mones. By analogy only, it has been suggested that the effect of aldosterone in the toad bladder (*14*) and cortisone in HeLa and strain L cells in culture (*15*) may be mediated by 3′,5′-AMP. Hechter *et al.* (*16*) showed that many biosynthetic processes in the rat uterus could be caused by 3′,5′-AMP, but that other nucleotides had similar effects. It also seems unlikely that an increased 3′,5′-AMP concentration is an absolute prerequisite for L-tyrosine-2-oxoglutarate aminotransferase (TAT) induction by glucocorticoids, as the effects of the N^6-2′-*O*-dibutyryl 3′,5′-AMP derivative and hydrocortisone on TAT were additive (*17*), and dexamethasone produced a 10- to 15-fold increase in TAT activity in cultured hepatoma tissue in the absence of detectable cell concentrations of adenyl cyclase (*18*).

In the tissue models described, in which effects of steroid hormones on growth or differentiation have been well characterized, the data suggest that these effects are not mediated by cyclic 3′,5′-AMP. Progesterone, however, caused a delayed and progressive activation of adenyl cyclase and an increase in concentration of tissue 3′,5′-AMP in the chick oviduct. The relation of this observation to the mechanism of progesterone action and the synthesis of avidin is not clear at present.

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Inside-Out Red Cell Membrane Vesicles: Preparation and Purification

Abstract. *Plasma membranes purified from human red cells were converted into small vesicles by disruption in alkaline buffer of low ionic strength. Most of these vesicles were inside-out. The presence of divalent cations prevented this inversion. The inside-out vesicles were separated from right-side-out vesicles by centrifugation to equilibrium in dextran density gradients.*

The plasma membrane deals with two distinctly different compartments, separates them, and mediates between them. Investigation of the biochemical specialization across this membrane has been limited by the inaccessibility of its inner surface to direct examination. We have approached this problem by promoting the budding of red cell plasma membrane ghosts into their cytoplasmic space, thereby generating inside-out vesicles whose outer faces are the cytoplasmic aspects of the parent membranes. Conversely, normally oriented vesicles are formed when a surface membrane buds into the extracellular space. We now present methods for the preparation and purification of inside-out and right-side-out red cell membrane vesicles suitable for the direct comparative analysis of the membrane's two faces.

Ethylenediaminetetraacetate (EDTA) was added to a concentration 0.010*M* to freshly drawn human blood. The red cells were washed three times with cold 0.15*M* NaCl in 0.005*M* sodium phosphate buffer, *p*H 8.0. Each milliliter of packed red cells was lysed by resuspension in 40 ml of cold 0.005*M* phosphate buffer, *p*H 8.0. The plasma membranes were centrifuged at 20,000*g* for 10 minutes to form pellets, which were washed twice more with the same buffer. The resulting pellets were white and were comprised of intact membrane ghosts.

Each pellet was resuspended in 25 ml of cold $5 \times 10^{-4}M$ sodium phosphate buffer, *p*H 8.0, for 1 hour or more, and then sedimented at 10^5g for 30 minutes. As judged by phase contrast light microscopy and thin-section electron microscopy the membranes appeared to be budding spontaneously into the ghost interior (endocytosis), leading to an accumulation of many small vesicles within each parent ghost. Gentle homogenization by pestle or by passage through a 27-gauge hypodermic needle reduced the residual ghost membrane to small vesicles and liberated the entrapped vesicles. The prior addition of divalent cations, such as $1 \times 10^{-4}M$ $MgSO_4$, to the $5 \times 10^{-4}M$ phosphate buffer stabilized the ghosts against spontaneous vesiculation. Homogenization of these stabilized ghosts caused them to vesiculate, primarily by budding into the extracellular space (exocytosis).

Right-side-out and inside-out vesicles were separated on linear gradients (density 1.01 to 1.07 g/cm^3) of Dextran-110 (Pharmacia Fine Chemicals), containing 5×10^{-4} phosphate buffer, *p*H 8.0, and $1 \times 10^{-4}M$ $MgSO_4$ (added to stabilize the vesicles). Homogenates were layered on the gradients and centrifuged to equilibrium at 10^5g for 16 hours. Roughly 90 percent of the membrane protein was recovered in three zones (Fig. 1): (i) a bottom band at the density of intact ghosts, 1.050 to 1.065; (ii) a top band at a density of approximately 1.01; and (iii) a scant zone spreading diffusely between 1.020 and 1.035. [The middle zone becomes enriched at the expense

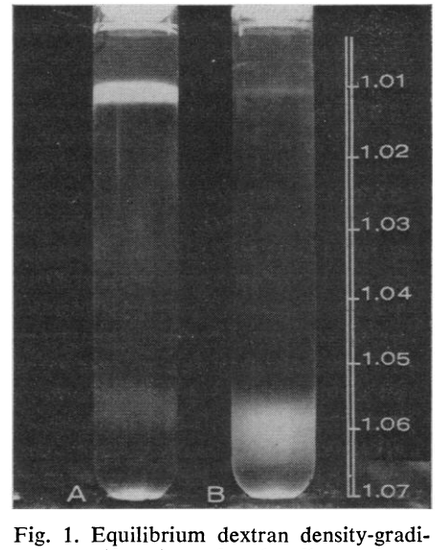

Fig. 1. Equilibrium dextran density-gradient centrifugation of red cell membrane vesicles homogenized in the absence (A) and presence (B) of $1 \times 10^{-4}M$ $MgSO_4$. The scale indicates the approximate density distribution of the gradient.

Table 1. Characteristics of vesicle fractions isolated by dextran gradient centrifugation of membranes homogenized in $5 \times 10^{-4}M$ phosphate buffer (*p*H 8.0) without $MgSO_4$.

Material	Protein* (% input)	Sialic acid†		
		Content‡ (nmole/mg of protein)	Percentage released by	
			Sialidase§	Trypsin‖
Homogenate	100	131		
Top zone (density 1.01–1.035)	62	142	15	12
Bottom band (density 1.050–1.065)	29	166	79	73

* Estimated fluorimetrically (*12*) and standardized against membrane fat-free dry weight. † Measured as in Warren (*13*). ‡Amount released by incubation in $0.1N$ H_2SO_4 at 80°C for 1 hour. §Performed with *Clostridium perfringens* sialidase (E.C. 3.2.1.18) (Worthington Biochemical Corp., 40 μg per milligram of membrane protein) in $0.1M$ tris acetate (*p*H 5.7) for 1 hour at 37°C (*14*). ‖ Performed with trypsin (E.C. 3.4.4.4) (Worthington Biochemical Corp.; 125 μg per milligram of protein) in 5 mM phosphate buffer (*p*H 8.0) for 1 hour at 37°C. The released sialopeptide was separated from the membranes by centrifugation, and its sialic acid was liberated with H_2SO_4 and determined.

of top band material when NH_4HCO_3 or tris(hydroxymethyl)aminomethane-hydrochloride (tris–HCl) is substituted for phosphate buffer throughout the procedure.] About 80 percent of the vesicles formed by homogenization in the presence of $MgSO_4$ were recovered in the bottom band, while 60 percent of the vesicles formed in the absence of $MgSO_4$ were found in the upper two zones (density 1.01 to 1.035).

Almost all of the vesicles equilibrating between the densities of 1.01 and 1.035 were inside-out, according to the following biochemical and morphologic criteria. It has been reported that the sialic acid of red cells is localized on the outer aspect of its plasma membrane (*1*). We reasoned that right-side-out vesicles would present no barrier to the enzymatic hydrolysis of this surface marker, but a sealed, inside-out vesicle would shield the internalized sialic acid from enzyme attack. We therefore characterized the vesicle fractions by the accessibility of their sialic acid to release by sialidase and their sialoprotein to trypsin hydrolysis (*2*). The vesicles in the upper zone had nearly the same specific sialic acid content as the input homogenate and the bottom band, but only 15 percent of this marker was released by sialidase and 12 percent by trypsin (Table 1). In contrast, the material in the bottom band was quite sensitive to hydrolysis by both enzymes.

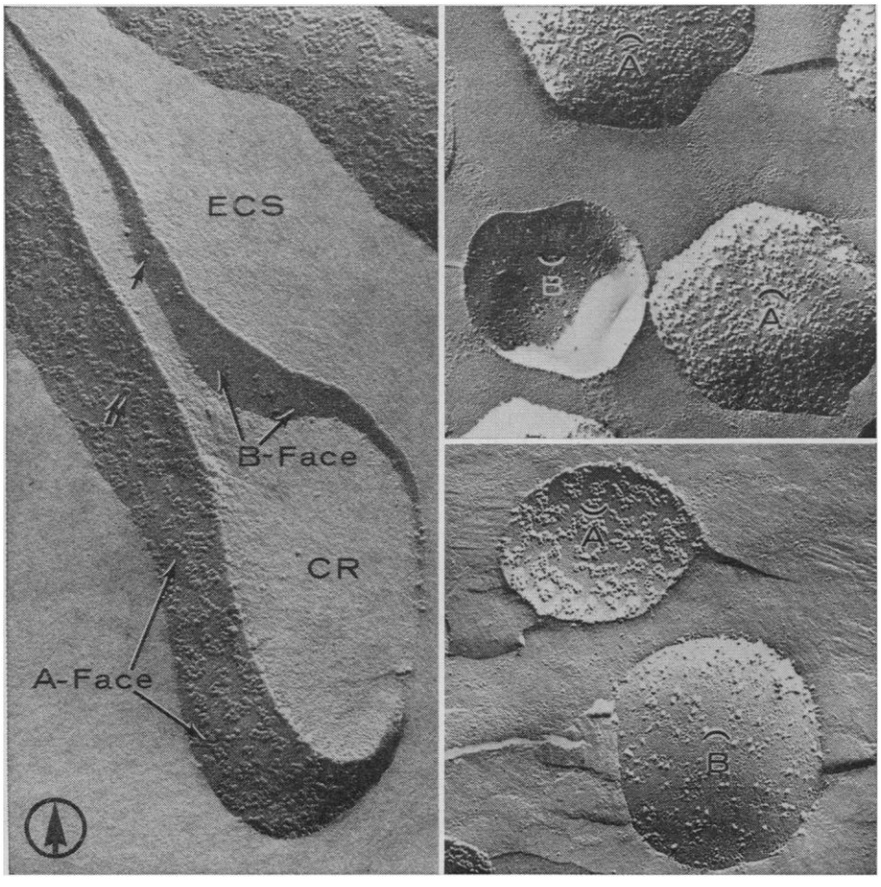

Fig. 2. Electron micrographs of carbon-platinum replicas of freeze-cleaved red cell ghosts and vesicles. (Left) Two distinct surfaces are visualized for ghost membranes. Fracture face A is covered with many 100-Å particles (double arrows) and is oriented toward the extracellular space (*ECS*). Face B has fewer particles and is oriented toward the cytoplasmic region (*CR*). The encircled arrow indicates the direction of carbon-platinum shadowing (× 60,000). (Upper right) Vesicles in the bottom band formed by exocytosis. Face A is convex (Ä) and faces outward while face B is concave (B̈) and faces the vesicle's interior (× 50,000). (Lower right) Vesicles from the top band formed by endocytosis. The usual morphologic relationships are now reversed. Face A is concave (Ä) and face B convex (B̈), indicating membrance inversion (× 60,000).

Morphologic evaluation of the orientation of vesicle membranes was carried out by freeze-cleave electron microscopy (*3*), because it was demonstrated by this technique that the red cell membrane has two readily distinguished surfaces (*4*). The fracture face normally oriented toward the extracellular space is covered by many 100-Å particles (face A; Fig. 2, left), while the surface oriented toward the cell interior bears only 20 to 25 percent as many particles (face B; Fig. 2, left). Although the precise location and function of the membrane-associated particles is unknown, their distribution nevertheless serves as a distinctive indicator of membrane orientation (*5*). Thus, right-side-out vesicles are those which retain the orientation of the parent membrane (Fig. 2, upper right), while the inside-out species show an inversion of these morphologic markers (Fig. 2, lower right).

By these criteria, 98 percent of the top-zone vesicles were inside-out. Evaluation of the bottom band was complicated by the strong tendency of these vesicles to continue inverting during preparation for electron microscopy. Thus, vesicles in the bottom band generated in the presence of $MgSO_4$ appeared 60 to 80 percent right-side-out, while most vesicles in the bottom band formed without $MgSO_4$ were inside-out (*6*).

The morphologic data suggest greater purity of the top (inside-out) fraction than do the biochemical results. Other than reflecting cross-contamination, the sialic acid released from the vesicles in the top zone might represent

(i) the presence of small amounts of this marker on the inner aspect of the red cell membrane or (ii) partial permeability of the vesicles to the enzymes. The slight inaccessibility of the sialic acid in the bottom band to enzymatic release seems related to the observed tendency of the vesicles in the lower band to continue inverting after removal from the dextran gradient. In any case, by both biochemical and morphologic criteria, more than 85 percent of the vesicles in the upper zone are inside-out.

The conditions for vesicle separation were predicted by a theoretical analysis of the factors determining the buoyant equilibrium of vesicles in density gradients (*7*). According to the proposed model, the prevalence of fixed charges on the inner membrane surface is a major determinant of the vesicle density in discerning gradient systems. The outer surface of the red cell membrane is enriched in sialic acid anions. It was reasoned that internalizing this excess of negative charge would produce vesicles of lower density than the parent ghost. The model further indicates that resolution is optimal in gradients of low osmotic activity, such as dextran. Consistent with the prediction, neither sucrose nor glycerol gradients have afforded satisfactory separation of right-side-out and inside-out vesicles.

Another useful theoretical prediction was that the size of the vesicle membrane should not influence the equilibrium density of the vesicle, within its elastic limits. This was borne out by our observation that intact ghosts and vesicles sharing their membrane orientation achieved buoyant equilibrium at the same density (*7*).

Various treatments have been reported to promote inversion of the red cell membrane (*8*) and the inner mitochondrial membrane (*9*). An electrostatic mechanism appears to be involved in our endocytosis procedure. We have looked for alterations other than topological in the inverted vesicles but have detected no significant perturbation in their fine structure, their sialic acid content, and the specific activity of certain enzymes. The protein electrophoretic patterns of the inside-out and right-side-out vesicles were the same, but both species were greatly depleted of a major, high-molecular-weight protein ["spectrin" (*10*)] found in intact ghosts. The release of spectrin to the medium occurred only under conditions of *p*H, ionic strength, divalent cation concentration, temperature, and incubation time that fostered endocytosis (*11*).

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Immunotherapy of Cancer: An Experimental Model in Syngeneic Guinea Pigs

Abstract. *Successful treatment of a solid tumor was accomplished by repeated intradermal injection of living tumor cells.*

Strain-2 guinea pigs immunized by intradermal injection of living transplantable syngeneic tumor cells acquire a state of systemic immunity. This observation has made it possible to design relatively simple experiments with the aim of establishing conditions for effective immunotherapy. By immunotherapy we mean that immunization is started some time after inoculation of a lethal dose of tumor cells. In these experiments, living tumor cells were injected intramuscularly, and 5 days later the first of three weekly intradermal inoculations with live tumor cells was started. The effect of the intradermal immunization was dependent upon the dose of the tumor cells inoculated intramuscularly. At the two lower doses four of 12 animals were free of tumor and survived for more than a year. It was also noted that the number of tumor cells inoculated intramuscularly may affect the growth of the immunizing inoculum which was injected intradermally.

Age-matched male guinea pigs (Sewall-Wright NIH strain-2) were used in these experiments. The induction of primary hepatomas in random-bred guinea pigs by the administration of the water-soluble carcinogen diethylnitrosamine in drinking water has been described (*1*). We have described the antigenic and biologic properties of transplantable solid and ascites tumors derived from primary hepatomas induced in strain-2 guinea pigs by the carcinogen (2–4). In the experiments described in this report solid tumor line 1, an adenocarcinoma (19th transplant generation) and an ascites variant of tumor line 1 (25th to 27th, 39th to 41st, and 60th to 62nd transplant generations) were used. An ascites variant of tumor line 4 (fifth transplant generation) and tissue culture line 7 (18th ascites transplant generation) were used. Inoculation of tumor cells intradermally resulted in immunization of the host (*3*, *5*, *6*). When normal strain-2 guinea pigs were inoculated with 3×10^6 line-1 ascites tumor cells a papule was formed which increased in size for a few days and then regressed. A second intradermal injection of tumor cells produced a