Table 1. The conversion of β-leucine to α-leucine in extracts of *P. vulgaris*. The reaction mixtures (*1*) contained (in 1.99 ml) 100 m*M* triethanolamine-HCl buffer (*p*H 8.5), 0.5 μ*M* flavine adenine dinucleotide, 0.5 m*M* reduced coenzyme A, 0.5 m*M* nicotinamide adenine-dinucleotide, 0.5 m*M* pyridoxal phosphate, 1 mg of crude extract protein, and the indicated amounts of DL-β-leucine. When added, the concentration of coenzyme B_{12} was 3.4 m*M*. Reaction mixtures were incubated under argon at 30°C. After 60 minutes the reaction was stopped by the addition of 0.05 ml of 6*N* HCl followed by 0.10 ml of 10 percent (weight to volume) sodium tungstate. Portions (0.05 ml) were taken to dryness and derivatives were obtained with 0.1 ml of BSTFA and 0.1 ml of acetonitrile as described in the text. Portions (2 to 5 μl) were taken for gas chromatography.

DL-β-leucine added (μmole)	Actual α-leucine formed (nmole)	
	No addition	Coenzyme B_{12} added
0 (Control)	0	0
5	153	379
10	392	825
20	355	697
30	355	1327

material caused by the addition of coenzyme B_{12}. This result prompted an examination of the yield of α-leucine itself. Table 1 shows the results of another experiment in which the addition of coenzyme B_{12} caused a twofold increase in the yield of α-leucine as measured by direct, specific assay.

The effect of the corrin-binding mucoprotein, intrinsic factor, is shown in Table 2. The extract was prepared separately from that shown in Table 1, and its activity was greatly inhibited by the addition of intrinsic factor; the addition of coenzyme B_{12} prevented the effect of intrinsic factor. These results are consistent with the inhibition being caused by the effective removal of the corrinoid cofactor from the leucine 2,3-aminomutase.

The presence of a cobalamin-dependent enzyme in the bean is surprising, since it has long been supposed that plants make no corrin compounds, nor do they require them from exogenous sources. It is interesting to speculate that the apoenzyme is produced by the leguminous plant and the corrin cofactor is supplied by nodular bacteria. *Rhizobium meliloti* and *R. trifolii* have been shown to synthesize vitamin B_{12} 5 to 14 times as well when isolated from pink root nodules as when grown in vitro (*5*). There were no obvious nodules on the seedlings used in these experiments. In another experiment, the beans were surface-sterilized with sodium hypochlorite solution and sprouted on sterile vermiculite kept wet with sterile distilled water. Extracts prepared from these beans were as active as those prepared under nonsterile conditions. Bacterial plate counts from tissue of the aseptically sprouted beans showed this tissue to be free of bacteria while the nonsterile beans had up to 2×10^6 bacterial cells per gram of tissue. The endogenous activity shown in Table 1 may represent either cobalamin-independent activity or the supplying of cofactor to the seed from the nodules of the parent plant. It is possible that the natural cofactor is not a corrin compound, but is one for which added coenzyme B_{12} substitutes. This is a remote probability, for such cofactors have not been described.

It is unlikely that the activity noted in these extracts could have arisen from contaminating bacteria, since the percentage of protein that could be ascribed to bacterial sources was necessarily small. Bacteria grown from the nonsterile beans possessed the cobalamin-dependent mutase activity but did not release any of the activity into the soluble fraction when subjected to homogenization in a Waring blender; sonic disruption was required instead. This indicates that any contaminating bacterial cells would have been removed from the homogenized plant extracts along with the insoluble plant material.

Cobalamin-dependent leucine 2,3-aminomutase activity appears not to be limited to leguminous plants. Seed of the annual ryegrass (*Lolium* spp.) was sprouted in a manner similar to the beans and showed a similar level of activity.

Table 2. The effect of intrinsic factor on the activity of leucine 2,3-aminomutase in bean seeds. Reaction mixtures and conditions were as described in Table 1 except that 20 μmole of DL-β-leucine and 0.9 mg of crude extract protein were added; 0.1 mg of intrinsic factor was added where indicated. Zero time blank of 53 nmole has been subtracted from all values.

Addition	α-Leucine formed (nmole)
None	76
Coenzyme B_{12}	296
Intrinsic factor	14
Coenzyme B_{12} + intrinsic factor	295

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Plasma Membrane: Rapid Isolation and Exposure of the Cytoplasmic Surface by Use of Positively Charged Beads

Abstract. *Erythrocytes were ionically attached to polylysine-coated beads 30 micrometers in diameter. The binding of the cells was so tenacious that lysis or disruption of the attached cells left the beads covered by plasma membranes whose cytoplasmic surface was exposed and accessible for further analysis.*

Many of the properties of a plasma membrane are determined by its composition and its interactions with intracellular elements. Composition can be analyzed once the membrane is isolated and purified; interaction with intracellular elements can be assayed if the protoplasmic surface of the isolated membrane remains freely accessible after purification. We have devised a method of isolating cell membranes which readily yields purified plasma membranes whose protoplasmic surfaces are fully accessible to further probing. This method employs specially coated polyacrylamide or glass beads whose charged surfaces promote the nonspecific adherence of negatively charged cells (*1*). We test and illustrate its use to isolate the plasmalemma of the human erythrocyte because this membrane has been well characterized after conventional isolation procedures.

One milliliter of well-washed, packed human erythrocytes was added to 1 ml of packed polylysine-coated polyacrylamide beads 30 μm in diameter (Fig. 1). Because of their net negative charge (*2*), the erythrocytes, present in excess, adhered to and completely covered the positively charged, polylysine-coated beads. A sucrose-phosphate buffer of low ionic strength (Fig. 1, legend) favored the initial bead-cell interaction and promoted maximal coverage. Attraction between beads and cells was such that many cells bound two beads simultaneously (Fig. 1a), effectively agglutinating them. Be-

cause the cells adhered so firmly to the relatively massive beads, those in contact with two beads were easily stretched (Fig. 1a), ruptured, and lysed (Fig. 1b), even by gentle agitation of the cell-bead mixture. After dilution with 8 ml of sucrose-phosphate buffer, the red erythrocyte-coated beads settled to the bottom of the tube within 5 minutes at 1*g* or within 15 seconds at 900*g*. The supernatant, containing unattached erythrocytes and any debris, was removed by vacuum aspiration. Washing was repeated two or three times, and the cell-covered beads were resuspended in 4 ml of the sucrose-phosphate buffer. The cells that adhered to the beads were disrupted and the beads were washed several times to remove unattached membranes and cell debris. Brief sonication (3 to 10 seconds at 25 watts with the microprobe of a Heat Systems Sonifier model W185) and vigorous swirling (5 to 20 seconds on a vortex mixer) were equally effective in disrupting the cells. Either treatment ruptured nonadherent portions of the cell, leaving on the bead primarily the portions of the plasma membrane that had initially made contact with the bead. After sonication for 2 seconds, nonadherent membrane edges were still visible and delineated the borders of the membrane pieces that remained firmly bound to the beads (Fig. 1c). After sonication for 5 seconds, these edges were removed and the adherent membranes could no longer be resolved by scanning electron microscopy. However, the adherent membranes could be discerned around the beads as a typical trilamellar structure by standard transmission electron microscopy, and intact membranes desorbed from the beads by high-salt treatment retained their usual lamellar structure and asymmetry (Fig. 1d).

Table 1. Phospholipid content of erythrocyte membranes on polylysine-coated polyacrylamide beads before and after sonication for 10 seconds. Ghosts were attached to beads in 20-milliosmolar phosphate buffer, *p*H 7.5. Cells were attached to beads and sonicated as described in the text. Lipids on the beads were extracted overnight in a mixture of chloroform and methanol (2 : 1). Nonlipid material was removed by washing the extract first with ¼ volume of 0.9 percent NaCl and then with ¼ volume of water. After most of the solvent was evaporated in a stream of N_2, lipids were spotted on 0.25-mm silica gel–coated thin-layer plates and developed in a mixture of chloroform, methanol, acetic acid, and water (65 : 43 : 3 : 1). Spots were located with iodine vapor and analyzed for phosphate.

Treatment	Phospholipid (%)			
	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine
Unsonicated ghosts on beads	28	34	31	8
Sonicated membranes on beads	30	34	27	9
Sonicated cells on beads	32	34	20	14

Starting with coated beads and washed erythrocytes, the entire procedure required approximately 20 minutes. One milliliter of polylysine-coated polyacrylamide beads, 30 μm in diameter, provided a surface area of approximately 800 cm^2, sufficient to harvest 300 μg of erythrocyte membrane protein (*3*, *4*). The number of erythrocytes actually bound to the beads was estimated from the amount of hemoglobin the bound cells released on lysis. In a typical experiment, 1 ml of beads bound 1.42×10^9 cells. (Once covered, beads did not attract further cells, and additional erythrocytes could not be bound.) Since one cell has a surface area of 145 μm^2 (*5*), the 1.42×10^9 cells bear 2.1×10^{11} μm^2 of membrane surface. After sonication and washing to remove unattached membranes and cell contents, the beads yielded 350 μg of protein, an amount of membrane protein equivalent to $0.89 \times 10^{11}\mu$m^2 of erythrocyte membrane (*3*, *4*). Thus, as might be expected from the manner in which these hemoglobin-filled erythrocytes contacted the

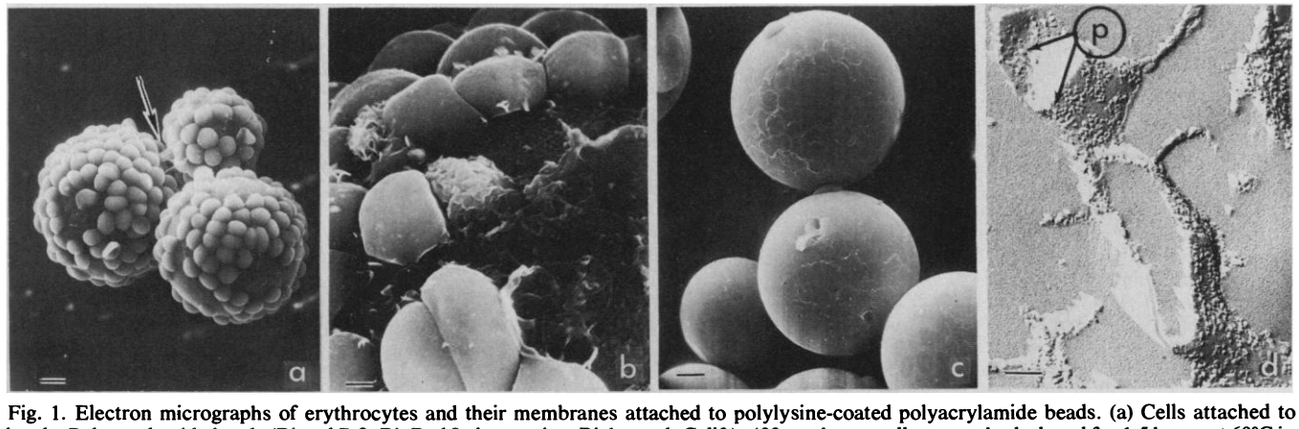


Fig. 1. Electron micrographs of erythrocytes and their membranes attached to polylysine-coated polyacrylamide beads. (a) Cells attached to beads. Polyacrylamide beads (Biogel P-2, BioRad Laboratories, Richmond, Calif.), 400 mesh or smaller, were hydrolyzed for 1.5 hours at 60°C in 0.5*M* Na_2CO_3. After thorough washing in water, 20 ml of wet carboxylated beads were incubated 1.5 hours at 20°C with 6 ml of 1*M* pyridine containing 600 to 800 mg of poly-L-lysine, 185,000 daltons (Sigma Chemical Co., St. Louis, Mo.). Six milliliters of 2.6*M* pyridine-HCl, *p*H 5.1, was then added. After 5 minutes, four 0.5-ml portions of freshly prepared 1*M* 1-ethyl-3,3-(dimethylaminopropyl)-carbodiimide-HCl were added at 1-minute intervals, and the mixture was left on a shaker at 20°C. At the end of 40 hours, the beads were allowed to settle and the supernatant was withdrawn. Ten milliliters of 1*M* NH_4Cl was then added, followed by 1 ml of freshly prepared 1*M* 1-ethyl-3,3-(dimethylaminopropyl)-carbodiimide-HCl. After 1.5 hours of shaking at 20°C the beads were washed once in 4*M* NH_4Cl, four times in water, and finally once in 10 to 20 m*M* tris-HCl, *p*H 7.5. They were stored in this last buffer with 0.02 percent NaN_3 (*13*). Just before use, both the erythrocytes and the beads were washed separately in a 220 m*M* sucrose solution containing 90 milliosmolar sodium phosphate buffer, *p*H 7.5. Unattached erythrocytes were removed by two washes in this sucrose-phosphate buffer. Some of the cells bind to more than one bead (arrow). (b) At higher magnification the collapsed membranes of lysed cells are clearly seen on regions of beads that appear bare at the lower magnification shown in (a). (c) After sonication for 2 seconds most of the cells have ruptured and only the edges of adherent membranes are visible. (d) After sonication for 5 seconds, the adherent membranes could be seen by first desorbing them from the beads. Membranes were desorbed by 10-minute exposure to 0.6*M* K_2HPO_4, *p*H 8.2, 0°C, with periodic agitation and were then freeze-fractured. The desorbed membranes appeared as sheets and vesicles with characteristic intramembrane particles (*P*). The scale is 10 μm for (a) and (c), 2 μm for (b), and 0.1 μm for (d).

bead surface (Fig. 1a), roughly 43 percent of their surface membranes were harvested on the beads (*6*). When erythrocyte ghosts were used rather than whole cells, they flattened themselves on the bead surface and yielded a higher percentage of their surface membrane (Fig. 2).

The protein (Fig. 3) and phospholipid (Table 1) composition of the membranes harvested from beads was indistinguishable from that of erythrocyte ghosts harvested from hypotonic lysates according to the procedure of Dodge *et al.* (*7*). These biochemical data, together with the electron microscopic observations (Fig. 1), indicate that the bead-bound membrane is biochemically and morphologically similar to the unbound membrane. Once on beads, the membranes remained firmly attached even during sonication (Fig. 2). However, solutions of low ionic strength, known to release spectrin from the cytoplasmic surface of the erythrocyte ghosts (*4*, *8*), also released spectrin from the bead-bound membrane (*9*). This was expected because the external surfaces of the intact cells contacted and adhered to the beads. Therefore, after cell rupture and washing, the cytoplasmic surfaces of the bead-harvested membranes faced outward and were freely accessible to components of the suspension medium.

Other experiments have shown that a wide variety of cells and organelles, including *Dictyostelium*, *Amoeba proteus*, rod outer segments, and mitochondria, can be bound to the beads (*10*). Although the exact procedures required to effect successful adherence vary, experiments with isolated mitochondria, HeLa cells and L cells (*10*) have already shown that the beads can be used as a rapid method to isolate and selectively enrich the plasmalemma or outer membrane of a variety of cells or organelles. The most important requirement for purification is that coverage of the beads be complete before any of the cells lyse or are ruptured.

Others have proposed the use of various surface ligands as density-perturbing agents to aid in membrane isolation (*11*), as well as the use of polylysine-treated surfaces to anchor cells for electron microscopy (*12*). Our technique, using relatively large, covalently coated beads, represents a novel extension and combination of these proposals. Because membranes, once bound, remain firmly attached to the beads (Fig. 2) and have their cytoplasmic surface exposed to the solution, the bead isolation technique provides not only a rapid method for membrane isolation but a tool that should be ideal for biochemical experiments designed to probe the interaction between cytoplasmic components and the cytoplasmic surface of the cell membrane.

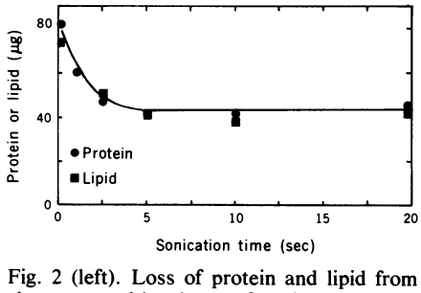
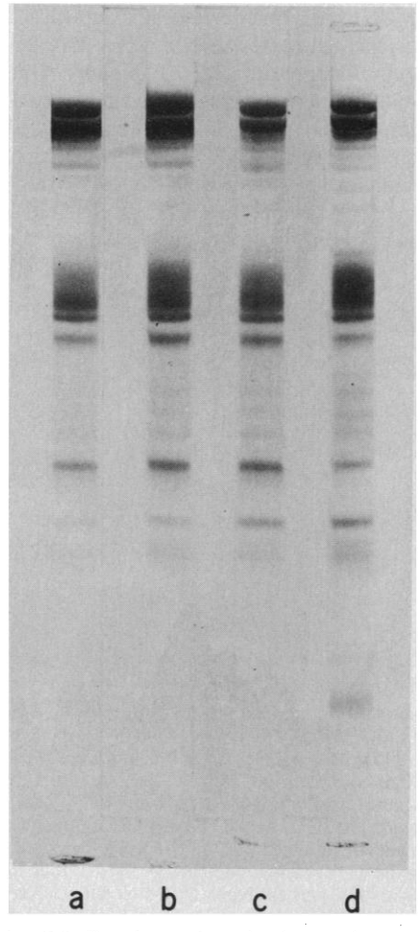


Fig. 2 (left). Loss of protein and lipid from ghost-covered beads as a function of sonication time. Packed ghosts (100 μl) (*7*) in 20 milliosmolar sodium phosphate buffer, *p*H 7.5, were added to 150 μl of packed polylysine-coated polyacrylamide beads that had been washed in the buffer. All of the measurements represent the amount of material remaining on the beads after sonication for the time indicated followed by three washes with the buffer. Ghosts, rather than cells, were used for these experiments so that the initial protein and lipid content represent only membrane-associated material. Fig. 3 (right). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of membrane proteins from ghosts or erythrocytes attached to polylysine-coated polyacrylamide beads. Cells or ghosts were attached to the beads as described in the text. (a) Unattached ghosts, control; (b) ghosts attached to beads; (c) membranes remaining on beads after attached ghosts [as in (b)] were disrupted by sonication for 8 seconds and the debris was washed away; and (d) membrane remaining on beads after attached erythrocytes were disrupted by sonication for 8 seconds and the debris was washed away. Attached erythrocytes were lysed in 20 volumes of 20 milliosmolar phosphate buffer, *p*H 7.5, before sonication and washing (*14*). For electrophoresis, the membranes associated with 250 μl of packed beads were solubilized by two washes in a solution containing 1 percent sodium dodecyl sulfate and 4 percent 2-mercaptoethanol. The protein was concentrated by combining these washes in an Amicon B-15 concentrator (molecular weight cutoff = 15,000). A 50-μl sample of this concentrate or of a similar control solution of ghosts membranes (*7*) was used for electrophoresis (*15*).



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