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Application of Radioisotopes in Biochemical Analyses: Metal Binding Proteins and Metal Transporters

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1. Introducition

Radioisotopes (RI) such as ^{3}H, ^{14}C, ^{32}P, and ^{45}Ca are excellent tools in biological research. Most RI are used as tracers in studies of primary and secondary metabolism, drug metabolism, transcription, translation, post-translational modifications such as protein phosphorylation, association of proteins with metals, and transport of metals across biomembranes. Furthermore, some experiments have used neutrons for mutagenesis of microorganisms, animals, and plants. Recent progress in the biological sciences has resulted in novel probes and labeling reagents, which has decreased the need for RI. Experiments with RI require experimental space specialized for RI, careful experimental procedures, and training. Although these are disadvantages, RI are still useful and powerful tools with high resolution compared with non-RI methods. Here, we describe the advantages of RI in biochemical assays, and detailed experimental procedures of metal-binding assays and membrane transport measurements of metal cations, especially calcium and zinc.

2. Advantages of radioisotopes as tracers

Most metabolic pathways that are described in biochemistry textbooks, in various organisms including humans, plants, and microorganisms, could not have been determined without RI such as ^{14}C, ^{35}S, ^{32}P, and ^{3}H. Biochemical experiments with RI provide information on the fates of metabolites, nutrients, and inorganic ions at each periodic stage of living organisms or cells. In the early era of molecular biology, ^{32}P was used as an essential tool in a large number of laboratories to determine DNA sequences and to identify target DNAs or mRNAs. Phosphorylation of serine and/or tyrosine residues is a key covalent modification of proteins. ^{32}P ([γ-^{32}P]ATP) is still used to investigate this biochemical process. ^{35}S ([^{35}S]glutathione) is also used to investigate protein *S*-glutathiolation, which regulates the redox state of cells or detoxifies xenobiotics and natural products.

There are several advantages of RI in biochemical analyses compared with non-RI experimental procedures as follows:

1. High sensitivity: Trace amounts of RI can be detected by using a scintillation counter, X-ray film (autoradiography), or imaging plate. For example, labeling with ^{32}P-nucleotides such as [α-^{32}P]ATP or [γ-^{32}P]ATP is frequently used to label DNAs. In addition to the RI method, labeling of DNA with digoxigenin (DIG) has been used as a non-RI method. DIG labeling can be done by polymerase chain reaction (PCR), and DIG-labeled DNA can be detected by immunochemical methods. Reagents for DIG labeling are available from Roche Diagnostics GmbH (Nonnenwald, Penzberg, Germany). The DIG method can be done without specific equipment and space for RI. However, sensitivity of the RI method is higher than the DIG method. In particular, RI methods have advantageous sensitivity in northern and Southern analyses.
2. High accuracy: A good example is protein phosphorylation in cells. Immunochemical analyses such as immunoblotting are also used to detect phosphorylated proteins. Antibodies specific to phosphoserine, phosphotyrosine, or peptides containing phosphorylated amino acid residues are prepared and used. The accuracy and sensitivity depend on the quality and specificity of the antibodies. In most cases, researchers must pay attention to artifactual signals. In contrast, labeling proteins with RI provides clear and quantitative information on protein phosphorylation. In RI methods, proteins are phosphorylated with [γ-^{32}P]ATP in cell free systems (*in vitro*) or in experiments using cells, tissues, or organisms (*in vivo*), and then proteins are extracted and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins in the gel are transferred onto a transfer membrane such as polyvinylidine fluoride (PVDF). The membrane is dried and subjected to autoradiography by contact with an X-ray film at –80°C for a few days. Imaging plates are now generally used for the detection of phosphorylated proteins. Imaging plates have several advantages compared with autoradiography: (i) high sensitivity (quick detection), (ii) good linearity between the content of ^{32}P and the signal, (iii) digital imaging, and (iv) no requirement of a dark room.
3. Reflection of natural conditions: Organic and inorganic compounds containing RI and radioactive elements have the same chemical properties as normal compounds and elements in most cases. Therefore, we can determine and follow the compounds and elements in cells, tissues, and organisms without artificial conditions. This is a critical advantage of RI.

3. Typical radioisotopes used in biochemical and molecular biological studies

3.1 Physicochemical properties of typical isotopes

As mentioned in other chapters, several isotopes are used for biochemical and molecular biological studies. The physical and chemical properties of typical isotopes that are frequently used in the biological sciences are summarized in Table 1. In most laboratories, ^{3}H, ^{14}C, ^{35}S, ^{32}P, and ^{125}I are used for labeling of amino acids, peptides, and proteins. These RI, except for ^{125}I, emit β-rays with different energies (^{3}H, low; ^{14}C, medium; ^{35}S, high). In most cases, ^{35}S is used as L-[^{35}S]-methionine or L-[^{35}S]-cysteine for labeling peptides or proteins in *in vitro* and *in vivo* experiments of protein synthesis. For example, L-[^{35}S]-

methionine is added into *in vitro* translation mixtures that lack cold methionine. In certain experiments, ^{35}S is used for sulfation of peptides or proteins through tyrosine modification by an enzyme tyrosylprotein sulfotransferase (Moore, 2003; Hoffhines et al., 2006). ^{32}P is used for monitoring protein phosphorylation of serine and/or threonine residues.

RI	Radiation Energy (keV)	Half life	Shielding (1)	Measure-ment (2)	Moni-toring (3)	Application (4)
H-3	β, 18.6	12.3 Y	Not required	ARG, LSC	SM+LSC	As a tracer for metabolites
C-14	β, 156	5730 Y	Not required	ARG, LSC	SM+LSC	As a tracer for metabolites and CO_2
S-35	β, 167	87.5 D	Not required	ARG, LSC	SM+LSC	As a tracer for protein synthesis
P-32	β, 1711	14.3 D	Acryl plate	ARG, LSC	GM	DNA labeling, protein phosphorylation
P-33	β, 249	25.3 D	Acryl plate	ARG, LSC	GM	DNA labeling and macro-array
Ca-45	β, 257	162 D	Acryl plate	ARG, LSC	GM	As a tracer for Ca in organisms and for characterization of Ca-binding proteins and kinetic assays of Ca transport
Zn-65	β, 329 γ, 511 γ, 1116	244 D	Lead blocks	γ counter, ARG, LSC	NaI (T1)	As a tracer for Zn in organisms and membrane transport
I-125	γ, 35.5 X, 27.5	59.4 D	Acryl plate contain-ing lead	ARG, γ counter	NaI (T1) for I-125	Protein labeling
Co-60	β, 310 β, 1480 β, 1173 γ, 1332	5.27 Y	Lead blocks	γ counter	NaI (T1)	Sterilization of medical equipment, radiation source for food irradiation and for mutation of seeds and organisms

(1) Shielding required for safety during experiments. (2) Adequate measurement methods for radioactivity: ARG, autoradiography; LSC, liquid scintillation counter. (3) Detection methods for pollution on the surface of experimental benches with RI: SM, smear method; LSC, liquid scintillation counter; GM, GM survey meter; NaI (T1), NaI (T1) survey meter. (4) Typical application in biochemical research.

Table 1. Physicochemical properties and quantification of typical RI in biological sciences

3.2 Quantification of radioisotopes and measurement of pollution

Several methods have been developed for quantifying RI and measuring pollution with RI during experiments. Here, we introduce two instruments, a Geiger-Müller (GM) survey meter (also known as a GM counter) and a liquid scintillation counter.

A GM survey meter is usually used to monitor β- and γ-rays, but not neutrons, from the surface of substances. Two electrodes are set in a cylinder filled with inert gas, such as neon, helium, or argon. A voltage is applied to the electrodes, i.e., the anode (a central wire or needle) and the cathode (the inside surface of the cylinder). GM survey meters operate under a high voltage of more than several hundred volts. When the ionizing radiation passes through the cylinder, ions and electrons are generated from some of the gas molecules. This reaction generates an electrical current pulse of constant voltage. GM survey meters are usually used for monitoring the surface pollution of RI that radiate β- or γ-rays. The meter cannot count γ-rays efficiently and does not distinguish each isotope generating β-rays.

As an efficient and practical means of quantifying β-ray radiation, liquid scintillation counters are commonly used for biochemical analyses. A liquid scintillation counter measures β-radiation in a solution containing a RI, fluorescent compounds (scintillators), and organic solvents such as xylene, dioxane, or toluene. As a scintillator, 2,5-diphenyloxazole (DPO) and 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP) are used. The energy of β-rays (β particles) from RI excites the scintillator, and then the excited fluorescent molecules dissipate the energy by emitting fluorescence. Therefore, radiation of β particles causes a pulse of fluorescent light. Liquid scintillation counters are used for measuring β-ray-emitting RI including ^{3}H, ^{14}C, ^{32}P, ^{45}Ca, and ^{65}Zn because the counting efficiency is high even for nuclides emitting low energy β-rays.

4. Analyses of Ca-binding proteins with ^{45}Ca

There are many types of Ca-binding proteins, such as calmodulin, calreticulin, and annexin (Berridg et al., 2003). Identification and quantitative characterization of Ca-binding proteins provide key information about their biochemical roles in living cells. Here, we briefly introduce biochemical methods to identify and characterize these proteins.

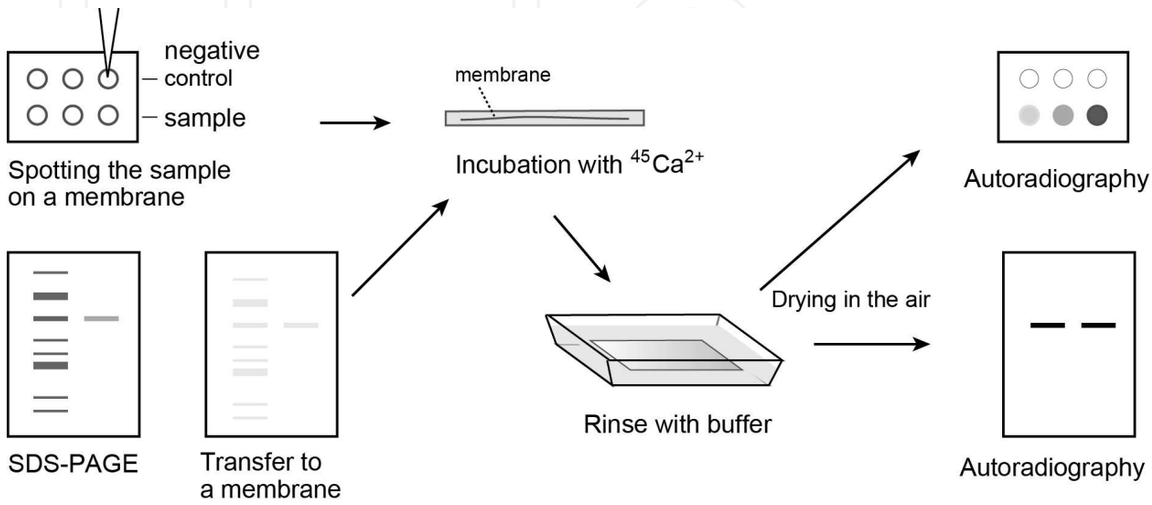
4.1 Identification of Ca-binding proteins

Staining of SDS-PAGE with Stains-all (commercially available from reagent companies such as Sigma Aldrich) is a conventional non-RI method to identify Ca-binding proteins (Campbell et al., 1983). Stains-all is a metachromatic cationic carbocyanine dye that tends to bind acidic proteins. Ca-binding proteins in particular are stained blue relatively stably (Yuasa & Maeshima, 2000). Therefore, this is a useful method to detect candidates for Ca-binding proteins in crude samples prepared from organisms.

Radioisotope ^{45}Ca is necessary to confirm that the protein(s) of interest can bind calcium. The ^{45}Ca overlay assay is one convenient method (Campbell et al., 1983; Yuasa & Maeshima, 2002; Ide et al., 2007; Kato et al., 2010). If a purified preparation is available, aliquots of the purified sample are spotted onto a membrane filter such as a PVDF membrane (ca. 30 × 40 mm). Then the membrane is incubated in a small volume (1 mL) of medium containing ^{45}Ca as $CaCl_2$, 5 mM $MgCl_2$, 60 mM KCl, and 10 mM Mes-KOH, pH 6.5, for 30 min at 25 to 30°C, and then washed with 10 mL of 50% (v/v) ethanol to remove unbound ^{45}Ca (Figure 1) (Nagasaki et al., 2008). The membrane is dried in air at room temperature. $MgCl_2$ and KCl are added into the reaction medium to mimic physiological conditions. An autoradiogram

of the $^{45}Ca^{2+}$-labelled proteins on the membrane can be obtained by exposure to an X-ray film for 3 days at –80°C.

If the purified protein(s) is not available, proteins separated by SDS-PAGE are transferred onto a transfer membrane such as PVDF as is usually used for immunoblotting (Figure 1). The Ca-binding protein(s) can be detected by the same method as the ^{45}Ca overlay assay mentioned above.

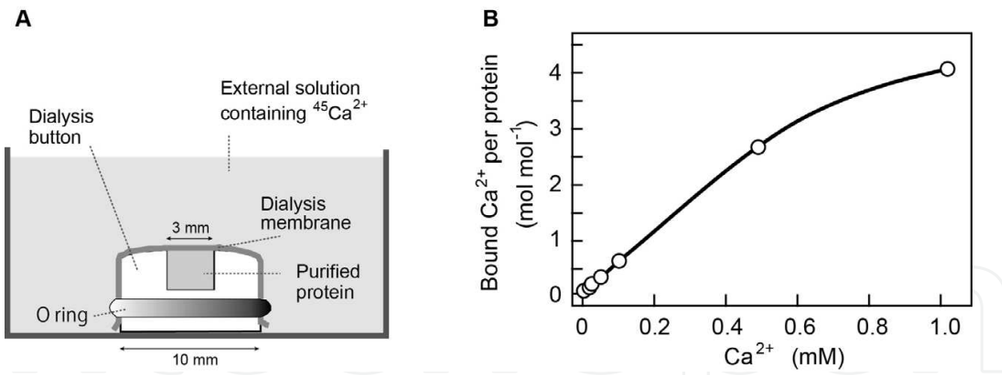


Purified proteins are spotted on a PVDF membrane (left, upper panel). In another method, a protein fraction that contains the Ca-binding protein is subjected to SDS-PAGE and then transferred to a PVDF membrane (left, lower panel). The membrane is incubated with buffer containing $^{45}Ca^{2+}$, rinsed, and then dried in air. By autoradiography, the $^{45}Ca^{2+}$-binding capacity is monitored (right, upper panel). From the membrane blotted after SDS-PAGE, the Ca-binding protein(s) is identified by autoradiography (right, lower panel).

Fig. 1. Detection of Ca-binding protein(s) by ^{45}Ca-overlay assay

4.2 Characterization of kinetic properties of Ca-binding proteins

The dissociation constant (K_d) for Ca^{2+} and the calcium-binding number are important kinetic parameters for understanding these proteins. Several assay methods can be used to measure the Ca-binding kinetics of Ca-binding proteins. For example, there is equilibrium dialysis, flow dialysis, membrane microassay, and spectrophotometry. The former two methods are carried out using $^{45}Ca^{2+}$. Most methods require a relatively large amount of the purified Ca-binding protein. Here, we introduce a special equilibrium dialysis using small dialysis buttons (Figure 2A). A small well of dialysis button is filled with the Ca-binding protein(s) and is sealed with a dialysis membrane. The protein solution in the well is dialyzed against 40 mL of buffer containing $^{45}Ca^{2+}$ at different concentrations. The Ca-binding protein binds the $^{45}Ca^{2+}$ entered into the well. After dialysis for 16 hr at 25°C, the protein solution in the well of each dialysis button is collected with a needle and syringe. An aliquot of the solution is spotted on a nitrocellulose membrane (13 mm in diameter), and then the membranes are dried in air. Total radioactivity associated with the filter membrane is measured with a liquid scintillation counter. Unbound Ca^{2+} is measured from the radioactivity of the external solution. The amount of Ca^{2+} bound to the Ca-binding protein increases in proportion to the concentration of Ca^{2+} as shown in Figure 2B.



(A) Diagram of the equilibrium dialysis assay with a dialysis button. Twenty microliters of purified protein is put in the well of a dialysis button of 3 mm in diameter, sealed with a dialysis membrane fixed with an O ring, and then dialyzed against 40 mL of 25 mM Mes-KOH, pH 6.0, 150 mM KCl with the indicated concentrations of $^{45}CaCl_2$. The volume of the external solution must be in excess of the sample volume to keep a constant level of Ca^{2+} during the assay. (B) Ratios of Ca^{2+} bound to the Ca-binding protein. The number of Ca^{2+} bound per Ca-binding protein and the K_d value for Ca^{2+} can be calculated from a Scatchard plot (a method of analyzing the binding of a ligand to a macromolecule). The result of radish Ca-binding protein is shown here (Yuasa & Maeshima, 2002).

Fig. 2. Equilibrium dialysis of Ca^{2+}-binding of purified Ca-binding protein

5. Measurement of membrane transport of zinc and calcium

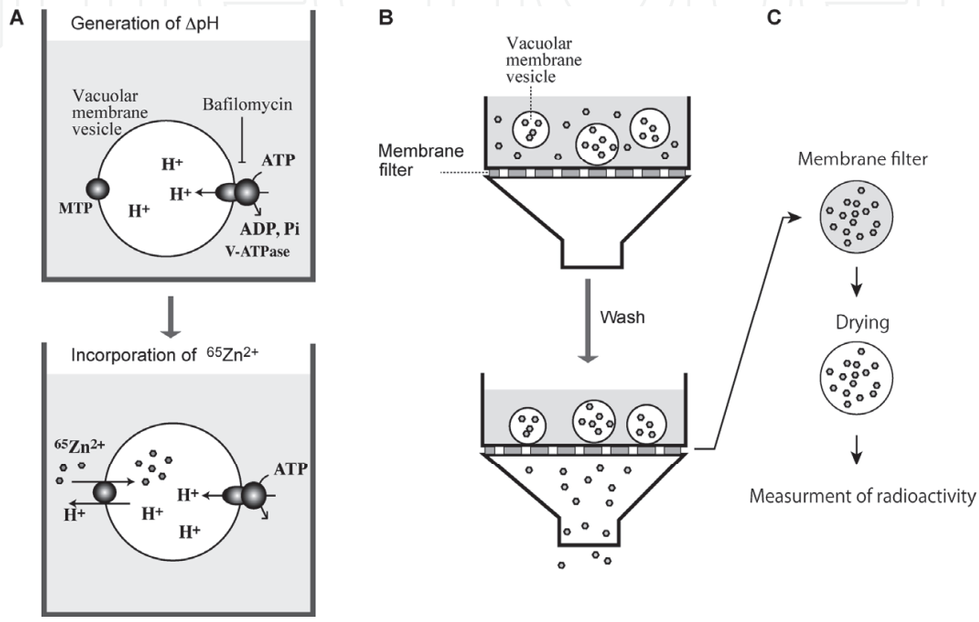
Radioactive elements such as Ca^{2+} and Zn^{2+} are commonly used in ion transport experiments because they provide direct evidence and quantitative information. Here, we introduce a Zn^{2+} transporter and a Ca^{2+} transporter, which work as metal/proton exchangers.

5.1 Determination of kinetic parameters of a Zn transporter across biomembranes

Transporters implicated in Zn transport include members of the metal tolerance protein (MTP), ZRT1/IRT1-like protein (ZIP) (also known as zinc-iron permease), and heavy-metal ATPase (HMA) (P_{1B} subgroup of P-type ATPase) families (Krämer et al., 2007). Here, we introduce an assay procedure for an MTP-type zinc transporter that works as a Zn^{2+}/H^+ exchanger. *Arabidopsis thaliana* MTP1 is localized in the vacuolar membrane, which has two types of proton pumps, vacuolar H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (Enrico et al., 2007). AtMTP1 actively transports excessive zinc in the cytoplasm into vacuoles (Kawachi et al., 2008; Kawachi et al., 2010).

The assay procedure of AtMTP1 expressed in *Saccharomyces cerevisiae* cells is shown in Figure 3. In this case, an *S. cerevisiae* mutant that lacks endogenous zinc transporters COT1 and ZRC1 is used as a host cell for heterologous expression. When ATP is added into the vacuolar membrane vesicle suspension, a pH gradient (ΔpH) is formed across the membrane by yeast endogenous V-ATPase. Then radioactive $^{65}Zn^{2+}$ is added into the reaction mixture as $ZnCl_2$. Under these conditions, AtMTP1 actively incorporates $^{65}Zn^{2+}$ into membrane vesicles using a ΔpH (Figure 3A). The reaction medium contains 300 mM sorbitol, 5 mM MES-Tris pH 6.9, 25 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.2 mM NaN_3, 0.1 mM Na_3VO_4, and 3 mM ATP-Tris. The uptake reaction is started by adding 5 μM $^{65}ZnCl_2$. Vacuolar membranes from plants and yeast contain metal-translocating ATPases that have the ability to transfer Zn^{2+} into vacuoles. Therefore, the activities of these ATPases

must be inhibited. Sodium azide and vanadate are potent inhibitors of the F-type and P-type ATPases, respectively. The K_m value for Zn^{2+} has been reported to be 0.30 μM for AtMTP1 (Kawachi et al., 2008). The value is comparable to the *S. cerevisiae* endogenous zinc transporter ZRC1 (0.16 μM) (MacDiarmid et al., 2002), *Escherichia coli* ZitB (1.4 μM) (Anton et al., 2004), and human hZIP4 (2.5 μM) (Mao et al., 2007). This method is applicable to assay the zinc transport activity of vacuolar membrane vesicles from plant tissues. Vacuolar membrane vesicles can be prepared from plant tissues such as mung bean hypocotyls by conventional differential centrifugation (Maeshima and Yoshida, 1989).



(A) Vacuolar membrane vesicles prepared from yeast cells or plant tissues are activated by adding ATP into the suspension. Vacuolar H^+-ATPase (V-ATPase) acidifies membrane vesicles and generates a pH gradient across the membrane.Bafilomycin A_1 is a potent inhibitor of V-ATPase and used to assess the V-ATPase-dependent (ΔpH-dependent) activity of theZn^{2+}/H^+ exchanger. When radioactive $^{65}Zn^{2+}$ is added, membrane vesicles actively uptake $^{65}Zn^{2+}$ using a pH gradient in a Zn^{2+}/H^+ exchanger-dependent manner. (B) Membrane vesicles are filtrated and washed with the buffer. (C) The radioactivity of $^{65}Zn^{2+}$ membrane vesicles trapped on the membrane filter is determined by a scintillation counter.

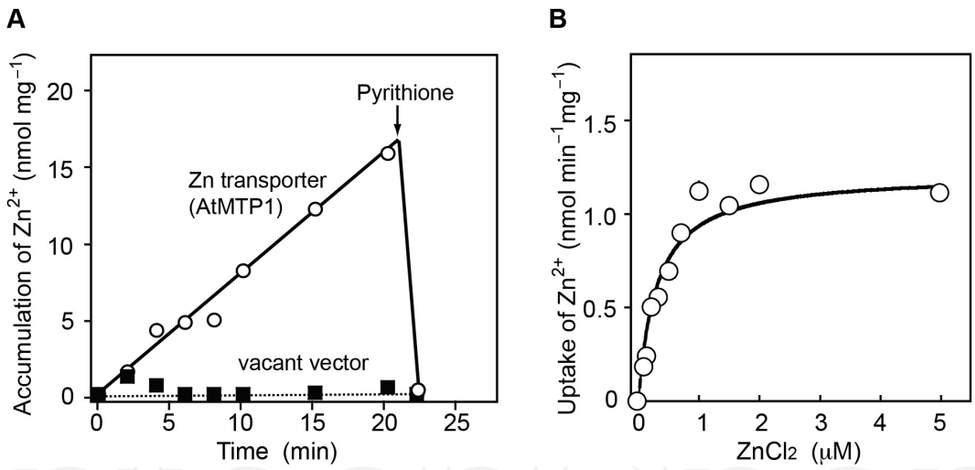
Fig. 3. Assay of Zn^{2+} transport into membrane vesicles through a Zn^{2+}/H^+ exchanger

The catalytic domain of V-ATPase (V_1 sector) is exposed to the cytoplasm. Therefore, only right-side-out membrane vesicles, in which the V_1 sector faces to the reaction mixture, can be energized by V-ATPase. Approximately half of the membrane vesicles are right-side-out when plant tissues and yeast cells are homogenized. In the remaining half, the V_1 sector faces the vesicle lumen and cannot utilize ATP. The inside-out vesicles have no ability to uptake $^{65}Zn^{2+}$ or to export zinc under this assay condition. Therefore, this experiment determines the Zn^{2+} uptake activity of the right-side-out membrane vesicles. The inside-out membrane vesicles do not interfere with the Zn^{2+} transport of right-side-out vesicles.
After an adequate period of uptake reaction, incorporated $^{65}Zn^{2+}$ must be separated from the un-incorporated ions. Aliquots (for example, 100 μL) of the membrane vesicle suspension

are transferred to funnels with nitrocellulose membrane filters that are presoaked with the buffer at appropriate intervals. Filter units with a 0.45-μm nitrocellulose membrane of 13 mm in diameter are easy to use for assays of multiple samples. The filter units are washed with 1.5 mL of cold wash buffer without $^{65}Zn^{2+}$. The wash buffer contains 300 mM sorbitol, 5 mM MES-Tris pH 6.9, 25 mM KCl, and 0.1 mM $ZnCl_2$. The addition of cold $ZnCl_2$ is essential to remove $^{65}Zn^{2+}$ from the surface of the membrane vesicles thoroughly. Finally, the radioactivity of $^{65}Zn^{2+}$ is determined by a γ scintillation counter.

When measuring the zinc transport activity of plant vacuolar membranes, vacuolar H^+-pyrophosphatase (V-PPase) also works as a useful proton pump (Maeshima, 2001). V-PPase hydrolyzes pyrophosphate (diphosphate) instead of ATP as a substrate. Therefore, metal-translocating ATPases do not work in the assay medium when assayed with V-PPase.

To demonstrate the active translocation of Zn^{2+} by exogenous zinc transporters, the membrane sample from the yeast mutant with a vacant vector is assayed as a control. Zinc ionophore pyrithione is usually used to collapse the concentration gradient of Zn^{2+} across the membrane at a concentration of 5 μM (MacDiarmid et al., 2002). If Zn^{2+} is actively incorporated into the membrane vesicles, the addition of pyrithione releases Zn^{2+} from membrane vesicles as shown in Figure 4A. Also, uptake experiments without ATP or with 0.2 μM bafilomycin A_1 is done by the same protocol. The bafilomycin A_1-sensitive zinc uptake activities are plotted as V-ATPase-dependent zinc uptake in time-course or substrate saturation analysis.



The vacuolar type Zn^{2+}/H^+ exchanger of *Arabidopsis thaliana* (AtMTP1) was heterologously expressed in a yeast (*Saccharomyces cerevisiae*) mutant that lacks endogenous vacuolar membrane zinc transporters (*zrc1 cot1* mutant). The vacuolar membrane-enriched fraction was prepared from yeast cells expressing AtMTP1 (circles) or vacant vector (closed squares) and assayed for zinc uptake activity. (A) Membrane vesicles were pre-incubated in uptake medium (1.0 mL) containing 3 mM ATP for 10 min at 25°C to generate a pH gradient across the membrane as shown in Figure 3A. The same reaction media supplemented with 0.2 mM bafilomycin A_1 were also prepared and assayed to measure bafilomycin A_1-sensitive zinc activity. The reaction was started by the addition of 5 μM $^{65}ZnCl_2$ at time 0 and continued for the indicated period. Aliquots (100 μL) of the reaction suspensions were filtered though a nitrocellulose membrane and washed with 1.5 mL of cold wash buffer. The radioactivity of $^{65}Zn^{2+}$ in the membrane vesicles was determined. The bafilomycin A_1-sensitive zinc uptake activities are plotted as V-ATPase-dependent zinc uptake. A zinc ionophore pyrithione is added into the reaction medium to make a final concentration of 5 μM to confirm the active transport of zinc. (B) Zinc uptake activity is measured at the indicated concentration of $^{65}ZnCl_2$ and shown as a substrate-saturation curve.

Fig. 4. Assay of zinc uptake by a zinc transporter

5.2 Determination of kinetic parameters of Ca transporters across biomembranes

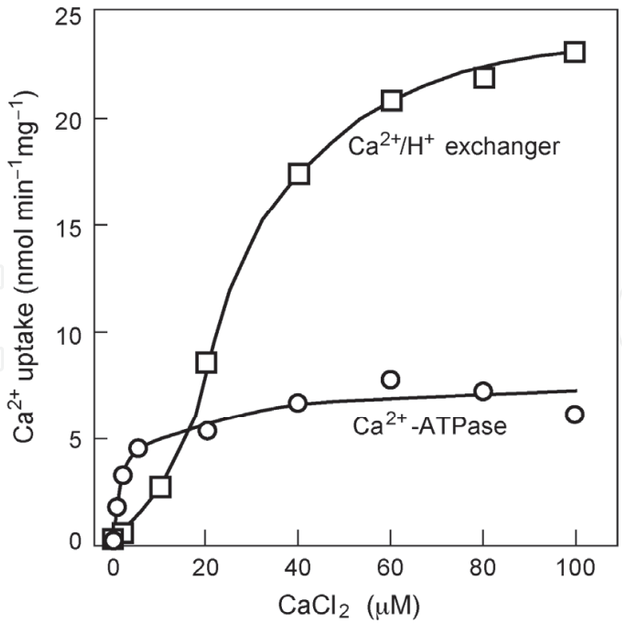
The Ca^{2+}-ATPase (calcium pump) belongs to the P-type ATPase family that includes the Na^+,K^+-ATPase (Morth et al., 2011), and actively translocates Ca^{2+} across the membrane coupled with ATP hydrolysis. Ca^{2+}-ATPases in eukaryotes are divided into to the ER-type and calmodulin-activated plasma-membrane-type Ca^{2+}-ATPases. The Ca^{2+}-ATPase is localized in the plasma membrane, ER, Golgi apparatus, and vacuole, and maintains calcium homeostasis in the cytoplasm and lumen spaces.

The Ca^{2+}/H^+ exchanger is the other type of active Ca^{2+} transporter (Ueoka-Nakanishi et al., 2000; Kamiya & Maeshima, 2004). As an energy source, the exchangers use a pH gradient across the membrane that is generated by proton pumps. Plant and fungal cells have the P-type H^+-ATPase in their plasma membranes, and H^+-ATPase in their vacuolar membranes as primary proton pumps. In plants, an additional proton pump, H^+-pyrophosphatase (V-PPase), functions as an efficient proton pump (Martinoia et al., 2007). Thus, the Ca^{2+}/H^+ exchanger is categorized as a secondary active Ca^{2+} transporter and exports excess Ca^{2+} from the cytoplasm to the extracellular space or vacuoles.

The physiological roles of Ca^{2+}-ATPase cannot be understood without information of their kinetic parameters. Quantitative analysis of calcium pumps and other active calcium transporters is usually performed by using $^{45}Ca^{2+}$. Assay procedures for plant vacuolar Ca^{2+}-ATPase and Ca^{2+}/H^+ exchanger are similar to the Zn^{2+}/H^+ exchanger. Aliquots (10 μg of protein) of vacuolar membranes prepared from plant tissues are suspended in 100 μL of assay medium consisting of 0.25 M sorbitol, 5 mM Mes-Tris pH 7.2, 50 mM KCl, 0.5 mM dithiothreitol, 3 mM $MgCl_2$, 1 mM Tris-ATP (ATP solution neutralized with Tris), and 100 μM $CaCl_2$ ([^{45}Ca], 37–220 kBq mL^{-1}) for the Ca^{2+}-ATPase assay. The uptake reaction is started by adding $CaCl_2$. After an adequate reaction period, the mixture is filtered through a presoaked 0.45-μm nitrocellulose filter (13 mm in diameter) as described for the Zn^{2+}/H^+ exchanger. The filter is washed twice with 200 μL of 0.25 M sorbitol, 5 mM Mes-Tris pH 7.2, 50 mM KCl, 0.5 mM dithiothreitol, 0.25 mM $MgCl_2$, and 1 mM ethylene glycol tetraacetic acid (EGTA). EGTA has a higher affinity for Ca^{2+} than for Mg^{2+} and is added to remove the unabsorbed $^{45}Ca^{2+}$ in the medium. Radioactive Ca^{2+} incorporated into membrane vesicles is trapped on the nitrocellulose membrane. The radioactivity associated with the filter membrane is measured with a liquid scintillation counter. The reaction medium for the V-PPase-dependent Ca^{2+}/H^+ exchanger contains 1 mM sodium pyrophosphate (Na_2PPi) instead of Tris-ATP. The membrane vesicles are pre-incubated with Na_2PPi for 3 min, and then the Ca^{2+} transport reaction is started by adding $CaCl_2$.

Background values resulting from incubations without ATP or Na_2PPi are subtracted from the corresponding values in the presence of ATP or Na_2PPi. Bafilomycin A_1 and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) dissolved in dimethyl sulfoxide (DMSO) are used to inhibit V-ATPase and collapse the pH gradient, respectively. The DMSO concentration in the assay medium should be less than 1% (by volume) to avoid artificial effects of the solvent. Calcium ionophore A23187 is also dissolved in DMSO and used to confirm the active transport of Ca^{2+} through Ca^{2+}-ATPase or Ca^{2+}/H^+ exchanger. A23187 is a mobile Ca^{2+} carrier produced by *Streptomyces chartreusensis* as an antibiotic.

Figure 5 shows typical substrate-saturation curves of the Ca^{2+}-ATPase and Ca^{2+}/H^+ exchanger of vacuolar membranes prepared from mung bean hypocotyls (Ueoka-Nakanishi et al., 1999). Experiments with radioisotope ^{45}Ca provide quantitative information of their transport kinetics. Ca^{2+}-ATPase is recognized as a high-affinity, low-capacity transporter, while the Ca^{2+}/H^+ exchanger is low-affinity, high capacity. These two active transporters maintain calcium homeostasis in the cytoplasm through their characteristic properties.



Activity of Ca^{2+}-ATPase (circles) in the vacuolar membrane was determined in the presence of 1 mM ATP, 0.1 mM bafilomycin A_1, and indicated concentrations of $^{45}CaCl_2$. Ca^{2+}/H^+ exchanger activity (squares) was determined after pre-incubation with 1 mM NaPPi for 3 min. V_{max} values of Ca^{2+}-ATPase and the Ca^{2+}/H^+ exchanger were 6.9 and 21 nmol min^{-1} mg^{-1} of protein, respectively. Apparent K_m values of Ca^{2+}-ATPase and the Ca^{2+}/H^+ exchanger were 2.6 and 25 μM, respectively.

Fig. 5. Calcium transport activity of Ca^{2+}-ATPase and Ca^{2+}/H^+ exchanger in vacuolar membranes.

6. Conclusion

High sensitivity and accuracy in quantitative assay are the actual merits of RI. Information concerning kinetic parameters of ion transporters cannot be obtained without RI, such as ^{65}Zn and ^{45}Ca, as described for Zn^{2+}/H^+ exchanger and Ca^{2+}/H^+ exchanger, and Ca^{2+}-ATPase. The obtained values of K_m and V_{max} are fundamental to evaluate physiological importance of individual transporters quantitatively. The data presented here are typical examples, which show advantages of RI in biochemical analyses. Although the use of RI is regulated by the laws established in each country, university and research institute, these rules keep the safety for the users and people. RI in biochemistry is one of the peaceful use of atomic energy and will be utilized as an essential tool to develop our scientific knowledge.

7. References

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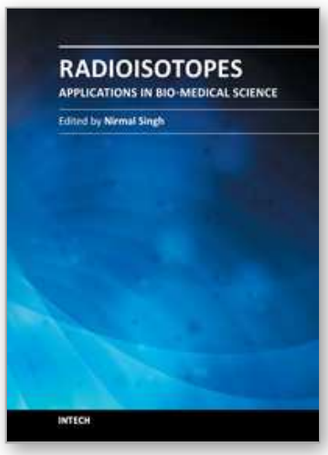

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The book Radioisotopes - Applications in Bio-Medical Science contains two sections: Radioisotopes and Radiations in Bioscience and Radioisotopes and Radiology in Medical Science. Section I includes chapters on medical radioisotope production, radio-labeled nano-particles, radioisotopes and nano-medicine, use of radiations in insects, drug research, medical radioisotopes and use of radioisotopes in interdisciplinary fields etc. In Section II, chapters related to production of metal PET (positron emission tomography) radioisotopes, 3-dimensional and CT (computed tomography) scan, SS nuclear medicine in imaging, cancer diagnose and treatments have been included. The subject matter will by highly useful to the medical and paramedical staff in hospitals, as well as researchers and scholars in the field of nuclear medicine medical physics and nuclear bio-chemistry etc.

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