

# ISOLATION AND PROPERTIES OF THE PLASMA MEMBRANE OF KB CELLS

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## ABSTRACT

Plasma membranes from KB cells were isolated by the method of latex bead ingestion and were compared with those obtained by the  $ZnCl_2$  method. Optimal conditions for bead uptake and the isolation procedure employing discontinuous sucrose gradient centrifugation are described. All steps of preparative procedure were monitored by electron microscopy and specific enzyme activities. The plasma membrane fraction obtained by both methods is characterized by the presence of the  $Na^+ + K^+$ -activated ATPase and 5'-nucleotidase, and contains NADPH-cytochrome *c* reductase and cytochrome  $b_6$ . The latter two enzymes are also present in lower concentrations in the microsomal fraction. Unlike microsomes which are devoid of the  $Na^+ + K^+$ -activated ATPase and which contain only traces of 5'-nucleotidase activity, the plasma membrane fraction contains only trace amounts of the rotenone-insensitive NADH-cytochrome *c* reductase but no cytochrome P-450, both of which are mainly microsomal components. Morphologically the plasma membrane fraction isolated by the latex bead method is composed of vesicles of 0.1–0.3  $\mu m$  in diameter. On the basis of the biochemical and morphological criteria presented, it is concluded that the plasma membrane fraction isolated by the above methods are of high degree of purity.

## INTRODUCTION

KB cells have been extensively used in our laboratory for the study of active amino acid transport, and the role of membrane phosphoinositides on various plasma membrane functions (1–5). It became apparent to us that the availability of a pure preparation of plasma membranes of KB cells would enable further in depth studies of the above phenomena. To this end we undertook the isolation of plasma membranes from KB cells utilizing various known methods. In our experience the most successful method for the isolation of the plasma membrane of KB cells was that based on the ingestion of polystyrene latex beads as originally developed by Wetzel and Korn (6) and by Heine and Schnaitman (7) for the iso-

lation of plasma membranes from *Acanthamoeba* and L cells, respectively. Another method also employed was a modification of the  $ZnCl_2$  method described by Warren and Glick (8) which makes no use of beads. The material presented in this paper deals with the methods of isolation and some of the enzymatic and morphological characteristics of the plasma membrane of KB cells.

## MATERIALS AND METHODS

### *Culture Conditions*

The KB cells used in these studies (certified line no. 17) were obtained from the American Type Culture Collection. They were grown in suspension cul-

ture in Eagle's minimal essential medium with 10% horse serum (9). The cell density was maintained between  $2 \times 10^5$  and  $5 \times 10^5$  cells per ml.

### Various Media

For bead uptake the cells were incubated in Krebs-Ringer bicarbonate buffer (KRB) of the following composition, in mM concentrations: KCl, 4.74;  $\text{CaCl}_2$ , 2.53;  $\text{KH}_2\text{PO}_4$ , 1.19;  $\text{MgSO}_4$ , 1.19; NaCl, 118.5;  $\text{NaHCO}_3$ , 24.9; and glucose, 2.0. The solution, equilibrated with a mixture of 95%  $\text{O}_2$ -5%  $\text{CO}_2$ , had a pH of 7.4.

The phosphate buffered saline (PBS) contained, in mM concentrations: sucrose, 250; EDTA, 1.0; NaCl, 137; KCl, 2.7;  $\text{Na}_2\text{HPO}_4$ , 8.1;  $\text{KH}_2\text{PO}_4$ , 1.47; and 0.5 g of bovine serum albumin per liter. The pH was 7.4.

The sucrose solutions used in the isolation of plasma membranes by the latex bead method were made in Tris-EDTA buffer (0.02 M Tris, pH 6.8, containing 1 mM EDTA), whereas those used in the  $\text{ZnCl}_2$  method were made in distilled water. The concentrations are based on wt/wt.

### Materials

The following materials were purchased from Sigma Chemical Co. (St. Louis, Mo.): NADPH, NADH, cytochrome *c* from horse heart type III, ATP, AMP, *p*-nitrophenylphosphate, rotenone, Tris, and EDTA. Polystyrene latex beads of 1.01  $\mu\text{m}$  in diameter were purchased as a 10% aqueous suspension from Dow Chemical (Midland, Mich.), and [ $1\text{-}^{14}\text{C}$ ] $\alpha$ -aminoisobutyric acid from Calbiochem (San Diego, Calif.). All other materials were of reagent grade.

### Enzymatic Assays

The following enzymatic activities were assayed according to the methods indicated:  $\text{Na}^+ + \text{K}^+$ -activated ATPase (10), 5'-nucleotidase (11), NADPH-cytochrome *c* reductase (12), NADH-cytochrome *c* reductase and rotenone-insensitive NADH-cytochrome *c* reductase (13), succinate-cytochrome *c* reductase (14), cytochrome *c* oxidase (15), acid phosphatase using *p*-nitrophenylphosphate as substrate (16), phosphoprotein phosphatase (17), and cathepsins (18).

The concentrations of cytochromes *aa*<sub>3</sub>, *b*, *c*, and *c*<sub>1</sub> were calculated from difference spectra (reduced minus oxidized) according to the method of Williams (19). Cytochrome *b*<sub>5</sub> was calculated from difference spectra (reduced minus oxidized) using an extinction coefficient of  $20.0 \text{ mM}^{-1} \text{ cm}^{-1}$  at 556 minus 575 nm (20). Cytochrome P-450 was calculated from difference spectra (reduced CO minus

reduced) using an extinction coefficient of  $91 \text{ mM}^{-1} \text{ cm}^{-1}$  at 450 minus 490 nm (21).

### Kinetics of Transport of $\alpha$ -Aminoisobutyric Acid

These were performed according to methods published earlier (2, 3).

### Analytical Methods

Inorganic phosphate was determined by the method of Fiske and SubbaRow (22), and protein by the method of Lowry et al. (23). Radioactive samples were counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) using as scintillation fluid a mixture of 792 ml dioxane, 30 ml toluene, 4.5 g 2,5-diphenyloxazole (PPO), and 90 g naphthalene. The latex beads are completely soluble in this mixture and do not affect the counting efficiency.

### Isolation of Plasma Membranes by the Latex Bead Method

**PREPARATION OF LATEX BEADS:** 10 ml of a 10% suspension of latex beads were centrifuged at 6000 *g* for 15 min, and were suspended in 4.0 ml of KRB medium. In order to break up bead clumps the suspension was sonicated twice for 15 s each with a 30-s interval, using a Branson model W-185C sonifier (Branson Instruments Co., Stamford, Conn.) at an output setting of 4.

**UPTAKE OF BEADS BY KB CELLS:** The harvested cells ( $5 \times 10^8$ ) were washed with 20 vol of ice-cold 0.15 M NaCl and were resuspended in 10 ml of KRB medium. To the cell suspension 4 ml of beads prepared as described above were added, and the mixture was incubated in a 50-ml Erlenmeyer flask for 30 min at 37°C with gentle agitation. At the end of this period 20 ml of 0.3 M sucrose were added and the mixture was centrifuged at 400 *g* for 4 min. The excess beads were removed by washing the cells five times with 20 ml each of 0.3 M sucrose, followed by a final wash with 30 ml of PBS.

**ISOLATION OF MEMBRANE-ENCLOSED BEADS:** The cell pellet from the previous step was suspended in 10 ml of PBS and was homogenized in a Dounce, type B, homogenizer employing 25 full strokes. The extent of cell breakage, which was complete, was monitored by phase microscopy. The cell homogenate was mixed with an equal volume of 60% sucrose, and 6-7 ml of the resulting mixture were placed at the bottom of each of three cellulose nitrate tubes of the SW-25.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and were then overlaid with approximately 6-7 ml each of 25, 20, and 10% sucrose. The tubes were centrifuged at 79,000

*g* for 90 min. The membrane-enclosed beads concentrated at the interface between the 10 and 20% sucrose layers were carefully collected after removing the overlying sucrose solution. The suspension of the membrane-enclosed beads was diluted with an equal volume of the Tris-EDTA buffer and was centrifuged at 30,000 *g* for 15 min. The resulting pellet was resuspended in 6–7 ml of 30% sucrose and was centrifuged once more in the discontinuous sucrose gradient described above. The band containing the membrane-enclosed beads was recovered from the gradient, and after dilution with an equal volume of the Tris-EDTA buffer it was centrifuged at 30,000 *g* for 15 min. The pelleted material was washed with 20 ml of 10% sucrose and was finally suspended in 1–3 ml of 10% sucrose or 0.02 M Tris, pH 6.8.

**ISOLATION OF MEMBRANES:** The suspension of the membrane-coated beads from the previous step was sonicated for twelve 10-s pulses in an ice bath in the Branson Sonifier (model W-185C) at an output setting of 4 with 1-min intervals in between each pulse. The temperature of the suspension did not rise above 5°C. The sonicated sample was centrifuged at 15,000 *g* for 20 min to remove the latex beads. The supernatant fluid contains the membranes which can be pelleted by centrifugation at 100,000 *g* for 1 h in the Spinco rotor no. 40.

#### *Isolation of Plasma Membranes by the ZnCl<sub>2</sub> Method*

This procedure is a modification of the ZnCl<sub>2</sub> method described by Warren and Glick (8). KB cells ( $5 \times 10^8$ ) were harvested by centrifugation at 400 *g* for 5 min, washed twice with 50 vol of cold 0.15 M NaCl, and resuspended in cold NaCl solution. To 1 ml of this suspension 3 ml of 0.001 M ZnCl<sub>2</sub> were added and the mixture was incubated at 25°C for exactly 6 min. The incubation was terminated by chilling in ice, and the cells were homogenized manually in a Dounce homogenizer (type B) at 4°C using about 150 strokes. The cell breakage, monitored by phase microscopy, was 80–90%. The homogenization step was repeated in the same manner until all of the original cell suspension was homogenized. The combined homogenate (40 ml) was mixed with an equal volume of 60% sucrose, and 40-ml portions of the resulting mixture were layered over discontinuous sucrose gradients consisting of 30 ml each of 55, 50, 48, 45, 43, 40, and 35% sucrose solutions in 250-ml polycarbonate centrifuge bottles. The gradients were centrifuged at 1400 *g* for 30 min in the Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Newton, Conn.) using the HS-4 rotor. The membrane-enriched fraction recovered from the 43 and 45% sucrose layers of each gradient contained mainly whole membrane envelopes, large membrane frag-

ments, and some unidentified particulate matter. These fractions were combined to give the "principal membrane fraction." In order to recover more membranes from the sucrose gradient the fractions recovered between 45 and 50% and between 40 and 43% sucrose were diluted with water to 35% sucrose and centrifuged separately at 5000 *g* for 12 min. The resulting pellets were suspended in 2 ml of 10% sucrose and each suspension was further purified on a discontinuous sucrose gradient consisting of 2 ml each of 45, 40, 35, and 30% sucrose. The gradients were centrifuged at 250 *g* for 30 min, and the membrane-enriched fractions recovered from the 35 and 40% sucrose layers were combined and centrifuged at 5000 *g* for 12 min. The pellet was suspended in 1 ml of 35% sucrose and was combined with the principal membrane fraction obtained from the first sucrose gradient (3.6 ml total volume). The combined membrane fraction was further purified by layering 0.6-ml aliquots over each of six similar sucrose gradients having the following composition: 0.5 ml of each of 65, 60, and 40% sucrose, and 1 ml each of 55, 50, and 45% sucrose. The gradients were centrifuged at 50,000 *g* for 1 h in the SW50-1 rotor (Beckman Instruments, Inc., Spinco Div.). The membranes recovered from the 55% sucrose layer of the 6 gradients were diluted with water to 35% sucrose and were pelleted by centrifugation at 6000 *g* for 12 min. After the membranes were washed in 10 ml of 0.25 M sucrose they were resuspended in 3 ml of the same sucrose solution and were used promptly in the various assays.

#### *Isolation of Mitochondria and Microsomes from KB Cells*

The method is a modification of the centrifugal fractionation procedure of Schneider (24) using 0.25 M sucrose. All operations were performed at 0–4°C. Solutions: 0.25 M sucrose; 0.25 M sucrose in 0.01 M triethanolamine, 1 mM EDTA, of pH 7.2 (STE); imidazole-glycylglycine buffer, 0.015 M, pH 7.4.

**PREPARATION OF CELL HOMOGENATE:** The cells ( $5 \times 10^8$ ) were harvested by centrifugation at 400 *g* for 4 min, washed with 0.15 M NaCl, and resuspended in 16 ml of 0.25 M sucrose. They were then homogenized in a Dounce homogenizer employing about 150 full strokes with a B pestle. Cell breakage was monitored by phase microscopy and was usually greater than 95%.

**ISOLATION OF MITOCHONDRIA:** The homogenate was centrifuged twice at 700 *g* for 10 min to remove nuclei and unbroken cells. The supernatant fluid was centrifuged in the HB-4 Sorvall swinging bucket at 8000 *g* for 20 min and the supernatant was centrifuged once more at this speed. The two pellets

thus obtained are the crude mitochondrial fraction whereas the supernatant fluid is the *postmitochondrial fraction* containing the microsomes.

The two pellets comprising the crude mitochondrial fraction were overlaid with 2 ml of STE and the "fluffy top layer" was removed by gentle agitation. The two pellets were combined by suspending them in 20 ml of STE and were centrifuged at 8000 *g* for 20 min. The pellet was washed twice more in the same manner and each time any fluffy layer was removed as described above. The washed pellet was suspended in 2–3 ml of imidazole-glycylglycine buffer to give the *final mitochondrial fraction*.

**ISOLATION OF MICROSOMES:** The postmitochondrial fraction obtained in the previous step was centrifuged twice at 15,000 *g* for 15 min in the HB-4 rotor, and the small amount of precipitated material was discarded. The microsomes were precipitated by centrifuging the supernatant fluid at 100,000 *g* for 90 min in the no. 40 rotor of the Spinco centrifuge. The pelleted microsomes were resuspended in 20 ml of STE and were centrifuged again as above. The resulting pellet was suspended in 4–5 ml of imidazole-glycylglycine buffer to give the *final microsomal fraction*.

### Electron Microscopy

After two washes in 0.15 M NaCl at 4°C, the cells were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1–3 h at 4°C. The fixed cells were centrifuged for 10 min in a desk top centrifuge and postfixed in 1–2% osmium tetroxide in 0.1 M cacodylate buffer for another hour at 4°C. Dehydration was carried out in ethanol and cells were embedded in Araldite omitting dehydration with propylene oxide which extracts the ingested latex beads. Sections, cut on a LKB microtome (LKB Instruments, Inc., Rockville, Md.) with diamond knives, were stained in uranyl acetate and lead citrate according to standard procedures. A similar procedure was followed with subcellular fractions, but in order to obtain packing of the pellet, centrifugations were carried out in a Sorvall RC2-B centrifuge at 5,000–10,000 rpm. in a swinging bucket rotor. Some membrane preparations were stained in block with 3% aqueous uranyl acetate. Electronmicrographs were taken with an Elmiskop IA electron microscope.

## RESULTS

### Kinetics of Bead Uptake by KB Cells

In a number of preliminary experiments the optimal conditions for maximal bead uptake were determined with respect to the composition of the incubation medium, the time and temperature of incubation, the size of the latex beads, and the

ratio of number of beads per cell. The amount of beads ingested was quantified as follows: At the end of the incubation excess beads were removed as described in the section on the "Isolation of Plasma Membranes," and the cell pellet was extracted with dioxane (2 ml/ $1 \times 10^6$  cells) for 4 h at room temperature. This time was found sufficient for extracting all of the ingested beads. After removing insoluble material by centrifugation the absorbance of the supernatant fluid at 259 nm was used to calculate the amount of polystyrene beads ingested (25). Similar incubations without beads were used as controls.

Two different incubation media were tried, the growth medium and Krebs-Ringer bicarbonate. Among various size beads the following were tried: 0.795, 0.81, 1.01, and 1.10  $\mu\text{m}$  in diameter. The ratio of number of beads per cell was varied from 500 to 4000. The results of these experiments showed that bead uptake was maximal when the cells were incubated with beads of 1.01  $\mu\text{m}$  in diameter in KRB medium at a bead multiplicity of 2000–4000 beads per cell. The time-course of bead uptake is shown in Fig. 1. The uptake of beads was linear with time for the first 15 min and was complete by 30 min. The rate of bead uptake was proportional to the cell concentration up to

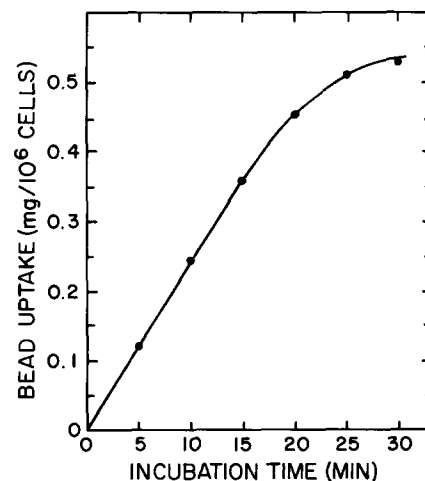


FIGURE 1 The uptake of latex beads by KB cells. 20 ml of KRB medium containing, per milliliter,  $1 \times 10^7$  cells and 16 mg of latex beads were incubated at 37°C. At the times indicated aliquots were removed and processed as described in the text for the determination of the amount of latex beads ingested. Similar incubations without beads served as cell blanks. Each point represents the average of triplicate samples after correction for the corresponding cell blanks.

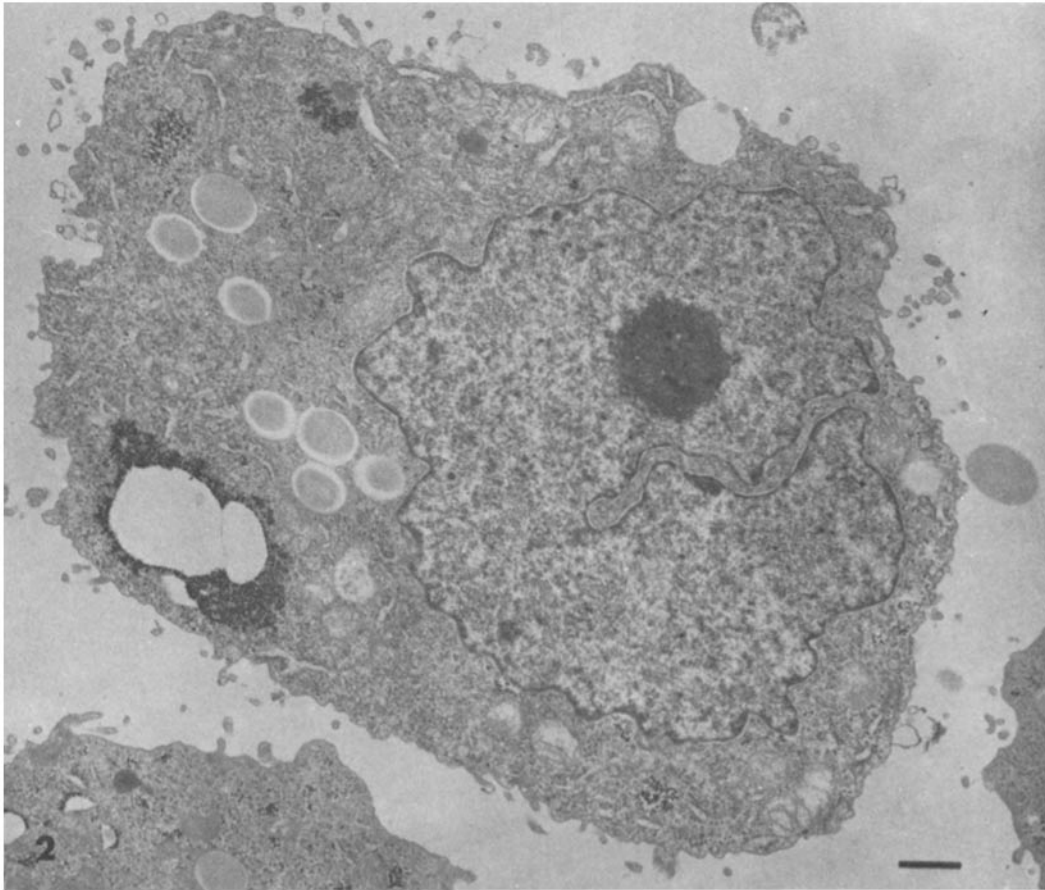
$1.8 \times 10^7$  cells per ml. Lowering the incubation temperature to  $0^\circ\text{C}$  abolished bead uptake.

### *Morphological Studies*

Cells incubated for 30 min with latex beads showed many intracytoplasmic round or oval beads with an average cross-section diameter of  $0.7\text{--}1\ \mu\text{m}$ . The beads appeared randomly scattered throughout the cytoplasm and were surrounded by a membrane  $75\text{--}100\ \text{\AA}$  thick; the profile of the membrane surrounding the bead was usually fuzzy but occasionally a sharp trilaminar profile was easily distinguishable (Fig. 3). The cells containing the beads showed no unusual alterations, with the exception of large numbers of glycogen particles around individual or "fused" beads (Fig. 2). Although cisterns of the rough endoplasmic reticulum were frequently close to the ingested beads,

there was no evidence of continuity or fusion of the membrane of the rough ER with the limiting membrane of the bead (Fig. 3). A few beads were seen in the cytoplasm of cells in mitosis.

Electron microscope study of isolated membrane-enclosed beads showed that the amount of membranous material, not surrounding, or bound onto the bead surface was negligible (Fig. 6). The membranous material observed in between the membrane-coated beads most likely represents membrane detached from the beads. The thickness of the membrane surrounding the beads was  $75\text{--}100\ \text{\AA}$ ; a trilaminar structure ( $75\text{--}100\ \text{\AA}$  wide) was occasionally identifiable (Figs. 3-5). The membrane surrounding the isolated beads was continuous, but the outer lamella (corresponding to the inner surface of the plasma membrane) was usually ruffled (Figs. 4, 5). The thickness of the trilaminar membrane varied from  $90$  to  $160\ \text{\AA}$ , and



**FIGURE 2** KB cell incubated for 30 min with latex beads. Lower left: confluent beads surrounded by glycogen granules. Bar,  $1\ \mu\text{m}$ .  $\times 8,000$ .

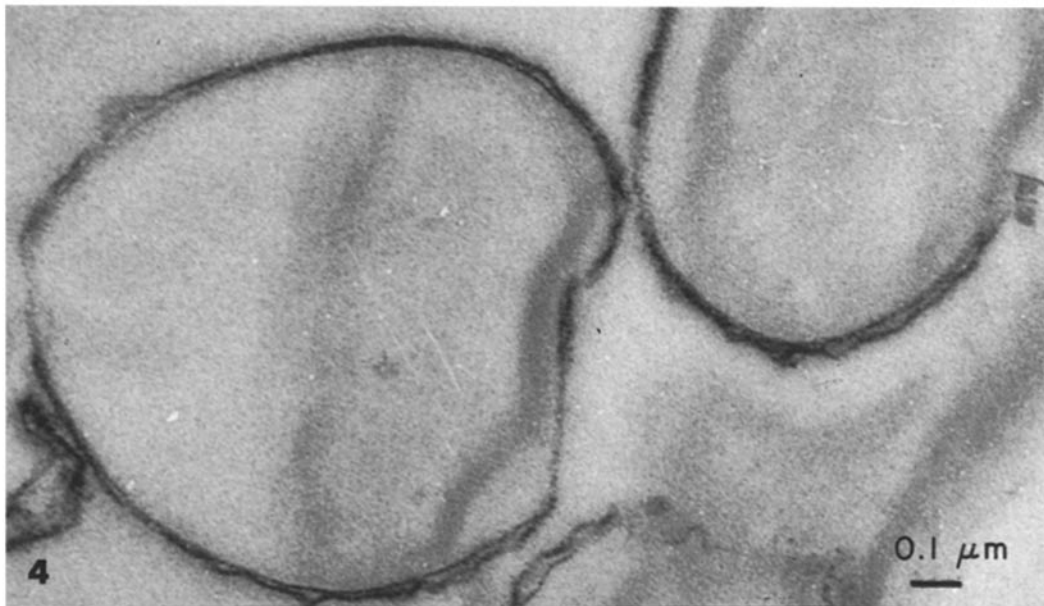
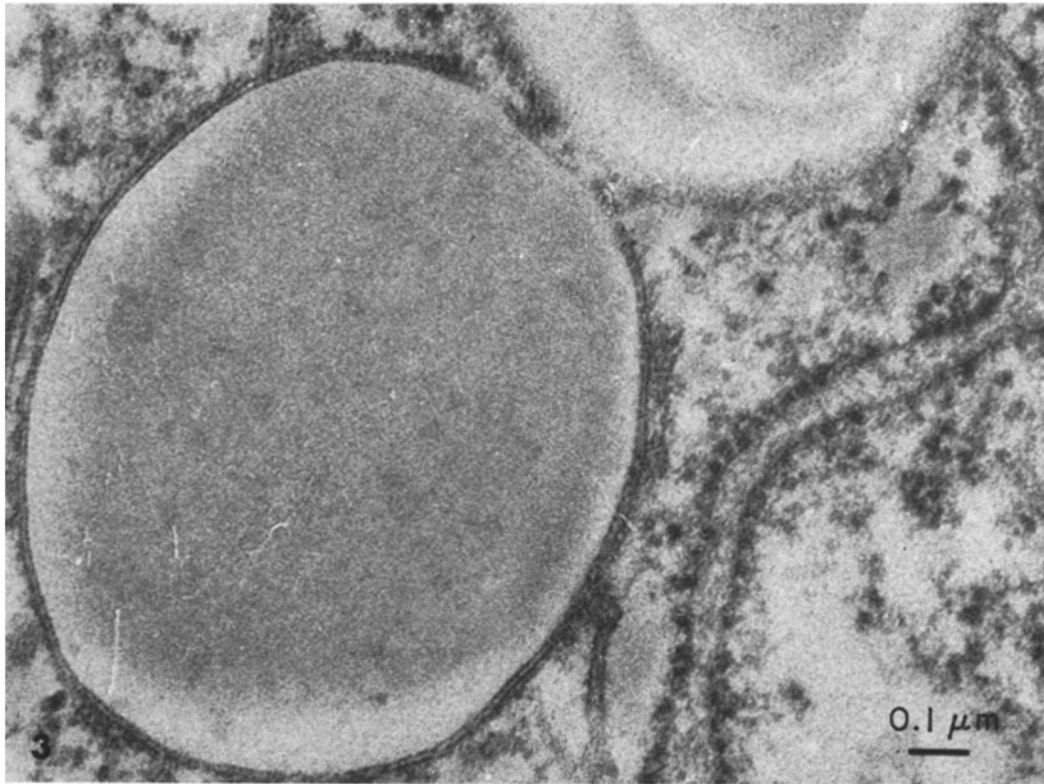


FIGURE 3 Trilaminar membrane (unit membrane), 80-100 Å thick, surrounding intracellular bead. Bar, 0.1 μm. × 80,000.

FIGURE 4 Isolated beads with surrounding membrane which occasionally shows trilaminar (unit membrane) structure. Bar, 0.1 μm. × 65,000.

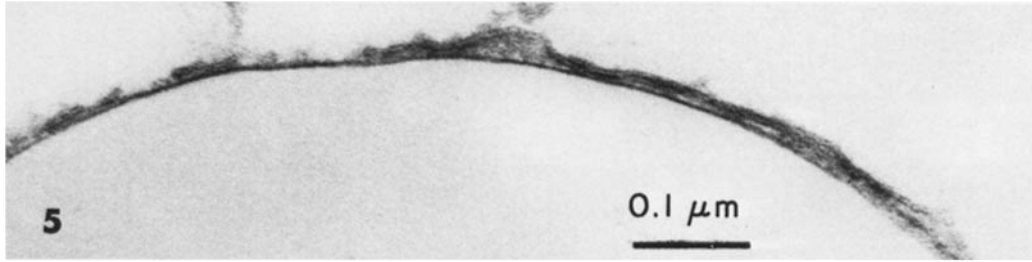


FIGURE 5 Higher magnification of a segment of the membrane surrounding an isolated bead. The outer lamella can be resolved to a trilaminar structure 45–50 Å wide. Note ruffled appearance of outer lamella and osmiophilic material in the space between inner and outer lamellae. Bar, 0.1 μm.  $\times 155,000$ .

occasionally the outer lamella could be resolved to a triple-layered structure 45–50 Å wide (Fig. 5). The interspace between the inner and outer lamellae was electron dense suggesting that the ruffled appearance of the outer lamella is not an artifact but possibly the result of tangential sectioning.

**PLASMA MEMBRANE PREPARATION:** Numerous single membrane-bound vesicles of round, oval, and occasionally tubular profile were seen in this preparation (Figs. 7, 8); the largest diameter of these structures was 0.1–0.3 μm, but occasionally larger profiles 0.5 μm in diameter were seen. Mitochondria, free ribosomes, ribosome-studded membrane, glycogen granules, or other identifiable components were not found in this fraction. The vesicles were empty or appeared to contain an amorphous material which probably was due to tangential sectioning. A trilaminar structure of the limiting membrane could be resolved occasionally.

**MICROSOMAL PREPARATION:** This preparation (Fig. 9) was characterized by numerous smooth vesicles (0.3–0.6 μm) which were somewhat larger than those of the plasma membrane preparation; most of these vesicles had no ribosomes; with the exception of many glycogen granules, this preparation was clean of other cytoplasmic constituents. Despite the proximity of glycogen granules to the ingested beads (Fig. 2) glycogen granules were absent from the plasma membrane preparation.

### Biochemical Studies

**CONCENTRATION OF VARIOUS ENZYME ACTIVITIES IN MITOCHONDRIA, MICROSOMES, AND THE PLASMA MEMBRANE OF KB CELLS:** Table I summarizes the specific activities of various enzymes in these fractions and in the unfractionated cell homogenate. The values repre-

sent the averages from three to five separate experiments. Maximal deviation of the individual values from the average was  $\pm 10\%$ .

The specific activities of the *sodium pump* ( $\text{Na}^+ + \text{K}^+$ -activated ATPase) and *5'-nucleotidase* are highest in the plasma membranes. These activities were concentrated in this fraction 21–23-fold over the concentration in cell homogenates. Microsomes are devoid of sodium pump and only trace amounts of this activity are present in the mitochondria, reflecting, very likely, marginal contamination by plasma membranes. The specific activity of 5'-nucleotidase in the microsomal and mitochondrial fractions was not very different from that of cell homogenates. It is concluded, therefore, that these two enzymes are unique components of the plasma membrane of KB cells.

The *NADPH-cytochrome c* reductase considered to be a microsomal system (26) and *cytochrome b<sub>5</sub>* reported to be present in both microsomes (27) and the outer mitochondrial membrane of rat liver cells (28) are also present in the plasma membrane. The specific activities in the latter fraction are, respectively, 1.6 and 2.0 times greater than the corresponding values of the microsomes. Vassiletz et al. also reported the presence of *NADPH-cytochrome c* reductase and *cytochrome b<sub>5</sub>* in the plasma membranes of liver cells (29). However, in this case the specific activities in the plasma membrane were 40% and 10%, respectively, of the corresponding values of the microsomal fraction.

*Cytochrome P-450* present in the microsomes was not detected in the plasma membranes, in agreement with similar findings with the plasma membranes of liver cells (29).

The *rotenone-insensitive NADH-cytochrome c reductase* had the highest specific activity in the microsomes as compared with the mitochondria. It was

virtually absent in the plasma membranes (1.7% of the specific activity found in microsomes). This activity has been also found in the outer mitochondrial membrane of rat liver cells (28).

*The mitochondrial enzymes* cytochrome oxidase and cytochromes *aa<sub>3</sub>*, *c<sub>1</sub>*, *c*, and *b* were not found in the microsomal or the plasma membranes isolated by the latex bead method. The presence of

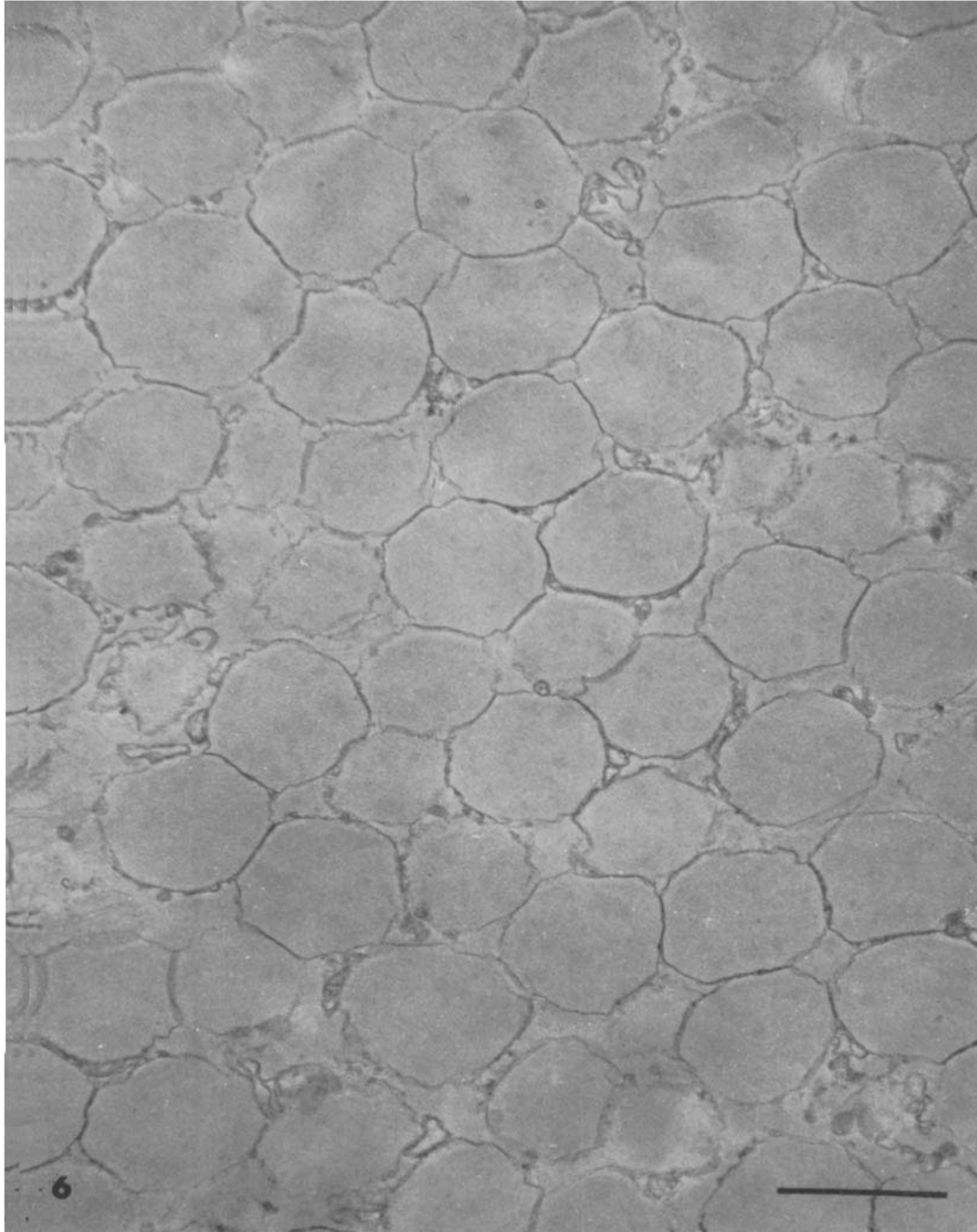


FIGURE 6 Isolated membrane-enclosed beads. Bar, 1  $\mu$ m.  $\times$  24,000.



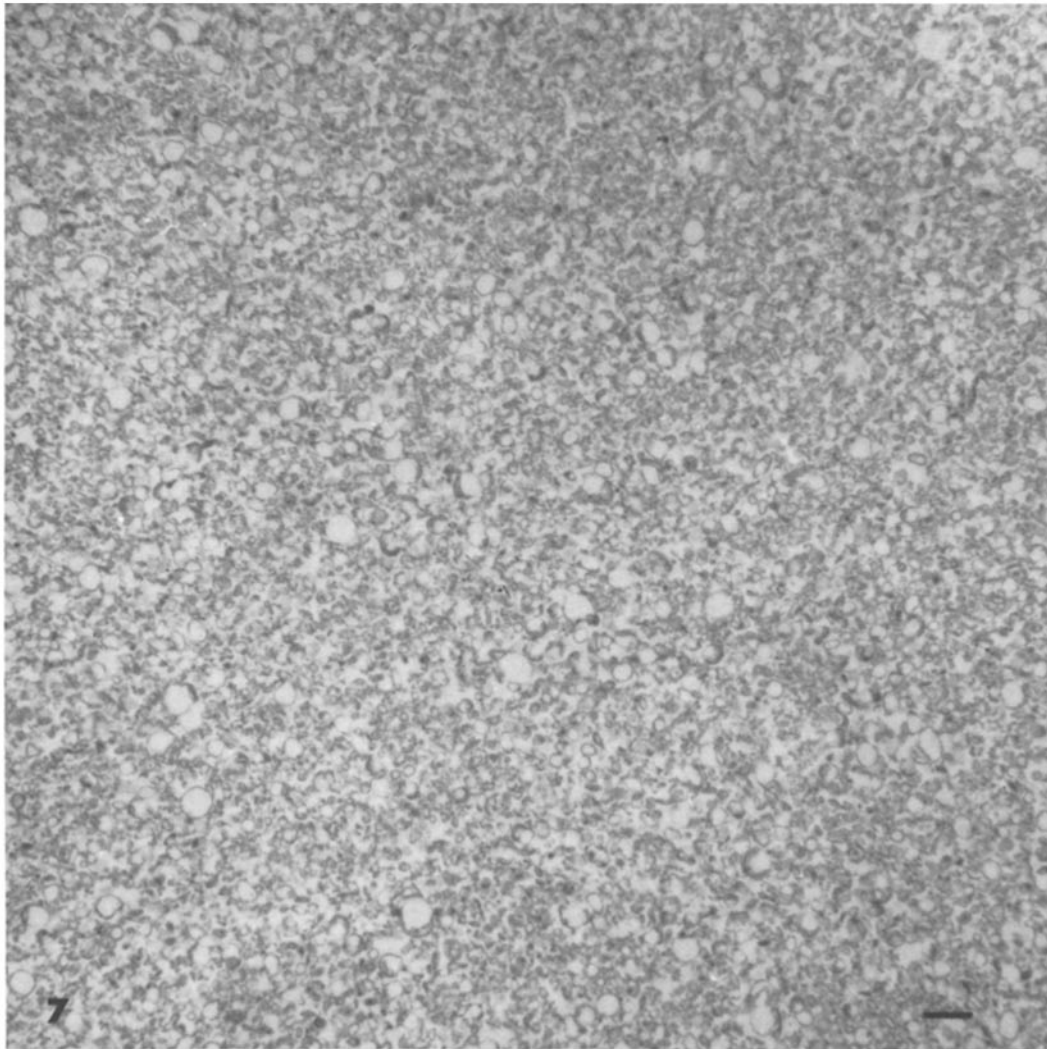


FIGURE 7 Plasma membrane preparation isolated by the latex bead method. Note homogeneity of fraction. Bar, 1  $\mu\text{m}$ .  $\times$  6,000.

these cytochromes was determined by difference spectrophotometry (reduced minus oxidized spectra) at the temperature of liquid nitrogen under conditions which could have detected amounts equivalent to 1% of the corresponding specific activities of the mitochondrial fraction. Succinate-cytochrome *c* reductase activity was detected in these membranes but its specific activity was 1.7% of that of the mitochondrial fraction.

The plasma membranes isolated by the  $\text{ZnCl}_2$  method exhibited traces of cytochrome oxidase activity and low levels of succinate-cytochrome *c* reductase the specific activity of which was 3.2%

of that of the mitochondrial fraction. They also contained trace amounts of cytochrome *c*<sub>1</sub> at a concentration equivalent to 1% of that of mitochondria, but they were free of cytochromes *b* and *c*.

The *acid phosphatase* activity used as a marker for the lysosomal fraction was found in all the fractions at approximately similar specific activity which was comparable to that of the unfractionated homogenate. Other lysosomal enzymes, i.e. cathepsins A, B, C, and D, and phosphoprotein phosphatases could not be detected in the homogenates or any of the other fractions.

KINETICS OF TRANSPORT OF  $\alpha$ -AMINOISO-

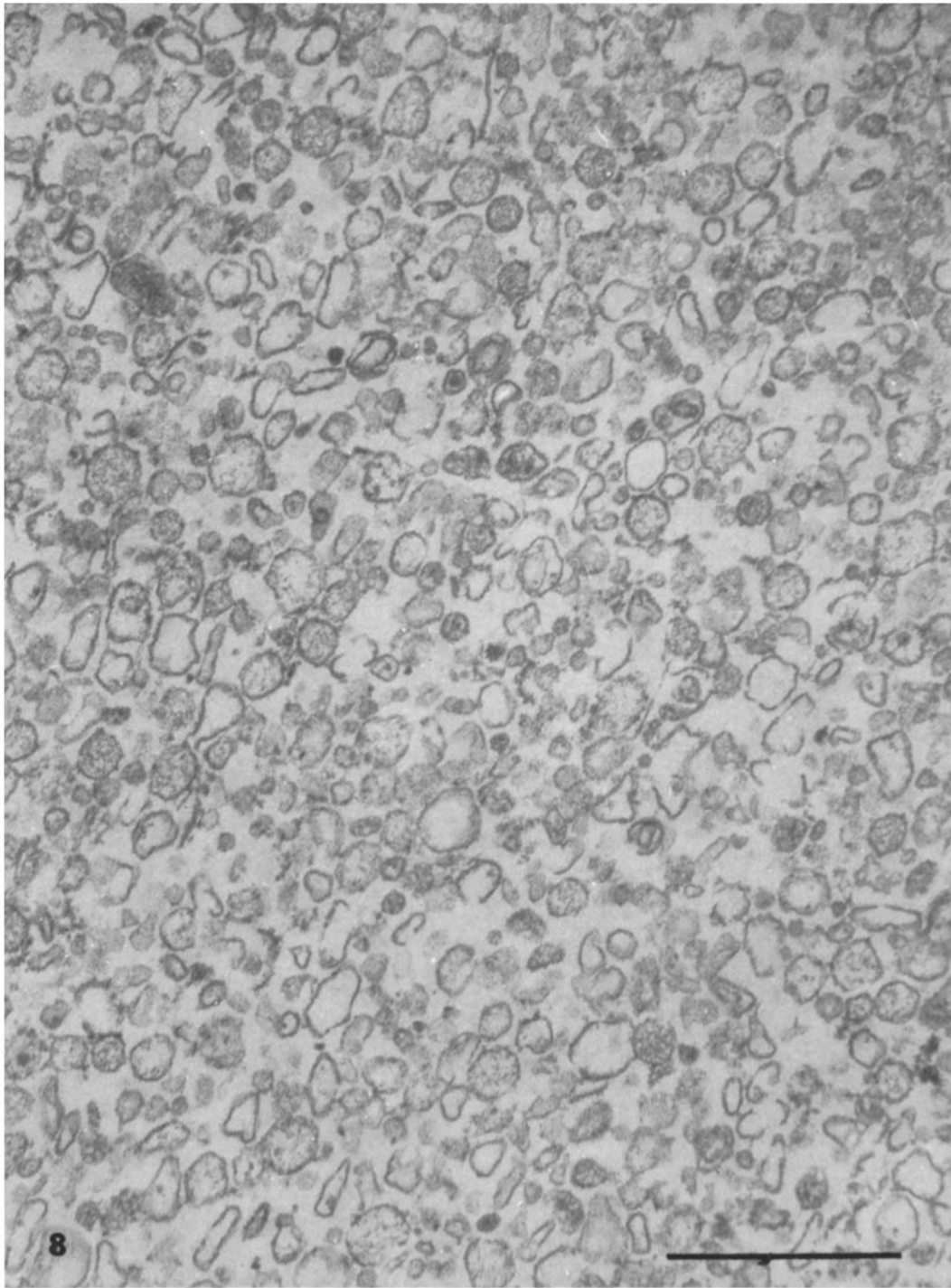


FIGURE 8 Plasma membrane preparation isolated by the latex bead method. Note vesicular nature of membranes and absence of glycogen granules. Bar, 1  $\mu$ m.  $\times$  34,000.

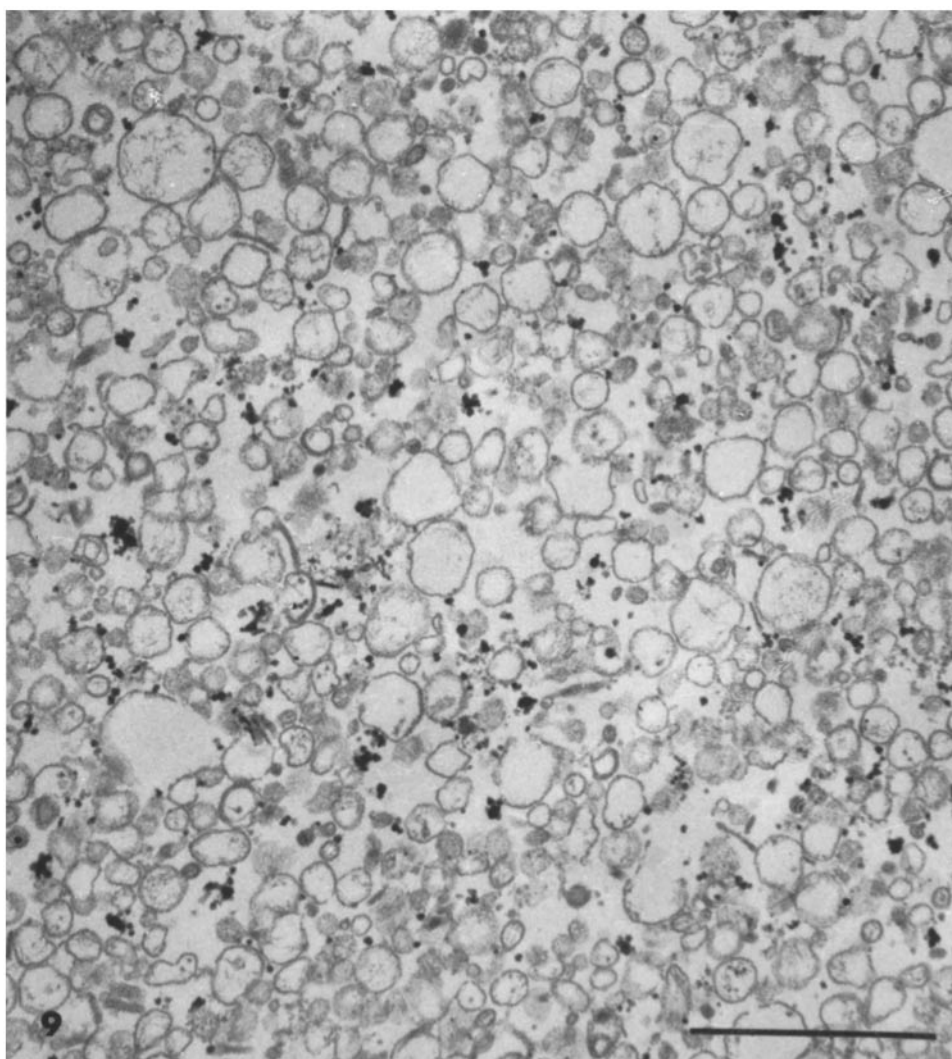


FIGURE 9 Microsomal fraction. Note clusters of glycogen granules. Bar, 1  $\mu\text{m}$ .  $\times 32,000$ .

BUTYRIC ACID (AIB) IN KB CELLS AFTER INGESTION OF LATEX BEADS: In these studies the kinetics of uptake of [ $^{14}\text{C}$ ]AIB were investigated with cells after ingestion of latex beads. It was reasoned that if the plasma membrane sites involved in AIB transport are interiorized during phagocytosis of latex beads such cells will exhibit decreased rate of AIB uptake and lower  $V_{\text{max}}$  for AIB influx as compared with cells not exposed to beads. If, on the other hand, the kinetics of AIB transport are unaffected by bead ingestion it would indicate that the transport sites of the plasma membrane are either not interiorized or that they are regenerated following their interiorization. The results are shown in Fig. 10. From the Lineweaver-Burk plots of the data the

$V_{\text{max}}$  (nanomoles of AIB taken up per min per  $10^6$  cells) and  $K_m$  (mM) values were derived. Cells not exposed to beads have a  $V_{\text{max}}$  of 18.0 as compared with 7.9 for cells after bead ingestion. The  $K_m$  was 1.0 mM for both types of cells. These results show conclusively that the number of AIB transport sites of the plasma membrane is decreased after bead ingestion as a consequence of plasma membrane interiorization accompanying phagocytosis.

#### DISCUSSION

##### *Purity of the Plasma Membranes*

In assessing the purity of the plasma membranes (or of any other fraction) it is desirable to employ

TABLE I  
Subcellular Distribution of Certain Enzyme Activities in KB Cells

Enzyme	Homog- enate	Mitochon- dria	Microsomes	Plasma membranes	
				Beads*	ZnCl <sub>2</sub> *
Na <sup>+</sup> + K <sup>+</sup> -activated ATPase	4.3	1.6	0.0	100.0	98.0
5'-Nucleotidase	65.0	87.0	77.0	1,400.0	1,375.0
Succinate-cytochrome <i>c</i> reductase	9.4	216.0	0.4	3.6	7.0
Cytochrome oxidase	2.1	19.6	0.0	0.0	0.005
NADPH-cytochrome <i>c</i> reductase	10.0	0.9	31.2	50.4	51.0
Rotenone-insensitive NADH-cytochrome <i>c</i> reductase	—	11.5	103.0	1.7	1.9
Cytochrome <i>b</i> <sub>5</sub>	—	—	0.04	0.08	0.076
Cytochrome P-450	—	—	0.003	<0.0003‡	<0.0003‡
Acid phosphatase	20.5	22.4	20.8	30.0	not done

The values represent specific activities which in all cases, except for cytochrome oxidase and cytochromes *b*<sub>5</sub> and P-450, are expressed as nanomoles of substrate used or product formed per min per mg of protein. Cytochrome oxidase activities are expressed as  $k \times 10^{-3} \text{ s}^{-1}$  per mg of protein, where *k* is the first order rate constant for the oxidation of ferrocytochrome *c*. The values for cytochromes *b*<sub>5</sub> and P-450 are in nanomoles per mg of protein. The concentration of rotenone in the assay was 0.1 mM. All fractions were sonicated for 30 s at 0°C before their use in the assays since this treatment gave higher activities for cytochrome oxidase and succinate-cytochrome *c* reductase, without affecting the other enzyme activities.

\* This refers to the method of isolation of the membranes.

‡ Lowest detectable level.

both biochemical and morphological criteria. Ideally, the minimal biochemical criteria should include: (a) two or more markers which are present in the fraction under consideration and absent in all other fractions and (b) two or more markers present in each of the other fractions but absent in the fraction under consideration. The morphological criteria can exclude contamination by easily recognizable structures, i.e. mitochondria and nuclei, but they are less adequate in distinguishing among simpler membrane structures, i.e., isolated vesicles of smooth endoplasmic reticulum, of the Golgi apparatus, or of plasma membranes.

With regard to the plasma membranes prepared by the latex bead method the results presented in Table I and in the text, as well as the electron micrographs, show that this fraction satisfies the above criteria of purity. The plasma membranes contain sodium pump and 5'-nucleotidase the specific activities of which increased by the same factor (22-23-fold) over the corresponding values in the unfractionated homogenate. Furthermore, the sodium pump is not detectable in the other fractions, and the 5'-nucleotidase is present at very low levels in both mitochondria and microsomes. The latter levels do not differ

significantly from the level in the whole homogenate.

Of the five mitochondrial enzyme markers, i.e. succinate-cytochrome *c* reductase, cytochrome oxidase, and cytochromes *b*, *c*, and *c*<sub>1</sub>, four were absent in the plasma membrane fraction and only the presence of trace amounts of succinate-cytochrome *c* reductase activity would suggest a maximal contamination of 1.7% by mitochondrial components.

Of the microsomal markers cytochrome P-450 was not detectable in the plasma membranes, and the specific activity of the rotenone-insensitive NADH-cytochrome *c* reductase of the plasma membranes was 1.7% of that of the microsomes, indicating a maximal possible contamination by microsomes of 1.7%. It is interesting to note that with respect to the NADPH-cytochrome *c* reductase and cytochrome *b*<sub>5</sub>, both of which have been considered primarily as microsomal components, their specific activities are significantly higher in the plasma membrane of KB cells than in the microsomes. It must be concluded, therefore, that these enzymes are present in both the plasma membrane and the microsomes of the KB cells.

With regard to the acid phosphatase the profile of its specific activity among the various fractions

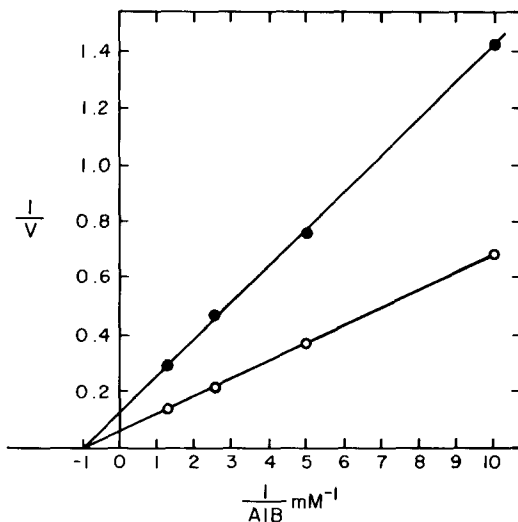


FIGURE 10 Lineweaver-Burk plots of the initial influx rate of AIB versus AIB concentration. The cells were first incubated for 20 min with, ●—●, or without, ○—○, latex beads in Krebs-Ringer bicarbonate buffer, and after removal of excess beads they were incubated at 37°C with different concentrations of [<sup>14</sup>C]AIB in the same buffer for 1 min. Velocity is expressed as nmoles of AIB uptake per 10<sup>6</sup> cells per min.

indicates no preferential concentration in any particular fraction, suggesting adsorption of this enzyme on the various fractions during their isolation.

The electron micrographs show the morphological homogeneity of the plasma membranes (Figs. 7, 8) and of the membrane-enclosed latex beads (Fig. 6) from which the plasma membrane fraction was derived.

With respect to the membranes isolated by the ZnCl<sub>2</sub> method the data of Table I show that the specific activities or concentrations of the various enzymes are practically identical to those of the latex bead membranes. An exception is the succinate-cytochrome *c* reductase the specific activity of which was twice as high in the ZnCl<sub>2</sub> membranes. This together with the presence of trace amounts of cytochromes *aa*<sub>3</sub> and *c*<sub>1</sub> in the latter membranes indicates that the degree of contamination of the ZnCl<sub>2</sub> membranes by mitochondrial enzymes, although small (3.2%), is twice that of the latex bead membranes. On the basis of these results the latex bead method appears to be as good, if not superior, to the ZnCl<sub>2</sub> method.

The isolation of plasma membranes by two

independent methods is very useful not only in assessing the purity of the membranes but also in evaluating the validity of the latex bead method. One question that can be raised concerns the possibility that the membranes isolated by this method represent a specialized portion of the plasma membrane engaged in phagocytosis rather than a representative sample of the whole membrane. That this is unlikely in the case of KB cells is evident from the excellent agreement between the specific activities of the four enzymes studied with the plasma membranes which were isolated by the two methods, i.e., the sodium pump, the 5'-nucleotidase, the NADPH-cytochrome *c* reductase, and cytochrome *b*<sub>5</sub>. Such agreement is improbable if the latex bead method was selecting specialized areas of the cell surface membrane. Additional evidence supporting the above conclusion comes from the kinetics of transport of α-aminoisobutyric acid. The results of Fig. 10 show that the number of transport sites (*V*<sub>max</sub>) of the plasma membrane was considerably decreased following phagocytosis of latex beads, a finding which is expected if these sites were interiorized. Hence, the plasma membrane interiorized as a result of phagocytosis carried with it amino acid transport sites and the specific enzyme markers of the plasma membrane. These findings agree with those of Lutton (30) who observed decreased binding of concanavalin A by the plasma membrane receptors of peritoneal macrophages following bead ingestion. Our results are not in agreement with those of Tsan and Berlin (31) who showed that in polymorphonuclear leucocytes and alveolar macrophages the rate of transport of various nonelectrolytes was the same before and after bead ingestion. They interpreted these results as indicating that the carrier sites involved in these transport systems were not interiorized during the internalization of plasma membrane accompanying phagocytosis. The differences between our results and those of Tsan and Berlin may result from differences in the structure of the plasma membranes of the cells used in these studies or they may reflect different mechanisms of phagocytosis operative in different cells.

The likelihood that other proteins or components of the plasma membrane may show significant quantitative differences between the two methods of its isolation employed in our studies cannot be ruled out, and only extensive chemical and structural characterization of the plasma

membranes obtained with these methods can provide a more detailed comparison. This applies not only to the latex bead method but to any other method of isolating the plasma membrane since selective removal of membrane components during the isolation is quite possible. It is relevant in this respect to note that Tris buffer causes a breakdown of the isolated plasma membrane of KB cells, and KB cells exposed to iso-osmotic Tris buffer (pH 7.0-7.4) lose progressively their viability and various plasma membrane functions, such as, concentrative amino acid transport, and the sodium pump. These effects of Tris have been observed with Tris from various sources even after repeated crystallizations. The presence of sucrose (10% or higher) in the Tris buffer protects the isolated plasma membrane against the Tris effect (unpublished observations). For these reasons methods employing Tris in the isolation of the plasma membrane (8, 32, 33) are not suitable for use with KB cells.

Another aspect concerning the validity of the latex bead method is the possibility that the plasma membrane surrounding the ingested beads undergoes modification following interiorization. Werb and Cohn (33) in their studies with macrophages observed that the membrane of the phagolysosomes isolated after ingestion of latex beads is similar to the plasma membrane in its content of 5'-nucleotidase, cholesterol, and phospholipid, provided that the phagolysosomes are isolated immediately after ingestion of the latex beads. Phagolysosomes isolated after various times following phagocytosis contained decreasing amounts of 5'-nucleotidase. They interpreted the disappearance of this enzyme as the result of possible modification of the phagolysosomal membrane after interiorization. In our studies the isolation of the membrane-coated beads was carried out immediately after phagocytosis (at the end of 30-min incubation with latex beads), and very rarely was fusion of ingested beads with themselves observed by electron microscopy. In KB cells fusion of beads among themselves is detectable usually after 1 h of incubation. Furthermore, the two enzyme markers of the KB plasma membrane (the sodium pump and 5'-nucleotidase) were enriched in the latex bead membrane by the same factor (22-23-fold), and the specific activities of the four enzymes studied with this membrane were the same as those in the plasma membrane isolated by the  $ZnCl_2$  method (without the use of beads). Of interest in this connection is the finding

that in liver cells the lipid composition of the lysosomal membrane is similar to that of the plasma membrane (34). These results give strong support to the conclusion that the plasma membrane isolated by the latex bead method was not modified after its interiorization, since it is highly improbable that such a modification would not also alter the profile of the specific activities of four enzyme components of this membrane or lead to the acquisition of eight non-plasma membrane enzymes that were investigated. Based on the above similarities of the plasma membranes isolated by the two methods the probability that the membranes are different from each other was statistically evaluated by the F-(Fisher) test to be less than 1%.

#### *Purity of the Mitochondrial and Microsomal Fractions*

Maximal contamination of the mitochondrial fraction by microsomes, as judged from the specific activity of the NADPH-cytochrome *c* reductase, is less than 3%. Contamination by plasma membranes amounts to 1.6% based on the level of the sodium pump activity. If, instead, contamination is calculated from the specific activity of the 5'-nucleotidase, and assuming that this enzyme is normally present only in the plasma membrane, the contamination of the mitochondrial fraction by plasma membranes is 6%.

The microsomes are free of any contamination by mitochondria as evidenced by the absence of cytochrome oxidase activity and of all the cytochromes of the mitochondrial electron transport system. The absence of sodium pump activity in the microsomal fraction indicates the absence of contamination by plasma membranes. However, the presence of low levels of 5'-nucleotidase activity could indicate 5% contamination by plasma membranes if this enzyme is an exclusive component of the latter fraction.

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