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Vesicle Formation during Reticulocyte Maturation

ASSOCIATION OF PLASMA MEMBRANE ACTIVITIES WITH RELEASED VESICLES (EXOSOMES)*

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Vesicles are released during the *in vitro* culture of sheep reticulocytes which can be harvested by centrifugation at 100,000 × *g* for 90 min. These vesicles contain a number of activities, characteristic of the reticulocyte plasma membrane, which are known to diminish or disappear upon reticulocyte maturation. The activities include acetylcholinesterase, cytochalasin B binding (glucose transporter) nucleoside binding (*i.e.* nucleoside transporter), Na^+-independent amino acid transport, and the transferrin receptor. Enzymes of cytosolic origin are not detectable or are present at low activity in the vesicles. Cultures of whole blood, mature red cells, or white cells do not yield comparable levels of these activities, supporting the conclusion that the activities arise from the reticulocytes. In addition, the lipid composition of the vesicles shows the high sphingomyelin content characteristic of sheep red cell plasma membranes, but not white cell or platelet membranes, also consistent with the conclusion that the vesicles are of reticulocyte origin. It is suggested that vesicle externalization may be a mechanism for shedding of specific membrane functions which are known to diminish during maturation of reticulocytes to erythrocytes.

We have reported that the transferrin receptor is lost from maturing sheep reticulocytes *in vitro* by the release of a small vesicle (1, 2). As the reticulocyte matures into the erythrocyte, the cell density increases (3) and a number of membrane associated activities are known to decrease (4–11). The question that arises is the fate of the activities known to decrease with maturation.

Earlier studies on the behavior of the vesicles indicated that the released transferrin receptor retained both transferrin and antitransferrin receptor antibody binding activities (12). The formation of vesicles was decreased under conditions of diminished metabolic activity (2), and cultures of mature cells did not show the formation of similar bodies (1, 2). Two major peptide bands were found in the released vesicles, the 94-kDa monomer of the transferrin receptor (1) and a 70-kDa peptide, now identified as the clathrin-uncoating ATPase (13).

An electron micrographic study (14), which followed the externalization of the transferrin receptor, suggested that after endocytosis of the surface receptor and fusion of endosomes to form larger structures, budding occurred at the internal surface of the vesicles. With the release of these small buds of ~50 nm diameter into the main body of the vesicles, multivesicular bodies were formed. The multivesicular bodies reached 0.5–1.0 μm in diameter and eventually fused with the plasma membrane releasing the 50-nm buds into the extracellular milieu. In the released vesicles, the transferrin-binding site and the antibody-binding site faced the external medium (1, 12), consistent with the proposal that two membrane inversions had occurred in the formation of the vesicles, one during the initial endocytosis and the second during the intravesicular budding phase.

Since general cell maturation and transferrin receptor loss occurred at approximately the same rate (12), we considered the possibility, examined in this report, that vesicle release may provide a general mechanism for loss of surface membrane components which are known to decrease during red cell maturation. This study provides evidence for selective membrane protein loss during *in vitro* maturation of reticulocytes.

MATERIALS AND METHODS

Reticulocytes were prepared as previously described from phlebotomized sheep (6, 11). Cells were cultured for 24–40 h at 37 °C in roller bottles in Eagle's minimal essential medium supplemented with glutamine (4 mM), adenosine (5 mM), inosine (10 mM), penicillin (200 units/ml), and streptomycin (200 μg/ml) (standard culture medium). Cells were collected by centrifugation at 8,000 × *g* for 10 min. The cell-free supernatant was recentrifuged for 90 min at 100,000 × *g* to collect the released vesicles. To assess whether cells other than reticulocytes gave rise to vesicles, washed, mature erythrocytes as well as washed, unfractionated blood cells from phlebotomized sheep and enriched fractions of white cells were cultured. Putative vesicle fractions were collected from all cultures after removal of the cells as described above. The pelleted vesicles were stored at −70 °C until required or used immediately after preparation.

Plasma membranes from reticulocytes and mature cells were prepared by osmotic lysis in 5 mM phosphate buffer, pH 8.0, containing 1 mM EDTA as described (15). The membranes were washed and stored at −70 °C in 20 mM phosphate buffer, pH 7.0. The membranes were centrifuged before use and resuspended in the appropriate medium as given in the text.

Labeling of Phospholipids

To label the phospholipids with ^{32}P, reticulocytes were incubated in P_i-free culture medium with [^{32}P]P_i (1 mCi/100 ml). For labeling with fatty acids, 280 nM [^3H]tetradecanoate, (36 Ci/mmol) was added to the medium. After 24–40 h incubation, cells and vesicles were collected by centrifugation. The lipids of the vesicles and the plasma membranes were extracted according to Folch *et al.* (16) and analyzed by thin layer chromatography (17) using chloroform: methanol:methylamine(40%):water (100:50:5:5) as solvent. The thin layer plates were autoradiographed and then sprayed with acid-molybdate to localize the lipids (18). Total phospholipid phosphorus was determined according to Lowry and Tinsley (19).

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Acetylcholinesterase

This was measured by standard procedures (20). The membranes and vesicles, suspended in 20 mM phosphate buffer, were incubated with acetylthiocholine (0.6 mM) and 0.25 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in a final volume of 1.0 ml containing 0.2 mM $MgCl_2$, 20 mM NaCl, and 10 mM NaHepes,[1] pH 7.4. The incubation was carried out in cuvettes at room temperature, and the change in absorbance at 417 nm followed continuously. To test for cryptic acetylcholinesterase, the reaction was followed in the presence and absence of 0.01% Triton. Triton was first added to the membranes at a concentration of 0.2% for ~5 min and diluted with the reagents to 0.01% prior to assay of activity.

Soluble Enzyme Assays

Membranes and vesicles were suspended in 20 mM phosphate buffer for all subsequent soluble enzyme assays. The vesicles were freeze-thawed three times before use as well as treated with Triton X-100 as given in the text to assure exposure of trapped enzymes. Unless otherwise specified, 50 μg of vesicle protein or 100 μg of plasma membrane protein were used for each assay.

Lactate Dehydrogenase

Measurement was by change in absorbance at 340 nm in a final volume of 1.0 ml using a standard protocol (21). The lactate concentration was 12.5 mM and 1 mM NAD, in 25 mM TRIS buffer at pH 9. The reaction was followed with and without 0.05% Triton X-100 at room temperature.

Glucose-6-phosphate Dehydrogenase

This was assessed by measuring the change in absorbance at 340 nm using glucose 6-phosphate (5 mM) as substrate in the presence of 1 mM NADP, 1 mM $MgCl_2$ in 10 mM TRIS buffer at pH 7.4 at room temperature (22). The final volume was 0.5 ml. The membranes were equilibrated with all reagents except glucose 6-phosphate, which was added to start the reaction.

Glyceraldehyde-3-phosphate Dehydrogenase

Measurement was by change in absorbance at 340 nm in the direction of NADH utilization as previously described (23). The mixture contained 50 mM TRIS buffer, pH 8.0, 10 mM $MgCl_2$, 8 mM ATP, 0.5 mM EDTA, 0.15 mM NADH, and 50 units of phosphoglycerate kinase in a final volume of 0.5 ml. After addition of phosphoglycerate kinase, incubation continued at room temperature until the change in absorbance was 0. Then 3-phosphoglycerate (10 mM, final concentration) was added and the change in absorbance measured.

6-Phosphogluconic Acid Dehydrogenase

This was measured according to the method described by Beutler (23) except that the concentration of NADP and 6-phosphogluconic acid were 1 and 3 mM, respectively, and the pH was 8.5. Twice as much vesicle protein was used for this assay as for the other enzyme assays (~100 μg of vesicle protein per assay).

Binding of [^{3}H]Nitrobenzylthioinosine (NBMPR)

Photoaffinity Labeling—Reticulocyte vesicles (~30–50 μg of protein) were exposed to 20 nM [^{3}H]NBMPR (specific activity 23 Ci/mmol) in the presence and absence of 100-fold excess of unlabeled NBMPR or adenosine in a quartz cuvette. The suspension was exposed to short bursts of irradiation with a mercury arc lamp (100 watts), interspersed with cooling on ice (2 min on and 2 min off). A 10-min period of exposure to light was used (8).

Equilibrium [^{3}H]NBMPR Binding—The method used is a modification of that described by Hammond and Martin (24) for benzodiazepine binding. Briefly, membranes or vesicles (~100 μg of protein) suspended in buffer containing 50 mM TRIS, 1 mM EDTA, soybean trypsin inhibitor (50 μg/ml), and 6 μM phenylmethylsulfonyl fluoride at pH 7.1, were incubated in a final volume of 1.0 ml with 5 nM [^{3}H]NBMPR with and without 10 μM unlabeled NBMPR to differentiate between specific and nonspecific binding. After an incubation of 30 min at room temperature, γ-globulin, sufficient to give a final concentration of 1.65 mg/ml was added, followed by 450 μl of 33% polyethylene glycol (PEG) 8000 dissolved in the buffer described above. The final PEG concentration was 10%. After vortexing the mixture and an additional 15 min incubation at room temperature, 4 ml of 8% PEG was added, followed by filtration through Whatman GF/B filters. The filters were washed once with an additional 4 ml of 8% PEG, dried, and counted.

Cytochalasin B

Labeling of cytochalasin B was carried out as described by Shanahan (25). Vesicles (~60–90 μg of protein) and plasma membranes (~200 μg of protein) from erythrocytes and reticulocytes were incubated with 1.5 μM [^{3}H]cytochalasin B (specific activity 155 Ci/mmol) without or with the following: 400 mM D-glucose, 400 mM L-glucose (control), 20 μM cytochalasin E in 20 mM phosphate buffer, pH 7.4. After 30 min on ice, the samples were irradiated in quartz cuvettes for 14 min, alternating 2 min of irradiation with 1 min of cooling. After irradiation, SDS-gel sample buffer was added, and the samples were heated for 3 min at 60 °C and prepared for electrophoresis. To test for specificity, the putative "vesicles" from a culture of white cells were used to measure cytochalasin B binding. The number of white cells cultured was approximately 2–2.5 times more than found by direct white cell counting in the volume of reticulocytes used to generate vesicles in the control reticulocyte culture.

Ouabain Binding

This was measured with [^{3}H]ouabain using a filter binding assay. Vesicles (~30–50 μg of protein/assay) suspended in 20 mM phosphate buffer were incubated with 1.3 μM ouabain (specific activity 11×10^6 cpm/nmol) at 37 °C for 45 min in a final volume of 0.06 ml. Nonspecific ouabain binding was assessed in the presence of excess unlabeled ouabain (800 μM). At the end of the incubation, 10 ml of ice-cold, 20 mM phosphate buffer was added, and the suspension was filtered on 0.22-μm Millipore GS filters and washed five to seven times with 2-ml aliquots of phosphate buffer, dried, and counted by liquid scintillation.

Amino Acid Transport

Vesicles (30–50 μg of protein/assay) were suspended in 0.15 M sodium chloride, buffered with 20 mM sodium phosphate buffer, pH 7.0, and incubated in a final volume of 0.07 ml for 15 min. Three types of assays were carried out: (*a*) net leucine uptake, (*b*) *trans*-stimulated leucine uptake, and (*c*) the effect of excess amino acids on leucine uptake. For net uptake, vesicles were preincubated in amino acid-free medium for 15 min at room temperature and then ^{3}H-labeled leucine was added to give a final concentration of 0.2 mM. For *trans*-stimulation, the vesicles were first preincubated with unlabeled leucine (0.2 mM) for 15 min, and the tracer [^{3}H]leucine was added to measure uptake. To assay specific inhibition of leucine uptake, the assay was as in *b*, except that the inhibitory amino acid (5 mM) was added simultaneously with the labeled amino acid. After 2 min at room temperature, the reaction was stopped by addition of excess, cold, phosphate-buffered isotonic NaCl, followed by rapid filtration on Millipore GS filters (0.22 μm). Preliminary experiments showed that net uptake reached steady state by 2 min and gave the peak of *trans*-stimulation at this time.

Protein was estimated by a modified Lowry procedure (27, 28).

Materials

Culture medium was obtained from GIBCO. ATP (disodium salt), NBMPR, ouabain, glucose 6-phosphate, 6-phosphogluconic acid, lactic acid, 3-phosphoglyceric acid, glycerate kinase, amino acids, and PEG 8000 were obtained from Sigma. NAD, NADP, and NADH were obtained from Boehringer Mannheim, Dorval, Canada. Labeled [^{32}P] P_i, [γ-^{32}P]ATP, [^{3}H]tetradecanoic acid (36 Ci/mmol), 3-*O*-[*methyl*-^{14}C]glucose (40 mCi/mmol), and EN^{3}HANCE spray were purchased from Du Pont-New England Nuclear. [^{3}H]Cytochalasin B (155 Ci/mmol) and L-[4,5-^{3}H]leucine (71 Ci/mmol) were purchased from Amersham Corp., Oakville, Ontario. The [^{3}H]nitrobenzylthioinosine was obtained from Dr. Simon Jarvis, Department of Pharmacology, University of Alberta, and from Moraveck Biochemicals, Brea, CA. For autoradiography, Kodak XAR-5 film was used. Other reagents were purchased from Fisher Scientific, Montreal.

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NBMPR, [^{3}H]nitrobenzylthioinosine; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate.

RESULTS

When reticulocytes, but not mature cells, are cultured *in vitro*, a population of vesicles is formed which is enriched in a limited number of peptides. The data in Fig. 1 compare the peptide composition of reticulocyte plasma membranes and mature cell plasma membranes with that of the vesicles. The two major peptides (94 kDa) and (70–72 kDa) were previously identified as the transferrin receptor (94 kDa) and the clathrin-uncoating ATPase (70 kDa) by showing their reactivity with antibodies directed against the transferrin receptor and the clathrin-uncoating ATPase, respectively (1, 13). It is evident that the vesicle is not merely a fragment of the plasma membrane. If only mature red cells are cultured, there is no evidence for vesicle formation, and the protein yields, measured in the 100,000 × *g* cell-free pellets, are barely detectable. For example, 1 ml of mature red cells after 40 h culture yields on average less than 10 μg of protein in the 100,000 × *g* pellet. In contrast, 120–150 μg of protein are derived from 1 ml of (60–80%) reticulocytes. Moreover, if 1 ml of an unfractionated sample of washed serum-free blood, containing all the cellular elements and a peripheral reticulocyte count of less than 10%, is cultured, the protein yield is ~30% of that seen with 60–80% reticulocytes. (It should be noted that the whole blood will contain all the youngest reticulocytes, since a percentage of the youngest cells is lost upon removal of white cells in the preparation of an enriched reticulocyte fraction.)

If vesicle formation occurs *in vivo*, high speed centrifugation of plasma from phlebotomized animals should yield a pellet containing the transferrin receptor, whereas normal serum would lack the receptor. In phlebotomized animals, the peripheral reticulocyte count is ~6% on average and the hematocrit is ~20%. We calculated that 30 ml of plasma should be sufficient to detect the transferrin receptor based on *in vitro* incubations with enriched reticulocytes. The 100,000 × *g* pellets obtained from normal and phlebotomized plasma were dissolved in 0.5% Triton X-100, immunoprecipitated, and processed for gel electrophoresis as previously described (12). The results (Fig. 2) demonstrate the receptor in immunoprecipitates of the high speed pellets from 30 ml of plasma from phlebotomized animals, but not from an equivalent volume of plasma from normal animals.

To substantiate the conclusion that the vesicles are membranous structures derived from the red cell population, their

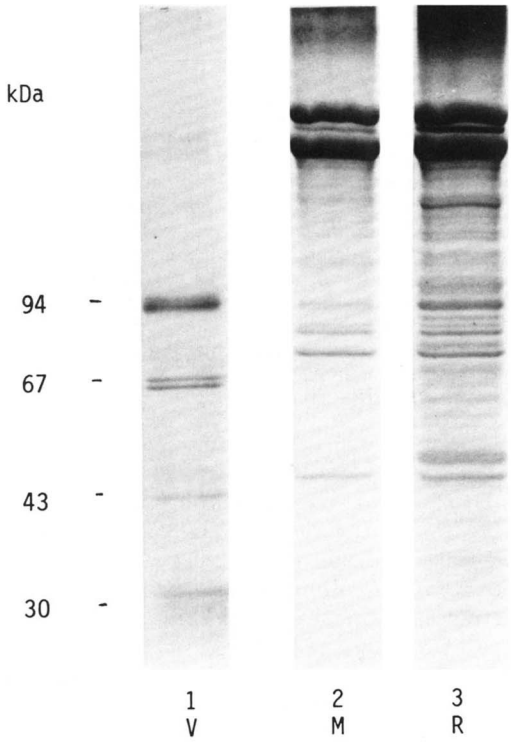

FIG. 1. **Peptide composition of vesicles and plasma membranes.** Plasma membranes derived from reticulocytes (*R*), mature cells (*M*), and vesicles (*V*) were suspended in SDS-gel buffer and heated for 5 min at 100 °C. The dissolved material was subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue as described under "Materials and Methods." *Lane 1,* total peptides of vesicles (~30 μg of protein) derived from culturing 0.3 ml of reticulocytes; *lane 2,* total peptides of mature cell membranes (~60 μg of protein) derived from 0.02 ml of packed cells; *lane 3,* total peptides of reticulocyte membranes (~130 μg of protein) derived from 0.02 ml of packed cells.

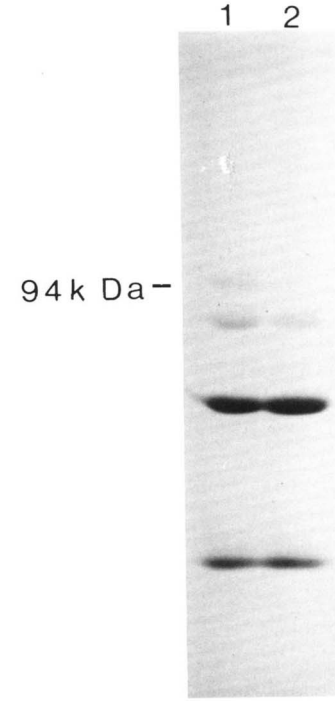

FIG. 2. **Recovery of transferrin receptor in 100,000 × *g* pellets of serum.** Fifty ml of heparinized blood drawn from an unbled and a bled sheep were centrifuged at 8,000 × *g* for 8 min to remove red cells. Thirty ml of plasma was removed and recentrifuged for 15 min at 8,000 × *g*. The cell-free, clear supernatant was diluted with an equal volume of phosphate-buffered saline and centrifuged at 100,000 × *g* for 90 min. The supernatant was removed, the tubes drained and wiped, and the pellets were dissolved in 0.5 ml of Triton X-100 followed by immunoprecipitation with the antitransferrin receptor antibody and protein A-Sepharose as described (12). Coomassie Blue stains of the immunoprecipitates are shown. *Lane 1,* plasma from bled sheep; *lane 2,* plasma from unbled sheep. The 94-kDa band is the transferrin receptor.

TABLE I

Phospholipid content of the vesicles and the plasma membranes

Vesicles and plasma membranes were isolated as described under "Materials and Methods." Lipid phosphorus was estimated by the Lowry and Tinsley (19) procedure and protein by a modified Lowry assay (28). The data from a single analysis are shown but are representative of three similar lipid/protein ratios.

	Phospholipid	Protein	Lipid/protein
	μg		
Vesicles	8.8	18.4	0.48
Reticulocyte membranes	35	82	0.43
Mature cell membranes	42	74	0.57

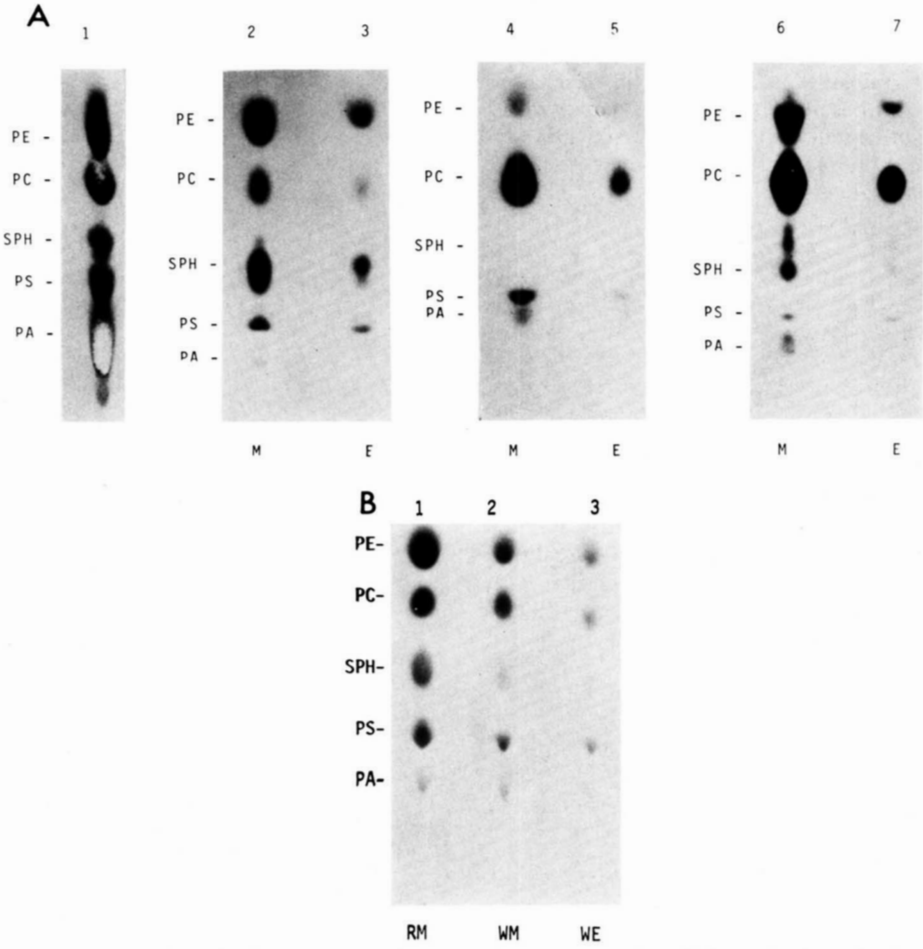

FIG. 3. *A*, Phospholipids of reticulocyte membranes and exosomes. After 40-h incubation of cells with [^{3}H] tetradecanoate or [^{32}P]P_i as described (12), cells or vesicles were harvested. Plasma membranes (100 μg of protein) and vesicles (30 μg of protein) were extracted with chloroform/methanol/water according to Folch *et al.* (16). The lipids were separated on TLC plates (Whatman K-5 silica gel) as described under "Materials and Methods." *Lane 1*, standards; *lanes 2* and *3*, total phospholipids of reticulocyte plasma membranes (*M*) and vesicles (*E*), respectively, as displayed after acid molybdate spraying (18). *Lanes 4* and *5* are radioautographs of ^{32}P incorporation into phospholipids of reticulocyte plasma membranes and vesicles, respectively. *Lanes 6* and *7* are radioautographs of [^{3}H]tetradecanoate incorporation into phospholipids of reticulocyte plasma membranes and vesicles, respectively. EN^{3}HANCE was used prior to autoradiography. *B*, Phospholipid composition of white cell membranes and vesicles. White cells, enriched by Percoll separation, were cultured overnight and vesicles collected. An aliquot of the cells as well as vesicles were extracted as above (16) and chromatographed. The plates were stained with acid molybdate (18). *Lane 1, RM*, reticulocyte membrane lipids; *lane 2, WM*, white cell membrane lipids; *lane 3, WE*, lipids from white cell vesicles. *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *SPH*, sphingomyelin; *PS*, phosphatidylserine; *PA*, phosphatidic acid.

phospholipid content and protein/lipid ratios were determined. The results in Table I show that the vesicles have phospholipid/protein ratios characteristic of sheep red cell or reticulocyte plasma membranes. It is known that in human red cells the protein/lipid ratio is the same in old and young red cells (29), with which the present findings are consistent. The phospholipid composition of the vesicles is also very similar to that of the red cell plasma membrane phospholipids, notably rich in sphingomyelin (30) (Fig. 3*A*, *lanes 2* and *3*). It is interesting to note that sheep platelets, unlike sheep red cells, are richer in phophatidylcholine (31) than sphingomyelin thus indicating that a major contamination from platelets in the vesicles is unlikely. Furthermore, white cells and 100,000 × *g* pellets derived from white cell cultures do not show the high sphingomyelin content characteristic of red cell membranes and vesicles (Fig. 3*B*), making it unlikely that the vesicles are derived from the white cell population.

If the vesicles originate from reticulocytes, precursor incorporation into lipids by reticulocytes should be reflected in the vesicles. Therefore, the incorporation of [^{3}H]tetradecanoate and [^{32}P]P_i into reticulocyte membranes and vesicles was measured during the culture period.

[^{3}H]Tetradecanoate incorporation into the phospholipids shows a similar pattern of labeling in both vesicle and reticulocyte membranes, the major lipid labeled being phosphatidylcholine. Although sphingomyelin is a major phospholipid of sheep red cell plasma membranes (30) (Fig. 3*A*, *lanes 2* and *3*), little new sphingomyelin synthesis occurs under these conditions (Fig. 3*A*, *lanes 6* and *7*).

Labeling studies with [^{32}P]P_i also show that the distribution of ^{32}P in the phospholipids of the vesicles is substantially the same as that in the plasma membrane (Fig. 3*A*, *lanes 4* and *5*), with phosphatidylcholine being the major labeled species. Overall, the data are consistent with the conclusion that exosomes are lipoprotein structures with a phospholipid composition characteristic of red cell membranes.

TABLE II

Absence of lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and gluconate-6-phosphate dehydrogenase in the externalized vesicles

Reticulocytes and mature cells were lysed with 30 times their volume of 5 mM phosphate buffer and 1 mM EDTA, pH 8.0, and centrifuged at 15,000 × *g* for 30 min to remove the membranes. A volume of 25 μl of the lysate was used for the assay. In determining specific activity, it was assumed that hemoglobin comprised 90% of the protein content. Representative experiments from three separate experiments are shown. The activities were measured by monitoring NADH formation at 320 nm using the conditions described under "Materials and Methods." The vesicles used came from 5×10^9 cells (~50 μg of protein), whereas the cell extracts represented $\sim 10^7$ cells, except for gluconate-6-phosphate dehydrogenase, where at least twice as much protein was used.

Enzyme source	Lactate dehydrogenase	Glucose-6-*P* dehydrogenase	Gluconate-6-*P* dehydrogenase
	pmol/μg protein[b]/min		
Reticulocyte lysate	300	60	8.5
Mature cell lysate	300	33	ND[a]
Vesicles	50	0.4	<0.9

[a] ND, not determined.
[b] Based on non-hemoglobin protein.

Presence of Soluble Enzymes in Exosomes—If the vesicles are formed in an environment in which they may entrap cytosolic enzymes, it might be possible to show the presence of "soluble enzymes" in the vesicles. Four enzyme activities known to be present in the cytosol were assayed, namely lactate dehydrogenase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase.

The results in Table II show that lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and gluconate-6-phosphate dehydrogenase activities can be detected in a dilute preparation of the stroma-free cytosol equivalent to $\sim 10^7$ cells, or ~30 μg of non-hemoglobin protein. (It is assumed that hemoglobin comprises 90% of the total protein.) In contrast, little activity could be detected in a population of fresh or frozen vesicles containing ~50 μg of protein, an amount of protein collected after culturing 5×10^9 cells. Per unit amount of protein, the vesicles have less of the four soluble enzyme activities than the corresponding plasma membranes (not shown). Since entrapment of the enzymes in the vesicles might prevent access to exogenous NAD or NADP, the vesicles were also assayed in presence of Triton in addition to being freeze-thawed before use. No evidence for entrapped activity was found with any of the dehydrogenases.

Glucose-6-phosphate dehydrogenase is known to lose activity rapidly in the absence of NADP (32); therefore, its apparent absence in vesicles could be due to the lack of NADP in the vesicles during the culture period. For this reason a second NADP-linked enzyme, 6-phosphogluconate dehydrogenase, which retains its activity (32), was measured. No 6-phosphogluconate dehydrogenase activity was detected in vesicles using 100 μg of vesicle protein per assay. An activity 10% of that in the lysate would have been detected in the vesicles.

Glyceraldehyde-3-phosphate dehydrogenase is a soluble enzyme which normally adheres to the cytoplasmic surface of the plasma membranes (33) but is inactive when bound to Band 3 of the red cell membrane (34). The results show that glyceraldehyde-3-phosphate dehydrogenase activity can be detected in reticulocyte membranes (*R*, Fig. 4). Similar results are obtained with mature cell membranes (now shown). No glyceraldehyde-3-phosphate dehydrogenase activity, however, is detected in vesicles (Fig. 4).

It is unlikely that the absence of glyceraldehyde-3-phosphate dehydrogenase in the vesicles is due to its inactivation by binding to Band 3 (33) for the following reasons: 1) There is no evidence for the presence of Band 3 in the vesicles (1). 2) At the ionic strength and phosphate content of the medium, the enzyme would dissociate from Band 3 (33). The latter conclusion is strengthened by data using plasma membranes as source of this enzyme, since the activity is readily detected in membranes of both erythrocytes and reticulocytes (Fig. 4).

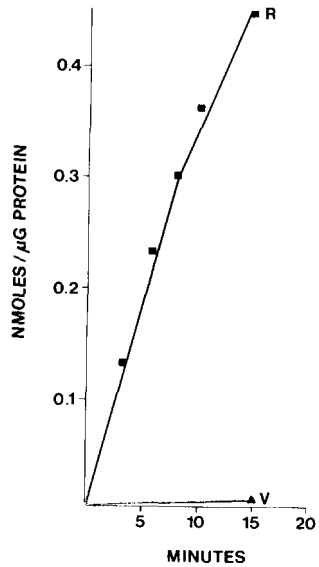

FIG. 4. **Glyceraldehyde-3-phosphate dehydrogenase.** This was measured in the direction of NADH oxidation in presence of glycerate kinase as described under "Materials and Methods." The activity was assayed in cell lysates, plasma membranes of mature cells, and reticulocytes, as well as vesicles. Only values with the reticulocyte membranes (*R*) and vesicles (*V*) are given. The membranes were prepared as described (12). The reaction was followed at room temperature. A representative experiment from three similar experiments is shown.

Hemoglobin does appear to be entrapped in the vesicles. This conclusion is based on the observation that the 100,000 × *g* pellets from reticulocyte cultures are red and they have an absorption at 540 nm. To distinguish between entrapped and external hemoglobin, the vesicles were treated with trypsin and then subjected to SDS-gel electrophoresis. There was little apparent loss of hemoglobin after trypsin treatment, suggesting that the hemoglobin is trapped in the vesicles.

Presence of Acetylcholinesterase in Vesicles—In contrast to the low levels of soluble enzymes, several plasma membrane-bound activities were found in vesicles. Acetylcholinesterase, a membrane-bound enzyme in erythrocytes (34, 35), is found in vesicles (Table III). The addition of Triton does not affect acetylcholinesterase activity. The right-side-out orientation of the acetylcholinesterase activity is consistent with earlier observations that both the transferrin-binding site and the anti-transferrin receptor-binding site are present in the vesicle in the same orientation as in the cell, *i.e.* right side out (1, 2, 12). The apparently lower specific activity of acetylcholinesterase in reticulocyte membranes compared to mature cells is probably due to the higher protein content of reticulocyte membranes per milliliter of packed cells (Fig. 1). It is evident in Fig. 1 that more protein is obtained from the membranes of reticulocytes than from an equivalent volume of mature red cells. Earlier studies expressed the loss of acetylcholinesterase during red cell maturation per cell number or hemoglobin content and not per milligram of membrane protein (34). The values have been recalculated on the basis of the cell number from which the membranes were derived (column 3,

TABLE III

Presence of acetylcholinesterase in vesicles and plasma membranes

Plasma membranes and vesicles were prepared as described under "Materials and Methods" and suspended in 20 mM phosphate buffer, pH 7.4. The reagents were added to give a final volume of 1.0 ml. To express the activity on a per-cell basis, a known number of cells (mature cells and reticulocytes) was lysed and the absorbance at 540 measured. An aliquot of the membranes derived from the lysed cells was used to assay acetylcholinesterase.

	Activity	
	nmol/min/μg protein	*nmol/min/10^9 cells*
Reticulocytes	0.28 ± 0.04 S.D. (n = 10)	7.3
Mature cells	0.34 ± 0.04 S.D. (n = 5)	4.3
Vesicles (from reticulocytes)	0.28 ± 0.10 S.D. (n = 5)	
"Vesicles" (from mature cells)	ND[a]	
"Vesicles" (from white cells)	ND	

[a] ND, not detectable.

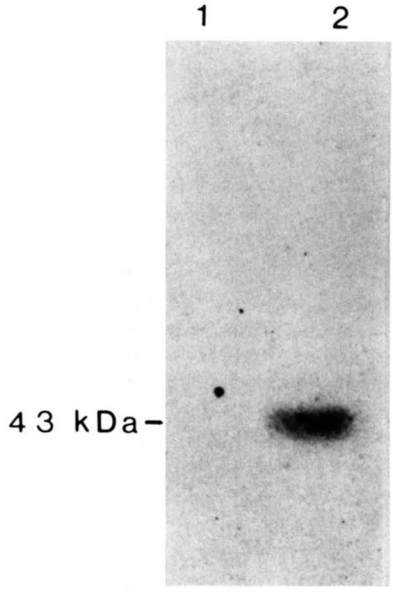

FIG. 5. **Binding of [^{3}H]nitrobenzylthioinosine to vesicles.** Vesicles were irradiated with UV with 20 nM [^{3}H]NBMPR as described under "Materials and Methods." After labeling, vesicles were washed, centrifuged, dissolved with SDS, and subjected to polyacrylamide gel electrophoresis according to Laemmli (26). Radioautographs of the gels are shown. *Lane 1*, with excess (2 μM) unlabeled NBMPR; *lane 2*, without excess NBMPR.

Table III). The reported decrease in acetylcholinesterase per cell during maturation of human red cells has been estimated at ~50% (4), a value consistent with our observation in sheep cells. It is clear that the acetylcholinesterase activity in the vesicles cannot be due to serum contamination since well-washed cells were cultured in serum-free media. Moreover, pellets derived from cultures of white cells or unfractionated blood show no acetylcholinesterase activity in the putative "vesicle" fraction.

Nucleoside Binding—In recent studies it has been shown that NBMPR can be used to label the nucleoside carrier of red blood cells in a highly specific manner (8, 36). Moreover, it is well known (8) that the nucleoside carrier diminishes as the sheep reticulocyte matures into the erythrocyte (8). Using the capacity of NBMPR to form a covalent linkage with the carrier upon photolysis (36), its binding to vesicles was determined. Only a single radioactive band at around 43 kDa was obtained (Fig. 5, *lane 2*), and labeling was abolished by the presence of excess thionucleoside (Fig. 5, *lane 1*) or excess adenosine (not shown).

TABLE IV

NBMPR binding in vesicles from reticulocytes and unfractionated blood cell cultures and in plasma membranes

Vesicles derived from cultures of reticulocytes and unfractionated whole blood cells were incubated with 5 nM [^{3}H]NBMPR (specific activity 40 dpm/fmol) for 30 min at room temperature in TRIS buffer, pH 7.1, and 10 mM EDTA as described under "Materials and Methods." Half the samples were incubated with 10 μM unlabeled NBMPR to measure nonspecific binding. In reticulocyte vesicles, the nonspecific binding was ≤10% of the total binding. With whole blood vesicles, nonspecific binding was ~25% of the total. The values given, which have been corrected for nonspecific binding, are the means ± the spread of three separate experiments, each done in duplicate with vesicles derived from three different sheep and on different occasions. In each of the three experiments, the same pool of blood was used to produce reticulocytes and the unfractionated blood cells which were cultured for exosome production. A duplicate experiment is shown in II. NBMPR binding was measured in plasma membranes from reticulocytes prior to incubation for 40 h and after 40 h of incubation. The population of vesicles formed was harvested and nucleoside binding measured. The values given have been corrected for nonspecific binding. The recovery of activity in the vesicles represents greater than 40% of the activity lost from the cells.

Vesicle origin	[^{3}H]NBMPR bound
	fmol/μg protein
I.	
Enriched reticulocytes	3.0 ± 1.0 (n = 3)
Unfractionated blood	0.6 ± 0.2 (n = 3)
Plasma membrane origin	
II.	
Fresh reticulocytes	1.2, 1.5
Matured reticulocytes	0.6, 1.0
Vesicles from reticulocytes	6.3, 4.6

Direct NBMPR binding was also measured in vesicles derived both from unfractionated blood cell cultures and from reticulocyte cultures (Table IV). It is clear that vesicles from unfractionated blood have a specific binding activity about one-fifth that from reticulocytes (Table IV). It may also be seen that, as reticulocytes mature, the membranes lose NBMPR binding capacity (Table IV, part II). That the vesicles show higher specific binding than the reticulocytes is consistent with a selective externalization of the nucleoside carrier. Forty percent of the lost binding activity may be recovered in the vesicles (not shown). The low binding of NBMPR in vesicles from unfractionated blood compared to vesicles from enriched reticulocytes supports the conclusion that the origin of the nucleoside-binding component is the reticulocyte and not other blood cells.

Cytochalasin B Binding—In sheep cells, glucose transport is diminished during maturation, although it is not completely lost (Fig. 6). Complete loss of this carrier has been reported for pig red cells (9) but no diminution of glucose transport in sheep cells has previously been reported.

Since cytochalasin B binds to the glucose carrier (25) and photoactivation brings about a covalent linkage with this carrier, binding studies were also carried out with plasma membranes and vesicles using excess D-glucose and cytochalasin E to judge the specificity of [^{3}H]cytochalasin B binding. The presence of L-glucose was used in the control. It is evident that cytochalasin B binding is observed with plasma membranes from mature cells (*M*), Fig. 7*A* (*lanes 1–3*), reticulocytes (*R*) (*lanes 4–6*), as well as with vesicles (*E*) (*lanes 7–9*). With plasma membranes from mature and immature cells, two peptides of similar molecular size (42 + 43 kDa) bind cytochalasin B (*lanes 2* and *5*). In vesicles, a single major

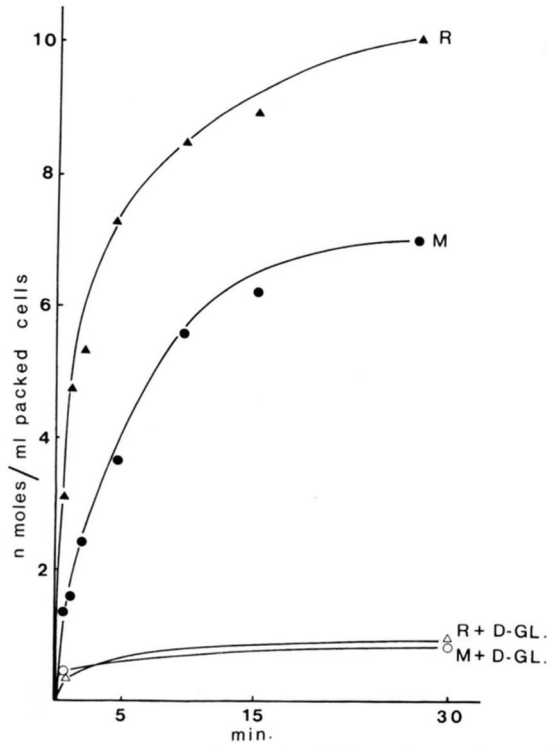

FIG. 6. **Transport of 3-[*methyl*-^{14}C]D-glucose by reticulocytes (*R*) and mature sheep red cells (*M*).** Cells in isotonic saline buffered with 20 mM phosphate were incubated with and without 4 mM D-glucose (*D-GL*) in presence of 16 μM 3-[*methyl*-^{14}C]D-glucose, (specific activity 4 μCi/nmol) in a final volume of 5.0 ml at room temperature. Aliquots of 0.5 ml were taken over a 30-min period and spun through 0.2 ml dibutyl phthalate, which was overlaid with 0.7 ml of phosphate-buffered isotonic saline. After removal of the supernatant and oil, the cell pellet was lysed, the protein precipitated with 5% trichloroacetic acid, and the clear supernatant counted.

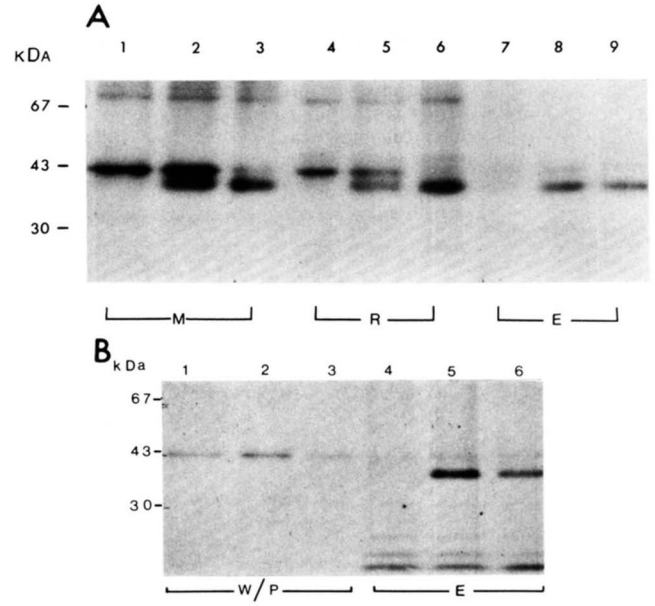

FIG. 7. **Labeling of membrane and vesicle proteins with [^{3}H] cytochalasin B.** *A*, red cell membranes and vesicles. Isolated plasma membranes and vesicles were irradiated in the presence of 1.5 μM [^{3}H]cytochalasin B as described under "Materials and Methods" in the presence of 500 mM D-glucose (*lanes 1, 4,* and *7*), 500 mM L-glucose (*lanes 2, 5,* and *8*) (control), and cytochalasin E (*lanes 3, 6,* and *9*). After labeling, the membranes were dissolved in SDS and processed by polyacrylamide gel electrophoresis according to Laemmli (26). Radioautographs of the gels are shown. *Lanes 1–3,* mature cell membranes; *lanes 4–6,* reticulocyte membranes; *lanes 7–9,* vesicles from reticulocytes. *B,* photoaffinity labeling of white cell/platelet vesicles and reticulocyte vesicles with cytochalasin B. The procedure was as above. *Lanes 1–3,* white cell vesicles (*W/P*); *Lanes 4–6,* reticulocyte vesicles (*E*). D-Glucose was present in *lanes 1* and *4,* L-glucose in *lanes 2* and *5,* and cytochalasin E in *lanes 3* and *6* during photolabeling.

band of 42 kDa binds cytochalasin B (*lane 8*). It has been shown that cytochalasin B binding to the glucose carrier is abolished by excess D-glucose, whereas binding to actin is abolished by cytochalasin E (37). It is noteworthy that, with the plasma membranes, the binding to the lower band (42 kDa) is inhibited by excess D-glucose (*lanes 1* and *4*) whereas binding to the upper band is inhibited by cytochalasin E (*lanes 3* and *6*). In vesicles, only the 42-kDa D-glucose-suppressible band is found (Fig. 7*A*, compare *lanes 7* and *9*), supporting the conclusion that the glucose carrier, not actin, binds cytochalasin B in the exosomes. That the glucose-sensitive cytochalasin B binding in exosomes arises from reticulocytes and not other cellular elements is shown by the fact that cultures of non-red cell elements (platelets and white cells), unlike reticulocytes, give rise to a protein pellet in which [^{3}H]cytochalasin B binding is suppressed by cytochalasin E but not by D-glucose (Fig. 7*B*, compare *lane 2* with *lanes 1* and *3*). Furthermore, cultures of unfractionated blood cells from phlebotomized sheep yield little D-glucose-suppressible cytochalasin B binding in the vesicles, and the amount of glucose-suppressible cytochalasin B binding is increased as the reticulocyte concentration is increased (not shown).

The low apparent molecular size of the D-glucose-sensitive, cytochalasin B-binding protein in sheep membranes compared to other systems may be due to differences in extent of glycosylation (37, 38). It has been estimated that glycosylation may change the apparent molecular size of the glucose carrier by 6 to 9 kDa (37, 38.)

Ouabain Binding—Red cell plasma membranes are known to contain both ATPase and phosphatase activities (39, 40),

TABLE V

Leucine transport in vesicles

Fresh or previously frozen vesicles were suspended in phosphate-buffered isotonic saline and incubated with 0.2 mM L-leucine for 15 min at room temperature. After the preincubation, a tracer amount of [^{3}H]leucine was added. In the non-preloaded samples, sufficient nonlabeled leucine was added simultaneously with [^{3}H]leucine to bring the final leucine concentration to 0.2 mM. Methionine or α-methylaminoisobutyrate (α-methyl-AIB) were added together with the [^{3}H]leucine. In column 3, a single experiment from 11 similar experiments is shown. A pooled average result is shown in column four. The percent stimulation (or inhibition) in each experiment was calculated using the respective control value as 100%. All measurements were done with 25–50 μg of vesicle protein. In any one experiment, the amount of protein was constant for the three or four conditions. All values given were corrected for retention of isotope by the filters in absence of vesicles (~150 cpm).

Preloaded with leucine	Amino acid inhibitor	Leucine uptake	Leucine uptake
		cpm/min	*% control ± S.D.*
+		816	200 ± 60 (n = 11)
−		537	100[a] (n = 11)
+	5 mM methionine	204	50 ± 20 (n = 11)
+	5 mM α-methyl-AIB	754	95 ± 5 (n = 3)

and it is known that Na/K-ATPase decreases in sheep reticulocytes during maturation (41). Although the vesicles contain low levels of ATPase activity (not shown), several attempts to measure the Na^+-activated, ouabain-sensitive component of the ATPase activity failed. To assess the presence of Na/K-ATPase in the exosomes, their ability to bind (^{3}H)ouabain

TABLE VI

Comparison of leucine uptake in vesicles from reticulocyte cultures, whole blood cultures, and whole blood/reticulocyte mixtures

The conditions were as described in the legend to Table V. Results from two different experiments (A and B) are shown in which the cells from the same bleeding were used to obtain the unfractionated whole blood cells, enriched reticulocytes, or 50/50 mixtures of the unfractionated blood and reticulocytes. In all cases, 1 ml of packed cells was cultured for 24 h, and all the exosomes released were used for the transport assay.

Vesicle origin	Preloaded with leucine	Inhibitor	Uptake	
			Exp. A	Exp. B
			cpm/min	
Reticulocyte (70–80%)	+		326	427
	−		167	91
	+	Methionine (5 mM)	75	41
50% unfractionated blood cells/50% reticulocytes (70–80%)	+		165	260
	−		199	149
	+	Methionine (5 mM)	52	87
Unfractionated blood cells	+		100	75
	−		100	34
	+	Methionine (5 mM)	80	78

was measured. The results show that the exosomes from reticulocytes bind 18 fmol of ouabain ± 0.1 (S.D.) ($n = 4$) per 50 μg of vesicle protein at an external ouabain concentration of 1.3 μM. Unlabeled ouabain completely suppressed the binding.

Leucine Transport—In earlier work we showed that maturing sheep reticulocytes lost the capacity to transport amino acids with time in culture (6). The data in Table V show that the exosomes transport leucine. The characteristics of Na^+-independent, energy-independent leucine transport are well known (42). The data in Table V show the characteristic *trans*-stimulation of leucine uptake by intravesicular leucine and inhibition by extravesicular methionine but not by α-methylaminoisobutyrate. Intravesicular isoleucine, but not glycine, can substitute for intravesicular leucine in obtaining *trans*-stimulation (not shown), in line with the known properties of the L-transport system (42). Vesicles from cultures of whole, unfractionated blood cells from phlebotomized animals show much less leucine transport activity than vesicles from cultures of reticulocytes (Table VI), again emphasizing the reticulocyte origin of the major portion of the externalized activity.

DISCUSSION

It is well known that within 24–48 h of being released into the circulation, the reticulocyte loses the last vestiges of the functions which distinguish it from the erythrocyte (43). In addition to the loss of remaining organelles, such as mitochondria and ribosomes, substantial changes also occur in the composition of the plasma membrane (4–11). These include loss or reduction of nucleoside transport (7, 8), glucose transport (9), Na/K-ATPase (41), amino acid transport (5, 6), and transferrin binding activity (10, 11, 44). The extent of loss of each of these functions depends on the species. For example, pig red cells, but not human red cells, lose all glucose transport (9) activity. Transferrin receptor is generally completely lost (10, 11, 44–46).

Zweig *et al.* (47) suggested that, after the clustering of reticulocyte-specific proteins at the cell membrane, the clustered components were removed by mechanisms which required the intervention of the spleen. Subsequent studies, however, have shown that the intervention of the spleen is not required for *in vitro* maturation of reticulocytes (1, 6–11, 46). Moreover, it has also been shown that, after the release of the nucleus, the major changes in the composition of the developing red cell membrane occur at the transition between reticulocyte and mature cell (48); that is, in the final hours before reaching the last stage of red cell development. In earlier studies, we showed that the 100,000 × *g* pellet of the cell-free supernatant of the culture medium contained a population of vesicles (1, 2). The transferrin receptor was found in this vesicular fraction, which we will refer to as exosomes. Harding *et al.* (45, 46) also concluded that, during reticulocyte maturation, transferrin receptor-containing vesicle are released.

The route of formation of exosomes does not appear to be related to the mechanism for vesicle release under adverse conditions (49) since metabolic inhibitors, including a reduction of temperature, reduce the formation of the vesicles bearing the transferrin receptor (1, 2) but result in an increased yield of membrane fragments. It is likely that the route by which plasma membrane proteins discussed here are packaged into exosomes follows the pathway described for the transferrin receptor (14), which involves endocytosis of specific membrane proteins followed by formation of multivesicular bodies. These bodies, upon fusion with the plasma membrane, release their contents (exosomes) into the medium as shown for the transferrin receptor (1, 14, 45, 46). It is not yet known how the specific membrane proteins are targeted for externalization. The exosomes are enriched in reticulocyte-specific proteins and devoid of some of the major plasma membrane proteins (*e.g.* Band 3) (1, 2), which are commonly found in membrane fragments formed under adverse conditions (49). There is no evidence that Band 3 (the anion exchange protein) disappears or diminishes during red cell maturation (50).

That exosomes arise primarily from reticulocytes and not from other cellular elements in the blood, such as mature red cells, platelets, or white cells, is shown by the following observations.

1) Long-term incubation of mature erythrocytes does not yield a population of vesicles, nor does the 100,000 × *g* residue from mature red cell cultures show any of the activities reported.

2) Cultures of unfractionated blood cells from phlebotomized animals containing platelets, white cells, and mature

red cells, but relatively low levels of reticulocytes, show little activity (amino acid transport [^{3}H]cytochalasin binding, [^{3}H] NBMPR binding, acetylcholinesterase, and transferrin receptor) in the putative exosome pellet in contrast with the activity arising from the culture of an equal volume of enriched reticulocytes. Since the contaminating cellular elements (white cells and platelets) are not enriched in the reticulocyte fraction, the activities must arise from the reticulocytes themselves.

3) Vesicles from white cell cultures showed cytochalasin B binding. This binding was suppressed by cytochalasin E, but not by D-glucose, suggesting the presence of actin, but not the glucose carrier, in the material of white cell origin. The presence of actin suggests that the material may originate from cellular degradation.

4) The amount of protein found in the 100,000 $\times g$ pellet after 24–40 h of culture was 2–3 times greater with reticulocytes than with unfractionated blood cell cultures.

5) The lipid composition of the vesicles was characteristic of the red blood cells with their high sphingomyelin content (30). White cells do not show the high sphingomyelin content of sheep red cells (Fig. 3*B*).

6) The transferrin receptor can be obtained in immunoprecipitates of the high speed pellets from plasma of phlebotomized animals and not unbled animals.

All these observations are consistent with the conclusion that the exosomes originate from reticulocytes during maturation *in vitro* and *in vivo*.

As cited above, during red cell maturation many enzymic activities are lost or diminished. Two mechanisms are established for intracellular degradation of proteins: proteolysis in lysosomes and cytoplasmic proteolysis of proteins tagged with ubiquitin (51, 52). The latter activity has been chiefly associated with degradation of soluble cellular proteins (51), particularly abnormal proteins. The presence of lysosomes can no longer be detected in reticulocytes (53) except by marker enzymes (54), and these activities also disappear with maturation. Thus neither mechanism is likely to account for the loss of membrane-bound activities.

If exosome formation represents a major route for externalization of "superfluous" membrane proteins, it should be possible to show that the activity found in the exosomes represents the majority of the activity lost. Such a balance sheet has not yet been successful since vesicles appear to lose activity during the culture period. Thus, we find that if freshly harvested vesicles are reincubated in fresh cell-free culture medium, 36–40% ($n = 3$) of the initial protein kinase activity and 46 ± 12% ($n = 6$) of the transferrin binding activity are lost after overnight culture. The recoverability of transferrin receptor protein (as measured by gel scanning of Coomassie Blue-stained gels) is generally better than the recovery of activity. Thus, in a series of three experiments in which 75% of the transferrin receptor was lost during maturation, 30–40% of the receptor protein was recovered in the exosomes but only 12% of the transferrin binding activity. The binding of NBMPR appears to be a more stable activity, and recoveries of ~40% of the lost activity may be found in the vesicles (Table IV). Further investigation is required before it can be concluded whether exosome formation is the major route for shedding of plasma membrane functions during maturation.

Additional investigation is also required to determine the signals which are used for vesicle segregation and externalization. The possibility that the 70-kDa protein associated with the vesicles acts as a signal has been considered (13).

It is also as yet unclear whether each exosome contains a mixture of all externalized components or if a mixed population is externalized. Early attempts to answer this question suggest that vesicles containing transferrin receptor also contain other activities. However, a definitive answer has not been obtained. Although several questions remain unresolved, the data nonetheless argue that significant amounts of plasma membrane-bound activities may be released in vesicular form during the maturation of reticulocytes and that exocytosis may play a significant role in red cell maturation.

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