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GLYCINE TRANSPORT BY MEMBRANE VESICLES FROM PIGEON RED CELLS

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SUMMARY

Small membrane vesicles were prepared from pigeon red blood cells. They took up glycine by a Na^+-dependent process resembling that of the intact cells. Also, the same modest inhibition of glycine uptake by alanine appears in both. Some 2–7% of the vesicle water space retained glycine. Taking into account this low retention space, and also the difference in surface: volume ratio, the calculated Na^+-dependent glycine uptake activity of the membrane vesicles is the same order of magnitude as that of the intact cells. Sedimentation in Ficoll gradients was examined with the aim of separating transport-active from inactive vesicles.

INTRODUCTION

Some properties of vesicular membrane transport systems depend on the size of the transporting body or organelle. To exploit size-dependent properties it is necessary to prepare small vesicles which retain the transport properties of interest.

Small membrane vesicles with transport activity have been prepared from fat cells[1,2] and sarcoplasmic reticulum[3]. Although the standard Kaback[4] membrane vesicles from bacteria are not much smaller than the original cells, their size can be reduced by sonication with retention of transport capacity*. Membrane "fragments" have been prepared from mammalian red cells. These fragments show ATP-dependent Ca^{2+} uptake but it is unclear whether uptake is due to accumulation or binding, and whether the fragments are smaller than whole cells[5]. Fragmentation of mammalian red cell membranes into vesicles and their fraction has been studied by Steck *et al.*[6,7], but transport by these vesicles has not been reported.

A specific active transport system for glycine in pigeon red blood cells has been extensively studied[8–13]. This system shows Michaelis–Menten kinetics with respect to glycine concentration, anion concentration and the square of the Na^+ concentration[8,11–13]. Hemolysed and restored cells can actively transport glycine[9,12].

The present report concerns the preparation of small membrane vesicles from pigeon red cells showing Na^+-dependent glycine uptake, and the description of some of their properties.

* H. R. Kaback, in response to a question from the floor at the 4th Annual Biochemistry–PCRI Winter Symposia, Jan. 10–14 (1972), Miami, Fla.

MATERIALS AND METHODS

Inorganic chemicals, sucrose, glycine and methanol were analytical reagent grade. Glucose was certified ACS grade. Ficoll was from Sigma Chemical Co., St. Louis, Mo., U.S.A. To remove Na^+ from Ficoll, 100-ml portions of 20% (w/v) Ficoll were dialysed against three 2-l portions of deionized water. Toluene was scintillation grade from Fisher Scientific Co., Fair Lawn, N. J., U.S.A. Aquasol, Omnifluor, [^{3}H]glycine and [1-^{14}C]glycine were from New England Nuclear, Boston, Mass., U.S.A. Nylon counting vials were from Amersham-Searle Corp., Arlington Heights, Ill., U.S.A. The solubilizer, BBS-3, was from Beckman Instruments, Inc., Fullerton, Calif., U.S.A.

Pigeon erythrocytes were prepared as described before[8]. For sonication, 8 ml of 25% (w/v) erythrocyte suspension in 154 mM KCl was mixed with an equal volume of solution containing 134 mM KCl, 3.75 mM D-glucose, 8 mM $MgCl_2$ and 4 mM $CaCl_2$. The mixture was sonicated with a 20 KHz Branson Sonifier cell disruptor (Model W185D, Heat Systems-Ultrasonics, Inc., Plainview, L.I., N.Y., U.S.A.) equipped with a microtip at Setting 4 for 25 s. The sample was in an ice–water bath during sonication. 4/5 of the sonicated suspension was centrifuged 30 min at $14500 \times g$. The supernatant was used to prepare a linear density gradient with 0.3 M sucrose. The upper translucent layer of the pellet was gently suspended in a few ml of uncentrifuged sonicated suspension and this mixed with the rest of the uncentrifuged sonicated suspension. This mixture was layered on top of the gradient and centrifuged in a clinical centrifuge 15 min at $1700 \times g$. Intact erythrocytes and nuclei were sedimented and discarded. The supernatant containing membrane fragments was centrifuged 30 min at $14500 \times g$, the sediment washed once with 154 mM KCl and resuspended in a solution containing 139 mM KCl, 4.5 mM KH_2PO_4 and 5.5 mM K_2HPO_4 at approx. 60 mg wet weight per ml. 12 ml of this was sonicated at Setting 4–6 for 60–85 s. Note that the sonic energy delivered per ml of suspension depends on the volume. Fragmented membranes were collected by centrifugation for 30 min at $14500 \times g$. The pellets can be stored overnight at 0 °C before or after annealing (below) without losing activity. For all experiments reported in this paper, if stored fragments were used, they were annealed after storage.

All operations were carried out in the cold.

For DNA, phospholipid and protein analysis, the membrane fragments, the nuclei-containing sediment from the sucrose density gradient and the $14500 \times g$ pellet from whole homogenate were extracted according to Zentgraf *et al.*[14]. Protein was determined by the biuret reaction[15], DNA with diphenylamine[16] and phospholipid according to Gomori[17].

Annealing of membrane fragments

Membrane fragments were incubated at 41 °C ("annealed") 15 min in a solution containing 129 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 5.5 mM K_2HPO_4 and 4.5 mM KH_2PO_4 (pH 6.9). This is later referred to as "standard" phosphate buffer. To assess the capacity to retain glycine which appears during annealing, labelled glycine, typically 0.24 μM [^{3}H]glycine (6.72 Ci/mmole) was added to the annealing solution. This was done for all experiments presented except those of Fig. 5 (and Table IV) and Fig. 6. After annealing, membrane fragments were centrifuged 15

min at 14500 × *g* and washed three times with annealing solution lacking labelled glycine. The ratio cpm per g wet weight: cpm per ml annealing medium of the isotope present in the annealing medium measures the fraction of the pellet water containing trapped glycine. We will later refer to this ratio as the value for glycine "trapped" or as the fraction of the fragments "sealed" to glycine depending on context.

Electron microscopy

Membrane fragment pellets were fixed overnight with 1% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7), washed with the same buffer, and fixed again for 2 h in 1% OsO_4 in the same buffer. After washing and dehydrating in a graded series of ethanol and propylene oxide mixtures, the samples were infiltrated with epon and polymerized. Silver sections were double stained with uranyl acetate and lead citrate. Sections were examined with a RCA EMU 3D modified electron microscope. The infiltration and subsequent steps were done for us by Dr K. W. Lee.

Na^+-dependent glycine uptake

Except where otherwise indicated (*e.g.*, Table II, 0 °C "annealed" fragments), annealed fragments were always used. Pairs of tubes with the equal amounts of membrane fragments, generally 50 mg each, were incubated; one of the pair had Na^+-rich medium, the other had a medium where K^+ replaced Na^+. The incubation media contained 137 mM KCl or NaCl, 3 mM KH_2PO_4 or NaH_2PO_4, 6 mM K_2HPO_4 or Na_2HPO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and labelled glycine. Typically 0.56 mM [1-^{14}C]glycine (6.26 Ci/mole) was used. Each membrane fragment pellet was suspended in 0.50 ml medium and incubated. Incubation was stopped by addition of 9.5 ml ice-cold 154 mM KCl and prompt centrifugation for 15 min at 14500 × *g*. If pellets of much less than 50 mg were used, the insides of the tubes were wiped. The pellets were weighed, extracted and aliquots of the extracts counted.

Extraction, counting and calculations

Weighed pellets were extracted with 1.00 ml methanol containing 25 mg/l unlabelled glycine. Aliquots of extract, generally 0.10 ml, were counted either in a Geiger counter (Nuclear Chicago) or a liquid scintillation counter (Ansitron division of Picker Nuclear). For scintillation counting, 0.10 ml extract was added to 15 ml of a counting mixture of 4 g Omnifluor and 20 ml BBS-3 diluted to 1 l with toluene.

For Ficoll density gradient fractions, 0.10 ml samples were mixed with 1.20 ml water and 13.7 ml Aquasol.

In all double-label experiments spillover corrections were made. Quench corrections were not necessary.

Na^+-dependent uptake was calculated as the difference between uptake by pellets incubated in Na^+ and K^+ media, divided by the wet weight of the pellet. Pellets are mostly water (Table I). For some calculations, corrections for "sealing" were made by dividing uptake/g pellet by the K^+ pellet value for fraction "sealed" to glycine. For short uptake incubations the loss of ^{3}H present at the start of incubation was too small to significantly affect our conclusions from these calculations. For a 5-min incubation at 40 °C, ^{3}H loss was 22.3 ± 5.1% (± S.E., $n = 4$).

Fractionation of membrane fragments by density gradient centrifugation

Linear density gradients were made with standard phosphate buffer solution and Ficoll solution in standard buffer. In some experiments the gradients were made hypertonic by the addition of sucrose. 1–4.5 ml of membrane fragment suspension was layered on the gradient and centrifuged in a S.W. 25.1 rotor, Spinco L-2 for 30 min at $45000 \times g$. Fractions of 6 drops were collected from the bottom of the tube. Aliquots of 0.10 ml were withdrawn and added to 4 ml 154 mM KCl and read at 600 nm. This wavelength is the absorbance minimum of hemoglobin. Absorbance is thus primarily due to light scattering. In some experiments (*e.g.* Fig. 5), the fractions were then pooled according to the light absorbance profile. 3 vol. of 154 mM KCl were added to the pooled fractions and the diluted suspensions centrifuged 30 min at $14500 \times g$. The pellets were then assayed for Na^+-dependent glycine transport activity as described above.

In another set of experiments (*e.g.* Fig. 6) the membrane fragments were annealed in the absence of labelled glycine and one half was incubated with [^{3}H]-glycine and the other half with [^{14}C]glycine. The suspensions were mixed and applied directly to the Ficoll density gradients. Fractions were collected and assayed for absorbance at 600 nm and ^{3}H and ^{14}C radioactivities. In these experiments the gradient fractions were not diluted or centrifuged but were mixed with Aquasol and water and counted.

RESULTS

Morphology of membrane fragments

Low intensity sonication was used in the early step of membrane fragment preparation. Inspection with a phase contrast microscope showed that there were many membrane fragments still retaining phase contrast as well as intact nuclei and a few ghosts. After separating the membrane fragments from the intact nuclei by centrifuging in the sucrose density gradient, a second sonication of higher intensity was used. The membranes were broken into tiny pink spheres, many of them beyond the resolution of light microscope. In the presence of divalent cations crumpling of particles appeared. From electron microscope examination, both annealed and un-annealed membrane fragments appeared to be mostly vesicles (Fig. 1) and will be so called from here on. The annealed vesicles seemed more irregular in shape. Their size varied from 0.5–0.02 μm. Intact cells are approx. 8–16 μm in diameter.

Chemical compositions of membrane vesicles

Table I shows the protein, phospholipid and DNA contents of the membrane vesicle preparation, the $14500 \times g$ pellet from the original homogenate and the nuclei-containing pellet from the sucrose gradient. The membrane vesicle preparation is depleted in protein and much depleted in DNA compared to the homogenate pellet. The membrane protein: phospholipid ratio is 1.86, similar to that reported for fragmented mammalian red cell membranes, 1.64[18], though less than that reported for hen erythrocyte membranes, 3.0[14].

Annealing of membrane vesicles

Table II shows that annealing greatly increased the trapping by the membrane

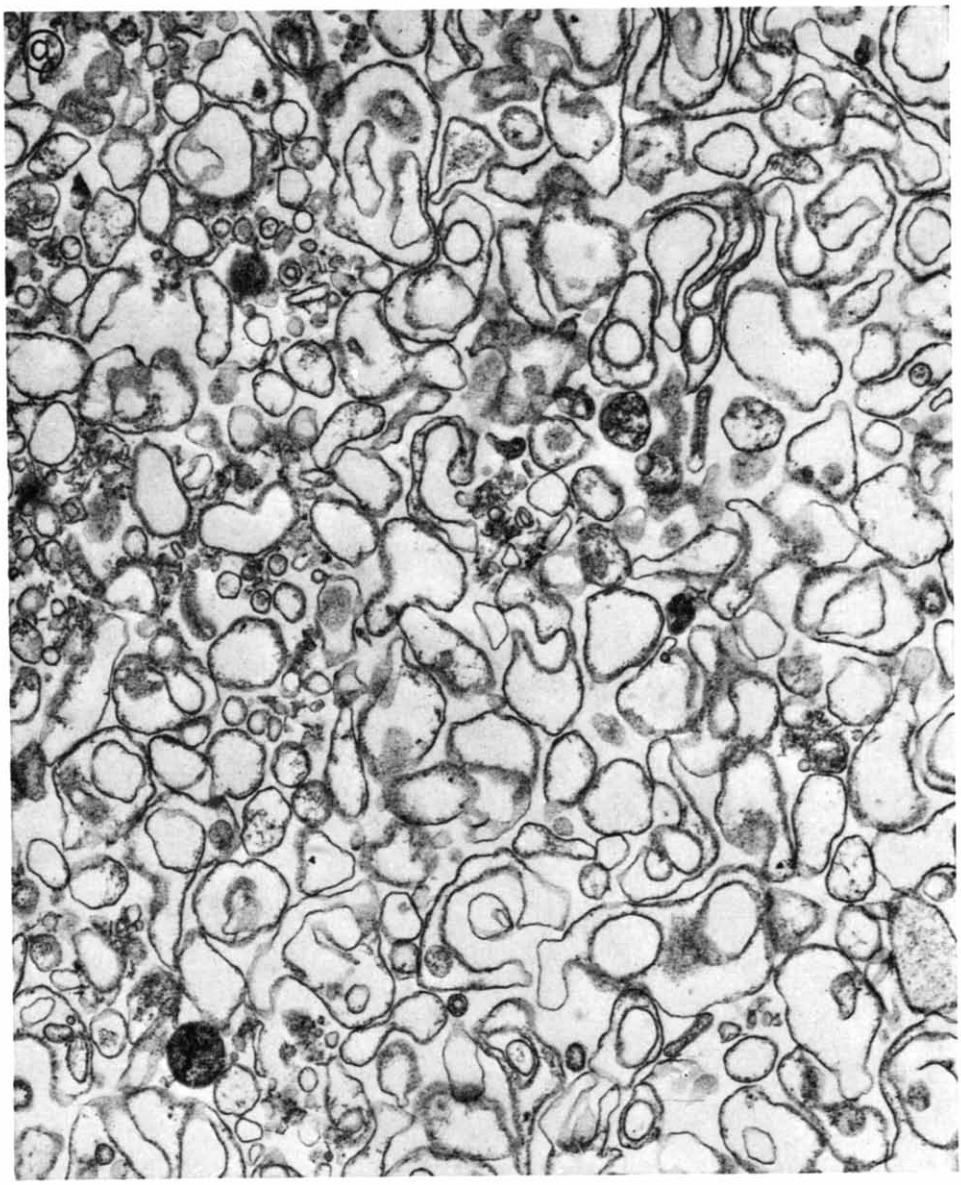

Fig. 1a.

vesicles of glycine from the annealing medium. It also shows that Na^+-dependent glycine uptake depends strongly on prior annealing. When unannealed membrane fragments were incubated with glycine, the amount of Na^+-dependent glycine uptake was approx. 32% of the annealed control fragments as shown by the results in Expts 6, 7 and 8. However, the uptake activity of unannealed vesicles was higher than we expected from values for trapped glycine. This observation suggests that a small amount of sealing of membrane fragments may have occurred during the processing

Fig. 1. Electron micrographs of membrane fragments after sonication at Setting 4 for 1 min. (a) Unannealed membrane fragments. Magnification 27000×. (b) After annealing at 41 °C for 15 min. Magnification 27000×.

steps prior to annealing at elevated temperature. Introduction of [^{3}H]glycine during sonication did not lead to much increase of the amount of glycine trapped.

When divalent cations were present in the sonication mixture, the trapping of glycine was decreased. EDTA in the annealing medium abolished the ability of the vesicles to trap glycine.

TABLE I

CHEMICAL COMPOSITION OF MEMBRANE FRAGMENTS

The cell homogenate pellet was taken after the first sonication of red blood cells at Setting 4 for 25 s. The nuclei fraction was taken from the sediment and membrane fragments from the supernatant of the sucrose density gradients. All samples were centrifuged, washed and extracted according to Zentgraf *et al.*[14]. Results are the average of two determinations.

Sample	*mg per g wet weight*			
	Protein	*Phospholipid*	*DNA*	*Phospholipid/ DNA*
Cell homogenate pellet	79.5	0.83	6.54	0.127
Nuclear fraction	12.4	0.57	9.07	0.063
Membrane fragments	3.5	1.88	0.49	3.84

Trapping of glycine upon annealing was 2–7%. In one experiment, trapping was 5% after 15 min annealing and increased only to 7% after 90 min. Therefore, a 15-min annealing time was employed for all later experiments.

Na^+-dependent glycine uptake by membrane vesicles

When annealed membrane vesicles were incubated with glycine at 30 °C in a Na^+-rich medium they took up considerable glycine in 5 min (Table II). There was little or no glycine uptake from a medium where K^+ replaced Na^+. The amount of glycine in a pellet incubated in K^+ medium is equal to that in an equal volume of the 20-fold diluted incubation medium from which it was sedimented. No Na^+-dependent glycine uptake was observed at 0 °C.

Fig. 2 shows the time course of Na^+-dependent uptake at 30 °C and 40 °C as well as the temperature dependence with a 5-min incubation time. Activation energy values calculated from Arrhenius plots of two such experiments were 24 and 22 kcal/mole. Activation energy values of 23.2[19] and (for V) 15.5[20] kcal/mole have been reported for glycine transport by Ehrlich ascites cells.

Table III shows that alanine in the incubation solution inhibits Na^+-dependent glycine uptake by about 17%. This is very similar to the inhibition observed with intact cells[10].

Fig. 3 shows a double reciprocal plot of Na^+-dependent glycine uptake against glycine concentration. Assuming linearity, the value for V is approx. 0.9 moles/3 min per ml "sealed volume". The K_m value obtained was 0.2 mM. This value is probably within a factor of 2 of the true value and is consistent with the K_m value for intact cells of 0.24 mM at the same Na^+ concentration[8]. Fig. 4 shows a double reciprocal plot of Na^+-dependent glycine uptake against the square of the Na^+ concentration. Assuming linearity, the slope: intercept ratio corresponds to that for intact cells[8]; the line shown in Fig. 4 is that for intact cells. As with intact cells[8], the intercept in Fig. 4 is that calculated from the K_m and V of Fig. 3 and the relationship

$$1/\text{intercept} = \{1/V\}\,\{(K_2/[\text{glycine}]) + 1\}$$

with $K_2 \approx K_m$ at high Na^+ concentration. Although these data show more scatter

TABLE II

ANNEALING OF MEMBRANE FRAGMENTS AND Na^+-DEPENDENT TRANSPORT OF GLYCINE

Membrane fragments were sonicated for the second time at Setting 4 for 1 min in 12 ml solution containing 139 mM KCl, 5.5 mM K_2HPO_4, 4.5 mM KH_2PO_4 (pH 6.9). When divalent cations were present, a solution containing 129 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 5.5 mM K_2HPO_4 and 4.5 mM KH_2PO_4 was used. After centrifuging at $14500 \times g$ for 30 min, the pellet was resuspended in the latter solution with 0.24 μM [^{3}H]glycine (6.72 Ci/mmole). This membrane fragment suspension was annealed, centrifuged, washed and incubated with [^{14}C]glycine for 5 min as described in Methods. In the column headed "% Glycine Trapped" the ratio is listed of labelled glycine from the annealing medium associated with the pellets (in cpm/g wet weight) to the labelled glycine in the annealing medium (in cpm/ml), times 100. Where annealing temperature is given as 0 °C, the 15-min exposure to high temperature was replaced by a 15-min exposure to 0 °C with no other change in procedure. In the columns designated "Na^+", "K^+" and "$Na^+ - K^+$" are listed, respectively, the μmoles of glycine/g wet weight of vesicles incubated in Na^+ medium (152 mM Na^+), K^+ medium (Na^+-free) and the difference between them.

Temperature (°C)		*Expt No.*	*μmoles glycine uptake per g wet weight*			
Annealing	*Incubation*		*% Glycine Trapped*	*Na^+*	*K^+*	*$Na^+ - K^+$*
0	0	1	0.008	0.025	0.027	−0.002
		2	0.000	—	—	—
		3	0.780	0.031	0.029	0.002
		Mean	0.25 ± 0.23	0.028	0.028	0
41	0	4	4.51	—	—	—
		5	6.23	0.026	0.024	0.002
		6	6.70	0.022	0.023	−0.001
		Mean	5.8 ± 0.9	0.024	0.023	0.001
41	0	4	2.61	—	—	—
(Mg^{2+} and Ca^{2+} present		1	2.40	—	—	—
during sonication)		2	2.54	—	—	—
		Mean	2.52 ± 0.06			
41	30	5	6.23	0.059	0.027	0.032
		6	6.70	0.065	0.029	0.036
		Mean	6.47	0.062	0.028	0.034
0	30	6	0.52	0.058	0.041	0.017
41	40	7	9.80	0.057	0.020	0.037
0	40	7	0.00	0.036	0.027	0.009
41	40	8*	2.7	0.215	0.041	0.174
0	40	8*	1.2	0.092	0.048	0.044

* In Expt 8, a modified method of membrane preparation was employed. A linear sucrose density gradient was made with 0.3 M sucrose and standard phosphate buffer. A 37.5% (w/v) erythrocyte suspension was sonicated and layered on top of the gradient. After separating from the nuclei and intact erythrocytes the membrane fragments were sonicated again at Setting 5 for 1.5 min. The membrane fragments were harvested by centrifugation and washed once with 25 ml/g wet weight solution containing 141 mM KCl, 2 mM EGTA, 1 mM $CaCl_2$, 5.5 mM K_2HPO_4 and 4.5 mM KH_2PO_4. The pellet was resuspended in the same buffer solution for annealing. Incubation time was 20 min.

than we would like, uptake by the vesicles and by intact cells appear to obey a similar kinetic equation

$$1/v = \{1/V\}\ \{(K_1/[Na^+]^2 + K_2)\ (1/[\text{glycine}]) + 1\}$$

with similar values for the kinetic parameters K_1 and K_2.

Fractionation of membrane vesicles on Ficoll density gradient

The profile of the membrane vesicles on a Ficoll density gradient is shown in Fig. 5. One major and three minor peaks were observed. When fractions were pooled and assayed, most of the Na^+-dependent glycine uptake activity was found in the main peak (Table IV). In another set of experiments one half of the membrane vesicles were incubated in 0.56 mM [^{14}C]glycine and the other half in 0.056 mM [^{3}H]glycine. Both incubations were in 68 mM Na^+ media. The two vesicle suspensions were combined and layered on a Ficoll density gradient. The results in Fig. 6 agree with previous ones in that most of the glycine uptake activity is in the main peak. Apparently Peak C represents a conglomerate of vesicles with different activities. More

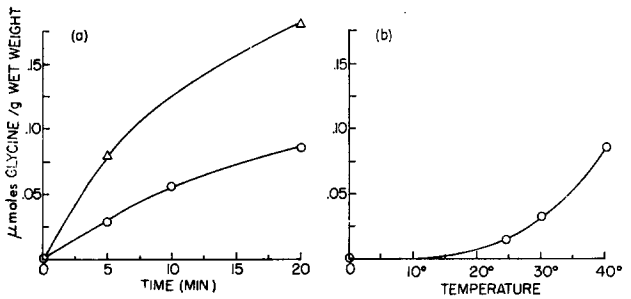

Fig. 2. Na^+-dependent glycine transport with varied time and temperature. The second sonication was at Setting 4 for 1 min. (a) Incubation at 40 °C (△) and 30 °C (○) for varied time. 0.56 mM [1-^{14}C]glycine (11.5 Ci/mole) and 136 mM Na^+ were used. (b) Incubation for 5 min at varied temperature.

TABLE III

Na^+-DEPENDENT GLYCINE TRANSPORT IN THE PRESENCE OF ALANINE

Membrane fragments were sonicated for the second time at Setting 6 for 75 s. Annealing and incubation were as described under Methods. [1-^{14}C]glycine, 0.14–0.17 mM (11.5 Ci/mole) was used. Time of incubation was 3 min.

Expt No.	*Glycine (mM)*	*Alanine (mM)*	*μmoles glycine taken up per g wet weight*				*% Inhibition*
			% Glycine "sealing"	*Na^+*	*K^+*	*$Na^+ - K^+$*	
1	0.14	2.0	2.9	0.173	0.064	0.109	16
	0.14	0		0.194	0.064	0.130	
2	0.17	2.0	1.8	0.156	0.077	0.079	18
	0.17	0		0.173	0.077	0.096	

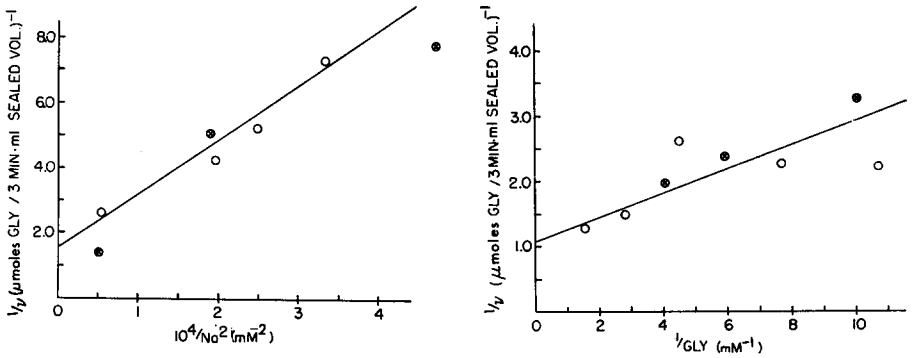

Fig. 3. Double reciprocal plot of Na^+-dependent glycine uptake against glycine concentration. The second sonication was done at Setting 6 for 75 s. Incubation time was 3 min with 136 mM Na^+. Different symbols show data from two experiments. Glycine spec. act. was 11.5 Ci/mole.

Fig. 4. Effect of Na^+ concentration on Na^+-dependent glycine uptake. The second sonication was done at Setting 6 for 75 s. Incubation time was 3 min and 0.27 mM glycine was used. Different symbols are used for data from different experiments. The straight line is that for intact cells[8].

than one [^{14}C]glycine peak appears under Peak C in Fig. 6. The first major one coincides with the [^{3}H]glycine and the absorbance peaks. A secondary [^{14}C]glycine activity peak is seen trailing the main absorbance peak. This and two other such experiments suggest that the trailing minor [^{3}H]glycine peak may precede the corresponding ^{14}C peak but the cpm values in this region were small and this feature has been difficult to reproduce.

DISCUSSION

Electron micrographs of intact avian erythrocytes do not clearly reveal any organelles except the nuclei. This has been ascribed to interference by hemoglobin. Turkey erythrocyte ghosts show a few mitochondria, but no endoplasmic reticulum or ribosomes[21]. However, the lysis conditions used[21] correspond to those used to liberate free ribosomes from mammalian reticulocytes[22]. Therefore, lysis may remove

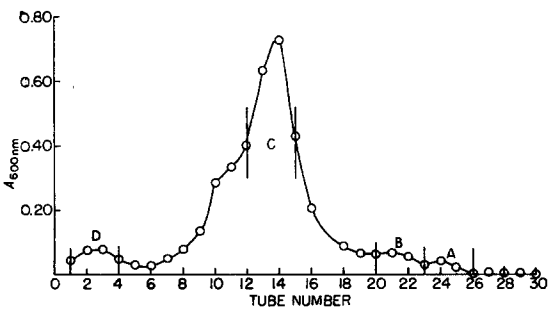

Fig. 5. Fractionation of membrane fragments on Ficoll density gradient. Linear density gradients were made from 0 and 24% Ficoll solutions in standard phosphate buffer. Membrane fragments were sonicated at Setting 4 for 1 min and annealed. 4.5 ml of this membrane preparation were layered on the gradient.

TABLE IV

Na^+-DEPENDENT GLYCINE UPTAKE OF FRACTIONS FROM FICOLL DENSITY GRADIENT

Fractions were pooled as shown in Fig. 5. A, Tubes 24–27; B, 20–23; C, 12–15; D, 1–4. Membranes were harvested by centrifugation and incubated in 0.56 mM glycine, 136 mM Na^+ for 20 min.

Fraction	*Expt No.*	*moles glycine* $(Na^+ - K^+)$/ *g wet weight*	*μmoles glycine* $(Na^+ - K^+)$/ *g phospholipid*
A	1	0.0116	29.3
	2	0.0000	0
B	1	0.0017	2.5
	2	0.0070	18.3
C	1	0.1944	74.6
	2	0.1884	74.0
D	1	0.0028	6.1
	2	0.0014	4.0

the ribosomes from these ghosts. Avian red cells do carry out a slow synthesis of hemoglobin.

Avian erythrocytes consume oxygen, although at much lower rate (1.3 mm^3/ml per h)[23] than avian liver and kidney slices (1700–2000 mm^3/ml per h)[24]. Avian erythrocytes also contain tricarboxylic acid cycle enzymes[25].

From the activity for malate dehydrogenase reported[25] for chicken red cells, and a value of 0.33 g dry weight/g wet weight[8] for pigeon red cells, we calculate

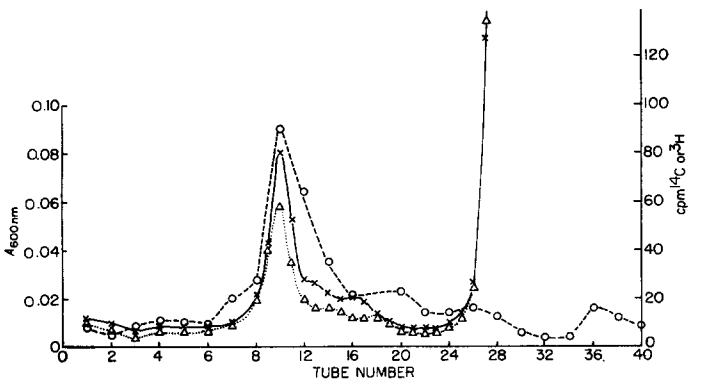

Fig. 6. Fractionation of membrane fragments on Ficoll density gradient. Linear density gradient was prepared from 0 and 17.5% Ficoll solutions in standard phosphate buffer made 7% hypertonic with sucrose. Membrane fragments were sonicated at Setting 6 for 75 s and annealed. Equal portions were incubated in 0.56 mM [^{14}C]glycine and 0.056 mM [^{3}H]glycine, 68 mM Na^+ for 20 min. These were then combined and 1 ml of this layered on the density gradient. o----o absorbance at 600 nm; ×—×, radioactivity of [^{3}H]glycine; △...△, radioactivity of [1-^{14}C]-glycine. Na^+-dependent glycine uptake of pooled fractions of Peak C was found to be 0.42 μmole glycine/g wet weight from duplicate density gradient fractions.

an activity of 27 μmoles/g wet weight per h. This may be compared with an activity value for malate dehydrogenase in pigeon breast muscle of $7 \cdot 10^4$ μmoles/g wet weight per h obtained from the data in Fig. 2 of ref. 26. (This is about twice the corresponding value for rat liver)[26]. On the basis both of Q_{O_2} and content of at least one mitochondrial enzyme, avian red cells appear to have a mitochondrial content about three orders of magnitude lower than actively metabolizing tissues. Thus avian erythrocytes, unlike mammalian erythrocytes do contain some intracellular membraneous structures. Expect for nuclei, however, they probably contain very little. Occasional mitochondrial structures were noted by Dr K. W. Lee on the electron micrographs of our vesicle preparations (*e.g.* Fig. 1a, lower left).

Glycine uptake by the vesicles resembles glycine transport by intact cells. Both processes require Na^+ in the medium. Both appear to obey similar kinetic equations with similar dependencies on glycine concentration and $[Na^+]^2$. In both systems a similar fraction of glycine uptake is inhibited by alanine, suggesting similar ratios of glycine to alanine–serine–cysteine–proline (ASCP)[11] transport routes.

Uptake by the vesicles probably represents transport and accumulation rather than simple Na^+-dependent binding. We have observed uptake of more than 0.3 μmole of glycine per ml pellet water. With 3.5 mg protein/g wet weight of pellet, this would correspond to an equivalent weight of 11000 daltons if all of the vesicle protein were binding protein. In addition, neither the temperature dependence nor the requirement for an annealing step would be expected for simple binding. The similarity of kinetic behavior between the vesicle system and intact cells, where transport and accumulation are unambiguous, also strongly supports this interpretation.

Active transport by intact or hemolysed and restored cells requires a Na^+ gradient[9,12,27–29]. There is no direct evidence that transport by the vesicle system does also, but the requirement for annealing suggests this. Our interpretation is that during annealing, some vesicles trap K^+. On subsequent incubation with glycine in Na^+ medium, a Na^+ gradient exists and these vesicles accumulate glycine. If vesicles are not annealed in K^+ medium first, during the subsequent incubation in Na^+ medium they trap Na^+-rich medium, have no Na^+ gradient and therefore do not accumulate glycine. Preliminary experiments indicate that vesicles annealed in Na^+ medium show little Na^+-dependent glycine uptake.

Annealing does not appear to be necessary in other vesicular transport systems[1–5]. It is not clear however whether this is the case because these systems do not require annealing to become capable of retaining small molecules ("sealing"), or because no ion gradient is required and sealing occurs during the incubation during which uptake occurs.

Vesicles unable to "seal" with respect to glycine cannot show glycine transport. We use the "fraction sealed" (Methods) in making approximate comparisons between the uptake activity of that fraction of the vesicles capable of retaining glycine with the activity of intact cells. This comparison cannot be very accurate. The value for "fraction sealed" should be the ratio of membrane area fully enclosing a volume to total membrane area. This ratio is related to the fraction of pellet volume enclosed by factors we cannot evaluate, *e.g.* the size distribution and exact geometry of the sealed fraction of the membrane vesicle population. We feel, however, that an order-of-magnitude comparison is worth making.

From electron micrographs we estimate that the diameter of membrane vesicles

was 0.5–0.02 μm compared to 8–16 μm for the intact cell. However the size distribution appears bimodal; there are large and small vesicles in comparable numbers but very few of intermediate size. Thus the large vesicles contain most of the membrane. Assuming these large vesicles to be spherical and to have the same membrane thickness as the intact cells, on the order of at least 200 vesicles could have been formed per cell. Since the ratio of surface area to volume of a sphere varies as the reciprocal first power of its radius, the transport activity per ml of the small vesicles should be roughly 20 times that of the intact cell. The V for the intact cells is 0.83 μmole glycine/15 min per ml pellet water[8]. The V for the membrane vesicles (Fig. 3) is on the order of 5.5 times that of the intact cell, or about $^1/_4$ of the calculated value. This low activity may be due to several causes: the calculation on which the comparison is based may be in error by this amount, the V calculation of the present experiments were taken from 3 min incubation which underestimates the initial rate, not all the sealed vesicles may be active in glycine transport, stimulating factors may have been extracted during vesicle preparation. We have found the activity to be quite variable from preparation to preparation and also that vesicles can be activated perhaps 2-fold. Our conclusion is that vesicle transport activity per unit area of membrane is the same order of magnitude as that of intact cells.

In our experiments with density gradient centrifugation of the vesicles, four different peaks were separated by the Ficoll density gradient. The Na^+-dependent glycine transport activity was found in the main peak. This result indicates that the membrane vesicles with glycine transport activity have not been separated from the main bulk of membrane vesicles.

The present experiments are a step toward the goal of separating membrane vesicles according to their ability to transport glycine. If vesicles are sufficiently small, some will contain and some lack a glycine porter. For spherical vesicles with constant transport activity per unit area of membrane, active transport will produce a percentage change in volume inversely proportional to the radius. Sufficiently small vesicles should thus be separable on the basis of their possession of a porter and comparison of their polypeptide compositions could, in theory, assist the identification and enumeration of all components of the porter. Such an identification and enumeration is not, in theory, possible with an extraction and reconstitution strategy until a set of characterized transport factors is obtained that can be incorporated into a synthetic lipid with reconstitution of transport activity.

Our attempts at separation into active and inactive vesicles have so far been suggestive at best (*e.g.* Fig. 6). However, we recently found that Ficoll causes these vesicles to lose previously accumulated glycine and Na^+.

We may also consider the effect of the subdivision of the membrane into smaller and smaller vesicles on the consequences of any lesions at which leakage occurs. If such lesions occur on the average in a few places in the membrane of a whole cell, most of the cell population will be leaky. The same lesion frequency per unit area in a vesicle population will leave most of the vesicles sealed. We have observed (Beygu-Farber, S. and Vidaver, G. A., unpublished) that vesicles can be frozen and thawed without much loss in previously trapped glycine. If the thawed vesicles are again annealed, they show appreciable glycine transport activity. This observation may show that such lesions are rare.

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