isolated by the procedure of Leelavathi *et al.* (14).

§ Isolated by the procedure of Ehrenreich *et al.* (5).

¶ The preparation was sonicated for 10 sec at 60 W (Biosonik III, microtip) at the beginning of the preincubation.

## Interaction of 5′-nucleotidase with antibody

The antibody prepared against 5′-nucleotidase inhibited the enzyme, but did not precipitate the protein. As judged by the binding of gamma globulin to an insoluble form of 5′-nucleotidase (16), the antiserum contained about 0.3 mg of antibody per ml.

5′-Nucleotidase was solubilized from the various Golgi fractions in 1% Triton X-100, 0.5% DOC. Each preparation was inhibited in an identical manner by serum containing antibody to the purified enzyme, and no inhibition was observed with control serum, taken from the same rabbit before immunization (Fig. 6).

Golgi cisternae were incubated with purified gamma globulin containing antibody to 5′-nucleotidase; 85–90% of the enzyme activity was inhibited either when the antibody was present during incubation, or when the cisternae were reisolated and assayed in the presence of Triton/DOC (Table 1). In contrast, when secretion vacuoles were incubated in the presence of antibody (so that about 75% of the activity was latent) only 35% of the activity was inhibited; after reisolation and solubilization in Triton/DOC, only 22% of the total activity was inhibited by antibody (Table 1). Sonication of secretion vacuoles in the presence of antibody resulted in the inhibition of a major fraction of the enzyme (Table 1); the higher specific activity in controls after sonication reflects the partial loss of content from the vacuoles.

## Interaction of 5′-nucleotidase with concanavalin A

5′-Nucleotidase is a glycoprotein (17, 18), and the enzyme activity is inhibited by concanavalin A (19, 20). The inhibition of the solubilized enzyme from secretion vacuoles and from Golgi cisternae by concanavalin A was very similar (not shown). The enzyme in intact secretion vacuoles, however, was considerably less inhibited by the lectin than the

enzyme in Golgi cisternae, and again, sonication of the vacuoles rendered the enzyme accessible to inhibition (Table 1). In these experiments it was necessary to incubate the fractions with concanavalin A under suboptimal conditions for binding (21) in order to preserve the morphology of the secretion vacuoles; it was thus not possible to obtain the maximum inhibition. In all these experiments no inhibition by concanavalin A was observed when 10 mM methyl-α-D-mannopyranoside was included in the medium.

These results show that 5′-nucleotidase in secretion vacuoles from the Golgi complex is relatively inaccessible to substrate, antibody, and concanavalin A. Because of the apparent fragility of the membrane, it is possible that the low enzyme activity and the low level of reaction to antibody and the lectin observed in the intact vacuoles is caused by damage to some of the structures during isolation; a major fraction of the vacuoles, however, remained impermeable to macromolecules when incubated under carefully controlled conditions. The cytochemical results show that $P_i$ released by the enzyme is trapped on the inside of secretion vacuoles. In contrast, in cisternal elements of the Golgi complex the enzyme is almost completely accessible to substrate, antibody, and concanavalin A, and $P_i$ released by the enzyme is trapped on the cytoplasmic side of the membrane.

Taken together, the results seem to establish that the substrate binding site, the site of release of $P_i$ from the enzyme, the antibody binding site, and the lectin binding site of 5′-nucleotidase are all localized on the cytoplasmic side of Golgi cisternae and on the inside of secretion vacuoles. It is possible that the antibody binding site and the lectin binding site are in close proximity, especially since no additive inhibition was observed when saturating amounts of antibody and concanavalin A were added together. To explain the results for secretion vacuoles on the basis of a localization of the enzyme on the cytoplasmic side of the membrane, it would be necessary to assume (*a*) that an inhibitor that can be removed by mild sonication is present in the membrane masking the substrate binding site, antibody binding site, and concanavalin A binding site, and (*b*) that the enzyme is capable of vectorial release of $P_i$ across the membrane. Since no evidence for such vectorial release of reaction products has been obtained in careful studies of the enzyme in leukocyte plasma membranes (22) and since extracts of sonicated secretion vacuoles had no effect on 5′-nucleotidase in Golgi cisternae, such an explanation would seem unlikely. It is also unlikely that antibody or concanavalin A bound to the enzyme in secretion vacuoles without inhibiting enzymatic activity: if this occurred, inhibition should have been observed after solubilization of the membrane in detergent. The results in Table 1 show that enzyme-antibody and enzyme-concanavalin A complexes do not dissociate during detergent treatment.

It is reasonable to suggest that the cytochemical procedure used in this work represents a useful technique for studying the sidedness of membrane enzymes, even though at present the technique is limited to unfixed, isolated cell fractions. Because 5′-nucleotidase is exceedingly sensitive to fixation (8, 9), the confirmation of the localization in intact tissue will only be possible when a fixation procedure that does not inactivate the enzyme has been devised. $P_i$ was sometimes trapped at some distance from its site of release from the membrane, since in secretion vacuoles lead precipitates were not always associated with the membrane (Fig. 3; see also ref. 9), even though at least 90% of the enzyme was firmly bound to the membrane. However, it is clear, both from the studies of Farquhar *et al.* (9) and from those reported here, that $P_i$ was trapped on only one side of the membrane in both secretion vacuoles and Golgi cisternae. It is thus unlikely that $P_i$ diffused across the membrane of either fraction before it was precipitated as lead phosphate. An asymmetric localization of lead precipitate has been observed for several membrane-bound phosphatases (9, 11, 23).

Secretion vacuoles are derived from the Golgi cisternae (1–3, 5). An inversion of the membrane, which could cause the apparent movement of the enzyme from one side of the membrane to the other, is unlikely since it would lead to the release of content; there is no evidence that very low density lipoproteins can be found free in the cytoplasm (1–6). It is thus necessary to postulate that either the entire enzyme, or at least the part of the polypeptide chain that contains the active site, antigenic site, and lectin binding site, is translocated across the membrane.

This interpretation clearly depends on the assumption that the molecules of 5′-nucleotidase that are observed in secretion vacuoles were previously localized in the Golgi cisternae. A study of the labeling kinetics of the pure enzyme, which is in progress in our laboratory, should permit a direct test of this hypothesis. The assumption does seem reasonable, however, since the enzyme was apparently concentrated at the periphery of the Golgi cisternae (Fig. 5 and ref. 9) and since the reorientation of 5′-nucleotidase was observed in buckled cisternae (Fig. 3), i.e., before the complete maturation of secretion vacuoles.

Singer and Nicolson (24) have suggested, on the basis of thermodynamic considerations, that the movement of proteins, and particularly glycoproteins, across lipid bilayers is unlikely. However, 5′-nucleotidase could exist in the membrane as a large aggregate, perhaps complexed with other proteins, as has been suggested for proteins in the erythrocyte membrane (see ref. 25). It would then be possible for the enzyme to move from one side of the membrane to the other through a milieu of protein rather than lipid, which might present less of an obstacle thermodynamically.

The mechanism by which the translocation of 5′-nucleotidase occurs has not been established. In preliminary experiments using polyacrylamide gel electrophoresis in sodium dodecyl sulfate, we have observed that the protein composition of secretion vacuole membranes is apparently different from that of Golgi cisternae. It is conceivable that the orientation of 5′-nucleotidase might be controlled by other proteins in the membrane. Although the immunochemical properties of the enzyme were apparently the same in Golgi cisternae and secretion vacuoles, it is also possible that some minor modification of the polypeptide chain (phosphorylation, acetylation, or glycosylation) was associated with the change in orientation.

In the case of 5′-nucleotidase, the physiological significance of the apparent movement of the protein across the membrane is obscure; it is evident, however, that the possibility that such movement can take place could have considerable importance both in the mechanism of membrane transport and in the regulation of membrane enzymes. Although it has been suggested that protein translocation is not involved in anion transport in erythrocytes, (26), it has been implicated in adenine nucleotide transport in mitochondria (27), and was proposed as part of a general mechanism of membrane transport by Danielli as early as 1953 (28).

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