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Elution and Uncoating of Coxsackievirus B3 by Isolated HeLa Cell Plasma Membranes

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Plasma membranes isolated from HeLa cells on discontinuous sucrose gradients were assayed for their capacity to elute and uncoat coxsackievirus B3 at 37 C. Because the viral receptors are limited to the surface of HeLa cells, the addition of radioactively labeled virus to the cells prior to cell homogenization provided a useful marker for locating the plasma membranes during the fractionation procedure. Four bands were formed on the discontinuous sucrose gradients with approximately 70% or more of the membrane-associated viral label being recovered in the most dense bands, designated as bands 3 and 4. Bands 3 and 4 also possessed the plasma membrane marker enzymes, Na^+, K^+ adenosine triphosphatase and 5'-nucleotidase and revealed typical structures characteristic of plasma membranes as revealed by electron microscopy. Pelleted and washed membranes from band 3 both eluted and uncoated B3 ^{32}P-labeled virus, whereas membranes from band 4 eluted virus but failed to uncoat it. The membranes from band 4 were shown to inhibit the viral uncoating activity when mixed with membranes of band 3. Characteristically, unfractionated homogenates of cell membranes eluted but did not uncoat virus. The finding of a naturally occurring inhibitor of virus uncoating provides for the first time a way to distinguish between the membrane activities of virus elution and virus uncoating. The inhibitor remains to be characterized.

It has been well established that the presence of specific receptors on the surface of cells is a major determinant controlling cell susceptibility to given virus infections (13, 14, 17). Previous research in this laboratory has been directed toward defining the early interactions between enteroviruses and their specific receptors on susceptible cells (5-8, 16, 28-30). In the course of these studies, Crowell and Philipson (6) found that a major portion of radioactively labeled coxsackievirus B3, which was attached to HeLa cells in the cold, eluted from the surface of the cells at 37 C in a manner similar to that found for poliovirus by others (9, 15, 20). Thus, when coxsackievirus and polioviruses attach to HeLa cells, 50 to 80% of the cell-associated virus (CAV) elutes spontaneously from the cell surface as noninfectious virions. About 5 to 10% of the CAV penetrates the cell membrane and is uncoated, while the remainder of the CAV remains unaltered (19). The significance of the divergent pathways of elution or uncoating of enteroviruses at the cell surface remains unexplained.

In this communication, we are reporting on the isolation of plasma membranes from HeLa cells by using a modification of the method of Bosmann et al. (2). The use of radioactively labeled virus attached to receptors is introduced as a marker to aid in the recovery of plasma membrane fractions. These procedures were applied in studies designed to determine whether isolated plasma membranes would retain their receptor activities for eluting and uncoating coxsackievirus B3. We use the term "uncoating" to designate the process whereby viral RNA is rendered susceptible to digestion by pancreatic RNase after the incubation of plasma membrane-associated virus (MAV) at 37 C. This designation is made possible because neither native nor eluted virions containing RNA are subject to RNase activity. (Presented in part before the annual meeting of the American Society for Microbiology, Miami, Fla., 8 May 1973).

MATERIALS AND METHODS

HeLa cells. HeLa cells were propagated in suspension with double strength Eagle minimal essential medium supplemented with 5% horse serum, 2% calf serum (CaS-2) and 2 μg of insulin per ml. HeLa cells for virus plaque assays were cultured as monolayers in plastic petri dishes (60 by 15 mm) as described previously (6).

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Viruses and virus purification. The origin of the Nancy strain of coxsackievirus B3 and the Mahoney strain of poliovirus T1 used in these studies was described earlier (8). The viruses were grown in suspension cultures of HeLa cells by using a multiplicity of infection of 10 to 50 PFU/cell. Viruses were recovered and then purified by two consecutive bandings in cesium chloride gradients as described elsewhere (7). The general characteristics of the purified viruses were similar to those described previously (6).

Preparation of membrane fractions. Cells grown in suspension were removed, counted, and pelleted by centrifugation at 400 × *g* for 15 min at 0 C. Cells (2.0×10^8) were resuspended in 40 ml of phosphate-buffered salt solution devoid of divalent cations (7) and again pelleted. Cells were treated once in 10 ml of 0.02% EDTA dissolved in phosphate-buffered salt solution and incubated at room temperature for 10 min. The cells were then washed twice more in 10 ml of a solution containing calcium acetate (10^{-3} M) in saline buffered with 0.05 M Tris, pH 7.0. After a final wash consisting of 10 ml of 0.01 M EDTA in 0.02 M Tris (EDTA-Tris) buffer, the cell pellet was resuspended in 5 ml of EDTA-Tris, incubated for 15 min at 4 C, and placed in a tightly fitting, 15-ml capacity Dounce homogenizer. The cells were ruptured usually with 18 downward strokes of the plunger, with the number of cells ruptured being monitored microscopically. The homogenate was transferred to a polystyrene centrifuge tube and centrifuged in an International centrifuge model PR-2 at 4 C for 5 min at (900 × *g*) to remove whole cells, nuclei, and any large aggregates from the membrane-containing supernate. The supernatant fluid (5.0 ml) was delivered to the bottom of a cellulose nitrate centrifuge tube (38.5 ml capacity) and mixed with 10 ml of 75% sucrose (wt/wt) in 0.002 M Tris, pH 7.0, to give the bottom layer a final sucrose concentration of about 50%. Then, 7.5 ml of 35% sucrose, 7.5 ml of 30% sucrose, and 5.5 ml of 25% sucrose were layered onto the gradient, respectively. Finally, 2.0 to 2.5 ml of 0.05 M Tris buffer was added, and over this was layered a few drops of mineral oil. All procedures were carried out at 0 to 5 C. The discontinuous gradient was centrifuged at 70,000 × *g* in an SW27 rotor for 17 h at 0 C. After centrifugation, four definitive bands were seen macroscopically. Each of these discrete regions were collected separately by direct aspiration from above. Fractions were diluted approximately 1:3 with 0.05 M Tris buffer, and the membranes were pelleted by centrifugation at 70,000 × *g* for 1 h at 0 C in an angle head rotor (Beckman No. 50). On occasion, pellets were washed once before being resuspended in appropriate solutions to be tested for virus receptor activities. This procedure was derived from the method of Bosmann et al. (2) and was modified for our use as follows. We scaled down the fluid volumes used in the procedure 10-fold to accommodate a reduced number of cells (2×10^8). To minimize the loss of plasma membrane in the pellet during the initial slow speed centrifugation of the cell homogenate, we reduced the rate and time of centrifugation from 10 min at 4000 × *g* to 5 min at 900 × *g*. The SW27 rotor with 38.5-ml capacity tubes rather than the SW25.2 rotor with 60-ml capacity tubes (Beckman Instruments Co.) was used for the preparation of sucrose gradients. The concentration of sucrose in the bottom layer of the gradients was increased from 45 to 50% (wt/wt).

Preparation of CAV. The early steps in preparing the CAV were identical to those described above for membrane preparation including the two calcium acetate buffer solution washes and one wash with EDTA-Tris. At this point the cells were again washed in 10 ml of phosphate-buffered salt solution and pelleted. The cell pellet was resuspended in 0.1 to 1.0 ml of phosphate-buffered salt solution containing the appropriate virus concentration, usually at a multiplicity of infection of 100 PFU/cell or less and incubated 90 min at 4 C for virus attachment. The amount of unattached virus was reduced by washing the pellet once in phosphate-buffered salt solution and twice more in 0.01 M EDTA in 0.02 M Tris (EDTA-Tris) buffer pH 7.0. All procedures were carried out at 0 to 5 C. The amount of CAV radioactivity was determined by difference between the amount of radioactivity of input virus and that found unattached in all wash fluids. In addition, the amount of cell-associated label was determined directly and found to be in close agreement with that determined by calculation from unattached virus radioactivity.

Preparation of plasma MAV. This procedure first included the preparation of CAV, and the preparation of HeLa cell membrane fractions from cells with attached virus. All steps were done as indicated previously, except that 8-drop fractions were collected from the bottom of the discontinuous sucrose gradients into small plastic tubes or directly into liquid scintillation vials.

Determination of elution and uncoating. Treatment of replicate preparations of MAV at 37 C or 0 C for 1 hour in 0.02 M *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethane-sulfonic acid (HEPES)-buffered saline, PH 7.0, containing 100 μg of RNase per ml provided experimental and control environments, respectively. After incubation, the tubes were centrifuged for 20 min at 3700 × *g* at 4 C to pellet the membrane fragments and membrane-associated virus. The supernatant fluids (1 ml) were decanted into new test tubes to which 1 ml of 10% cold trichloroacetic acid was added; a few drops of bovine serum albumin (5 mg/ml) was used as carrier. After centrifugation as above, pellets and supernates were separated and assayed for radioactivity. Virus labeled with ^{32}P was used in all elution experiments containing RNase and was counted in 10 ml of 0.1 NaOH by Cerenkov radiation, as noted below. Radioactive counts remaining in the MAV pellet were considered to be attached virions which were neither eluted nor uncoated. Counts in the trichloroacetic acid pellet were considered as "eluted" virions that remained intact, since they were not susceptible to digestion by RNase (7). Counts in the trichloroacetic acid supernatant fluids were considered as "uncoated" virus because they were susceptible to digestion by RNase.

Assay for radioactivity. Samples containing ^{32}P were counted by Cerenkov radiation (4) in 10 ml of 0.1 M NaOH by using the ^{3}H channel setting in a liquid scintillation spectrometer (model LS-133, Beckman

Instrument, Inc.). Samples labeled with either ^3H or ^{14}C were solubilized in 0.5 ml of 0.1 M NaOH and 10 ml of a toluene-liquid scintillation mixture. This mixture contained 1 gallon (about 3.8 liters) of "scintanalyzed" toluene (Fisher Scientific Co.), to which was added 400 ml of Bio-Solv Solubilizer BBS-3 and 32.2 g of Fluoralloy dry mix TLA (equal to 8.0 g of butyl PBD and 0.5 g of PBBO per liter; Beckman Inst. Co.). In experiments with doubly labeled virus, appropriate replicate samples employing each isotope separately were assayed for correction of spillover counts in the respective channels.

Protein and enzyme assays. Protein estimations were made with a modification of the method of Lowry et al. (18). 5'-Nucleotidase was assayed with 5'-AMP by the method of Heppel and Hilmoe (12), while the Na^+, K^+ ATPase was assayed with 5'-ATP by the method of Pullman et al. (24). Ouabain (10^{-5} M) and oligomycin (5×10^{-5} M) were used as inhibitors. The method of Fiske and SubbaRow (11) was used for inorganic phosphate determinations.

Radioactive reagents. L-[^{14}C]valine at 1.8 mCi/mg, and carrier-free ^{32}P-labeled phosphate were purchased from New England Nuclear, Boston, Mass. Reconstituted protein hydrolysates of ^3H- and ^{14}C-labeled amino acids were purchased from Schwarz/Mann, Orangeburg, N.Y., as 1 mCi per ml in 0.01 M HCl.

Preparation for electron microscopy. The samples to be studied (virus, cells, or membranes) were pelleted by centrifugation, fixed in 5% glutaraldehyde solution for 15 min at 4 C, and thereafter treated with 5% osmium tetroxide at room temperature and then dehydrated. The samples were embedded in epoxy resin (Araldite) (10), stored overnight, rotated 3 h at 40 C, and polymerized at 60 C for 40 to 48 h. Sections were cut on a Porter-Blum microtome, stained first with 10% uranyl acetate for 30 min then in lead citrate for 30 min (25) and examined in a Siemens Elmskop I electron microscope.

RESULTS

Receptor-associated virus as a marker for the recovery of HeLa cell plasma membranes. In a study of the distribution of coxsackiervirus and poliovirus receptors of HeLa cells, Zajac and Crowell (29) found that these viral receptors were limited to the cell surface. Thus, attached, radioactively labeled virus would serve as a marker of the plasma membranes during subcellular fractionation, provided the procedures were carried out at low temperature (0 to 4 C) to prevent elution and uncoating of virus (6, 9, 15). An attempt to provide visual evidence of the presence of coxsackievirus B3 virions attached to the surface of intact HeLa cells at 0 C is described as follows.

HeLa cells from a suspension culture were enumerated, and 5×10^6 cells were placed in each of two plastic tubes and centrifuged at 400 $\times$ *g* for 15 min at 0 C. The fluid phase was discarded, the tubes were drained, carefully wiped free of residual moisture, and placed on ice. One cell pellet was resuspended in 0.2 ml of purified coxsackievirus B3 containing 2×10^{10} PFU to give a multiplicity of infection of 4×10^3 PFU/cell and a cell concentration for virus attachment of 25×10^6 cells/ml. The remaining cell pellet was resuspended in 0.2 ml of phosphate-buffered salt solution to serve as a cell control. The suspensions were placed in small polypropylene tubes and incubated on ice for 24 h. After virus was allowed to attach, the cells were centrifuged into a pellet, the supernate was discarded, and the cells were resuspended in gluteraldehyde-osmium fixative, and embedded, sectioned, and prepared for electron microscopy. Figure 1 reveals the presence of coxsackievirus B3 virions at the surface of a HeLa cell. Note that the virions are limited to the external surface of the plasma membrane and that they are attached to discrete areas of the surface. This is, portions of membrane are devoid of virions even though a saturating amount of virus was applied to the cells (5). No comparable virus-like particles were seen in uninfected HeLa cell control preparations. Figure 2a depicts a section of a HeLa cell with attached virus at higher magnification, and Figure 2b shows an uninfected cell at the same magnification. Figure 3 is included to compare with the previous preparations the appearance of purified coxsackievirus B3, which was pelleted, fixed, embedded, sectioned, and photographed in a similar manner to that given in the preceding figures. Note the crystalline arrays of virions, which give evidence of a rather high degree of purity of the coxsackievirus preparations employed in this study.

To obtain plasma membranes from HeLa cells the method of Bosmann et al. (2) was selected for use with modification as outlined above. HeLa cells grown in suspension cultures were prepared for disruption and homogenized, and the cytoplasmic membranes were recovered on discontinuous sucrose gradients. The appearance of a typical gradient after centrifugation is seen in Fig. 4. The cytoplasmic contents were separated into four bands, designated numerically from top to bottom, with band 4 being located in the more dense region from 1.16 to 1.21 g/cm^3. On occasion, a small pellet was obtained and its radioactive content was determined after solubilizing the pellet in 0.1 M NaOH. The gradients were fractionated by puncture of the tube bottom, and 8-drop fractions were collected. The fractions comprising each of the four bands were pooled, respectively, diluted 1:3 in 0.05 M Tris buffer, pH 7.0, and centrifuged at 70,000 $\times$ *g* for 1 h. The pellets

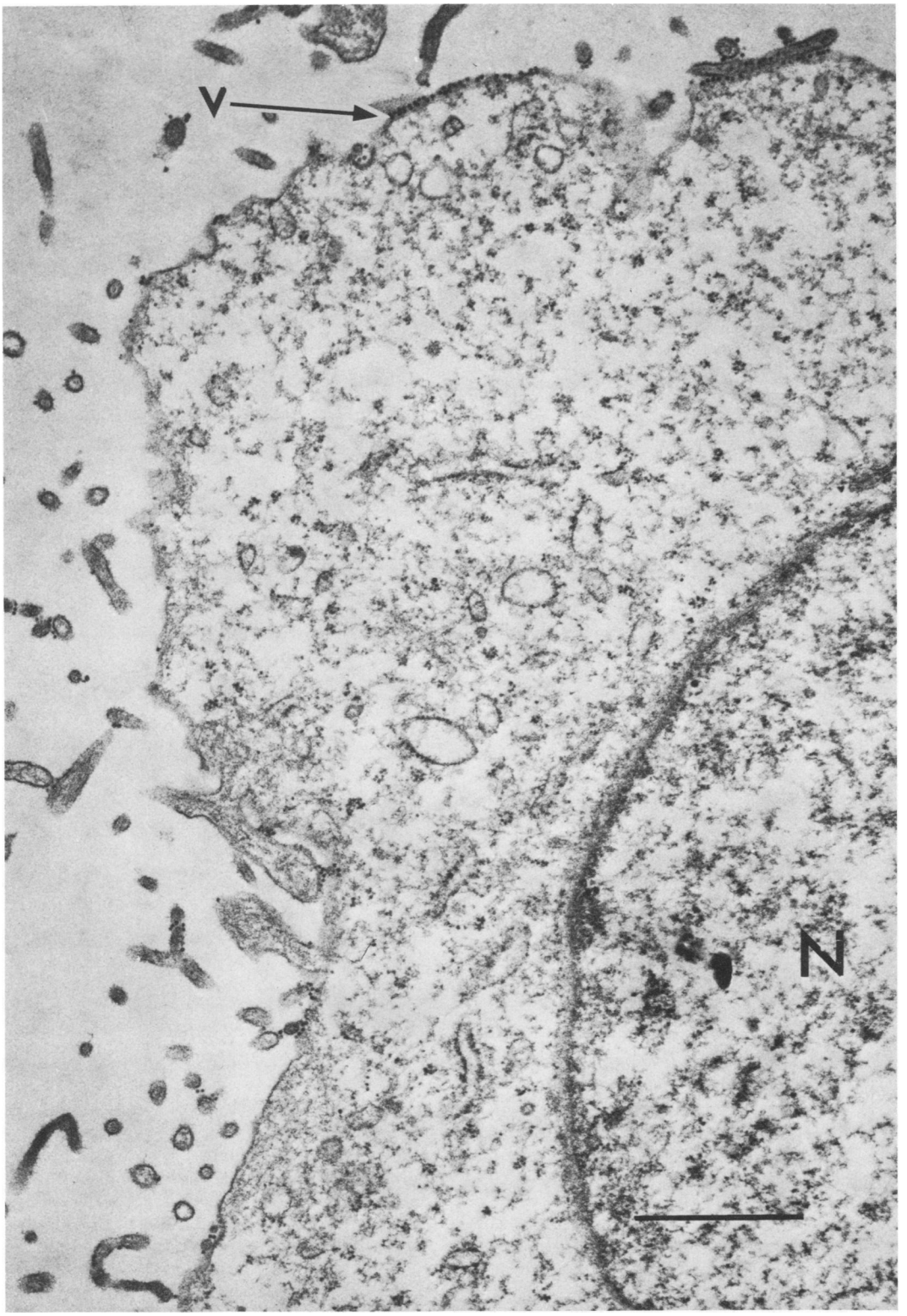

FIG. 1. *Low-magnification electron micrograph of a HeLa cell preparation infected with a saturating amount of coxsackievirus B3. Note that the virions (V) are limited to the external surface of the plasma membrane and that they are attached to discrete areas of the cell (N) nucleus. Bar, 1.0 μm.*

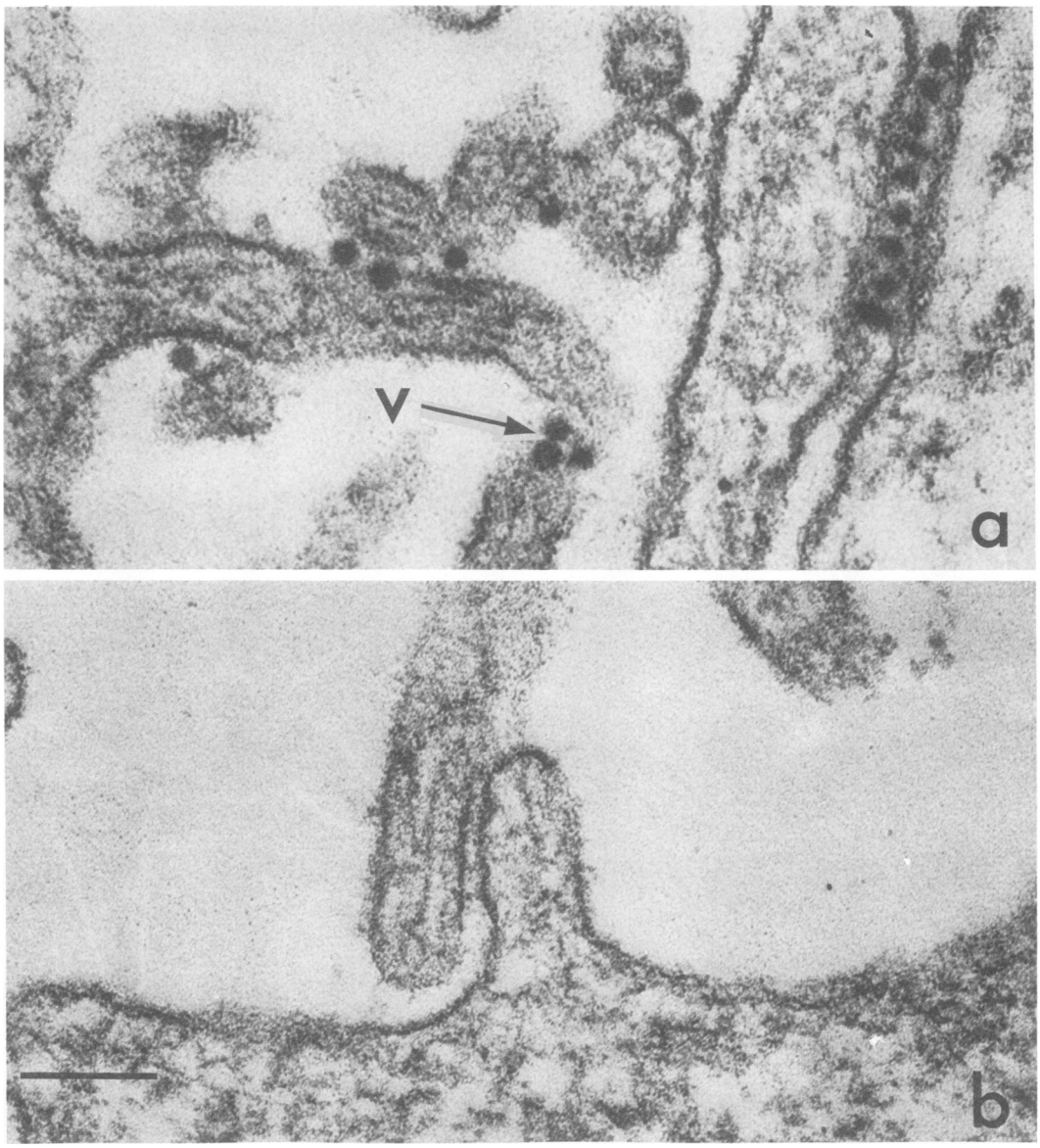

FIG. 2. *(a) Higher magnification electron micrograph of a section of a HeLa cell with attached coxsackievirus B3 (V). (b) Higher magnification of a section of a normal HeLa cell. Bar, 0.1 μm.*

were resuspended separately in 1-ml volumes and aliquots were assayed for protein, enzyme content, and the capacity to inactivate coxsackievirus B3.

The results of three replicate experiments are summarized in Table 1. The data show that bands 3 and 4 contained both the plasma membrane marker enzymes, (ouabain-sensitive Na^+, K^+ ATPase, and 5′-nucleotidase) and the coxsackievirus B3 receptor activity (29). In a separate experiment, the fractions from bands 3 and 4 were combined, pelleted, resuspended, and applied to a fresh sucrose gradient. A comparable visible separation of the two bands again was obtained with both bands retaining their respective virus receptor activities. Thus, the modification of the technique of Bosmann et al. (2) employed here allowed the recovery of the plasma membranes in two bands rather than in the pellet.

To facilitate the study of elution and uncoating of virus by isolated plasma membranes, virus was allowed to attach to HeLa cells prior to the disruption of the cells. Experiments were

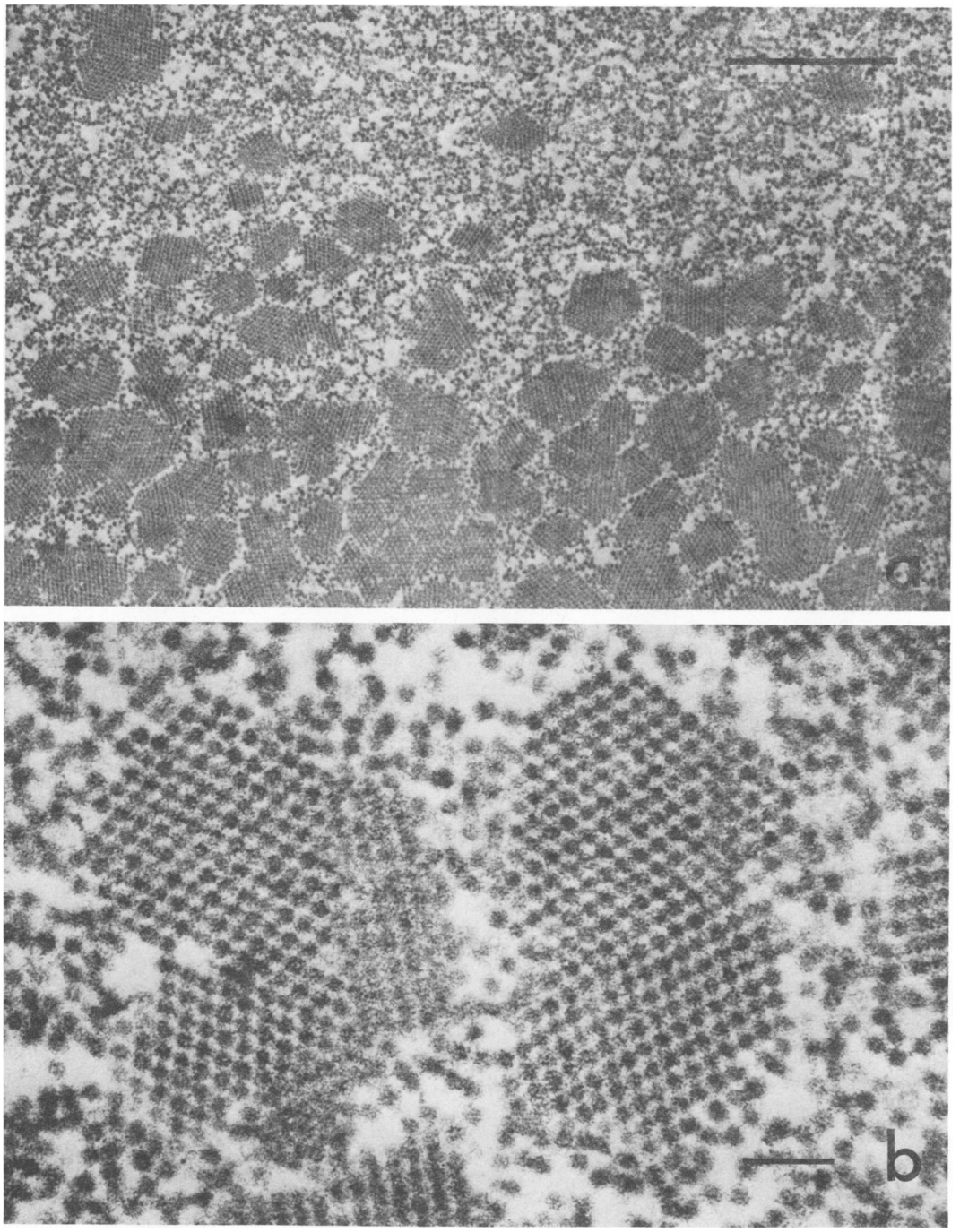

FIG. 3. (a) *Electron micrograph of purified coxsackievirus B3 seen at low magnification. Note the crystalline arrays of virions.* (b) *Higher magnification of purified coxsackievirus B3. The virions aggregated in the lattice measured 20 nm in diameter, with a center to center distance of 25 nm. Virions not aggregated in the lattice measured 25 nm in diameter. Bar, 0.1 μm.*

performed to monitor the distribution of receptor bound virus radioactivity in subcellular fractions produced during the preparation of isolated plasma membranes. B3 virus labeled with ^{14}C-valine was added to a HeLa cell population before homogenization at a multiplicity of infection of less than 100 PFU/cell and was incubated for 90 min at 0 C. A homoge-

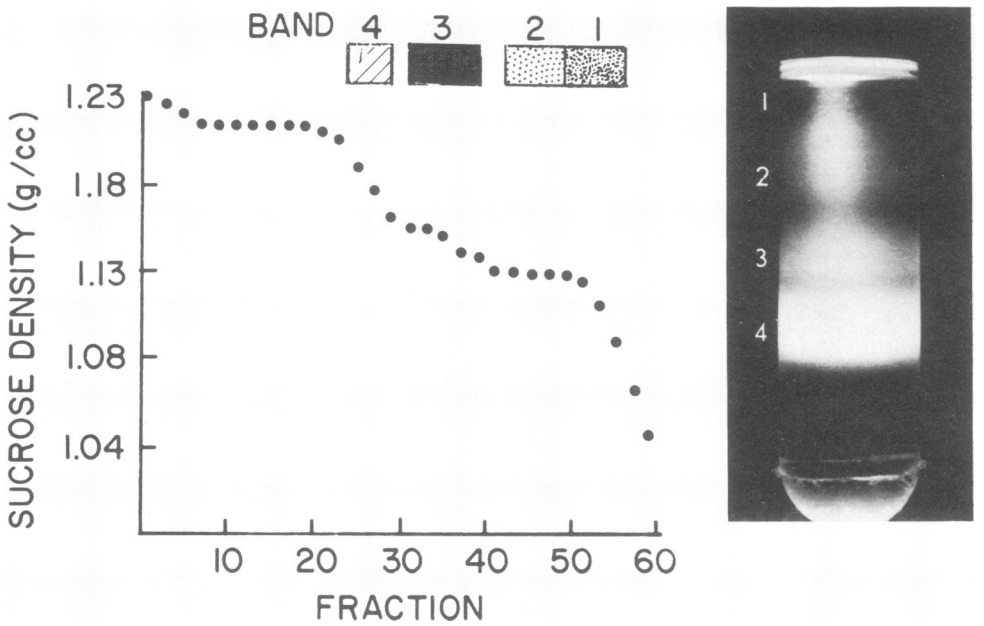

FIG. 4. *HeLa cell membranes banded on a discontinuous sucrose gradient. Cells were homogenized, nuclei and unbroken cells were removed by slow speed centrifugation, and the homogenate was placed on a discontinuous sucrose gradient. The gradient was centrifuged at 70,000 × g for 16 h at 0 C. The resultant bands are designated as 1 to 4 above, with the sucrose densities of each fraction represented on the graph.*

TABLE 1. *Protein content, plasma membrane marker enzyme, and virus receptor activities of HeLa cell cytoplasmic extracts fractionated on discontinuous sucrose gradients*[a]

Gradient band	Protein (mg/ml)	ATPase[b]		5'-Nucleotidase		Coxsackievirus B3 inactivated (% PFU)[c]
		U/ml	U/mg of protein	U/ml	U/mg of protein	
1	0.08	0	0	0	0	1
2	0.11	0	0	2	18.2	6
3	0.17	20	117.6	20	117.6	76
4	1.71	34	19.9	26	15.2	38

[a] Average amounts compiled from three separate experiments with each measurement performed in duplicate.

[b] One unit (U) of enzyme activity = 1 μmol of Pi liberated per h per 0.5 ml of membrane fraction. The ATPase is the Na^+, K^+ ATPase which was ouabain sensitive and oligomycin insensitive.

[c] Infectious virus, equivalent to an input multiplicity of less than 100 PFU/cell, was incubated for 60 min at 37 C and plaque assayed for the amount of surviving virus.

nate prepared from the washed cells was processed for plasma membrane recovery on discontinuous sucrose gradients as described before, using virus label as marker.

Analysis of the recovery of viral radioactivity from a representative experiment revealed that approximately 99% of the input viral radioactivity was recovered (Table 2; Fig. 5). Since only about 1% of the radioactivity appeared in the slow-speed pellet (900 × *g*), it appears probable that the cells were thoroughly disrupted and that no appreciable amount of virus was bound to the nuclei. Seventy percent of the radioactivity in the sucrose gradient was distributed in bands 3 (42%) and 4 (28%), coinciding with the results described above, which showed that these two bands possessed receptor activity (Table 1). On numerous occasions, more than 70% of the radioactivity was found distributed between bands 3 and 4.

Figures 6 and 7 depict the appearance of bands 3 and 4, respectively, as viewed by electron microscopy. It is evident that typical membrane structures are present in each of

these bands. Although virions are definitely seen attached to the membranes, attempts to distinguish between those vesicles which are right-side out from those which are inverted by locating attached virions were not very rewarding (Figs. 6a and 7a). Thus, from the results of these studies, we concluded that labeled virus, which is attached to receptors of intact cells, provides a useful marker for locating plasma membranes and thereby aids in their recovery from cellular homogenates.

Elution and uncoating of coxsackievirus B3 by HeLa cell plasma membranes recovered from sucrose gradients. To ascertain whether the two membrane bands recovered from our discontinuous sucrose gradients might show differing activities for elution and uncoating of coxsackievirus B3 the following experiments were conducted.

First, it was determined whether the membrane fractions would elute attached B3 virus. Coxsackievirus B3 [^{14}C]valine was allowed to attach to intact HeLa cells for 2 h at 0 C at an MOI of 17 PFU/cell, and the plasma membrane fractions with attached virus (80% of the input virus) were recovered on sucrose gradients as described previously. The fractions under the two peaks, designated bands 3 and 4, were pooled separately and tested for virus elution at 37 C. A small sample of each preparation was kept at 0 C to serve as a control, since B3 virus will not elute in the cold (6, 7). After incubation for 60 min, the membranes were pelleted by centrifugation and the amount of virus radioactivity in the supernatant fluids (membrane-dissociated virus) and the pellets (membrane-associated virus) was determined (Table 3). It

TABLE 2. *The distribution of coxsackievirus B3 [^{14}C]valine radioactivity after attachment to HeLa cells and subsequent homogenization in preparation for recovery of plasma membranes on discontinuous sucrose gradients*

Preparation	Virus radioactivity	
	Counts/min	% of total
Virus added	35,795	100
Cell wash no. 1	7,898	21.9
Cell wash no. 2	1,822	5.0
Cell wash no. 3	1,509	4.2
Residual on tube	145	0.4
CAV	24,151	67.4
Pellet (900 × *g*)	319	0.6
MAV[a]	23,832	66.5

[a] MAV was the supernatant fluid (5 ml) obtained after centrifugation at 900 × *g* for 5 min and which was fractionated on a sucrose gradient.

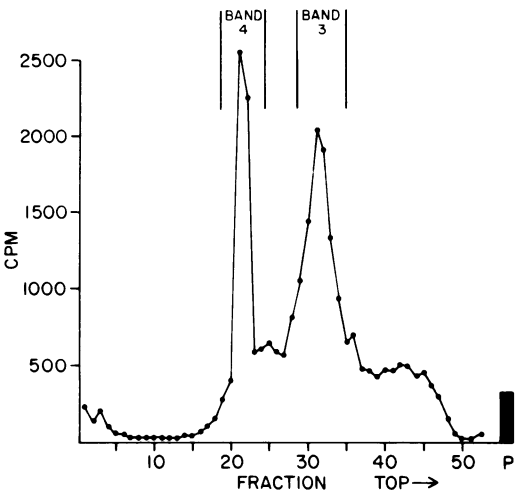

FIG. 5. *Distribution of coxsackievirus B3 [^{14}C]valine radioactivity in HeLa cell membrane fractions isolated on a discontinuous sucrose gradient. Note that 70% of the total counts/min put onto the gradient was recovered in bands 3 and 4 while total recovery was approximately 100%.*

was evident that more than 70% of the MAV eluted from both membrane preparations at 37 C, which was comparable to the amount of virus eluted from unbroken HeLa cells (6, 7). In a separate experiment of similar design, the addition of reduced glutathione (0.01 M) inhibited elution from each membrane preparation at 37 C. This inhibitory effect on elution coincided with that found previously for intact cells (unpublished observations). To determine that the virus eluted from the membrane bands was comparable to virus eluted from intact HeLa cells, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of pelleted eluted virus was performed as described previously (7). Analysis of the gel profiles (not shown) revealed that the lowest-molecular-weight virion polypeptide, VP 4, had been removed from the membrane-eluted virions, as was shown previously for virions eluted from cells (7).

Virus labeled with ^{32}P also was found to elute from HeLa cell plasma membranes under similar conditions. Since eluted virus is resistant to RNase (7), it was possible to test the membrane-dissociated virus radioactivity for RNase sensitivity and thereby obtain a measure of the amount of uncoated virus. In experiments using B3 ^{32}P-labeled coxsackievirus cell-associated virus was prepared at 0 C, the cells were washed and disrupted, and plasma membrane fractions were collected from discontinuous sucrose gradients. The profile of a representative gradient collected in 8-drop fractions is shown in Fig. 8

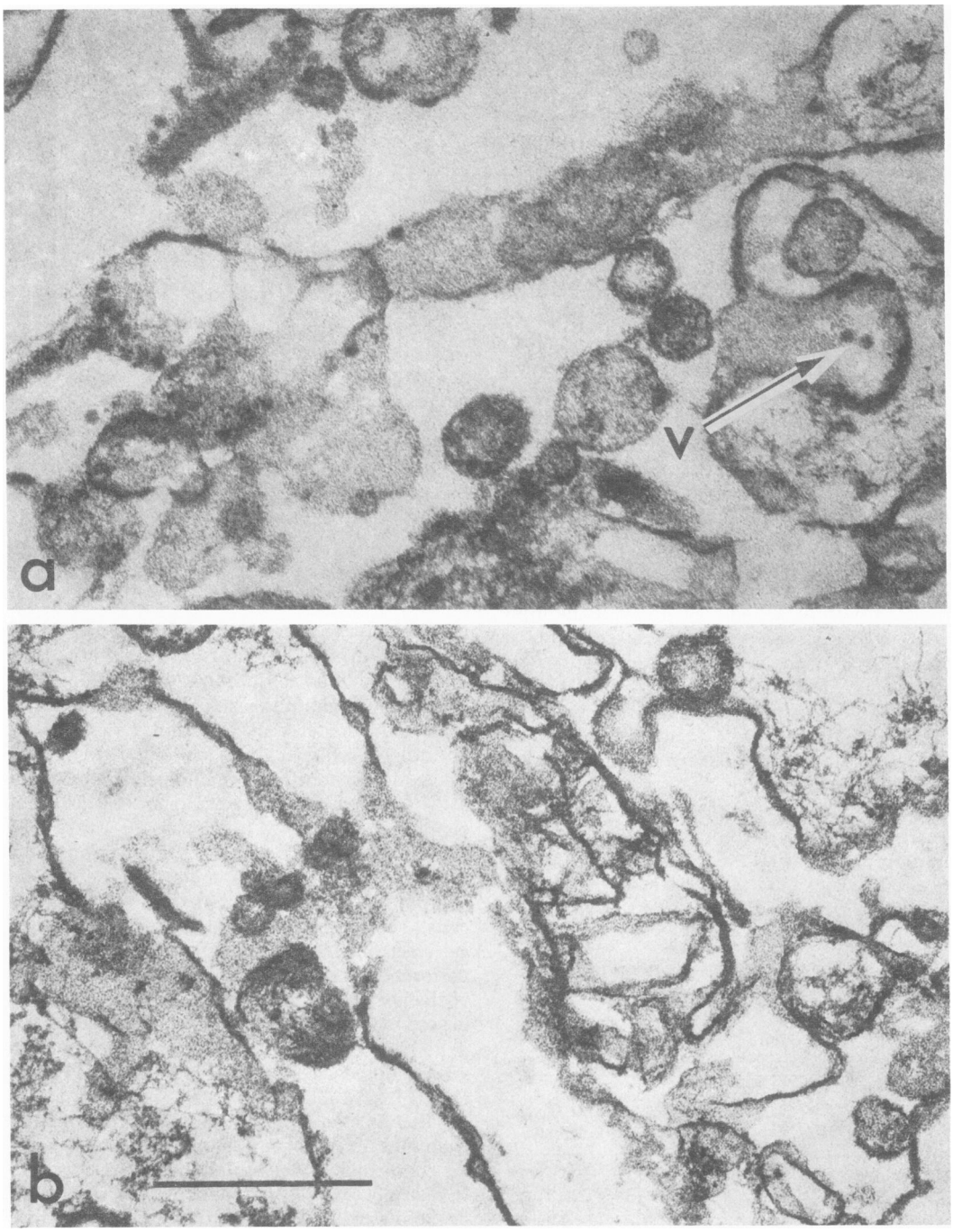

FIG. 6. (a) *Electron micrograph of HeLa cell membrane band 3 isolated from a discontinuous sucrose gradient. Coxsackievirus B3 (V) was attached to the HeLa cells prior to homogenization and centrifugation and can be seen in the plasma membrane preparation.* (b) *Membrane band 3 as it appeared when virus was not added to the HeLa cells. Bar, 0.5 μm.*

with the 2 major bands indicated. Fractions under the peaks were pooled, respectively, the membranes were pelleted by centrifugation, and each was resuspended in 1 ml of 0.02 M HEPES-buffered saline (pH 7.0) for assay of elution and uncoating activities. Samples of the preparations were incubated at 37 C and 0 C for 1 h, as before, and the membranes were resedi-

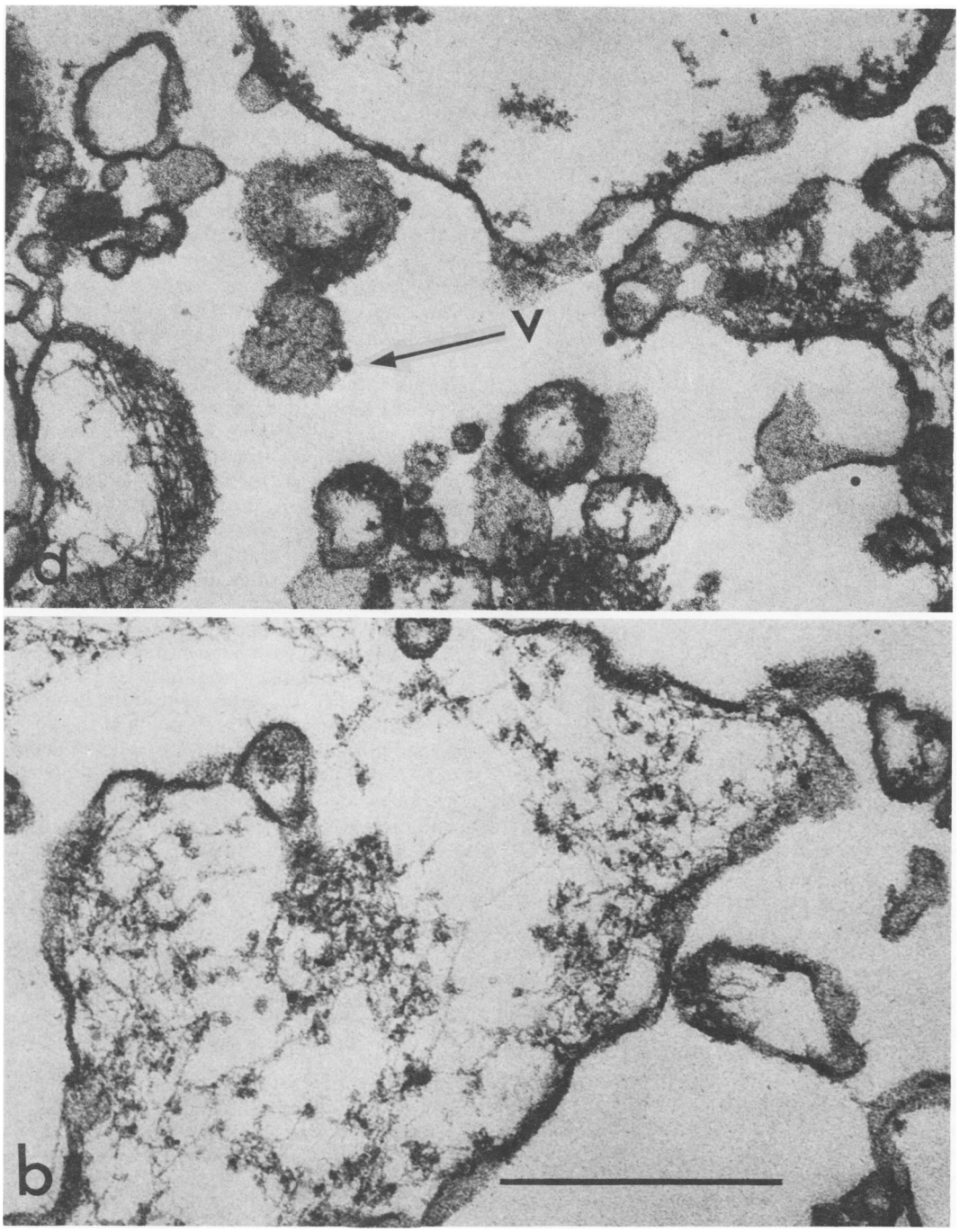

FIG. 7. *(a) Electron micrograph of HeLa cell membrane band 4 isolated from a discontinuous sucrose gradient. Coxsackievirus B3 (V) was attached to the HeLa cells prior to homogenization and centrifugation and can be noted in this preparation. (b) Membrane band 4 as it appeared when no virus was added to the HeLa cells. Bar, 0.5 μm.*

mented at 3700 × *g* for 20 min. The supernatant fluids were removed and RNase (100 μg) was added to each tube. The samples were incubated for an additional 30 min at room temperature and precipitated with cold trichloroacetic acid at a final concentration of 5%. The radioactivity obtained in the supernatant fluids after trichloroacetic acid precipitation was consid-

TABLE 3. *Elution of coxsackievirus B3 [^{14}C]valine by HeLa cell membranes isolated from discontinuous sucrose gradients*[a]

Sucrose gradient band	MAV[b]		Membrane-dissociated virus			
			Counts/min		%	
	0 C	37 C	0 C	37 C	0 C	37 C
3	120	686	20	2,289	14	77
4	760	629	21	1,723	3	73

[a] The amounts of radioactivity tabulated were obtained after incubation of the membrane preparations (MAV) for 1 h at the temperature indicated.

[b] Counts per minute.

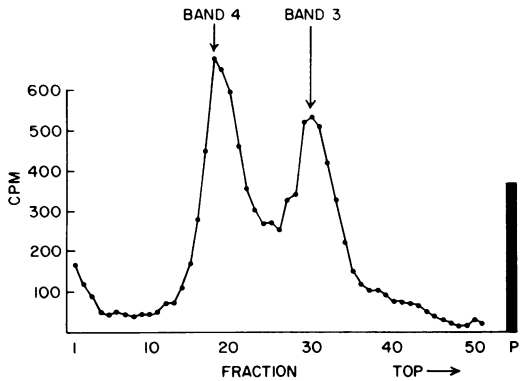

FIG. 8. *Distribution of ^{32}P-labeled coxsackievirus B3 radioactivity after virus attachment and HeLa cell fractionation on a discontinuous sucrose gradient. Eight-drop fractions were collected and the two major peaks were found to contain over 80% of the virus radioactivity which was added to the gradient.*

ered to be uncoated virus, whereas the radioactivity in the trichloroacetic acid precipitates was designated as eluted virus. The counts remaining with the membrane pellet after incubation were considered to be virus which remained receptor associated.

The results given in Table 4 reveal that greater than 90% of the viral radioactivity associated with membranes in each of the bands remained as MAV at 0 C, while more than 50% was dissociated at 37 C. The virus population which became dissociated from the membranes of gradient band 3 consisted of almost equal amounts of RNase-sensitive and RNase-resistant virions. The virus radioactivity which dissociated from gradient band 4 membranes, however, was predominately RNase resistant. Thus, the membranes from band 3 both eluted and uncoated B3 virus, whereas those from band 4 only eluted virus and did not appear to uncoat it. These observations were confirmed during several repeated experiments. Additional experiments were done as a control to determine the stability at 37 C of ^{32}P-labeled coxsackievirus B3. It was found that the virus was resistant to RNase activity and, therefore, the uncoating of virus by band 3 membranes was considered to be due to a specific effect on the virion capsid.

Inhibition of coxsackievirus B3 uncoating by HeLa cell plasma membranes. The finding that one of the plasma membrane-containing bands (band 4) would elute attached B3 virus, but not uncoat it, suggested the possibility that band 4 contained an inhibitor of virus uncoating. Experiments were designed to test this possibility even though no previous reports had revealed or suggested an inhibitor which could inhibit uncoating without inhibiting elution.

Two separate homogenates of HeLa cells were prepared, one with attached ^{32}P-labeled coxsackievirus B3 and the other without virus. The homogenates were fractionated on discontinuous sucrose gradients in parallel as described previously and fractions containing bands 3 and 4 were pooled separately from each gradient. An equal portion of the uninfected band 3 was mixed with band 4 from the infected homogenate and, likewise, uninfected band 4 was mixed with the virus containing band 3. These mixtures and equal amounts of the virus containing bands 3 and 4 were pelleted separately and the membrane-containing pellets were suspended in HEPES-buffered saline for incubation at 0 C or 37 C for 1 h. The amounts of virus radioactivity which became membrane dissociated and sensitive to RNase were determined as before, and are presented in Table 5.

TABLE 4. *Elution and uncoating of ^{32}P-labeled coxsackievirus B3 by HeLa cell membrane fractions recovered from a discontinuous sucrose gradient*[a]

Sucrose gradient band	MAV (% counts/min)		Membrane dissociated virus (% counts/min)			
			RNase resistant (eluted)		RNase sensitive (uncoated)	
	0 C	37 C	0 C	37 C	0 C	37 C
3	93	40	6	31	2	28
4	94	48	5	46	1	5

[a] Results compiled from four separate experiments. Fractions within the gradient bands were pooled, divided into two portions, pelleted, resuspended in HEPES-buffered saline, pH 7, and incubated at either 0 C or 37 C for 1 h. The amount of radioactivity as MAV prior to incubation was taken as 100%.

TABLE 5. *Inhibition of uncoating of ^{32}P-labeled coxsackievirus B3*

Sucrose gradient band	MAV (37 C)		Membrane dissociated virus (37 C)			
			RNase resistant (eluted)		RNase sensitive (uncoated)	
	%	Counts/min	%	Counts/min	%	Counts/min
3	34	235	37	252	29	196
3 and 4[a]	40	408	58	588	1	15
4	54	1,759	42	1,375	4	145
4 and 3[a]	49	1,730	46	1,629	4	154

[a] Fractions were pooled from the virus-containing gradient bands designated 3 or 4, respectively, and a portion of each received an equal amount of similarly prepared membrane fractions from noninfected cells. The mixtures were pelleted, resuspended in HEPES-buffered saline, pH 7, and incubated at either 0 or 37 C for 1 h. The amount of radioactivity as MAV prior to incubation was taken as 100%. At 0 C over 91% of the counts/min remained as MAV.

The data show that about 50 to 60% of the membrane-associated virus was eluted from the membranes in each of the four preparations. The membranes in band 3 also uncoated a significant porportion of the virus as shown previously. Of interest was the observation that the addition of membranes from band 4 (uninfected) to the virus-containing band 3 inhibited the uncoating activity of band 3. It was also interesting to note that the addition of band 3 from the uninfected preparation to the virus-containing band 4 did not enhance uncoating by the band 4 membranes. Thus, it was concluded that band 4 membranes contained an inhibitor of coxsackievirus B3 uncoating activity. The nature of this inhibitor is not known, but it did not inhibit the action of RNase on heat-disrupted B3 ^{32}P in the presence of membranes from band 4 when tested in a separate experiment. In fact, the amount of RNase used in all of these experiments was in excess by more than 10-fold the concentration needed to make the viral RNA soluble, as determined in control experiments using heat disrupted virus.

DISCUSSION

Studies of uncoating of enteroviruses by susceptible cells have uncovered a phenomenon of virus elution (6, 9, 13, 15, 20, 21). This phenomenon is characterized by the dissociation of radioactively labeled virions from the cell surface when cell-virus complexes are incubated at 37 C. Eluted virions differ from native virions in that the former are rendered noninfectious, do not reattach to susceptible cells, and have lost their lowest-molecular-weight polypeptide, VP4 (7). The present report reveals that isolated plasma membrane preparations of HeLa cells retained their capacity to elute coxsackievirus B3. The membrane-eluted virus was considered to be comparable to that obtained from intact cells, since VP 4 was lost from the eluted virions (7) and the elution activity of both cellular and membrane preparations was inhibited by low temperature and glutathione (9, unpublished observations).

The observation that uncoating of CAV is a temperature-dependent process, which is inhibited by low pH (6) and glutathione (9, unpublished observations) suggested that both elution and uncoating of virions are related activities of the viral receptors on the cell surface (6). However, results of the studies described in the present report provide evidence that viral elution and uncoating are more likely separate and distinct activities of receptors, even though both processes may have a common basis in causing an initial alteration of the virion manifested by the loss of VP 4. This interpretation of receptor function was based on our finding of a naturally occurring inhibitor which blocks virus uncoating without inhibiting virus elution.

This finding was made possible by modifying the method of Bosmann et al. (2) for the isolation of plasma membranes of HeLa cells. The most significant modification was to increase the sucrose concentration at the bottom of the gradient so that the plasma membranes were distributed in the two lower bands rather than in the pellet. The two lower bands, designated 3 and 4 according to their increasing densities, contained the two major plasma membrane marker enzymes, 5'-nucleotidase and Na^+, K^+ ATPase in addition to the receptors for poliovirus (unpublished data) and coxsackievirus. If one totals the units of enzyme activities from the 2 bands and calculates the specific activity of each enzyme, the results are comparable to those found by Bosmann and co-workers (2) for these enzymes in their pellet fraction which contained the plasma membranes. Electron micrographs of the band 3 and 4 preparations provided further evidence of their membrane composition.

In keeping with the thoughts of Warren et al. (27) and Wallach and Lin (26), we have introduced the use of radioactively labeled virus to serve as a marker (1) of plasma membrane location during subcellular fractionation. Ear-

lier studies from our laboratory had shown that the receptors for polioviruses and group B coxsackieviruses are found only on the outer surface of HeLa cells (29). Thus, labeled virus was allowed to attach to the receptors of cells prior to cell homogenization and fractionation. Since whole cells are readily washed free of unattached virus, the procedure allowed for labeling of the receptors and therefore of the plasma membrane, with minimal levels of background virus radioactivity. An essential consideration in using labeled virus as a marker of receptor location was the maintenance of low temperature during the entire procedure to prevent premature virus elution and uncoating. The results of experiments designed to monitor the distribution of virus radioactivity during the cell fractionation procedure and the subsequent separation of plasma membranes on discontinuous sucrose gradients revealed that almost all of the radioactivity was accounted for. Thus, we concluded that labeled virus which is made cell associated provides a useful marker for locating the plasma membranes for subcellular fractionation.

During this study, we were able to visualize a patchy distribution of attached B3 virions on the cell surface by aid of the electron microscope. The conditions of the experiment were adequate to saturate the available coxsackievirus B3 receptors on the cells (5). The clustered arrangement of receptors for coxsackieviruses B3 corrrelated well with results of our previous studies, which revealed that this virus attached to receptors that are different from those used to bind polioviruses (5, 6, 8, 16, 28–30). When poliovirus T1-^{3}Haa was added to HeLa cells prior to subcellular fractionation, as was done with coxsackievirus B3, a comparable distribution of virus label was found in the sucrose gradients. These results also were obtained by using double labels (poliovirus T1-^{3}Haa and coxsackievirus B3 [^{14}C]valine) on the same suspension of HeLa cells to indicate that either virus can serve equally well as a marker of plasma membranes.

In previous studies, Holland and Hoyer (13) have shown that HeLa cell plasma membranes will elute poliovirus and labilize a portion of attached virus which can be inactivated by a mixture of trypsin, chymotrypsin, and ribonuclease. Labilization to RNase activity was not accomplished in the absence of proteolytic enzymes and whole-cell homogenates were devoid of these activities presumably because they were comprised of a large "microsomal" component, and microsomes were without effect on disrupting virions. Chan and Black (3) also found that isolated plasma membranes from susceptible cells contained a labilizer for poliovirus, although they did not account for eluted virus. Philipson and Lind (23) solubilized a component from erythrocyte ghosts which labilized hemagglutinating strains of echovirus 7 and coxsackievirus B3 to RNase digestion, without showing activity against non-hemagglutinating strains of virus. The results reported herein for coxsackievirus B3 confirm and extend the above observations concerning the initial interactions of isolated plasma membranes on enteroviruses.

One of the most significant findings of our studies was that unfractionated cell homogenates failed to uncoat coxsackievirus B3, but that separation of the plasma membranes into two bands on sucrose gradients resulted in band 3 having uncoating capability. Additional experiments revealed that membranes in band 4 (the more dense band) possessed an inhibitor of virus uncoating when mixed with membranes from band 3. It is tempting to speculate that band 4 may be comprised primarily of the so-called microsomal fraction obtained by Holland and Hoyer (13) which attached poliovirus but failed to uncoat it. It is interesting to note that HeLa cells which were relatively resistant to poliovirus seemed to have a reduced capability of processing attached virus for viral replication (13). Perhaps these resistant cells have a higher level of inhibitor which prevents virus uncoating. The nature of the virus uncoating inhibitor remains to be determined.

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