Phospholipid Transfer from ER to the Peribacteroid Membrane in Soybean Nodules

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Organelle membranes subfractionated from nodules of *Glycine max* differed in lipid composition. Of the enzymes assembling phospholipids, choline kinase (EC 2.7.1.32) was recovered in the cytoplasmic fraction whereas CDP choline 1,2-diacylglyceride choline phosphotransferase (EC 2.7.8.2) was located in the ER. When methyl [^{14}C]CDP choline was supplied to nodules *in vivo* radioactivity was found in the ER. On longer labelling periods [^{14}C]phosphatidylcholine spread into denser regions of the gradient, including the peribacteroid membrane (which surrounds the *Rhizobium japonicum* symbiont). [^{14}C]Phosphatidylcholine was a notable component of such membranes.

Adenosine triphosphatase (EC 3.6.1.3) activity in the peribacteroid membrane fraction was compared to that of other major organelle fractions. The peribacteroid membrane ATPase was equally active on pH 6.0 and 8.0, was stimulated by K^+ and inhibited by DES, DCCD and MoO_4. In these respects it was more similar to the ATPase activity in the ER and mitochondria than in tonoplast or cytoplasm.

The results are discussed in relation to modes of peribacteroid membrane biogenesis, including enzyme modification.

Introduction

After the initial invasion of *Glycine max* root cells by effective strains of *Rhizobium japonicum* the membrane which surrounds the prokaryote undergoes massive proliferation as the bacteroids divide and fill the mature symbiotic cell [1]. This so-called peribacteroid membrane appears to be initially derived from the plant plasmamembrane [2] but evidence has accumulated from both electron microscopic [3] and biochemical [4, 5] sources that this huge increase in membrane area is supported directly by the biosynthetic parts of the endomembrane system independently of the plasma membrane.

Since these membranes are regarded as being important in cellular compartmentation and the organization of the exchange between host cell and endosymbiont [6], we are continuing to characterize them in biochemical terms.

Abbreviations: CDP, choline diphospho; DCCD, N–N′ dicyclohexylcarbodiimide; DES, diethylstilbestrol; IDP, inosine diphosphate; MES, 2N-morpholino ethane sulphonate; EDP, phospho(enol)pyruvate; t.l.c., thin layer chromatography; TRIS,Tris (hydroxymethyl) amino methane.

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Materials and Methods

1) Growth of tissue

Glycine max var. Mandarin (Kurt Behm GmbH, Hamburg) was grown and infected with *Rhizobium japonicum* strain 61-A-101 (Nitragin Company, Milwaukee) exactly as described before [4]. 21 d after infection nodules were picked and used immediately.

2) Preparations of subcellular fractions

The scheme detailed in Fig. 1 was routinely followed. Tissue was chopped to a fine paste with a single razor blade and, following a filtration and low-speed centrifugation step, the cell- and cell-debris-free homogenate was layered over the first sucrose density gradient (adopted from the scheme of Mellor and Lord [7]). Marker enzyme activities through such a gradient have been given before [4]. Gradients could be fractionated into 1 ml fractions or bands could be withdrawn by syringe. For lipid extraction procedures endoplasmic reticulum (ER) and mitochondria were repurified on subsequent 60% – 30% gradients. Bacteroids, many still enclosed within peribacteroid membranes (for electron micrographs, see [5]) were fractionated into clean bac-

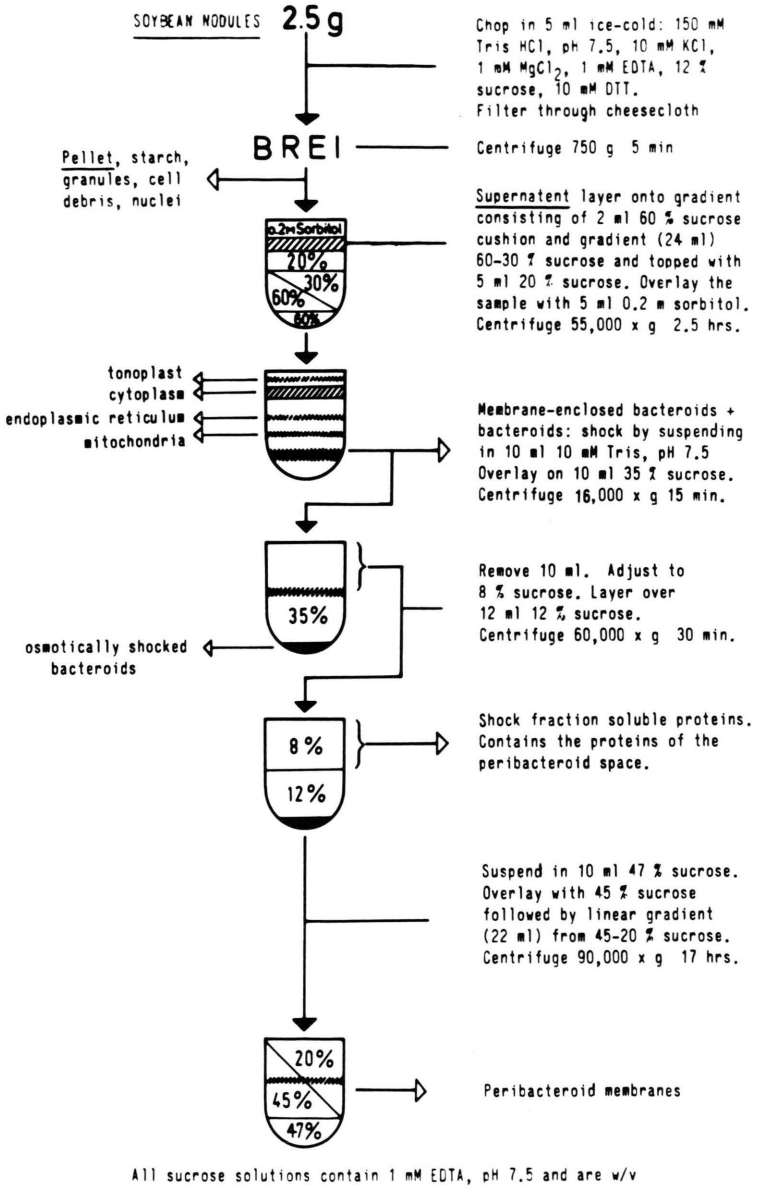

Fig. 1. Flow diagram of cellular fractionation scheme followed using soybean root nodules. For further details see text.

teroids and peribacteroid membranes following the scheme outlined (modified from [8]).

3) Radioactive labelling

Prior to homogenization 1 g of roughly halved nodules were incubated at 25 °C with 1.0 µCi methyl [^{14}C]CDP choline in 2 ml homogenization buffer (see Fig. 1) for the appropriate length of time in the dark. [^{14}C]Phosphatidylcholine was later removed from collected gradient fractions as described by Lord *et al.* [9].

4) Analysis of lipids

Total lipids were extracted from subcellular fractions by shaking 1 ml of sample with 2 ml of chloroform : methanol (2 : 1). The aqueous place was re-extracted with 2 ml $CHCl_3$ and the pooled organic phases were washed with 2 ml chloroform : methanol : water (3 : 48 : 47) then dried at 20 °C overnight. Extracted lipids were redissolved in 20 µl $CHCl_3$: MeOH (2 : 1) and spotted onto a corner of a pre-coated silica gel 60 t.l.c. plate (Merck, Darmstadt). Chromatograms were run in the first dimension

in a solvent consisting of $CHCl_3$:MeOH:NH_4OH (65:25:4) and then in the second dimension in a solvent containing $CHCl_3$: acetone: MeOH: acetic acid: H_2O (3:4:1:1:0.5). Such plates, developed in iodine vapor, were compared to those made from known standard mixtures (Supelco, 8399 – Griesbach, FRG). Autoradiograms were obtained by spraying the t.l.c. plate with a fine mist of toluene-based scintillator then incubation with Kodak AR10 autoradiograph film at −60 °C for 10 days.

5) Enzyme assays

5.1. Choline Kinase (EC 2.7.1.32)

The assay was performed basically as described by Lord *et al.* [9] except that samples were desalted by passage through a column of sepharose CL-6B equilibrated with 10 mM TRIS HCl (pH 7.5) and 1 mM $MgCl_2$ prior to assay as we found that high sucrose concentrations hindered the chromatographic separation of choline from phosphorylcholine [10].

5.2. Choline phosphotransferase (EC 2.7.8.2)

250 µl aliquots of enzyme were incubated with 0.1 µCi methyl-[^{14}C]CDP choline for 45 min whereupon the reaction was stopped and [^{14}C]Phosphatidylcholine was extracted and estimated as described by Lord *et al.* [9].

5.3. Inosine diphosphatase (EC 3.6.1.6)

The assay method followed that of Ray *et al.* [11] as modified by Mellor and Lord [12]. Phosphate released after 1 h incubation at 25 °C was estimated by the method of Fiske and Subba Row [13].

5.4. Adenosine triphosphatase (EC 3.6.1.3)

ATPase activity was routinely measured using an ATP-regenerating system at 28 °C [14]. Standard reaction mixtures contained 50 mM TRIS MES (pH 6.0 or 8.0), 2.5 mM $MgCl_2$, 5 mM TRIS ATP, 1.5 mM PEP, 0.2 mM NADH, 10 units lactate dehydrogenase (EC 1.1.1.27), 10 units pyruvate kinase (EC 2.7.1.40) and enzyme to a volume of 1 ml. ATPase activity was calculated from the rate of NADH oxidation followed at 334 nm in the time period between 5 and 20 min following the initiation of the reaction at which time the reaction rate was linear. For studies on cytoplasmic ATPase the method of Walker and Leigh [15] was used which follows the rate of phosphate release from TRIS ATP. With this method strict controls were kept which made allowances for sorbitol and sucrose content of the samples. At all times activity due to non-specific phosphatases was followed in parallel experiments using PNP release from disodium *p*-nitrophenyl phosphate. Free PNP was estimated as by Bassarab *et al.* [16].

6) Chemicals and isotopes

[^{14}C]choline chloride (51 Ci mol^{-1}) and methyl-[^{14}C]CDP choline (42 Ci mol^{-1}) were from Amersham Buchler, Braunschweig. All other chemicals and biochemicals were from either Boehringer, Mannheim or Sigma, München.

Results and Discussion

1) Phospholipid synthesis

Enzymes of the CDP choline pathway are all present in root nodules formed by *Glycine max* in response to symbiosis with *Rhizobium japonicum*. These enzymes are namely choline kinase (EC 2.7.1.32) which catalyses the ATP-dependent phosphorylation of choline to phosphorylcholine;

A) Choline + ATP
$\rightleftarrows$ phosphorylcholine + ADP

phosphorylcholine cytidyl transferase (EC 2.7.7.15) which produces CDP choline from CTP and phosphorylcholine;

B) CTP + phosphorylcholine
$\rightleftarrows$ CDP-choline + pyrophosphate

and choline phosphotransferase (EC 2.7.8.2) which synthetizes phosphatidylcholine from CDP-choline and diglyceride;

C) CDP-choline + diglyceride
$\rightleftarrows$ phosphatidylcholine + CMP.

The cytidyl transferase is generally regarded as being an ER-localized enzyme [17], but was not investigated in this study. Fig. 2a shows that after sucrose density centrifugation, all of the choline kinase activity was recovered in the cytoplasmic fractions. This is in good agreement with earlier work [9]. From the point of view of membrane formation the enzyme which provides phosphatidyl-

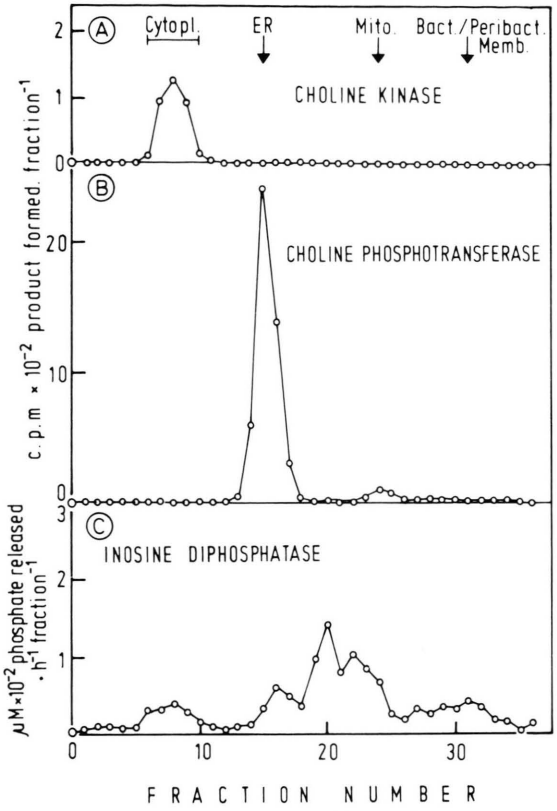

Fig. 2. Distribution of a) choline kinase, b) choline phosphotransferase and c) inosine diphosphatase in collected fractions after sucrose density gradient centrifugation of soybean root nodule homogenates. "cytopl.", "ER" and "Mito." mark the position of host (plant) cell cytoplasm, ER and mitochondria respectively. "Bact./Peribact. Membr." marks the position of bacteroids and bacteroids retaining their peribacteroid membranes.

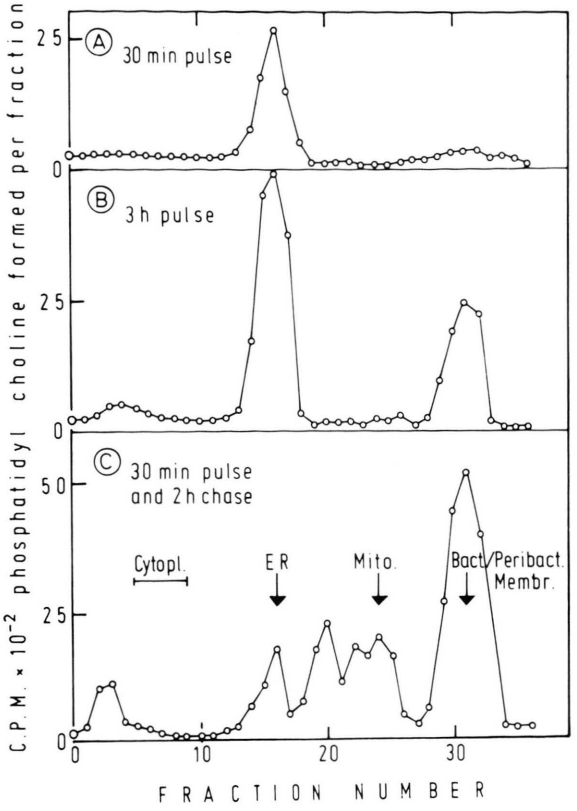

Fig. 3. Distribution of [^{14}C]Phosphocholine in fractions after sucrose density gradient centrifugation of homogenates prepared from soybean nodules which had been incubated with CDP [^{14}C]choline for: A) 30 min, B) 3 h or C) 30 min with a subsequent 2 h chase period using a one thousandfold excess of cold CDP choline. "cytopl.", "ER" and "Mito." mark the position of host (plant) cell cytoplasm, ER and mitochondria respectively. "Bact./Peribact. Membr." marks the position of bacteroids and bacteroids retaining their peribacteroid membranes.

choline, phosphocholine transferase, is very important. Fig. 2b shows that over 90% of the activity is confined to the ER in this tissue. Most of the remaining (10%) activity cosedimented with mitochondria. Whether this activity is integrally associated with mitochondria is not clear. Previous reports of this enzyme in mitochondrial fractions were assigned to contamination, however Sparace and Moore [18] have characterized an authentic phosphocholine transferase from plant mitochondria inner membrane. These authors believe however that their enzyme is involved only in a low level activity maintaining the mitochondrial membrane and that in fact the enzymes in the ER produce the overwhelming majority of phospholipids.

2) In vivo labelling

The use of continuous gradients overlayed with crude homogenates enables comparisons to be drawn directly about subcellular compartmentation without false indications of purity common with stepwise procedures, or differential loss of organelles from potentially damaging pelleting and resuspending steps. Thus we were able to perform accurate pulse-chase experiments. When nodules were treated with CDP [^{14}C]choline for 30 min most of the extracted [^{14}C]phosphatidylcholine from separated organelles appeared in the ER fractions (Fig. 3a). Fig. 3b shows that after longer labelling periods radioactivity also appeared in denser parts

of the gradient. These peak fractions corresponded with those containing bacteroids, many of which are surrounded by a peribacteroid membranes [5]. Osmotically shocked bacteroids were not labelled (data not shown). Autoradiographed t.l.c. maps of lipids from such labelled peribacteroid membrane preparations contained only a single radioactive spot (data not shown), which cochromatographed with phosphatidylcholine (Fig. 4). When tissue was incubated for 30 min with CDP [^{14}C]choline and then chased for 2 h with a thousandfold excess of cold CDP choline, the ER fractions were found to contain substantially less ^{14}C than as before without chase. A concomittent rise in the [^{14}C]Phosphatidylcholine content of the bacteroid and peribacteroid membrane fractions was however evident (Fig. 3). We interpret these results as meaning that ER-assembled phosphatidylcholine has been chased into peribacteroid membrane. Other subcellular sites of chased [^{14}C]Phosphatidylcholine accumulation were: 1) Mitochondrial fractions (marked by fumarase, EC 4.2.1.2); 2) A broad band around 1.16 g ml^{-1} which may be Golgi because of the presence of IDPase in that region (Fig. 2c) [11]; 3) In a light band which floated in 0.2 M sorbitol. On physical basis [19], and also by marker enzyme activity [20] we have tentatively regarded this light band as containing tonoplast material [4]. The recovery of such a band may well be possible because the gentle chopping of this tissue in an osmotically-adjusted buffer enables the vacuoles, which in infected cells never properly develop and remain small and dispersed through the cell [21, 1], to be released largely intact.

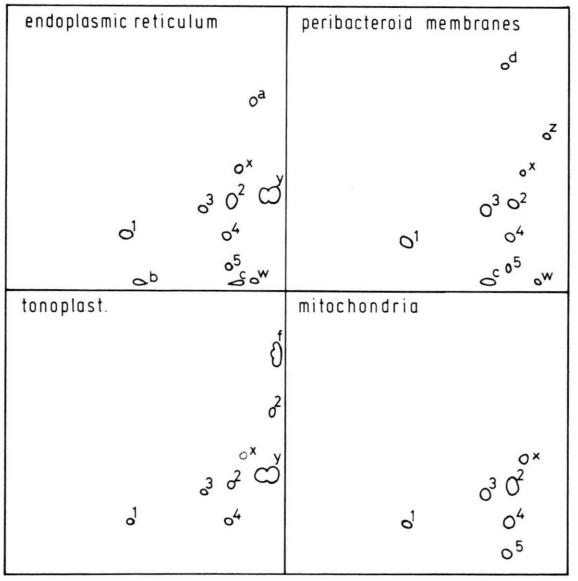

Fig. 4. Two dimensional t.l.c. analysis of constituent lipids from major gradient fractions. The first dimension is vertical and the second horizontal from origin in the bottom left-hand corner. The numbers mark the identified lipids diphosphatidyl glyceride (1), phosphatidyl choline (2), phosphatidyl ethanolamine (3), phosphatidyl inositol (4) and phosphatidyl serine (5). a–f constitute unidentified components which are unique to a particular cell fraction. w–z constitute unidentified components which are shared between cell fractions.

These dynamic analyses support the generally accepted theory that tonoplast membranes, Golgi and mitochondria (at least partly) receive phospholipids from ER [22, 23]. Peribacteroid membranes, known to accumulate glycoconjugates from ER [4] must now be added to that list.

3) Lipid distribution

Fig. 4 shows an analysis of lipid constituents of four membrane types from soybean nodules. The simplest composition was found in mitochondria which conisted of diphosphotidyl glycerol, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol and phosphatidylserine. An ubiquitous unidentified lipid "x" was also present. At the opposite extreme, ER showed the most heterogeneity in lipid composition, as would be expected from its proposed biosynthetic role. From the 12 discernable ER-derived lipids a high proportion (3 unknowns) were acidic. One of these, "w" was shared with the peribacteroid membrane, which itself had another unknown acidic lipid. ER shared a major component ("y", a double spot) with tonoblast membranes. This may be a cebroside or fatty acid [24]. The tonoplast, as well as having another large unidentified component, shared a lipid with the peribacteroid membranes ("z").

Although all these membranes contain phosphatidyl choline as one would expect since they were all labelled by CDP [^{14}C]choline, it appears that mitochondria are quite distinct from the other types. Whilst the other three membranes possess more similarities, peribacteroid membranes are markedly closer to ER than to any other fraction tested.

This static analysis is suggestive of a relationship between ER and peribacteroid membranes, but a final conclusion is hindered by the lack of informa-

tion about Golgi and plasma membrane lipid composition. Such data must wait until we can overcome the technical difficulties of isolating pure Golgi and plasma membrane from nodules.

4) ATPase studies

Verma *et al.* [6] used histochemical localization of ATPase activity in conjunction with inhibitors to demonstrate a unique similarity between the ATPase's of peribacteroid membranes and plasma membrane. In order both to characterize this ATPase further and compare it to other membrane ATPases we undertook an extensive survey of this enzyme activity (Table I). The inhibition of ATPase by 50 mM K^+ at pH 6.0 in the fraction which we regard as tonoplast is not readily explainable, however the stimulation by K^+ at pH 8.0 and the effects of DES, DCCD and MoO_4 at this pH value are in good agreement with the figures reported by Leigh and Walker [25] and Walker and Leigh [15] for tonoplast from red beet. The occurrence of an acid ATPase which is insensitive to K^+ in cytoplasm concurs with the findings of Leonard *et al.* [26] working with oat roots, as is our finding of DCCD and DES sensitive alkaline ATPases in ER and mitochondria.

In our hands significant ATPase activity was lost between the pelleting and floatation gradient stages of membrane preparation. We do not yet know whether this is due to loss of contamination or instability of the enzyme.

From our results on surviving ATPase in peribacteroid membranes however, activity was almost equal to pH 6.0 and pH 8.0, was stimulated by K^+, but not to the same extent as either our ER or mitochondrial fractions or putative plasma membrane ATPase [26]. It was also sensitive to some extent to all the inhibitors used, which may serve to differentiate it from tonoplast ATPase (DCCD insensitive at pH 6.0).

In animal tissues membrane bound ATPases are known to be altered by membrane lipid composition [27, 28]. Plant enzymes are known to require some phospholipids for activity [29], including ATPase [30]. Alam and Alam [3] have shown ATPase activity to be allosterically modified according to membrane fluidity. It is known that peribacteroid membranes have a very high lipid/protein ratio [8]. The lipid composition differs also from other major membrane types (Fig. 4). The range of variation between ATPases from either our ER and peribacteroid membrane fractions and published data on oat root plasma membrane ATPase fall within that range described by Alam and Alam [31] for the submaxillary gland plasma membrane ATPase from rats fed with differing lipid diets. Thus the possibility exists that there are not as many distinct ATPases as types of cell organelles but fewer, whose characteristics are affected by the lipid compositions of the membrane into which they are anchored. Such a hypothesis is in line with general theories of macromolecular processing and transport through

Table I. Percent change in ATPase activity by isolated subcellular fractions from soybean root nodules when treated with the following reagents at pH 6.0 and pH 8.0. Values are mean figures from at least 10 independent experiments and showed a variation of less than 15%.

	Tonoplast		Cytoplasm		E.R.		Mitochondria		PBM	
	6	8	6	8	6	8	6	8	6	8
K^+ 50 mM	−24	+50	−30	+10	+40	+52	+62	+84	+27	+36
DES 100 μM	−63	−46	−60	−17	−38	−53	−58	−59	−42	−50
DCCD 10 μM	0	−25	−16	−22	−44	−50	−44	−67	−20	−31
MoO_4 100 μM	−48	−19	−70	−35	−38	−28	−60	−20	−35	−40
Ratio of activity at $\frac{\text{pH 6.0}}{\text{pH 8.0}}$	10.6		7.0		0.7		0.7		0.95	
Specific activity[a]	5.0		9.1		4.6		1.8		0.7	

PBM, peribacteroid membranes; DES, diethylstilbestrol; DCCD, N−N′ dicyclohexylcarbodiimide; MoO_4, ammonium molybdate.
[a] μmol ATP hydrolyzed · mg protein^{-1} · h^{-1} at pH 8.0.

the cell [22] and underline the differences between membrane differentiation (the structural and/or chemical modification of existing membranes) and membrane flow (the physical flux of membrane constituents from one compartment to another) [23]. If this speculation is true, the argument of Verma *et al.* [6] that peribacteroid membrane is provided by recycling from plasmamembrane is seriously weakened. This hypothesis, based on ATPase staining of thin tissue sections, a technique critisized by Quail [32], need not be invoked due to the ability of a direct-flow/differentiation model to explain this phenomenon. Such a notion that ATPase, in vesicles derived from Golgi, share the properties of plasmamembrane ATPase from grounds of membrane flow has been published previously [33].

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