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A METHOD FOR DEMONSTRATING THE HETEROGENEITY OF PIGEON RED CELL MEMBRANE VESICLES BASED ON THEIR GLYCINE TRANSPORT ACTIVITY

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Summary

A procedure is described which is capable of detecting the changes in size and/or density of small membrane vesicles resulting from solute uptake. Vesicles which have taken up solute sediment more slowly in a density gradient, the ratio of glycine uptake/vesicle-trapped space is not uniform in the vesicle population, and vesicles with higher uptake/space ratios are preferentially retarded upon centrifugation.

Alanine transport activity is associated with glycine transport activity in that retardation of vesicles due to glycine uptake equally retards vesicles possessing alanine uptake activity.

Introduction

The aim of the work to be described was to devise a method capable of separating transport-active membrane vesicles from less active or inactive vesicles.

The effect of a transport process on the amount of solute enclosed within a vesicle should increase with the surface/volume ratio of the vesicle and therefore vary inversely with vesicle size. With small vesicles, significant percentage changes in volume and mean density should result from the transport activity of the vesicle shell. Both the increase in volume and the decrease in mean vesicle density should act to retard the sedimentation of vesicles in a density gradient. In a population containing vesicles of different transport activities, the more active vesicles should be more retarded.

The membrane vesicle preparation used is a modified form of that previously described [1]. The vesicles have the Na^+-dependent glycine active transport system characterized in earlier work (ref. 2 and refs. therein). They also have

an Na^+-dependent alanine uptake activity (Lee and Vidaver, unpublished) like intact cells [3,4]. Vesicles prepared by a slightly different procedure also have an ATP-dependent Ca^{2+} transport system (Ting and Vidaver, unpublished). The glycine uptake activity was used to produce the retardation of active vesicles on density gradient centrifugation. We also found an association between alanine and glycine uptake activities in the vesicle population by using glycine uptake to cause a retardation of vesicles which had been allowed to take up a small amount of labelled alanine.

Materials and Methods

Bovine serum albumin (Cohn fraction V), ethyleneglycol bis(β-aminoethyl-ether) *N,N'*-tetraacetic acid (EGTA) and Tween-80 were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Tween-80 was kept refrigerated. Bovine serum albumin was dialysed against deionized water and adjusted to pH 7.2 with 1.36 M KOH. Human hemoglobin was from Worthington Biochemical Corp., Freehold, N.J., U.S.A. It was mostly methemoglobin and was reduced by sodium dithionite under N_2, passed through a G-25 Sephadex column and dialysed against 2 mM phosphate buffer, pH 7 [5]. [2-^{3}H]glycine and L-[3-^{3}H]alanine were from New England Nuclear, Boston, Mass., U.S.A. [1-^{14}C]glycine, ^{22}NaCl, [*Me*-^{3}H]choline chloride and [*Me*-^{14}C]choline chloride were from Amersham/Searle, Arlington Heights, Ill., U.S.A. Scintisol, the solubilizer used for liquid scintillation counting was from Isolab, Arkon, Ohio, U.S.A. The scintillator 2a70, and scintillation grade toluene, were from Research Product International Corp., Elk Grove Village, Ill., U.S.A. All other chemicals used were analytical reagent grade.

Solutions used and their compositions were as follows. Solution A, composition: 146 mM KCl, 1.8 mM D-glucose, 4 mM $MgCl_2$, 2 mM $CaCl_2$. Solution B, composition: 136 mM KCl, 2 mM EGTA (K^+ salt), 1 mM $CaCl_2$, 4.5 mM KH_2PO_4, 5.5 mM K_2HPO_4 adjusted to pH 8.0 with 0.16 M KOH. Solution C (annealing solution), composition: same as solution B, except 4 mM $MgCl_2$, 30 mM sucrose and 113 mM KCl replaced 136 mM KCl. Labelled choline was added in some experiments. Bovine serum albumin, composition: 30 mM sucrose, 1 mM EGTA (K^+ salt) 5—16% bovine serum albumin (neutralized with KOH) and KCl or NaCl to give a calculated total osmolarity of 0.308 M. The osmotic contribution of bovine serum albumin was ignored since bovine serum albumin concentration did not affect red cell volume in such solutions. Bovine serum albumin solutions used in linear density gradients also contained 0.2% Tween-80 and 0.05% hemoglobin. Solution (incubation), composition: 137 mM NaCl or KCl, 2 mM EGTA (K^+ salt), 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM Na^+ or KH_2PO_4, 6 mM Na^+ or K_2HPO_4; labelled glycine and/or alanine at the appropriate concentrations. Liquid scintillation, composition: 2.4 g 2a70, 100 ml scintisol and 500 ml toluene counting cocktail.

The membrane vesicles were prepared by a modification (Sorensen and Vidaver, unpublished) of our earlier procedure [1]. All operations were at 0—5°C except where otherwise indicated. The washed erythrocytes, with the white cells removed by aspiration, were resuspended in solution A to give a 15% w/v cell suspension. 100 ml was sonicated with a Branson sonifier, model

W 185D, microtip probe at setting 4 for six 30-s intervals with 30-s cooling intervals. 45 ml of 0.3 M sucrose solution was added to the sonicated cell suspension. The mixture was centrifuged for 5 min at 5000 × *g* in a Sorval RC2B centrifuge in an SS-34 rotor. The resulting supernatant was centrifuged for 20 min at 23 500 × *g*. The loose upper layer of the pellet was carefully rinsed into another centrifuge tube with several approx. 5 ml portions of 154 mM KCl and the sticky brown bottom pellet was discarded. The suspension was centrifuged and the selective resuspension and centrifugation was repeated. The resulting pellet was resuspended in solution B, about 0.4 ml/g of original cells, and sonicated in 12 ml portions at setting 5 for two 45-s intervals in an aluminum cup in an ice bath with 30-s cooling periods. The suspension was centrifuged for 5 min at 6000 × *g* and the pellet discarded. The supernatant was centrifuged 45 min at 32 800 × *g* and the resulting pellet was resuspended in solution C and incubated at 41°C ("annealed") for 15 min. In experiments where a marker for "trapped space" was used, the labelled compound (usually choline) was present in the annealing solution (solution C). The membrane suspension was stored overnight at 0°C. The suspension was reannealed for 5 min at 41°C on the next day and diluted with 9 volumes of solution C adjusted to pH 7.5 instead of 8.0. The diluted membrane suspension was annealed for another 5 min and centrifuged for 5 min at 6000 × *g*. The pellet was discarded and the supernatant centrifuged for 30 min at 32 800 × *g*. The pellet, approx. 200 mg wet weight, was resuspended with 0.7 ml 7.5% bovine serum albumin solution and layered on a discontinuous bovine serum albumin "sizing" density gradient with steps of 7.5, 9.0 and 10.5% bovine serum albumin. After centrifugation in a Beckman L5-65 ultracentrifuge for 35 min at 275 000 × *g* in a SW41 Ti rotor at 5°C, the fraction of membrane vesicles banding at the 9.0–10.5% bovine serum albumin interface was carefully withdrawn.

Three basic kinds of experiments were performed to study the effect of glycine uptake on sedimentation of membrane vesicles in a bovine serum albumin density gradient.

(1) "Space-uptake" experiments. Vesicles for these experiments were annealed in the presence of [^{3}H]choline chloride which served as a trapped space marker. The "sized" membrane vesicle suspensions were prepared as described above. 2 0.5 ml aliquots, usually from one sizing density gradient, were each mixed with an equal volume of solution D (150 mM in Na^+) containing either a "high" (e.g. 2.0 mM) or "low" (e.g. 0.05 mM) concentration of [^{14}C]glycine. Each sample was incubated 7 min at 40°C, chilled, diluted with 0.75 ml 154 mM KCl containing Tween-80 to give a final concentration of approx. 2.5% bovine serum albumin and 0.2% Tween-80. ^{22}NaCl was added to the chilled suspension. This was to label with ^{22}Na any incubation medium that moved down into the gradient during the subsequent centrifugation. The mixture was layered on top of a linear 5–13% bovine serum albumin density gradient containing 0.2% Tween-80 and 0.05% hemoglobin and centrifuged for 45–55 min at 275 000 × *g* in a SW41 Ti rotor at 5°C. Usually 6 drop fractions were collected from the bottom of the centrifuge tube into counting vials containing 12 ml of counting cocktail and counted in a Packard or Ansitron liquid scintillation counter for ^{3}H, ^{14}C and ^{22}Na. The counts were spill-

corrected. Quench corrections were unnecessary. If the ^{22}Na counts indicated that more than 20% of the ^{14}C or ^{3}H counts came from contamination by incubation medium, the data was not used. Otherwise the data was corrected for this contamination.

(2) "Space-space" experiments. Two separate portions of membrane vesicles were annealed in solution C. One contained [^{3}H]choline chloride, the other contained [^{14}C]choline chloride. After "sizing" on separate gradients, each sample was divided into two aliquots. One of the aliquots was incubated with a high concentration and the other with a low concentration of unlabelled glycine in Na^+-rich medium as described for "space-uptake" experiments. The resulting four samples contained: (i) [^{3}H]choline chloride plus high glycine; (ii) [^{3}H]choline chloride plus low glycine; (iii) [^{14}C]choline chloride plus high glycine; (iv) [^{14}C]choline chloride plus low glycine. The samples were combined, (i) with (iv) and (ii) with (iii) and each combined sample centrifuged in a linear bovine serum albumin density gradient etc. as described for the "space-uptake" experiments.

(3) "High-low uptake" experiments. No radioisotopes were added during the annealing step. Two aliquots of "sized" membrane vesicles from one sizing density gradient were incubated separately, one with a high concentration of [^{14}C]glycine (e.g. 2.0 mM, which is greater than K_m [6]), the other with a low concentration of [^{3}H]glycine (e.g. 0.01 mM, much less than K_m). After incubation, both aliquots were chilled, combined, and centrifuged in a linear density gradient etc. as described above.

Calibration experiments were also made in which two or three aliquots of membrane were annealed, each with a different isotope as space marker (e.g. [^{3}H]choline, [^{14}C]choline and ^{22}Na), and each with a different concentration of KCl. These were centrifuged on separate "sizing" gradients, each gradient having a KCl concentration equal to the KCl concentration used for annealing the corresponding membrane aliquot. The three "sized" vesicle samples were then combined, diluted and centrifuged in a linear density gradient etc. Note that these vesicles were not incubated for glycine uptake after the annealing step.

Results

In the "high-low uptake" experiments the two co-centrifuged populations of vesicles differed only in that one had taken up solute from a high glycine medium and the other from a low glycine medium. The ^{14}C and ^{3}H curves (Fig. 1) mark the distribution in the gradient of the uptake activity of these two populations. In Fig. 1a, K^+-filled vesicles were incubated in Na^+ media. The retardation of vesicles incubated in high glycine is evident. Vesicles from high glycine medium took up 80 times as much glycine as those from low glycine medium. In Figs. 1b and 1c there was no transmembrane Na^+ gradient; in Fig. 1b only K^+ was present on both sides of the membrane and in Fig. 1c, Na^+ was on both sides. In both these cases the retardation was negligible. These data also show the Na^+-gradient requirement for glycine uptake by these vesicles. In the experiment shown, uptake of glycine from high glycine (2.0 mM) Na^+ medium into K^+-filled vesicles was 4.5-times the uptake from high glycine K^+

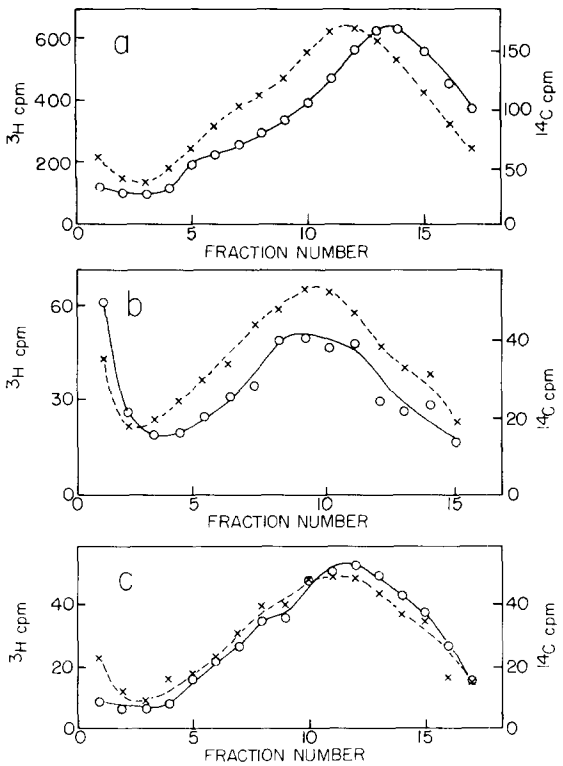

Fig. 1. Sedimentation of membrane vesicles in bovine serum albumin density gradients; "high-low uptake" procedure. Annealed membrane vesicles from one "sizing" density gradient were divided into two portions. One portion was incubated with 2.0 mM [^{14}C]-glycine, 5 μCi/ml, the other with 0.01 mM [^{3}H]-glycine, 17 μCi/ml for 7 min at 40°C. The portions were combined and centrifuged in a linear 5—13% bovine serum albumin density gradient. In (a), membrane vesicles were annealed in K^+ medium and incubated in Na^+ medium (77 mM Na^+). In (b), membrane vesicles were annealed in K^+ medium and incubated in K^+ medium. In (c) vesicles were annealed and sized in 77 mM Na^+ medium and incubated in 77 mM Na^+ medium. o———o, radioactivity of [^{14}C]glycine. X- - - - -X, radioactivity of [^{3}H]glycine.

medium into K^+-filled vesicles and 3-times the uptake from high glycine Na^+ medium into Na^+-filled vesicles. The corresponding ratios from low glycine media are 9- and 10-times. Thus most of the glycine uptake in the presence of the cation gradient is Na^+ gradient-driven glycine accumulation.

"High-low uptake" experiments show only the gradient position of uptake activity. Inactive vesicles would not be seen, nor would differences in transport activity within the vesicle population be seen. In the "space-uptake" experiments, vesicles are marked by the space they contain as well as their uptake activity. Since some vesicles in a population might be more active than others, and vesicles with different activity/space ratios might move to different positions in the gradient in the absence of solute uptake, it is necessary to use two density gradients of "space-uptake" experiments. One gradient is needed to measure the uptake/space ratio distribution of the minimally shifted population (low glycine) and one to measure the change in this distribution produced by extensive solute uptake. Fig. 2 shows such an experiment. Fig. 2a (low glycine) shows that the uptake/space ratio is greater in the higher density

region of the peak. Figs. 2b and 2c (high glycine) show that the migration of vesicles with the higher uptake/space ratio is preferentially retarded by solute uptake. Similar amounts of retardation are produced by solute uptake from 2.0 and 0.76 mM glycine. This similar retardation was also found with the "high-low" procedure. The significance of this similar retardation is considered in the discussion.

The shift of the uptake curve relative to the "space" curve does not give a true picture of the relative amounts of active and less active or inactive vesicles. The counts taken up are the product of the amount of active vesicles times their uptake activity. The "space-space" experiments can discriminate between space enclosed in more and less transport-active vesicles. Two density gradients are required for this experiment also, since the vesicles combined and co-sedimented in one gradient are themselves prepared from two different "sizing" gradients which might not yield populations with exactly the same migration distributions. In the experiment shown in Fig. 3 they did. The retardation of the high glycine vesicles relative to the low glycine vesicles was similar whether the [^{3}H]choline vesicles (Fig. 3a) or the [^{14}C]choline vesicles (Fig. 3b) were incubated in the high glycine medium. This was not always the case. In some experiments one of the pair of gradients showed more ^{14}C-^{3}H displacement than the other. (In no case did the retarded isotope curve contain the same

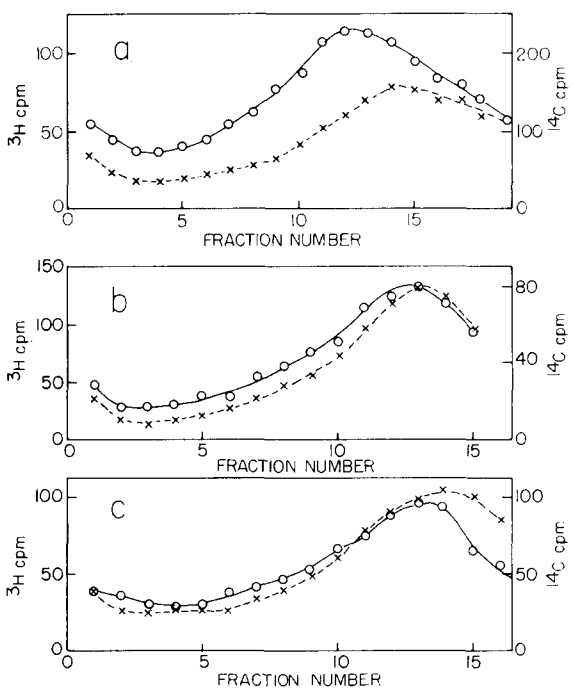

Fig. 2. Sedimentation of membrane vesicles in bovine serum albumin density gradients; "space-uptake" procedure. Membrane vesicles were annealed in K^+ medium in the presence of 4 mM [^{3}H]choline chloride, 56 μCi/ml. "Sized" membrane vesicles were incubated in 77 mM Na^+ medium with [^{14}C]-glycine, 5 μCi/ml, at (a) 0.05 mM, (b) 2.0 mM or (c) 0.76 mM for 7 min at 40°C. Samples were centrifuged as for Fig. 1. ○———○ radioactivity of [^{14}C]glycine; X------X, radioactivity of [^{3}H]-choline chloride.

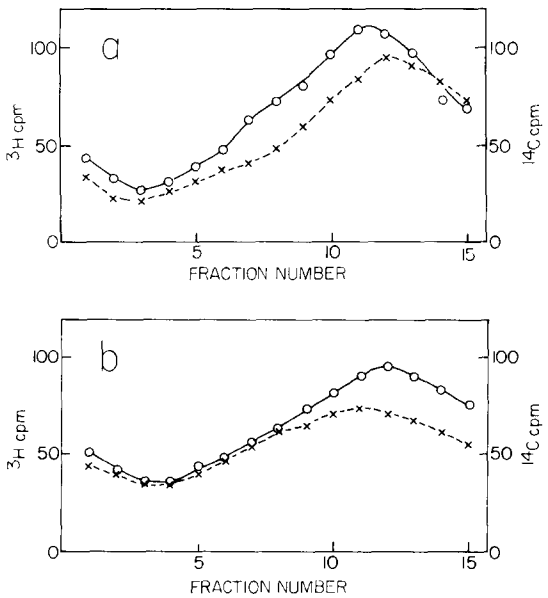

Fig. 3. Sedimentation of membrane vesicles in bovine serum albumin density gradients; "space-space" procedure. Two portions of membrane vesicles were annealed in K^+ medium; one in the presence of [^{3}H]-choline chloride, 4 mM, 56 μCi/ml, the other with [^{14}C]choline chloride, 4 mM, 28 μCi/ml. Each sample was "sized" and divided into two aliquots which were incubated separately in either 2.0 mM or 0.01 mM unlabelled glycine in the presence of 77 mM Na^+ at 40°C for 7 min. In (a) [^{3}H]choline containing vesicles were incubated with 2.0 mM glycine and [^{14}C]choline containing vesicles were incubated with 0.01 mM glycine. They were then combined and centrifuged in a linear bovine serum albumin density gradient. In (b) [^{3}H]choline containing vesicles were incubated with 0.01 mM glycine and the [^{14}C]choline containing vesicles were incubated with 2.0 mM glycine. They were then combined and centrifuged in a linear density gradient as for Fig. 1. o———o, radioactivity of [^{14}C]choline, X- - - - -X, radioactivity of [^{3}H]-choline.

isotope in both gradients.) The ratio of the total areas under the ^{14}C and ^{3}H curves is 1.207 for Fig. 3a and 1.1805 for Fig. 3b. In a duplicate experiment, the ratios were 0.5693 and 0.5637. That is, no trapped choline was lost as a consequence of solute uptake from high glycine media.

We do not usually see two resolved peaks, * so accurate quantitation is impossible. However, the two limiting cases, (a) all vesicles equally active or (b) most vesicles inactive, are excluded. If all vesicles were equally active the "space-uptake" experiment would show no displacement of "uptake" relative to "space". If only a small fraction of the vesicles were active, the "space-space" experiments would show no significant curve displacement.

The "calibration" experiments (Fig. 4), in which vesicles were prepared with different amounts of KCl trapped during annealing, show how much retardation is produced by changes in the amount of vesicle-enclosed solute. An 11 mM excess or deficit of KCl produces large shifts in position. In the "space-uptake" experiments estimates of both vesicle-enclosed space and glycine uptake are available, e.g. in the experiment of Fig. 2, glycine uptake

* A referee asked whether further modification of our procedure might allow actual separation of different vesicle populations. We think this might be done to some extent by using a higher density fraction from the sizing gradient.

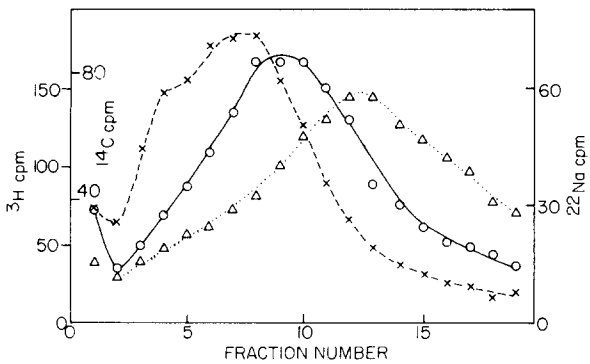

Fig. 4. Calibration curves. Co-sedimentation of vesicles containing different amounts of enclosed solute. Three portions of membrane vesicles were annealed in solution C containing different concentrations of KCl and different isotopes: (i) 113 mM KCl, [^{3}H]glycine, 57 μCi/ml, (ii) 124 mM KCl, [^{14}C]glycine, 37 μCi/ml, and (iii) 135 mM KCl, ^{22}NaCl 37 μCi/ml. Each preparation was "sized" on a bovine serum albumin step density gradient containing the same KCl concentration as the corresponding annealing medium. The three "sized" vesicle samples were combined and layered on a linear 5—13% bovine serum albumin density gradient, 124 mM in KCl. ○———○, radioactivity of [^{14}C]glycine; X- - - - -X, radioactivity of [^{3}H]glycine, △———△, radioactivity of ^{22}NaCl.

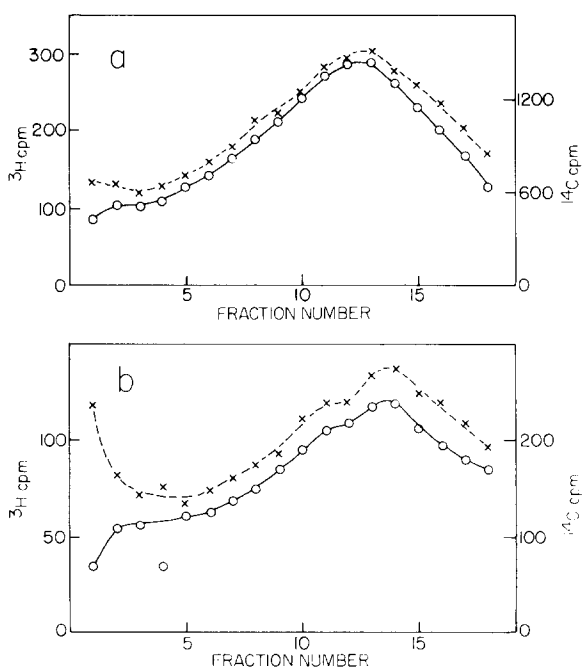

Fig. 5. Co-sedimentation of glycine and alanine transport activities on a bovine serum albumin density gradient. Membrane vesicles were annealed in solution C plus 2.0 mM alanine without isotopes. (Alanine uptake by the vesicles appears to be a Na^{+}-dependent exchange process, Lee and Vidaver, unpublished.) "Sized" vesicles were incubated in the presence of (a) 0.05 mM [^{14}C]glycine, 5 μCi/ml and 0.02 mM [^{3}H]alanine, 25 μCi/ml, (b) 2.0 mM [^{14}C]glycine and 0.02 mM [^{3}H]alanine. Each sample was centrifuged in a density gradient as for Fig. 1. ○———○, radioactivity of [^{14}C]glycine. X- - - - -X, radioactivity of [^{3}H]alanine.

was 22.5 μmol/ml, presumably accompanied by 2 NaCl per glycine [6—9]. The position shift produced by solute uptake appears less than the shift produced by trapping of an equivalent amount of solute during annealing. However, the ratio, total solute uptake/space, might be overestimated in the "space-uptake" experiments because during the 41°C incubation in which glycine and Na^+ are taken up, solutes may also be lost. The solutes lost might be Na^+ taken up in the course of the incubation, or labelled choline trapped at the annealing step. The apparent solute uptake is at least sufficient to produce the observed position shifts on the analytical gradients.

With a method capable of shifting the gradient position of transport-active vesicles, we could test for association between glycine transport activity and other membrane properties. We measured alanine transport activity to see if this was present in the same vesicles that had glycine transport activity. These experiments were made like the "space-uptake" experiments except that 2.0 mM alanine and no isotope were present during annealing and either 2.0 mM or 0.05 mM [^{14}C]glycine plus 0.02 mM [^{3}H]alanine were in the incubation media. We found (Fig. 5) that alanine and glycine uptake activities coincided whether high or low glycine concentrations were used. That is, the vesicles retarded by glycine transport had alanine transport activity proportional to their glycine uptake activity. A "space-uptake" experiment was also made at the same time with the same vesicle preparation with 2.0 mM alanine present inside to verify that the normal space-uptake shift was occurring (data not shown).

Discussion

The data presented show that uptake of glycine (and presumably Na^+) causes small vesicles to be retarded on density gradient centrifugation. The calibration curves (Fig. 4) show that the position of vesicles on the gradient is quite sensitive to the amount of enclosed solute. The apparent solute uptake on incubation with high glycine concentrations is more than sufficient to produce the observed retardation. The only difference in treatment associated with the shift is the glycine concentration in the incubation medium and this is only effective if there is simultaneously an inwardly direct Na^+ gradient (Fig. 1). Vesicles with high activity/space ratios are preferentially retarded (Fig. 2), and the shift is observed when space markers are used to label both vesicle populations (Fig. 3). These properties are those expected if solute uptake causes the retardation. However the isotope profile shifts in the "high-low uptake" and "space-uptake" experiments might have been caused by other phenomena. Vesicles cannot take up an unlimited amount of glycine nor hold an unlimited amount of solute. With vesicles incubated in low glycine medium, higher uptake/space ratios are found in the faster sedimenting region of the peak (Fig. 2). If these "more active" vesicles could take up, from high glycine medium, no more glycine per ml of enclosed space than the less active vesicles in the slower migrating region, then the high glycine uptake profile would appear to be retarded relative to the low glycine profile. In this case however, no shift would be seen in the "space-space" experiments which is contrary to observation. Alternatively, the smaller and hence "more active" vesicles might

take up more solute than they can hold. Then selective loss of labelled solute could produce the apparent shift in the "high-low uptake" experiments. If glycine and choline were equally lost, the "space-uptake" experiments should show no shift. If only glycine were lost, the "space-space" experiments should show no shift.

The "space-uptake" experiments show that the "sized" vesicle population is heterogeneous, containing vesicles with different ratios of space to uptake activity. This is unlikely to be due to some vesicles being inside out and others being right side out since nearly the same retardation is produced by incubation with 0.76 mM glycine or 2.0 mM glycine (Fig. 2b vs. Fig. 2c). From the kinetic parameters for glycine entry and exit [6], entry and exit rates would be equal at 0.76 mM glycine with the Na^+ concentration (77 mM) used, so solute uptake should be independent of "sidedness".

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