A Simple and Sensitive Procedure for Measuring Isotope Fluxes through Ion-specific Channels in Heterogenous Populations of Membrane Vesicles*

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In this paper, we describe a simple and highly sensitive manual assay for isotope fluxes through ion-conducting pathways, particularly cation-specific channels, in heterogenous populations of small membrane vesicles. We measure uptake of tracer of the ion of interest, against a large chemical gradient of the same ion. As a result of the imposed chemical gradient, a transient electrical diffusion potential is set up across the membranes of those vesicles which are highly permeable to the ion of interest. The isotope tends to equilibrate with the diffusion potential and is therefore concentrated selectively and transiently into those vesicle containing the channels. Furthermore, when performed in this way, the time course of tracer equilibration occurs over several minutes, rather than the sub-second range expected for tracer equilibration into channel-containing vesicles in the absence of an opposing chemical gradient of the permeant ion.

The use of the procedure is demonstrated for three Na-conducting channels: gramicidin D incorporated into phospholipid vesicles, amiloride-blockable Na channels in toad bladder microsomes, and veratridine-activated tetrodotoxin-blockable Na channels in rat brain synaptic membranes. For all three cases, it proved simple to measure a specific 22Na uptake, in a minute time range, using very low concentrations of the channel-containing vesicles. By comparison with isotope flux measurements performed without an opposing Na gradient, the power of the present assay derives from both the very large gain in sensitivity and the convenient time course.

Functional properties of ion-conducting channels can be studied by electrophysiological techniques on native cells or phospholipid bilayers. For isolation of channel proteins and biochemical characterization, it is essential to assess the functional state in a well defined subcellular system, such as in membrane vesicles. But measurement of ion fluxes in small vesicles is difficult. First, the typical conductance of channels is in the order of $10^{-10}$-$10^{-12}$ mho/channel (1-6). Thus, one would expect that the equilibration time of a tracer added to a suspension of vesicles will be in the order of seconds (if not less) and therefore inaccessible to manual techniques. Furthermore, the density of ion channels in cell membranes can be as low as 0.5/μm2 (3, 4), and thus on isolation of native membrane vesicles with a radius of less than 0.4 μm, one can expect a ratio of less than one channel/cell membrane vesicle. Also, membrane vesicles are usually derived from heterogeneous populations of cells and are always contaminated with vesicles from internal organelles such as mitochondria, endoplasmic reticulum, etc. Thus, the volume of interest, i.e. that bounded by the membranes containing the channels of interest, can often be expected to be a relatively small fraction of the total vesicle volume. One is therefore faced with the problem of measuring an extremely fast flux into a small fraction of the vesicle population and distinguishing it from a nonspecific flux into the bulk of the vesicle volume.

For purification of channel proteins and their assay, one invariably wishes to reconstitute the protein into artificial phospholipid vesicles. In a number of cases where channel proteins have been isolated, binding of a specific ligand such as tetrodotoxin (7) or saxitoxin (8, 9) has been used as the criterion for preservation of function during purification, but not all channels show high affinity ligand binding. A transport assay would of course be preferable. However, the small diameter of phospholipid vesicles in the various reconstitution procedures (300-1000 Å) and the requirement for an excess of lipid compared to protein should lead to a very small fraction of the vesicle population containing channels and makes the problems of measurement even more severe, even in cell membrane vesicles. Assay of isotope uptake or release from reconstituted vesicles has proved to be possible only in favorable cases in which the channel protein is available in large quantities, e.g. acetylcholine receptors (10) and the tetrodotoxin receptor (11, 12). As expected, the tracer equilibration time with these systems is so fast as to preclude continuous monitoring of the time course with simple manual methods.

In this paper, we describe a simple, sensitive, and convenient flux assay for selective ionic channels in membrane vesicles and demonstrate its use to measure 22Na fluxes through gramicidin incorporated into phospholipid vesicles, amiloride-sensitive Na channels in membranes isolated from toad bladder, and tetrodotoxin-sensitive Na channels in brain synaptic membranes. This assay was conceived partly on the basis of an interpretation offered by Glynn and Warner (13) for the transient accumulation in human red cells of 42K flowing through Ca-activated K channels: the Gardos effect.
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EXPERIMENTAL PROCEDURES

Methods

Vesicle Preparations

Liposomes—80 mg of crude soybean lecithin were suspended in 2 ml of media containing 25 mM imidazole (pH 7), 5 mM EDTA, and 150 mM NaCl. The lipid suspension was vortexed for 5 min and then sonicated in a Branson 12 bath sonicator until it became transparent (~30 min).

Toad Bladder Microsomes—Toads (Bufo marinus, Dominican Republic) were obtained from National Reagents (Bridgeport, CT) and kept partially submerged in tap water. The animals were double pithed and perfused through the ventricle with about 500 ml of Ringer's solution. The bladders were then excised and immersed in ice-cold homogenizing buffer containing 55 mM NaCl, 87.5 mM sucrose, 12.5 mM imidazole (pH 7), 2.5 mM EDTA, and 0.1 mM amiloride. All the subsequent operations were carried out at 0°C. The preparation of microsomes is based on a procedure developed by Palmer and Edelman.1 The epithelial cells were scraped from the supporting tissue with a glass microscope slide and washed twice in the above homogenization buffer by centrifugation at 800 g for 10 min. Washed cells obtained from four to six hemibladders were suspended in 2 ml of homogenization buffer and disrupted by a single 5-s burst with a Polytron tissue grinder (Brinkmann Instruments) at setting 6. The homogenate was centrifuged at 800 g for 10 min, and the pellet (mainly nuclei and unbroken cells) was discarded. The supernatant was centrifuged at 8,000 g for 5 min, yielding a cloudy supernatant, a loose yellow pellet, and a hard brown pellet. The loose layer was separated from the tight pellet by gentle shaking and was combined with the supernatant. The combined fraction was centrifuged for 60 min at 27,000 g, yielding a clear supernatant and pellet. This pellet, “the microsomal fraction,” was suspended in the homogenizing buffer to a final concentration of 9.5–15 mg of protein/ml and used for transport measurements within 4 h.

Brain Synaptosome Membranes—Brain membranes were obtained from synaptosomal lysates. Synaptosomes were prepared from rat brain homogenates by a procedure similar to that described by Krueger et al. (14). All operations were carried out at 0–4°C. The synaptosomes were centrifuged for 60 min at 27,000 g, yielding a clear supernatant and pellet. The pellet, “the microsomal fraction,” was suspended in the homogenizing buffer to a final concentration of 9.5–15 mg of protein/ml and used for transport measurements within 4 h.

Transport Assay

Two different procedures were used to remove Na ions from the vesicles prior to the transport assay. For experiments with liposomes, a volume of 200 μl was centrifuged through a Sephadex G-50-40 column pre-equilibrated with 150 mM Tris-Cl, 25 mM imidazole (pH 7), and 5 mM EDTA, as described previously (15). This step exchanged the external NaCl by an equal amount of Tris-Cl without changing the total volume. The eluted vesicle suspension was mixed with 800 μl of the above solution to which gramicidin had been added. The transport assay was initiated 30 s later by adding 10 μl of 22NaCl (2 Ci) to the suspension.

For experiments with toad bladder microsomes and brain synaptic membranes, the external Na was removed by passing the vesicles through a cation exchange column (Dowex 50-X8 Tris form). Volumes of 100-200 μl of the vesicle suspension were applied to small Dowex columns (see below) and eluted with 1-1.5 ml of 175 mM sucrose. This step exchanged the external cations by Tris and diluted the suspension 5-15-fold. Various reagents (amiloride, veratridine, and tetrodotoxin) were added, as required, to the vesicle suspension. The assay was initiated 90 s later by adding 10-15 μl of 22NaCl (2–3 Ci).

The vesicle suspensions were incubated with the isotope in the different conditions and for the times indicated in the legends to the Figs. 1-4. In order to separate the vesicles from the medium, 100-μl aliquots of the vesicle suspension were applied to 5-6 cm columns of Dowex 50-X8 (Tris form) poured in Pasteur pipettes, and the vesicles were eluted into counting vials by addition of 1.5 ml of ice-cold suspension (175 mM sucrose). The vesicles were washed with 1-2 ml of 175 mM sucrose containing 25 mg/ml of bovine serum albumin and stored at 0°C. The amount of 22Na trapped in the vesicles (eluted on the Dowex columns) was estimated by scintillation counting. The 22Na content is expressed everywhere as a fraction of the initial total radioactivity in the vesicle reaction medium. Transport assays were performed at 0 or 4°C for experiments using cell membrane vesicles or liposomes, respectively.

Materials

Bovine albumin, (fraction V), soybean lecithin, gramicidin D, Dowex 50X-8 (50–100 mesh), tetrodotoxin, phenylmethylsulfonyl fluoride, pepstatin A, iodoacetamide, and Sephadex G-50-40 were obtained from Sigma. Veratridine (99% pure) was obtained from Aldrich. Amiloride was a gift from Merck Sharp and Dohme. 22NaCl (200 μCi/ml, carrier-free) was purchased from Amersham Radiochemicals. All conventional chemicals were of analytical grade.

RESULTS AND DISCUSSION

The Principle of the Measurement—The principle of the assay is as follows. The vesicles are prepared to contain a relatively high concentration of NaCl. Shortly before the assay, the external Na is replaced by a relatively impermeant ion such as Tris. As a consequence of the Na gradient, an electrical diffusion potential will be set up, the magnitude of which will be determined by the relative permeabilities of Na, Cl, and Tris through the membrane. Only in those vesicles containing the Na channel is the Na permeability likely to be much greater than the Cl and Tris permeabilities, and hence a Na diffusion potential of maximal size and interior negative will be formed. If an isotope that permeates through the channel (in our case 22Na) is added to the exterior solution, it will tend to equilibrate with the membrane potential without itself significantly affecting the potential. It will therefore accumulate selectively into that fraction of the vesicle population containing the channels. In time, the Na gradient will dissipate, as will the interior negative membrane potential, and so 22Na will leave the vesicles. It will be shown that by arranging the flux assay in this way, the measurement of 22Na uptake is highly sensitive due to its accumulation, the time course of the selective 22Na uptake is convenient (in the minute range), and one can distinguish permeability properties of the channels of interest from nonselective Na permeabilities.

Gramicidin Channels in Phospholipid Vesicles—The experiments in this section demonstrate the use of the transport assay for the case of a well characterized channel-forming ionophore, gramicidin (17). Fig. 1A shows the time course of 22Na uptake into soybean phospholipid vesicles prepared to contain 150 mM NaCl, in the presence and absence of gramicidin and a large Na gradient. In the presence of a Na gradient and the ionophore, a large amount of 22Na was taken up, and after reaching a maximal level of about 1% of the total added radioactivity, after about 8 min, the 22Na content of the vesicles declined slowly. If we assume that the average molecular weight of the phospholipids is 1000 and there are about 3000 phospholipid molecules/sonicated vesicle (18), then the molar concentration of vesicles is about 2–3 x 10^-7 M. Assuming optimally that the gramicidin molecules are all incorporated into the vesicles and one requires two gramicidin molecules to produce an active channel (19), then at the concentration of ionophore used, 5 x 10^-7 M, one could expect that not more than about one in every 100 vesicles contains the channel. The ratio of internal to external volume in our
It is of interest that the transient accumulation of tracer in the gramicidin-containing vesicles is reminiscent of the classical "counter transport" phenomenon observed for carrier kinetic mechanisms. Although the phenomenon of isotope accumulation against a chemical gradient has been taken as diagnostic of a "carrier" mechanism (21), the result with gramicidin, a well characterized channel-forming ionophore, shows that in the experimental conditions chosen, the distinction between carrier and "channel" kinetic mechanisms is not possible.

Amiloride-sensitive Na Channels.—A crude preparation of membranes was prepared from epithelial cells scraped from toad bladders. The apical surface of the epithelial cells contains the amiloride-sensitive Na-specific channel (3, 22). The membrane preparation used consists of a mixture of vesicles derived from apical and basolateral membranes of the cells and is also heavily contaminated with fragmented mitochondria and probably other internal organelles. In the experiment of Fig. 2, the cells were homogenized and the membranes prepared in a medium containing 55 mM NaCl and 100 μM amiloride. Shortly before the assay, the external Na was replaced with Tris as described under "Methods." Amiloride is a weak base (pK₈.7), and it is essentially all in the protonated form at this experimental pH of 7.5. External amiloride will therefore be removed on the Dowex column. Then 22Na uptake was monitored in the conditions described in the legend to Fig. 2. As seen in Fig. 2A, when unlabeled NaCl was added to the medium and the Na gradient was thus abolished, a small, slow, and monotonic uptake of 22Na was observed. Inhibition by externally added amiloride was not detectable. Conversely, when only 22Na was added to the medium and the transmembrane Na gradient was maximal, a very large uptake was found and it declined slowly from the maximum. Amiloride added to the external medium inhibited both the initial rate and the maximal extent of 22Na uptake by about 65%. In five different preparations, the average inhibition by amiloride was 69 ± 5% (mean ± S.E.). Since amiloride acts from the outside of epithelial cells, it is likely that the amiloride-sensitive flux is occurring in vesicles oriented right side out with respect to the cellular orientation. The purpose of adding amiloride to the membrane preparation media was that it might, when incorporated inside, block Na

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**Fig. 1.** 22Na uptake through gramicidin channels in liposomes. Sonicated phospholipid vesicles (40 mg/ml) were prepared in 150 mM NaCl solution, and the external Na was substituted by Tris as described under "Methods." A, the eluted vesicles (200 μl) were diluted 5-fold in one of the following solutions: 150 mM Tris-Cl, 25 mM imidazole (pH 7), 5 mM EDTA, 5 × 10⁻⁸ M gramicidin ( ), same solution but without gramicidin (△), same solution using a solution that contains NaCl instead of Tris-Cl ( ). About 30 s after the dilution, 10 μl of 22NaCl (2 μCi) were added to each suspension. The trace uptake at t = 0.5 min was measured as described under "Methods." B, the initial stages of this assay were as in part A, using a diluting solution containing 150 mM Tris-Cl and gramicidin ( ). At t = 4.5 min (indicated by the arrow), the suspension was divided into two portions of 600 and 100 μl, respectively. The larger volume of suspension was mixed with 80 μl of a solution containing 150 mM KCl, 25 mM imidazole (pH = 7), and 5 mM EDTA. 110-μl aliquots of this suspension were removed to the Dowex columns at the times indicated ( ). The smaller portion (100 μl) served as a control. This portion was mixed with 10 μl of Tris-imidazole buffer and was applied to a Dowex column at t = 10 min ( ).

Conditions is roughly 0.01. The fact that at the maximum about 1% of the total radioactivity was taken up suggests that the 22Na uptake occurred mainly in those vesicles that contained the ionophore. This calculation takes into account the finding (20) that gramicidin molecules do not migrate from vesicle to vesicle. Other experiments (not shown) indicated that it was easy to detect differences in isotope uptake with gramicidin concentration only 2-fold higher or lower than 5 × 10⁻⁸ M, a finding which supports the conclusion that the molar concentration of channels is much lower than that of the vesicles. The slow decline in internal 22Na content (Fig. 1A) was due presumably to dissipation of the Na gradient limited by the slow permeation of Tris ions into the vesicles or net loss of NaCl. A rapid loss of 22Na could be induced (Fig. 1B) by addition to the exterior medium of K ions, which permeate readily through gramicidin (17) and should thus bring about immediate depolarization of the membrane potential (for an analysis of the effects of the gradient on the time course, see Miniprint²). In the absence of a Na gradient or the ionophore, the uptake of isotope was very small and monotonic (Fig. 1A). This 22Na uptake is essentially that which occurs into the ionophore-free lipid vesicles. These results, compared to the 22Na uptake without a gradient, the imposition of the Na gradient produced at least a 50-fold increase in 22Na uptake and this occurred over minutes.

² Portions of this paper (including Figs. 5–7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1146, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

**Fig. 2.** Amiloride-blockable 22Na uptake in toad bladder microsomes. Membrane vesicles from epithelial cells were isolated as described under "Methods." Volumes of 100 μl (1 mg of protein/ml) were applied to Dowex 50-X8 columns and eluted with 1.0 ml of sucrose (175 mM). A, the eluted vesicles were diluted with an equal volume of four different reaction mixtures consisting of 175 mM sucrose ( ), 175 mM sucrose, 0.2 mM amiloride ( ), 110 mM NaCl ( ), 110 mM NaCl, 0.2 mM amiloride ( ). All reaction mixtures contained 10 μCi of 22Na (50 μl). Samples of 100 μl were removed at the indicated times and applied to Dowex columns. B. The experiment was initiated as in A, and the symbols have the same meaning. At the time indicated by arrows, the vesicles were diluted 1:1 with solution of 110 mM NaCl.
fluxes through channels in any membranes oriented inside out with respect to the cellular orientation. The uptake of 
\( ^{22}\text{Na} \) which persists in the presence of amiloride added to the assay medium indicates the presence of other Na- or cation-specific permeation pathways, possibly in a different population of vesicles. The fact that most of the tracer is retained even 120 min after imposition of the NaCl gradient implies that the relevant vesicle space is either highly impermeable to Cl or cannot contract to a great extent (see "Analysis of 
\( ^{22}\text{Na Flow} \) in Miniprint"). On the other hand, abolishing the Na gradient by addition of NaCl to the medium at the peak of 
\( ^{22}\text{Na} \) accumulation led to an immediate loss of 
\( ^{22}\text{Na} \) from the vesicles both in the absence and presence of amiloride (Fig. 2B). This phenomenon is quite similar to that described for gramicidin in Fig. 1B. The sensitivity of the 
\( ^{22}\text{Na} \) flux to externally added amiloride, in an experiment like that in Fig. 2, was examined in Fig. 3, which shows the initial phase of 
\( ^{22}\text{Na} \) uptake at different concentrations of amiloride. The inhibitor is effective at very low concentrations and similar to those required to inhibit the Na flux in the intact bladder (22, 23). We conclude that much if not all of the amiloride-blockable flux measured in the vesicles is passing through the same Na channels characterized previously in intact bladder (3, 22-24).

Recently, Chase and Al-Awati (25) have been able to detect an amiloride-blockable 
\( ^{22}\text{Na} \) efflux in an apical membrane-enriched fraction isolated from toad bladder cells. The 
\( ^{22}\text{Na} \) flux was measured using rapid flow equipment and occurred over a subsecond time scale. These properties of the 
\( ^{22}\text{Na} \) flux are expected for conditions without a Na gradient. The necessity of using the rapid flow equipment makes such measurements less convenient than the manual assay described in this paper. Labelle and Valentine (26) have reported a much slower Na flux in toad bladder microsomes. This flux was inhibited by amiloride but only at concentrations higher than 0.6 mM. The slow time course and relative insensitivity to amiloride make it doubtful that the apical Na channels were being observed.

**Tetrodotoxin-sensitive Na Channels**—The third system that we have utilized to demonstrate the efficacy of the assay is the veratridine-activated, tetrodotoxin-inhibitable Na channel in rat brain synaptic membranes (Fig. 4). For this experiment, sonicated synapticosomal membrane fragments, equilibrated with 150 mM NaCl, were prepared as described under "Methods." Fig. 4 shows the time course of 
\( ^{22}\text{Na} \) uptake into synaptic membranes and the effects of veratridine, tetrodotoxin, and a transmembrane Na gradient. It is expected that in the presence of veratridine these channels will be open, while tetrodotoxin should block them. The criterion of a successful measurement will therefore be inhibition by tetrodotoxin of a flux observed in the presence of veratridine. As seen in Fig. 4, when such measurements are made in synaptic membranes without a Na gradient, the isotope uptake is small and inhibition by tetrodotoxin is only just detectable. However, with a transmembrane Na gradient, 
\( ^{22}\text{Na} \) uptake into the veratridine-activated synaptic membranes was large and showed the by now expected biphasic kinetics. Here, tetrodotoxin inhibited the 
\( ^{22}\text{Na} \) uptake by about 60–70%, to the same level as that observed in synaptic membranes which were not preincubated with veratridine.

Stimulation of the Na Flux by veratridine could be observed at concentrations as low as 10^{-6} M. The concentration dependence of the flux followed a simple hyperbolic saturation curve with half-maximal activation at \( 1 \times 10^{-3} \) M veratridine. Tetrodotoxin inhibited the stimulation of the flux produced by \( 1 \times 10^{-4} \) M veratridine. The concentration of tetrodotoxin giving a half-maximal inhibition of the initial rate, \( I_{50} \), was about 3 \times 10^{-3} M. These affinities are similar to those observed in excitable cells (27). The 
\( ^{22}\text{Na} \) uptake in the absence of both veratridine and tetrodotoxin presumably reflects the presence in the vesicle preparation of a Na conductance which is neither activated by veratridine nor inhibited by tetrodotoxin.

The resolution of the tetrodotoxin-sensitive 
\( ^{22}\text{Na} \) flux shown in Fig. 4 is far better than that obtained in previous

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**Fig. 3. Sensitivity of 
\( ^{22}\text{Na} \) uptake to amiloride.** 
\( ^{22}\text{Na} \) uptake was measured in toad bladder microsomes as described in the legend to Fig. 2 and under "Methods." Increasing concentrations of amiloride (0–16 \times 10^{-4} M) were used.

**Fig. 4. Tetrodotoxin-blockable 
\( ^{22}\text{Na} \) uptake in brain synaptic membranes.** Rat brain synaptic membranes were obtained from synaptosomes as described under "Methods." The membranes in 100 mM NaCl, 0.4 mM MgCl\(_2\), 5 mM Tris-Hepes, and 1 mM iodoacetamide were sonicated for 20 s in a bath sonicator prior to use. The membranes were then incubated at room temperature for 15 min in 100 \( \mu \)M veratridine (triangles) or 100 \( \mu \)M veratridine + 1 \( \mu \)M tetrodotoxin (TTX) (squares) or with no extra additions (circles). Volumes of 100 \( \mu \)l (2 mg of protein/ml) were applied to Dowex 50-X8 columns and eluted with 1 ml of sucrose (175 mM). The eluted vesicles were diluted with an equal volume of five different reaction mixtures consisting of 100 mM Tris-Cl, 100 \( \mu \)M veratridine (\( \Delta \)), 100 mM Tris-Cl (\( \bigcirc \)), 100 \( \mu \)M veratridine, 1 \( \mu \)M tetrodotoxin (\( \bigcirc \)), 100 mM NaCl, 100 \( \mu \)M veratridine (\( \Delta \)), and 100 mM NaCl, 100 \( \mu \)M veratridine, 1 \( \mu \)M tetrodotoxin (\( \bigcirc \)). To 1 ml of each mixture were added 10 \( \mu \)Ci of \( ^{22}\text{Na} \) (50 \( \mu \)l), and samples of 100 \( \mu \)l were removed at the indicated times and applied to Dowex columns.

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\( ^{22}\text{Na} \) uptake was measured in toad bladder microsomes as described in the legend to Fig. 2 and under "Methods." Increasing concentrations of amiloride (0–16 \times 10^{-4} M) were used.

3 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
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studies using synaptic vesicles without an opposing Na gradient (11, 12, 28). Measurements of $^{22}$Na uptake into phospholipid vesicles reconstituted with the partially purified Na channel protein have failed to show any stimulation by veratridine, although in two reports evidence has been produced that active Na channels were incorporated into the vesicles (29, 30). By contrast, it has been shown\(^4\) that in vesicles reconstituted with partially purified Na channel protein, veratridine produces a 400–500% increase in $^{22}$Na flux when assayed in the presence of an opposing Na gradient. This flux is sensitive to tetrodotoxin and occurs in a time scale of minutes.

**Significance and Potential Uses of the Assay**—Comparison of the experiments described in this paper with previous work on membrane vesicles from both toad bladder and rat brain synaptic membranes emphasizes the convenient time course and tremendous gain in sensitivity of the flux measurements when performed according to the present procedure.

The success of this assay depends on the existence of a large differential membrane permeability between the ion of interest and the other ions present. It is therefore applicable to all transport systems involving net conductance of the ion of interest, but especially to the case of channel mechanisms. The method is particularly useful for work with heterogeneous membrane systems because the accumulation of the isotope into vesicles containing the channels of interest greatly magnifies the flux into those vesicles and separates functionally the different classes of vesicles. Assay of channels in a heterogeneous population of vesicles is the first step towards purification of channel proteins and biochemical characterization. Reconstitution of functionally active channel protein into artificial phospholipid vesicles will also make use of the advantages of the assay. The experiment of Fig. 1 clearly demonstrates that a successful reconstitution can be detected even if the channel of interest is incorporated into a small fraction of the lipid vesicles (less than 1%), and the assay provides also a sensitive measure of the concentration of the channels. By assaying channel fluxes in different vesicle fractions isolated from structurally complex tissues such as muscle, it should also be possible to use the procedure to localize channels to particular membranes.

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REFERENCES


\(^4\) B. Rudy, unpublished experiments.
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Analysis of the law of isotope fluxes

In this section, we attempt to analyze a semi-quantitative manner the time-course of Na uptake into the vesicles. The overall kinetic behavior is determined by the permeability and concentrations of all the ionic species, as well as by the dimensions of the vesicles (A/V and W/V). The initial concentrations of solutions and ions, and the initial values of A/V and W/V, are in the system with which the components fluxes thus determined. The effective concentrations of Na, W/V, and C are:

\[ E = \frac{C}{E_{\text{ref}}} \]

The initial fluxes of all ions are calculated in moles (nmol)/sec. The initial flux of Na is:

\[ J_{\text{Na}} = -\frac{C_{\text{Na}}}{E_{\text{ref}}} \exp \left( -\frac{1}{E_{\text{ref}}} \right) \]

The change in the active vesicle volume is calculated for the time increment at, assuming that the osmotic equilibrium holds:

\[ V_{\text{Na}} = \frac{C_{\text{Na}}}{E_{\text{ref}}} \exp \left( -\frac{1}{E_{\text{ref}}} \right) \]

and then new values of A/V and W/V are obtained at t + dt. We can neglect the increase in volume of Na ions and the increase in volume of the vesicle is calculated for a further increment at. The accumulation of increase in concentration of isotope and increments of time provides the full time-course. The choice of the particular small time increment is that such a 2-fold change in its value does not appreciably change the calculated time-course.

Using the above procedure, we analyze the time-course of Na uptake by vesicles under various conditions. For simplicity, we consider two restrictive situations. In the first, we assume that the only ions with appreciable permeabilities are Na and Tris. The membrane potential caused by the diffusion of Na ions does their gradient is dissipated by entry of Tris ions. Net salt transport will not occur and the volume of the vesicles is constant. In the second situation, we assume that the fluxes of Na, W/V, and C are equal at t = 0, and that in the initial phase of Na uptake, the vesicle has an external Na concentration of 30 mM and is in contact with a Tris solution of 10 mM Na (3) and that for Tris uptake Na/V Na. Assuming a Na/V Na (3) to per mg protein (24), the product concentrations of our particular channel model, micrometer is determined by the Na/V Na (3) to per mg protein (24), the product concentrations of our particular channel model, micrometer (31). It is likely that similar values of Na/V Na and A/V Na apply to other mucosal preparations. Without a Na gradient, the total fractional uptake is exactly equal to the annual Na/V Na, i.e., 10% for Na uptake was performed for several minutes (5). As in the first situation, the higher the Na/V Na, the higher the Na uptake and the factor is the rate of Na accumulation and final off. The opposite is the case for Tris uptake.

Fig. 5. Calculations of Na uptake by vesicles in conditions without volume changes

The time-course of Na uptake was simulated as described in the text assuming that Tris and Na are the only ions with appreciable permeabilities. The initial situation assumed the following: vesicles containing 300 mM NaCl are suspended in a solution containing 100 mM Tris and 1.0 mM Tris NaCl. The following values were used for the fluxes: Na/V Na = 1.5 x 10^-3 cm^2 sec^-1, W/V W = 5 x 10^-3 cm^2 sec^-1, and C/V C = 1.0 x 10^-3 cm^2 sec^-1.

A. The time course was calculated for 3 different values of Na/V Na (indicated in the figure) at fixed values of W/V W and C/V C.

B. The time-course was calculated for different values of W/V W and C/V C at fixed values of Na/V Na.

C. The time-course was calculated for different values of Na/V Na and C/V C at fixed values of W/V W.

The time interval at which the curves were 1 sec up to 3 min and 5 sec for longer times.

The second restrictive situation we consider is in which only Na ions have appreciable permeability in addition to Tris ions. In this case and the concentration of NaCl gradient will drive salt out from the vesicles. One can assume that in relatively large vesicles, such as those obtained from cells, that solute equilibration is maintained. Here the rate of salt is calculated by extrusion of the vesicles. The total amount of Na in the vesicles falls but the concentration is nearly constant. The driving force for Na entry, the membrane potential, falls much more slowly than in the absence of volume changes. The loss of Na Na occurs by net salt efflux and not contraction of the vesicles. The time course of Na Na entry, the membrane potential, falls much more slowly than in the absence of volume changes. The loss of Na Na occurs by net salt efflux and not contraction of the vesicles. For example as low as 50 for the case A/V A = 3 x 10^-3 cm^2 sec^-1. In practice of course it is very unlikely that the vesicles can contract to such an extent because they should become non-electric as their membrane decreases. If and when this occurs the net loss of Na Na (and NaNa) will diminish and there will be a level higher than chemical equilibration of the Na ions. An effect of this kind could explain the plateau level of NaNa reached in Fig. 4.

Fig. 6. Calculations of Na uptake by vesicles in conditions with volume changes

The time-course and magnitude of Na uptake was simulated for a situation in which Na and Cl are the only permeable ions and the vesicles are perfused with NaCl. The initial situation assumed the following: vesicles prepared at 300 mM NaCl are suspended in 10 mM Tris and 1.0 mM NaCl. The following values were used for the fluxes: Na/V Na = 1.5 x 10^-3 cm^2 sec^-1, W/V W = 5 x 10^-3 cm^2 sec^-1, and C/V C = 1.0 x 10^-3 cm^2 sec^-1. The time-course was calculated for different values of W/V W and C/V C at fixed values of Na/V Na.

A. The time course calculated for the different values of W/V W and C/V C indicated in the figure.

B. The changes in vesicular volume associated with the net Na Cl efflux calculated in A.

In the practice of experiments of Figs. 3, 4, and 5, we repeatedly involved both net Na loss and contraction of the vesicles as well as exchange of Na for Tris ions. The relatively slow time-course of Na accumulation and efflusions observed are similar to those predicted with the assumed ranges of values of Na/V Na, W/V W, and C/V C. We also consider, in vesicles assumed to be filled with 300 mM NaCl and suspended in 10 mM Tris. We explore the consequences of varying Na/V Na from 5 x 10^-3 to 5 x 10^-4 cm^2 sec^-1, W/V W from 5 x 10^-3 to 5 x 10^-4 cm^2 sec^-1, and C/V C from 1 x 10^-4 to 1 x 10^-5 cm^2 sec^-1. The range of Na/V Na has been based on the kinetics of sodium transport observed in intact bladder (8) and that for W/V W Na/V Na and A/V Na apply to other mucosal preparations. Without a Na gradient, the total fractional uptake is exactly equal to the annual Na/V Na, i.e., 10% for Na uptake was performed for several minutes (5). As in the first situation, the higher the Na/V Na, the higher the Na uptake and the factor is the rate of Na accumulation and final off. The opposite is the case for Tris uptake.

Fig. 7. Calculations of Na uptake into vesicles assuming non-independence of unidirectional and isotonic Na fluxes

Conditions assumed were those of Fig. 6 with W/V W = 3 x 10^-3 cm^2 sec^-1, and various values of Na/V Na (5 x 10^-4 to 5 x 10^-4 cm^2 sec^-1).
A simple and sensitive procedure for measuring isotope fluxes through ion-specific channels in heterogeneous populations of membrane vesicles.

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