Na+-dependent amino acid transport can be reconstituted by gel filtration of disaggregated plasma membrane and asolectin vesicles coupled to a freeze-thaw cycle. The resultant transport activity is markedly affected by the nature of the reconstitution medium. Reconstitution in K+ permits the formation of active liposomes, whereas reconstitution in Na+, Li+, or choline does not. Electron micrographs of K+ liposomes show a wide variation in vesicle sizes. Ficoll density gradient fractionation of K+ liposomes shows that the largest vesicles are lipid rich, have the lowest density, and have the highest level of Na+-dependent amino acid transport. Liposomes formed in Na+ have a 34% smaller trapped volume than K+ liposomes and lack a population of large vesicles. A second freeze-thaw in K+ restores activity to Na+ liposomes which now contain large low density active vesicles. Fluorescence measurements of freeze-thaw-induced mixing of vesicle lipids indicates that the absence of large vesicles in Na+ liposomes is due to inhibition by Na+ of lipid vesicle fusion events during freezing and thawing. The large vesicle fraction is enriched in a 125-kDa peptide. It has not yet been established whether this peptide is part of the transport system for neutral amino acids.

Amino acids are accumulated in mammalian cells by Na+-dependent and Na+-independent transport systems (1). Kinetically, these systems present a wide and overlapping substrate specificity (2-4), making estimations of the number of distinct amino acid carriers present in the membrane extremely difficult. In the absence of a variety of specific, high affinity inhibitors of particular amino acid transport systems, which might help in delineating this problem, the alternative vesicle fraction is enriched in a 125-kDa peptide. It has not yet been established whether this peptide is part of the transport system for neutral amino acids.

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Reconstitution of Amino Acid Transport

removed and dialyzed overnight against 100 volumes of 100 mM KCl, 5 mM Tris-HCl, pH 7.4, 0.1 mM MgCl₂, 0.1 mM CaCl₂ (K⁺ buffer) containing 0.25% cholic acid (w/v) and 1 mM phenylmethylsulfonyl fluoride. The dialyzed material, referred to as the solubilized membrane suspension, was stored at 4°C until used for reconstitution.

Reconstitution Procedure—Reconstitution was carried out in different buffers similar to the K⁺ buffer described above except that 100 mM KCl was replaced by the same concentration of Na⁺ ions. Different buffers which were similar to the K⁺ buffer described above, except that 100 mM KCl was replaced by the same concentration of Na⁺, were used. The asolectin suspension (250 µl containing 10 mg of asolectin) was mixed with 2 ml of the solubilized membrane suspension (approximately 15 mg of protein) and applied to a Sephadex G-50 column pre-equilibrated in the buffer that had been used to suspend the asolectin. Turbid fractions eluting in the void volume were collected, pooled, frozen in ethanol/dry ice and allowed to thaw at 22°C. The liposomes were collected by centrifugation at 40,000 x g (20 min) and suspended in 400-500 µl of K⁺ buffer. The liposome suspension was incubated for at least 40 min at 22°C before measurement of amino acid uptake.

In a previous report and in one experiment here (Fig. 1), solubilized plasma membranes were also dialyzed against Na⁺ buffer containing 0.25% cholic acid to prepare Na⁺ liposomes. This step is unnecessary since liposomes with the properties characteristic of reconstitution in Na⁺ buffer (5) are obtained from solubilized membranes dialyzed against K⁺ buffer, if the asolectin and the G-50 column used for reconstitution are both in Na⁺ buffer. Reconstitution in the other buffers was carried out in a similar manner. Both the asolectin suspension and the G-50 column were equilibrated in the buffer under study. After preparation, the liposomes were collected on filtration and extracted with 1 ml of 3:1 chloroform/methanol.

Ficoll Density Gradient Centrifugation—Liposome suspensions (800–900 µl) after one or two freeze-thaw cycles, were layered on Ficoll density gradients consisting of 1 ml of 10% Ficoll, 5 ml of 7% Ficoll, and 5 ml of 5% Ficoll. All Ficoll solutions (w/v) were made up in K⁺ buffer, pH 7.4. The gradients were centrifuged at 30,000 x g at 4°C for 30 min. Following centrifugation, three distinct liposome layers were generally visible: (a) at the interface of the load and the 5% Ficoll layer, (b) at the interface of the 5 and 7% Ficoll layers, and (c) at the 7 and 10% Ficoll interface. They are referred to as the top, middle, and bottom fractions, respectively. The top fraction, containing 2 ml of K⁺ buffer (4°C), was added, and the liposomes in each layer collected by centrifugation at 40,000 x g for 20 min. The pellets were suspended in small volumes of K⁺ buffer and amino acid transport measured as described below.

Measurement of Na⁺-dependent Amino Acid Uptake—Amino acid transport activity was measured as described previously (5). In brief, Na⁺-gradient-stimulated transport activity was measured by dilution of 50-µl aliquots of the liposome suspensions into 250 µl of Na⁺ buffer containing 0.1 mM [³¹CJ]AIB (1.2 x 10⁶ dpm/nmol). Samples of 50 µl were removed at timed intervals, diluted into 2 ml of Na⁺ buffer (4°C) containing 0.1 mM unlabeled AIB, and rapidly filtered through a Whatman GF/B glass-fiber filter under vacuum. The filter was washed with 10 µl of Na⁺ buffer (4°C), dried, and the radioactivity measured. Uptake in the absence of a Na⁺ gradient was measured by dilution of 50-µl aliquots of the liposome suspension into 250 µl of K⁺ buffer containing 0.1 mM [³¹CJ]AIB. Samples were removed and treated as described above.

Non-specific binding of [³¹CJ]AIB to the liposomes was determined by addition of aliquots of the liposome suspension to 2 ml of Na⁺ buffer (4°C) containing 0.1 mM AIB and [³¹CJ]AIB followed by filtration and processing as described above.

Measurement of 3-O-Methyl-D-glucose Uptake—Liposomes were prepared in K⁺ and Na⁺ buffers (described above) and suspended in K⁺ buffer, and uptake was measured by dilution of 50-µl aliquots of the suspensions into 250 µl of K⁺ buffer containing 0.1 mM ³¹CJ methyl-D-glucose (3.7 x 10⁶ dpm/nmol) at 22°C. Aliquots of 50 µl were removed at timed intervals, diluted into 2 ml of K⁺ buffer containing 5 mM MgCl₂, and filtered.

Non-specific binding was determined by addition of liposomes to 2 ml of K⁺ buffer containing 3-³¹CJ methyl-D-glucose and 5 mM MgCl₂ at 4°C. The material was filtered immediately and processed in the usual manner.

Most of the data are expressed as pmol or nmol of solute/mg of protein. However, when AIB and 3-O-methyl-D-glucose uptake are directly compared, results are expressed as pmol of solute/mg of protein (5, 14), to provide a direct comparison of the uptake of the two solutes in terms of equivalent volumes of medium cleared of solute.

All the experiments have been repeated at least three times and the results shown are from typical experiments. Results from different experiments, usually carried out with different membrane preparations, showed some quantitative variations in terms of the degree of amino acid transport. However, qualitatively, the results were identical when different experiments were compared.

Assay of Vesicle Fusion—50 nmol of NBD-PE was solubilized in 50 µl of 2.5% cholate, 4 µl were added to a 1-ml aliquot of solubilized membrane material containing 0.7–0.8 mg of protein. After dialysis, as described above, the extract was combined with 5 mg of sonicated asolectin vesicles, made up in K⁺ or Na⁺ buffer, one-fifth of which were labeled with 50 µg of Rhö-PE. The rest of the reconstitution process was carried out as described above in K⁺ or Na⁺ buffer. Aliquots of the reaction mixtures were taken at various stages, and their fluorescence was measured on a Perkin-Elmer LS-5 spectrophotometer. The amount of Rhö-PE fluorescence was determined with excitation and emission wavelengths of 470 and 637 nm, respectively, and slitwidths of 5 nm, while wavelengths of 525 and 596 nm were used for Rhö-PE. The efficiency of resonance energy transfer from NBD-PE to Rhö-PE in the vesicles before Triton disruption was calculated as (1 – 0.06) (NBD-PE fluorescence before Triton X-100/NBD-PE fluorescence after Triton X-100), where the factor of 0.06 corrects for a small effect of the detergent on the NBD-PE fluorescence in the absence of Rhö-PE. The maximum possible quenching (corresponding to complete intermixing of the fluorescent probes between vesicles) was determined by repeating the above measurements after freeze-thawing the samples an additional 2–4 times in K⁺ buffer, or after extracting and drying the total lipids (16) and redispersing them in buffer by bath-soxation. Equivalent maxima were obtained by the two methods.

Electron Microscopy—Liposomes were fractionated on Ficoll gradients, and the layers were removed, diluted with K⁺ buffer (4°C), and centrifuged at 40,000 x g for 20 min. The liposome pellets were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M Na⁺ cacodylate buffer, pH 7.3, washed twice in 0.1 M Na⁺ cacodylate buffer containing 6% sucrose, and fixed again in 1% OsO₄ in 0.1 M Na⁺ cacodylate buffer, pH 7.3. Samples were stained with 2% uranyl acetate, embedded in epoxy resin, sectioned to 700–800 A, and stained with 2% uranyl acetate and Reynold’s lead citrate. Sections were viewed in a Philips 500 Electron Microscope.

SDS Gel Electrophoresis—Gradient polyacrylamide gels (5–15%) were run according to Laemmli (17). Samples were boiled for 5 min in 3% SDS (w/v), 0.062 M Tris-HCl, pH 6.8, 5% β-mercaptoethanol (v/v), 10% glycerol (v/v), 0.3% bromphenol blue (v/v). Conventional staining and destaining methods were used. Standards for SDS gels were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin with molecular weights of 97,400, 67,000, 43,000, 30,000, 20,100, and 14,400, respectively.

Protein and Phospholipid Determination—Protein concentration was determined by a modified Lowry procedure (18, 19) using bovine serum albumin as standard protein. Phospholipid was measured as lipid phosphorus according to Lowry and Tinsley (20), extending the acid digestion time to ≥1 h to ensure quantitative conversion of lipid phosphorus to inorganic phosphate.

RESULTS

Previous studies from this laboratory (5, 6) have shown that Na⁺-dependent amino acid transport occurs in liposomes reconstituted from solubilized membranes in the presence of K⁺ but not in Na⁺. Preliminary evidence indicated that Na⁺ liposomes were smaller than K⁺ liposomes (5, 6). To address more fully whether the total volume available in the two populations of liposomes is different, two independent methods of measuring vesicle volume were used: [³¹CJ]AIB and 3-
steady state is reached rapidly and little further uptake is observed after 5 min of incubation. Significantly, liposomes prepared in K\textsuperscript{+} buffer show a larger steady state uptake than those prepared in Na\textsuperscript{+}, equivalent to intravesicular volumes of 3.85 and 2.5 \( \mu \)l of solute/mg of protein, respectively, after a 30-min incubation (Fig. 1). The accumulation of \( [\text{\textsuperscript{14}C}] \text{AIB} \) is considerably slower than that of 3-O-methylglucose and, at 30 min, the system has not yet achieved the final steady state. It is evident, however, that K\textsuperscript{+} liposomes after 30 min show a larger content of \( [\text{\textsuperscript{14}C}] \text{AIB} \), equivalent to 2.4 and 1.6 \( \mu \)l of solute/mg of protein for liposomes prepared in K\textsuperscript{+} and Na\textsuperscript{+} buffer, respectively. With both solutes, Na\textsuperscript{+} liposomes show an average available volume 34\% less than the K\textsuperscript{+} liposomes.

A difference of 34\% in the average vesicle volume cannot account for the difference seen in amino acid transport in Na\textsuperscript{+} and K\textsuperscript{+} liposomes. Therefore, additional factors must be involved such as inactivation of the transport proteins in the Na\textsuperscript{+} buffer or the presence of a population of transport-competent vesicles in K\textsuperscript{+} buffer, which are absent in Na\textsuperscript{+} buffer.

The average size of liposomes may be increased by additional freeze-thaw cycles, a process known to cause fusion of lipid and proteolipid vesicles (7-9). If the Na\textsuperscript{+} liposomes contain a denatured carrier, freeze-thawing will not restore activity. If, however, a population of vesicles is formed in K\textsuperscript{+} which is not formed in Na\textsuperscript{+}, an additional freeze-thaw cycle may restore activity to the Na\textsuperscript{+} liposomes. The results in Figs. 2 and 3 compare the effects of a second freeze-thaw cycle on K\textsuperscript{+} liposomes and Na\textsuperscript{+} liposomes, respectively. After the first freeze-thaw cycle, K\textsuperscript{+} liposomes (Fig. 2A) show Na\textsuperscript{+}-dependent amino acid transport. In the presence of Na\textsuperscript{+}, a steady state is reached at approximately 10 min at 22 °C. The initial rate of Na\textsuperscript{+}-dependent amino acid uptake can be estimated at 127 pmol/min/mg of protein with an accumulation at steady state ~7\% that of the medium. K\textsuperscript{+} liposomes freeze-thawed a second time show enlargement of the available intravesicular space, as indicated by a 2-fold increase in AIB uptake in K\textsuperscript{+} buffer (Fig. 2B). The Na\textsuperscript{+}-dependent uptake in these liposomes proceeds linearly for a longer period (presumably because there is a larger intravesicular volume), and steady state has not been achieved in 10 min. The initial rate of Na\textsuperscript{+}-dependent uptake, estimated from the difference between uptake in Na\textsuperscript{+} and uptake in K\textsuperscript{+} at 2 min, is 175 pmol/min/
mg of protein. The results shown in Fig. 2 are from a typical experiment. However, the effect of a second freeze-thaw on the initial rate of Na+-dependent uptake in K⁺ liposomes is variable, ranging from little or no effect (~10%) to an enhancement of up to 7-fold. This variability may be due to the relative extents of enlargement of the liposomes. The parallel experiment with Na⁺ liposomes is shown in Fig. 3, A and B. Na⁺ liposomes, after a single freeze-thaw, show no Na⁺-dependent amino acid uptake (Fig. 3A). Following a second freeze-thaw in K⁺ buffer, Na⁺-dependent amino acid transport reappears (Fig. 3B). Thus, the amino acid transport system is viable but unexpressed in Na⁺ liposomes after a single freeze-thaw step. After a second freeze-thaw in K⁺ buffer, the volume of Na⁺ liposomes increases (Fig. 3C). The parallel experiment with Na⁺ liposomes is shown in Fig. 3A, B and C. The initial rate of Na⁺-dependent uptake in Na⁺ liposomes varies, ranging from little or no effect (<10%) to an enhancement of up to 7-fold. This variability is due to the relative extents of enlargement of the liposomes. The parallel experiment with Na⁺ liposomes is shown in Fig. 3, A and B. Na⁺ liposomes, after a single freeze-thaw, show no Na⁺-dependent amino acid uptake (Fig. 3A). Following a second freeze-thaw in K⁺ buffer, high levels of Na⁺-dependent transport are recovered in Na⁺, Li⁺, and choline liposomes, but not in imidazole liposomes. In no instance, however, is the activity restored to the level seen in K⁺ liposomes (Table I). Only liposomes prepared in mannitol buffer show an appreciable Na⁺-dependent uptake (>30% of control) after the first freeze-thaw cycle. Liposomes reconstituted in Na⁺ have the smallest apparent volume and lowest transport. In all cases, restoration of Na⁺-dependent amino acid transport is accompanied by an increase in the size of the average intravesicular space (Table I). The one exception is that with imidazole liposomes, where expansion of the intravesicular space at the second freeze-thaw is not accompanied by a large increase in amino acid uptake.

Reincorporation of Proteins into Liposomes in the Presence of Various Solutes—The variation in the transport characteristics of the different liposomes do not appear to be due to a cation specific exclusion or partial incorporation of membrane protein components during reconstitution. The results in Fig. 4 clearly show that liposomes made in K⁺, Na⁺, Li⁺, and choline incorporate approximately equal amounts of identical peptides. The same peptides are present in imidazole liposomes, but with imidazole there is less selective reincorporation of proteins into the liposomes for reasons which are at present unknown. The reconstituted liposomes appear to be deficient in peptides with a molecular mass of ~60 kDa, with the exception of a peptide of 125 kDa, which appears to have been specifically incorporated into the liposomes (Fig. 4). Because of the exclusion of certain membrane proteins and the maintenance of high levels of AIB transport in K⁺ liposomes, the reconstitution acts as a “purification” step increasing the specific activity of amino acid transport.

Fig. 4. SDS gel electrophoresis of native, solubilized, and reconstituted membranes. Ehrlich cell plasma membrane was solubilized and reconstituted under varying conditions using procedures described in detail under “Materials and Methods.” Membrane and liposome suspensions (100 μg) were boiled for 5 min with 3% SDS, 5% β-mercaptoethanol and applied to 5–15% gradient polyacrylamide gels. Lane 1, native plasma membranes; Lane 2, solubilized plasma membranes; Lane 3, K⁺ liposomes; Lane 4, Na⁺ liposomes; Lane 5, Li⁺ liposomes; Lane 6, choline liposomes; Lane 7, imidazole liposomes.

Reconstitution of Amino Acid Transport

<table>
<thead>
<tr>
<th>Solute composition of reconstitution medium</th>
<th>AIB uptake in K⁺ and Na⁺ buffer</th>
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</thead>
<tbody>
<tr>
<td>Liposomes 1</td>
<td>Liposomes 2</td>
</tr>
<tr>
<td>Uptake (Na⁺-K⁺)</td>
<td>Net uptake (Na⁺-K⁺)</td>
</tr>
<tr>
<td>K⁺</td>
<td>Na⁺</td>
</tr>
<tr>
<td>Potassium</td>
<td>74</td>
</tr>
<tr>
<td>Sodium</td>
<td>57</td>
</tr>
<tr>
<td>Lithium</td>
<td>72</td>
</tr>
<tr>
<td>Mannitol</td>
<td>95</td>
</tr>
<tr>
<td>Choline</td>
<td>123</td>
</tr>
</tbody>
</table>

*Values in parentheses represent the fractional activity after reconstitution in other cations, based on reconstitution in K⁺ = 100%.

A number of other solutes replacing K⁺ behave in a manner akin to Na⁺. Liposomes prepared in Li⁺, choline, or imidazole show little or no Na⁺-dependent amino acid transport (Table 1) after the first freeze-thaw cycle. However, after a second freeze-thaw in K⁺ buffer, high levels of Na⁺-dependent transport are recovered in Na⁺, Li⁺, and choline liposomes, but not in imidazole liposomes. In no instance, however, is the activity restored to the level seen in K⁺ liposomes (Table I). Only liposomes prepared in mannitol buffer show an appreciable Na⁺-dependent uptake (>30% of control) after the first freeze-thaw cycle. Liposomes reconstituted in Na⁺ have the smallest apparent volume and lowest transport. In all cases, restoration of Na⁺-dependent amino acid transport is accompanied by an increase in the size of the average intravesicular space (Table I). The one exception is that with imidazole liposomes, where expansion of the intravesicular space at the second freeze-thaw is not accompanied by a large increase in amino acid uptake.

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Reconstitution of Amino Acid Transport

TABLE II
Distribution of protein and phospholipid following Ficoll density gradient centrifugation of K⁺ and Na⁺ liposomes

K⁺ and Na⁺ liposomes were made by procedures described under "Materials and Methods" incorporating a single freeze-thaw cycle in K⁺ and Na⁺ buffer, respectively. The liposomes were suspended in K⁺ buffer and applied directly to the Ficoll gradient described in Fig. 5. Following centrifugation (SW 40.1, 32,000 × g, 30 min, 4 °C) the vesicle layers were carefully removed, recovered by centrifugation, and suspended in K⁺ buffer.

<table>
<thead>
<tr>
<th>Liposome fraction</th>
<th>K⁺ liposomes</th>
<th>Na⁺ liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein mg</td>
<td>Phospholipid mg</td>
</tr>
<tr>
<td>Total</td>
<td>2.68 (100%)</td>
<td>9.2 (100%)</td>
</tr>
<tr>
<td>Top layer</td>
<td>0.03 (1.2%)</td>
<td>0.22 (2.4%)</td>
</tr>
<tr>
<td>Middle layer</td>
<td>0.02 (0.8%)</td>
<td>0.04 (0.4%)</td>
</tr>
<tr>
<td>Bottom layer</td>
<td>2.19 (82%)</td>
<td>6.76 (74%)</td>
</tr>
<tr>
<td></td>
<td>3.82 (100%)</td>
<td>8.5 (100%)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.036 (0.4%)</td>
</tr>
<tr>
<td></td>
<td>0.036 (0.4%)</td>
<td></td>
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</table>

*Values in parentheses show the fractional amounts of protein and lipid in vesicles after centrifugation based on the initial protein and lipid content of the original proteoliposome fraction = 100%.

Fig. 5. AIB uptake in fractions obtained after Ficoll density gradient centrifugation of K⁺ liposomes. K⁺ liposomes were made by procedures described under "Materials and Methods," incorporating a single freeze-thaw step. The liposomes were suspended in K⁺ buffer and 800 μl applied to a gradient consisting of 1 ml of 10% Ficoll, 5 ml of 7% Ficoll, and 5 ml of 5% Ficoll. The gradient was centrifuged in a SW 40.1 rotor at 32,000 × g for 30 min and the three liposome layers (top, middle, and bottom) carefully removed, diluted with K⁺ buffer, and recovered by centrifugation. The pellets were resuspended in K⁺ buffer and uptake was measured by dilution of 50-μl aliquots of the initial liposomes (A, 168 μg of protein), top layer liposomes (B, 8.2 μg of protein), middle layer liposomes (C, 6 μg of protein) and bottom layer liposomes (D, 126 μg of protein) into K⁺ (●) or Na⁺ (○) buffer containing [³⁵S]AIB as described in Fig. 2. Initial velocity values in Na⁺ media of 195, 1170, 255, and 97 pmol of AIB/2 min/mg of protein can be calculated for total liposomes (A) and top (B), middle (C), and bottom (D) layer fractions, respectively. When K⁺ liposomes are freeze-thawed a second time and fractionated on a similar Ficoll gradient, the initial rates (AIB uptake/2 min/mg of protein) for the same fractions are 341 (A, total liposomes, freeze-thawed twice), 2534 (B, top layer), 487 (C, middle layer), and 496 (D, bottom layer).

**Fig. 5** AIB uptake in fractions obtained after Ficoll density gradient centrifugation of K⁺ liposomes. K⁺ liposomes were made by procedures described under "Materials and Methods," incorporating a single freeze-thaw step. The liposomes were suspended in K⁺ buffer and 800 μl applied to a gradient consisting of 1 ml of 10% Ficoll, 5 ml of 7% Ficoll, and 5 ml of 5% Ficoll. The gradient was centrifuged in a SW 40.1 rotor at 32,000 × g for 30 min and the three liposome layers (top, middle, and bottom) carefully removed, diluted with K⁺ buffer, and recovered by centrifugation. The pellets were resuspended in K⁺ buffer and uptake was measured by dilution of 50-μl aliquots of the initial liposomes (A, 168 μg of protein), top layer liposomes (B, 8.2 μg of protein), middle layer liposomes (C, 6 μg of protein) and bottom layer liposomes (D, 126 μg of protein) into K⁺ (●) or Na⁺ (○) buffer containing [³⁵S]AIB as described in Fig. 2. Initial velocity values in Na⁺ media of 195, 1170, 255, and 97 pmol of AIB/2 min/mg of protein can be calculated for total liposomes (A) and top (B), middle (C), and bottom (D) layer fractions, respectively. When K⁺ liposomes are freeze-thawed a second time and fractionated on a similar Ficoll gradient, the initial rates (AIB uptake/2 min/mg of protein) for the same fractions are 341 (A, total liposomes, freeze-thawed twice), 2534 (B, top layer), 487 (C, middle layer), and 496 (D, bottom layer).

injured by fractionation on Ficoll gradients. Liposomes formed in K⁺, after a single freeze-thaw, are heterogeneous with three distinct populations of vesicle present (Table II). Measurements of amino acid transport capacity in the three liposome layers in Na⁺ and K⁺ provide estimates of carrier-mediated Na⁺-dependent AIB uptake and intravesicular volumes, respectively, in each layer. Comparisons of transport activity (Fig. 5) show that the low density top layer fraction has the highest rate of Na⁺-dependent AIB uptake. Initial rate values of 1170 pmol of AIB/2 min/mg of protein for top layer liposomes compare to values of 195 for total liposomes and 255 and 97 pmol of AIB/2 min/mg of protein for middle and bottom layer liposomes, respectively. The top layer liposomes also have the largest available intravesicular volume. After 10 min, AIB uptake in K⁺ medium (the nonmediated uptake) approaches 24 μl of solute/mg of protein into top layer vesicles. This compares to a value of 1.8 μl for the initial mixed liposomes, prior to gradient fractionation. The large size of the vesicles present in the top layer fraction, an observation confirmed by electron microscopy (see below), probably contributes to their low density, as does their high phospholipid:protein ratio (6.6:1, Table II). Although the top liposome layer makes up only 1.2% of liposomal protein applied to the Ficoll gradient, its high transport activity suggests that this fraction plays a significant role in determining the overall transport capacity of the total liposomes. The distribution of Na⁺ liposomes on the Ficoll gradient is consistent with this conclusion. Inactive liposomes, prepared with a single freeze-thaw in Na⁺, have no low density layer (Table II) and almost all the protein sediments in the high density layer (bottom
somes. Na' liposomes were made by procedures described under "Materials and Methods," incorporating a single freeze-thaw cycle, and finally suspended in K' buffer. The liposome suspension was frozen a second time in ethanol/dry ice, thawed at room temperature, and an aliquot applied to the Ficoll density gradient described in Fig. 5. Following centrifugation and removal of the liposome layers (Fig. 5), [3H]AIB uptake was measured by dilution of 50-μl aliquots of the initial liposomes (102 μg of protein), before (A) and after (B) the second freeze-thaw, top layer liposomes (C, 54 μg of protein), middle layer liposomes (D, 60 μg of protein), and bottom layer liposomes (E, 64 μg of protein) into K' (0) or Na' (O) buffer containing [3H]AIB (0.1 μM). Initial velocity values of 189, 598, 314, and 27 pmol of AIB/2 min/mg of protein in Na' media can be calculated from these data for total double freeze-thawed Na' liposomes (B) and top (C), middle (D), and bottom (E) layers, respectively.

layer). No major changes in either phospholipid:protein ratio or activity are seen after centrifugation. Thus, top and middle layer fractions, the high activity populations in K' liposomes, are absent in Na' liposomes. Activity can be restored to Na' liposomes by a second freeze-thaw in K' (Table I), and when these liposomes are fractionated on a Ficoll density gradient, a pattern characteristic of that obtained for K' liposomes appears. Thus, the activity induced by a second freeze-thaw of Na' liposomes in K' medium is correlated with the formation of active top and middle layer fractions. These fractions sediment to exactly the same positions as in the gradient described for K' liposomes (Table II). In addition, the phospholipid:protein ratios of the three layers are virtually identical to those obtained after fractionation of K' liposomes (Table II).

The data in Fig. 6 summarize the AIB transport characteristics of the various Na' liposome fractions obtained after Ficoll density gradient centrifugation of Na' liposomes freeze-thawed a second time in K' buffer. The top layer again has the largest available volume, based on AIB uptake in Na'-free medium. A comparison of these data (Fig. 6) with those obtained for singly freeze-thawed K' liposomes (Fig. 5) shows some similarities. It is noteworthy that the middle layer vesicles, which are similar in available volume in both Na' and K' liposome preparations, show similar levels of Na'-dependent amino acid uptake, while the most dense layers in both cases are almost devoid of transport activity. However, the top layer liposomes from singly freeze-thawed K' liposomes are larger (more available space) and more active than the corresponding population from doubly freeze-thawed Na' liposomes (1170 and 588 pmol of AIB/2 min/mg of protein for top layer fraction liposomes from single freeze-thawed K' liposomes and double freeze-thawed Na' liposomes, respectively).

When K' liposomes are freeze-thawed a second time and fractionated on Ficoll density gradients, there is a decrease in the fraction of protein sedimenting in the bottom layer and an increase in the fraction of protein recovered in the top and middle layers, compared to the recoveries after a single freeze-thaw cycle. The redistribution of protein in the three fractions after the second freeze-thaw is paralleled by a redistribution of phospholipid, resulting in similar phospholipid:protein ratios after the first and second freeze-thaw cycles. The top layer obtained after double freeze-thawed K' liposomes are fractionated still contains the most active population of vesicles which show an initial rate of 2534 pmol of AIB/2 min/mg of protein, compared to only 1170 pmol of AIB/2 min/mg of protein for the top layer removed after the first freeze-thaw. In addition, while the latter contains only 1.2% of the applied protein, the high activity top layer from double freeze-thawed K' liposomes now contains 18% of the protein. In contrast, the bottom layer in the same gradient contains only 31% of the applied protein compared to 82% in single freeze-thawed K' liposomes (Table II). However, the increasingly heterogeneous nature of K' liposomes, following a second freeze-thaw, is indicated by the appearance of a high density fraction that passes through the 10% Ficoll and pellets at the bottom of the tube. This pelleted material has little or no amino acid transport activity.

SDS gel electrophoresis of the fractions obtained after density gradient centrifugation of double freeze-thawed K' liposomes reveals an apparent enrichment of a 125-kDa peptide in the top layer liposome fraction (Fig. 7). The content of this peptide is less in the middle and bottom layers, and it is almost totally missing in the material sedimenting at the bottom of the tube. The enrichment of a 125-kDa peptide has already been noted after reconstitution of the membrane proteins (Fig. 4). The decrease in the quantity of the 125-kDa peptide in the three vesicle fractions from top to bottom, after density gradient centrifugation, is paralleled by the decrease in Na'-dependent amino acid transport in the respective fractions.

Fluorescence Assay of Vesicle Fusion—These findings suggest that vesicle fusions induced by freeze-thawing are important for the production of highly transport-competent vesicles, and that such fusions occur more readily when the freeze-thaw buffer contains K' than when it contains certain other solutes, such as Na'. To test these possibilities directly, two different fluorescent probes, NBD-PE and Rho-PE, were initially incorporated into the dialyzed membrane extract and into a portion of the asolectin vesicles, respectively. The extent of intermixing of the two probes, measured by the efficiency of resonance energy transfer between probe molecules, was then determined at various stages in the reconstitution process. The external conditions were chosen such that maximum efficiency of energy transfer would be expected only when an average vesicle contains the intermingled components of substantial numbers of the vesicles present in the initial reconstitution mixture.
Lipid mixing during preparation of K+ and Na+ liposomes

Solubilized and dialyzed plasma membrane extracts containing NBD-PE and asolectin prelabeled with Rho-PE were used in the preparation of K+ and Na+ liposomes using Sephadex G-50 chromatography. At the indicated stages of the reconstitution process, the percentage quenching of NBD-PE fluorescence by Rho-PE was determined as described under "Materials and Methods." The quenching values have been scaled to give $Q = 0\%$ in the void volume fractions before freeze-thawing and 100% after complete lipid mixing. The ratio of NBD-PE to Rho-PE in various fractions was determined by fluorescence measurements after dissolution of the samples in Triton X-100 (TX-100) to eliminate energy transfer. The ratios of the probe fluorescence intensities have been scaled to give values of unity for the void volume fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Void volume (freeze-thawed)</th>
<th>Supernatant*</th>
<th>Pellet*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quenching (% max)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K+ liposomes</td>
<td>0</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>Na+ liposomes</td>
<td>0</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>NBD$_{TX-100}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K+ liposomes</td>
<td>1.0</td>
<td>1.03</td>
<td>0.37</td>
</tr>
<tr>
<td>Na+ liposomes</td>
<td>1.0</td>
<td>1.01</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Supernatant and pellet fractions were obtained after centrifugation of the freeze-thawed void volume fractions at 40,000 × g. The liposome pellets were suspended in K+ buffer and resonance energy transfer was measured before (Footnote a) and after (Footnote b) a second freeze-thaw.

While freeze-thawing promotes increased intermixing of components initially present in the dialyzed membrane extract and in the asolectin vesicles, it is clear from the quenching data of Table III that a single freeze-thaw does not produce complete intermixing of the vesicle populations. This point is underscored by the observation that, when the reconstitution mixture is pelleted after a single freeze-thaw, the pelleted material is relatively enriched in NBD-PE and depleted in Rho-PE when compared to the unfractionated sample after the freeze-thaw, while the opposite is true for the supernatant. The liposome pellets may therefore be enriched in endogenous membrane phospholipid.

Electron Microscopy of Reconstituted Vesicles—That the average vesicle size is larger in top layer liposomes after freeze-thawing and gradient separation was confirmed by electron microscopy. The data (Fig. 8) clearly show that the vesicles from the top layer are larger than the liposomes found in the other two layers. Some large liposomes (≥150 nm in diameter) are present in the middle layer, but they are a minority in terms of the total number of vesicles. Only a small number of large liposomes are found in the bottom layer, while both the middle and bottom layers contain large numbers of small vesicles. These data are consistent with the "available space" measurements obtained with [14C]AIB uptake in Na+-free media (Fig. 5). The average diameter of vesicles in the top layer is $270 \pm 110$ nm ($n = 71$). A number of vesicles appear to have a bilamellar structure and this is particularly so in liposomes from the top layer fraction where 70% of the liposomes appear to be bilamellar. Many of the remaining vesicles are multilamellar (more than two membranes). It is unclear whether these apparent multilamellar structures are real or if they represent partial collapse and folding of large vesicles. In addition, it is unclear how a multilamellar structure, if it is a genuine feature of these vesicles, would affect their transport characteristics.

**DISCUSSION**

This study demonstrates the importance of using K+ for the effective reconstitution of amino acid transport by freeze-
thaw techniques. Reconstitution in buffers containing Na⁺, Li⁺, or choline results in the production of liposomes which show little or no Na⁺-dependent amino acid uptake. Reconstitution in mannitol buffer gives a moderate degree of transport, but the activity recovered is less than half that obtained with K⁺. The requirement for K⁺ has been linked to the formation of a population of large lipid-rich vesicles highly active in Na⁺-dependent amino acid transport. With Na⁺ in the medium, such a population of vesicles is not formed. The presence of an active population of vesicles, mixed with less active and inactive vesicles, was shown by gradient centrifugation of the mixed K⁺ liposome population, with the least dense, lipid-rich, layer possessing the highest activity. In addition, this layer had the largest intravesicular space, as measured by AIB uptake in Na⁺-free medium, and the largest average diameter, as measured by electron microscopy. All these data are consistent with the conclusion that, in the absence of K⁺, and particularly in the presence of Na⁺, fusion of the lipid vesicles to provide the active population is inhibited. The effect of ions on fusion was further confirmed by measurement of the resonance energy transfer between NBD-PE and Rho-PE, which permits fusion events to be quantitated (12). These experiments showed that liposomes reconstituted in Na⁺ consistently demonstrated lower levels of lipid mixing between vesicles than did K⁺ liposomes during the first and second freeze-thaw cycles. This inhibition of freeze-thaw-induced vesicle fusion by the presence of Na⁺ has earlier been observed in protein-free lipid vesicles (9, 10, 21). In these studies, as in the present study, both mixing of vesicle lipids (21) and the formation of large lipid vesicles (9) were inhibited by Na⁺ and a number of other agents including Li⁺, glycerol, and sucrose (9, 21). In contrast, K⁺, under certain conditions, was shown to promote vesicle fusion (9, 21). Although vesicle fusion is dependent on the concentration (21) and type (10) of phospholipid used, as well as the concentration of cation present (9, 21), Oku and MacDonald (9) have shown that freeze-thawing of protein-free lipid vesicles in the presence of K⁺, at the concentration used in the present study, results in the formation of a higher number of larger vesicles than occurs with Na⁺.

The mechanism of lipid vesicle fusion and the effect of ionic environment on this process are not clearly understood. It has been suggested (9, 10) that ionic effects on the degree of fusion induced by freeze-thawing are related to the eutectic temperatures of the salt solutions. Oku and MacDonald (9) present compelling evidence that a decrease in the eutectic temperatures of frozen alkali metal chloride solutions roughly parallels the decreasing ability to form large vesicles from these solutions. Probably the most important effect of variable eutectic temperatures would be on the rate and degree of water removal from lipid bilayers, an important prerequisite for vesicle fusion (10).

It is clear from SDS gel electrophoresis of the various liposome populations that the absence of activity in Na⁺, Li⁺, or choline liposomes is not due to a failure to reincorporate proteins into the vesicles, nor is it due to a differential reincorporation of proteins, since the peptide patterns obtained are identical to those for K⁺ liposomes. Reconstitution in imidazole buffer apparently does result in the incorporation of some additional membrane proteins not present with the other ions. Imidazole also appears to have a different effect on reconstitution with respect to transport activity, since a second freeze-thaw of imidazole liposomes only slightly improves Na⁺-dependent uptake.

Liposomes made in mannitol buffer were partially transport-competent, a surprising observation in view of the fact
that some sugars have been reported to inhibit the formation of large, protein-free, lipid vesicles (9, 21), presumably through a cryoprotective action (22). Whether this is a specific effect of mannitol or whether liposomes containing protein behave differently than protein-free lipid vesicles is unknown.

Freeze-thawing of G-50 void volume fractions involves the interaction of membrane protein, endogenous membrane phospholipid, preformed asolectin vesicles, and cations. Im and Spector (23) have reported the successful reconstitution of amino acid transport from Ehrlich cells by G-50 chromatography of solubilized membranes, without the addition of exogenous phospholipid and without a freeze-thaw step. Therefore, in the present study, G-50 chromatography probably induces the formation of some liposomes from endogenous phospholipid which may fuse either with each other or with preformed asolectin vesicles during the freeze-thaw.

There is some evidence from the fluorescence experiments to indicate a preference for the incorporation of endogenous lipid into the isolated reconstituted liposomes. Similarly, liposomes formed by reconstitution from Ehrlich cell plasma membranes by dialysis show a preference for endogenous over exogenous phospholipids (14). The protein insertion and membrane fusion reactions are such that certain proteins are excluded from the reconstituted liposomes recovered at 40,000 X g. This selective reincorporation of proteins during reconstitution has been observed in other systems (24, 25) and it may be looked on as a “purification” step in the identification of the transport protein. The various vesicle fractions obtained in subsequent stages of the reconstitution process exhibit very similar protein profiles, as one would expect if a transport-competent vesicle population. The quantity of this protein parallels the level of Na+-dependent amino acid transport in the various liposome populations, being highest in the top layer, and lowest in the fraction that pellets through 10% Ficoll. However, assignment of transport function to a particular protein species by these procedures must be interpreted with some caution.

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