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Interactions of Vesicular Stomatitis Virus with Murine Cell Surface Antigens

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The process of maturation of vesicular stomatitis virus (VSV) results in the loss of 70% of the H-2^k antigenic activity from L-cell plasma membranes. This phenomenon is also demonstrated during VSV infection of cells of the H-2^d haplotype. Using the method of inhibition of immune cytolysis, VSV-infected L5178Y tissue culture cells and VSV-infected METH A fibrosarcoma cells grown in vivo show a loss of H-2^d activity of 73 and 76%, respectively. Using monospecific antisera, it is seen that VSV infection results in a significant loss of antigenic activity of the gene products of both the H-2D and H-2K regions in cells of the H-2^d and H-2^k haplotypes. In hybrid cells expressing H-2^k as well as H-2^b, VSV infection results in the decrease of both H-2 antigenic activities to the same extent. VSV purified from L cells shows considerable H-2^k activity, but the reaction of this virus with anti-H-2^k serum does not prevent a normal subsequent infection with this virus. VSV may associate with H-2 antigen in the culture medium, but the results of mixing VSV with uninfected H-2-containing homogenates suggest that this association occurs only when the host cell and the cell homogenate share the same H-2 haplotype. Velocity sedimentation of VSV, which would remove contaminating cellular membrane fragments, does not separate H-2 activity from VSV. H-2 activity is also stably associated with VSV throughout sequential sucrose gradient centrifugation steps. It is possible that H-2 antigen is a structural component of VSV grown in murine cells.

Vesicular stomatitis virus (VSV) is an enveloped RNA-containing virus which matures at host cell surfaces (18, 33, 55). It has been established that the carbohydrate and lipid components of the virion envelope are host specified and are obtained from host cell enzymes or from the cell membrane during VSV maturation (2, 4, 22-24, 30). The protein components, on the other hand, are virus specific and are inserted into the cell membrane, forming an altered membrane site that becomes the envelope of the mature virion during the process of budding (7, 48, 49). Although it is certain that all newly synthesized protein in the virion envelope is virus specific, since VSV inhibits cell-directed protein synthesis by 3 h postinfection (p.i.) (32), it is not clear whether or not there is absolute exclusion of pre-existing host proteins from the virion envelope. Early attempts to determine if proteins of the viral envelope of budding viruses were synthesized by the host cell prior to infection have consisted of studies in which cells were radiolabeled with amino acids before infection and virus from these cells was analyzed for radioactive proteins (17, 38), or studies in which mature virus particles were reacted with ferritin-conjugated antibodies against uninfected host cell surfaces (12). These studies showed that whereas most of the protein of the virion is virus specific, a minimal contribution of host protein, approximately 2 to 5%, was always found.

In addition, the phenomenon of phenotypic mixing, that is, the assembly of the genome or nucleocapsid of one virus within the envelope or part of the envelope of a second, often unrelated virus within one cell, is now well known for many combinations of enveloped viruses (3, 5, 29, 51-54). The existence of phenotypic mixing implies that the process of virus maturation of enveloped viruses can tolerate some degree of error or nonspecificity, at least with respect to the envelope glycoproteins.

If the viral maturation process at the host cell plasma membrane can result in the inclusion into the virus envelope of proteins or glycoproteins not specified by that virus, then there is the possibility that proteins or glycoproteins of the host cell could also contribute to the maturing virus envelope.

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We have therefore studied the fate of a specific host cell membrane protein during VSV infection and the possible inclusion of that protein as a structural component of the virion. Using the method of inhibition of immune cytolysis, we have demonstrated the loss of 70% of the major histocompatibility antigen, the H-2 locus gene product, of L cells during infection with VSV and the association of the H-2^k antigenic activity of these cells with virus preparations from the culture medium (15). Although H-2 antigen is a glycoprotein, the antigenic activity resides within the protein moiety (35, 44, 45).

In this report, we extend our initial findings and demonstrate that the loss of H-2 activity from the cell surface during VSV maturation is a general phenomenon occurring in many different cell lines of various H-2 haplotypes and that the association of H-2 antigenic activity with virus particles is specific as well as stable throughout various virus purification steps.

MATERIALS AND METHODS

Cell cultures. L-cell suspension cultures and HeLa S_3 cells were grown in Joklik-modified Eagle minimal essential medium (Schwarz/Mann) plus 6% fetal calf serum (Grand Island Biological Co.), called complete minimal essential medium, at a concentration of 5×10^5 to 10^6 cells/ml and 3×10^5 to 6×10^5 cells/ml, respectively.

METH A cells, a chemically induced ascites fibrosarcoma, were maintained in BALB/c mice, and P815 cells, an ascites mastocytoma, were grown in DBA/2j mice.

The growth of L5178Y murine leukemia tissue culture cells (kindly provided by M. Nowakowski) derived from DBA/2 mice has been described (13).

HEL 3b, the cloned product of the Sendai virus-mediated fusion between a hepatoma cell line, Hepa la, lacking the enzyme hypoxanthine-guanine phosphoribosyltransferase (11) and an L cell line, LM(TK^-), deficient in the enzyme thymidine kinase, is a stable hybrid clone which was selected and maintained in monolayer culture in HAT selective medium (25, 26) consisting of Dulbecco-Vogt-modified Eagle medium (Grand Island Biological Co.) containing 10% fetal calf serum (Flow Laboratories) plus hypoxanthine, aminopterin, and thymidine, as detailed previously (40). These hybrid cells, as well as the parental cell lines, were generously donated by G. J. Darlington.

Growth of VSV. VSV of the Indiana serotype, originally grown in chicken embryo cells, was grown in the various suspension cell cultures by a method similar to that of Kang and Prevec (21). The procedure for the preparation of infected cells at 22 h p.i. for the assay of H-2 antigenic activity, as well as the method of concentration and partial purification of extracellular VSV from the culture medium of infected cells at 22 h p.i., have been detailed in a previous report (15). VSV preparations were either stored at −70°C in ET (1 mM EDTA-1 mM Tris-hydrochloride, pH 7.6) buffer containing 10% dimethyl sulfoxide (Me_2SO) (50) or were suspended in ET buffer without Me_2SO and further purified.

Purification of VSV. Various methods or combinations of methods of virus purification were used in these studies. For the purpose of isopycnic banding of VSV in sucrose, frozen suspensions of VSV were thawed, sonically treated for 2 min in a Bronson sonic oscillator at a setting of 3, and layered onto a continuous 30 to 50% (wt/wt) sucrose gradient prepared in ET buffer and centrifuged either in a Spinco SW27 rotor for 18 h at 27,000 rpm or in a Spinco SW41 rotor for 12 h at 35,000 rpm at 4°C. Gradient fractions were collected and analyzed for absorption at 260 nm in a continuous-flow cell by a Gilford recording spectrophotometer. The density of selected fractions was determined either by measurements with a Bausch and Lomb refractometer or by weighing 100-μl samples of the gradient fractions. The collected virus-containing fractions were pooled and either made 10% in Me_2SO and stored at −70°C or centrifuged to a pellet in ET buffer in a Spinco SW27 rotor at 27,000 rpm for 2 h at 4°C, resuspended in a small amount of ET buffer containing 10% Me_2SO, and stored at −70°C, or further purified.

For the purpose of VSV purification by velocity sedimentation in sucrose, linear 5 to 20% (wt/wt) sucrose gradients were prepared in ET buffer and calibrated for distance traveled versus sedimentation coefficient using ^{32}P-labeled T_2 bacteriophage (a gift of R. Kavenoff) having an S value of 700. Gradients were centrifuged in a Spinco SW27 rotor at 18,000 rpm for 40 min at 4°C and collected, and the position of the phage marker was determined. Thawed VSV preparations were sonically treated as above, and not more than 2 ml was layered onto 5 to 20% (wt/wt) sucrose gradients and centrifuged in a Spinco SW27 rotor under the conditions described for the calibration. After centrifugation and collection of the gradient, it was determined that less than 5% of the 260-nm absorbance was found in the region to which VSV T particles would sediment (19, 32), and from the position of the virus zonal band it could be stated that almost all the detectable virus consisted of B particles. Virus-containing fractions were pooled, centrifuged to a pellet in ET buffer as described, resuspended in a small volume of ET buffer, and either stored at −70°C with 10% Me_2SO or further purified.

Suspensions of VSV purified by equilibrium and/or zonal centrifugation in sucrose were layered onto a 20 to 30% (wt/wt) potassium tartrate gradient prepared in ET buffer and centrifuged in a Spinco SW27 rotor for 2.5 h at 27,000 rpm at 4°C. Gradient fractions were collected, and the density of selected fractions was determined as above. The virus-containing fractions were pooled, centrifuged to a pellet as described, resuspended in ET buffer plus 10% Me_2SO, and stored at −70°C. When gel electrophoresis of viral polypeptides was indicated, VSV-containing pellets from tartrate gradients were resuspended in 0.01 M phosphate buffer, pH 7.4, and dialyzed against the same buffer for 4 h before the

addition of 2% sodium dodecyl sulfate and 1% 2-mercaptoethanol (Eastman Organic Chemicals) as required.

Plaque assays of VSV samples were performed on monolayer cultures of L, LM(TK$^-$), or HEL 3b cells by the method of Jimenez et al. (20).

Polyacrylamide-sodium dodecyl sulfate gel electrophoresis. Radiolabeled virus polypeptides were analyzed by electrophoresis on 18-cm 7.5% polyacrylamide-sodium dodecyl sulfate gels as detailed previously (32, 43). Gels were crushed and collected in scintillation vials by means of an automatic gel fractionator and fraction collector (Savant; 31), scintillation fluid was added, and fractions were assayed for radioactivity in a Beckman liquid scintillation spectrometer.

Assay for H-2 alloantigenic activity. The production of H-2 alloantisera and the detection of H-2 activity on the surface of virus-infected and uninfected cells, as well as in purified VSV preparations, by the method of inhibition of immune cytolysis measured by the release of ^{51}Cr from target lymph node cells have been described in detail previously (15). Lymph node cells from B10.Br mice (H-2^k) and BALB/c mice (H-2^d) were used for assays of H-2^k and H-2^d antigenic activities, respectively. Both inbred strains of mice were purchased from Jackson Laboratories, Bar Harbor, Me. Lymph node cells from mice from the F_1 cross of C57B1/10 (H-2^b) and B10.Br (H-2^k) (a generous gift of F. Lilly), which we called H-2^k/H-2^b F_1 mice, were used to assay the antigenic activity of H-2^b on the surface of infected and uninfected Hepa la cells and both H-2^b and H-2^k activities on the surface of infected and uninfected HEL 3b cells. In these studies, antigen activity of cells is defined as units of antigen per milligram of protein, and antigenic activity of virus preparations is defined as either units of antigen per milligram of protein or units of antigen per PFU of virus. Total protein was determined by the method of Lowry et al. (27), with bovine serum albumin (Calbiochem) as the standard.

RESULTS

H-2^d antigenic activity of VSV-infected cells. We have previously reported the loss of greater than 70% of the H-2 activity of L cells (H-2^k) during infection with VSV and have shown that it is the process of viral maturation at the host cell surface that results in the loss of antigenic activity (15). To determine whether the loss of H-2 antigenic activity in VSV-infected cells is a phenomenon peculiar to L cells, or to cells of the H-2^k haplotype, cells of the H-2^d haplotype were similarly studied. Figure 1 shows the inhibition of immune cytolysis of VSV-infected and uninfected L5178Y cells at 22 h p.i. Although the culture medium of infected L5178Y cells yielded infectious titers of VSV of approximately 1 log lower than virus from an equal number of L cells, VSV-infected L5178Y cells showed a loss of 73% of the H-2^d activity compared with uninfected cells (Fig. 1, Table 1).

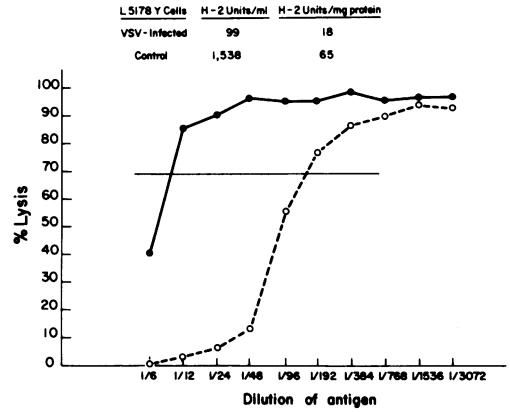

FIG. 1. *H-2^d antigenic activity of VSV-infected L5178Y cells. L5178Y cells were infected with VSV for 22 h at a multiplicity of infection of 10. Infected and control cells were prepared for the assay of H-2 antigenic activity as described previously (15). A 100-μl sample of cells (antigen) was serially diluted 1:2 and reacted with 100 μl of anti-H-2 antiserum appropriately diluted with a 1:10 dilution of guinea pig complement (Grand Island Biological Co.) and 100 μl of ^{51}Cr-labeled lymph node cells from a BALB/c mouse. In this case the anti-H-2^d antiserum used was raised in a mouse of the H-2^k haplotype. The reaction was terminated after 1 h at room temperature with 300 μl of 10 mM EDTA in phosphate-buffered saline, pH 6.9, cells were centrifuged, and samples of the supernatant fluid were counted for radioactivity. The percentage of lysis of lymph node cells was calculated for each dilution, and the reciprocal times 10 of the dilution which caused 50% lysis was defined as the number of H-2 units per milliliter. When the full-release control, containing diluted antiserum but no inhibiting antigen, was not 80% of the total counts released by undiluted antiserum, the 50% lysis level for determining H-2 units per milliliter was adjusted. Symbols: ●, VSV-infected L5178Y cells; ○, uninfected cells. The horizontal line is the adjusted 50% lysis level.*

P815 cells and METH A cells are both of the H-2^d haplotype and were taken directly from the mice in which they were grown. These tumor cells were shown to replicate VSV to high titers in preliminary experiments. Cells assayed for H-2^d activity at 22 h p.i. showed a 62 and 76% loss of H-2^d activity from P815 and METH A cells, respectively (Table 1), compared with uninfected cells.

H-2^b and H-2^k activities of VSV-infected hybrid cells. To determine whether the loss of antigenic activity during VSV infection of one set of H-2 specificities (H-2^k) would alter or prevent the effect of VSV infection on a second separate set of H-2 specificities (H-2^b), HEL 3b, a hybrid cell line expressing both H-2^k and H-2^b

activities as a result of concomitant expression of the H-2 locus genes from each of the parental cell lines, was assayed after VSV infection. Table 2 shows that the H-2^k and H-2^b activities of HEL 3b cells were affected identically during VSV infection. Assays of the parental cell line Hepa la showed that 58% of the H-2^b antigenic activity was no longer detectable after a 22-h VSV infection compared with uninfected Hepa la cells.

Assay of individual H-2 specificities of VSV-infected cells. The total H-2 activity of any cell is composed of the sum of activities contributed by a set of individual specificities from both the H-2K and H-2D regions, the two genes of the complex H-2 genetic locus, each coding for a separate membrane molecule (8, 9, 10, 46). By the use of monospecific antisera, the fate of individual specificities during VSV infection could be determined. L cells of the H-2^k haplotype were infected with VSV for 22 h and assayed for the activity of the H-2.11 and the H-2.32 specificities. Specificities 11 and 32 are products of the H-2K and H-2D genes, respectively, and therefore reside on separate glycoprotein membrane molecules (10). In VSV-infected cells, H-2.11 activity decreased approximately 59% (Fig. 2A), whereas H-2.32 activity decreased approximately 45% (Fig. 2B), compared with uninfected cells. In a comparable manner, VSV-infected METH A cells assayed for the individual specificities H-2.31 and H-2.4, representing the H-2K and H-2D gene products, respectively, showed similar decreases in antigenic activity compared with uninfected cells (unpublished data). Therefore, during infection with VSV there was a significant loss of

TABLE 1. *Effect of VSV infection on H-2 activity in cell lines of the H-2^d haplotype*

Cell line	VSV	H-2 units/mg of protein	% of loss of H-2^d activity in VSV-infected cells
L5178Y	Infected	18	73
	Uninfected	65	
P815	Infected	230	62
	Uninfected	607	
METH A	Infected	173	76
	Uninfected	712	

TABLE 2. *H-2^b and H-2^k activities of VSV-infected hybrid cells*

Cell line	H-2^b units/mg of protein[a]	% Loss of H-2^b activity	H-2^k units/mg of protein[a]	% Loss of H-2^k activity
HEL 3b				
Infected[b]	41.7	78.0	53.1	77.9
Control	189.4		240.0	
Hepa 1a				
Infected[b]	3.0	57.7		
Control	7.1			

[a] Both H-2^b and H-2^k antigenic activities of HEL 3b cells, as well as H-2^b activity of Hepa 1a cells, were assayed using target lymph node cells from H-2^k/H-2^b F_1 mice. To eliminate any cross-reaction between specificities comprising the H-2^b and H-2^k haplotypes, antisera against H-2^b specificities were raised in B10.Br mice (H-2^k) and antisera against H-2^k specificities were raised in C57Bl/10 mice (H-2^b).

[b] Both cell lines were infected with 10 PFU of VSV per cell in confluent monolayer cultures (10^7 cells/culture) in the absence of serum. At 1.5 h p.i., the medium (Dulbecco-Vogt-modified Eagle medium) was completed by the addition of 10% fetal calf serum and the cells were incubated for 22 h, removed from the flask, washed, and assayed for H-2 activity.

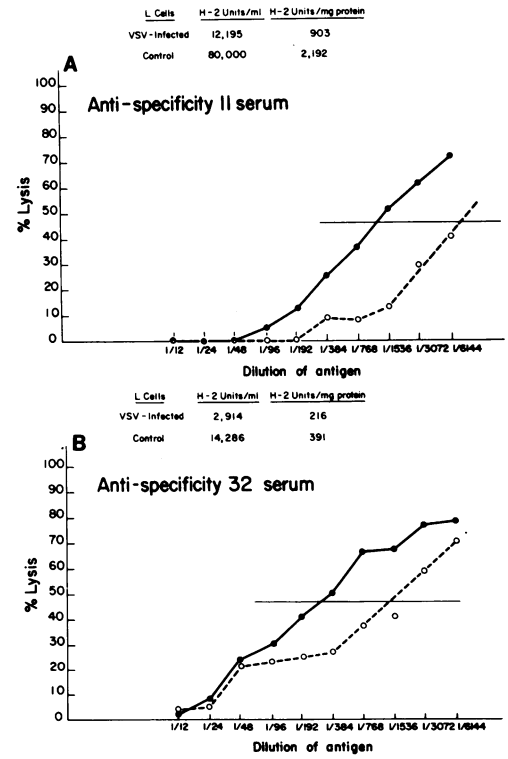

FIG. 2. *Antigenic activity of individual H-2 specificities of VSV-infected L cells. L cells, at 10^7 cells/ml, were infected with VSV for 22 h in the absence of actinomycin D. (A) Antigenic activity of specificity 11. Absorbed antiserum to specificity 11 of the H-2K gene was used in this assay. (B) Antigenic activity of specificity 32. Absorbed antiserum to specificity 32 of the H-2D gene was used in this assay. Symbols: ●, VSV-infected L cells; ○, uninfected L cells. The horizontal lines are the adjusted 50% lysis levels.*

antigenic activity of the gene products of both the H-2K and H-2D genes in cells of the H-2^k and H-2^d haplotypes.

H-2 activity of VSV. VSV grown in L cells and purified from the culture medium at 24 h p.i. has been shown to possess considerable H-2^k antigenic activity (15). This H-2 activity cannot be due to simple contamination of the virus preparation by cellular material during virus purification, since it has been shown that, when VSV was grown in L cells and in HeLa cells containing no H-2 activity, mixed with L cell homogenates, and purified, only the virus preparation grown in L cells contained H-2 activity (15). However, the antigenic activity of the L-cell-grown virus preparation mixed with an L-cell homogenate was greater than that virus preparation mixed with a HeLa cell homogenate (15) or purified directly from the cell culture medium (unpublished data). From these data, it was concluded that, at least in part, the association of H-2 with virus takes place extracellularly but that there is a specificity to this association since it occurs only if the virus was grown in an H-2-containing cell. We then determined whether the association of H-2 activity with VSV can take place between homogenates and virus grown in any H-2-containing cell line or if there is an additional specificity for haplotype. L5178Y cells, L cells, and HeLa cells (10^8) were infected with VSV for 22 h. The cells were then removed by centrifugation, and the virus-containing supernatant fluids were each mixed with 10^8 L5178Y cells, which were disrupted with a tight-fitting Dounce homogenizer. After 1 h at 0°C, cells and debris were removed by centrifugation and the virus preparations were purified by polyethylene glycol precipitation (15, 28) and isopycnic banding as described above. Virus pellets were resuspended in equal amounts of ET buffer and assayed for plaques on L-cell monolayers and for H-2^d activity. To insure that none of the individual specificities of the H-2^k haplotype present in L cells and common to both H-2^k and H-2^d would be detected by the anti-H-2^d serum, the antiserum used was one that was raised in H-2^k mice. The H-2^d activity of VSV grown in H-2^d cells and mixed with H-2^d-containing homogenates was greater than the activity of VSV grown either in H-2^k cells or in cells lacking H-2 antigen and mixed with H-2^d-containing homogenates (Table 3). The difference in H-2 activity between virus preparations grown in L5178Y cells and the other cell lines is smaller when antigenic activity was expressed as H-2 units per milligram of protein than when expressed as H-2 units per PFU. This may be due to the large amount of non-H-2 protein as well as H-2 antigenic protein derived from the host cell present in samples of VSV grown in L5178Y cells. This specific association of cellular components with virus could have occurred either during the maturation of VSV in L5178Y cells or during the mixing procedure. Although the infectious virus yield from L5178Y cells is 1 log less than the yield from L cells, the H-2 activity of this preparation (H-2 units per PFU) was 20 times as great as that of the preparation from L cells. We therefore concluded that the association of H-2 from cell homogenates with virus preparations is specific for H-2 haplotype.

Amount of H-2 associated with VSV. VSV-infected L cells, an equal number of uninfected L cells, and the virus purified from these cells by equilibrium density banding in sucrose were assayed for total H-2^k units in each preparation. Table 4 shows that, at 22 h p.i., VSV-infected cells retained only 13% of the total H-2 of control cells. However, VSV purified from these cells was found to have 75.7% of the total H-2^k of control cells. H-2^k activity not associated with virus could not be detected in the culture medium from VSV-infected cells even after a fivefold concentration of the fluid and subsequent dialysis (unpublished data). Approximately 11% of the H-2 activity cannot be accounted for (Table 4).

Infection of L cells with anti-H-2^k-coated cells. VSV was grown in L cells and assayed for plaques on L-cell monolayers yielding a titer of 2.6×10^8 PFU/ml. Two-tenths milliliter of this

TABLE 3. *H-2^d activity in VSV preparations grown in H-2^d or H-2^k cells and mixed with homogenates containing H-2^d activity*

VSV grown in:	H-2^d units/mg of protein	PFU/ml	H-2^d units/PFU ($\times 10^9$)	mg of protein/PFU
L5178Y cells	47	1.2×10^8	390	0.92
L cells	<10	1.7×10^9	<18	0.12
HeLa cells	<8	7.5×10^9	<4	0.05

TABLE 4. *Association of H-2 antigen with VSV grown in L cells*

Prepn	H-2 units/ml	Total H-2 units in the sample	% Total H-2 of uninfected L cells
VSV-infected L cells	149	298	13.5
Uninfected L cells	1,099	2,198	
Purified VSV[a]	833	1,666	75.7

[a] VSV was purified from 10^8 L cells at 22 h p.i. by isopycnic banding in sucrose.

virus preparation was reacted with a 1:28 dilution of anti-H-2^k serum or normal mouse serum for 1 h at 0°C. The entire reaction mixtures were then used to infect two separate cultures of 5×10^7 L cells in the presence of actinomycin D. The course of infection was followed by measuring the incorporation of [^{14}C]uridine (New England Nuclear Corp.) into trichloroacetic acid-insoluble material (Fig. 3A). When VSV pretreated with anti-H-2^k serum was used to infect L cells, the rate of RNA synthesis, determined by the slope of the cumulative incorporation curve and the maximal virus-specific RNA produced at 11 h p.i., as well as the amount of whole virus detected on isopycnic gradients at 22 h p.i. (Fig. 3B), was nearly equal to that of infections in which VSV was pretreated with normal serum. Therefore, the presence of antibodies directed against H-2^k in preparations of VSV purified from L cells did not interfere with a subsequent infection of VSV in L cells.

Effect of different purification methods on the H-2 activity of VSV preparations. Since the density in sucrose of VSV and of plasma membranes is similar, it is possible that, during the isopycnic banding of VSV, H-2 antigen on cellular fragments might have associated with or purified along with the virus. If H-2 activity of VSV preparations is due to cellular contamination, it might be eliminated or reduced by another method of virus purification such as velocity sedimentation.

After a 23-h infection of L cells with VSV in the presence of 0.5 μCi of ^{14}C-labeled amino acids per ml, virus was concentrated from the extracellular medium, and one-half of the preparation was subjected to velocity sedimentation in sucrose while the remainder of virus was subjected to equilibrium density banding in sucrose. Analysis of gradient fractions (Fig. 4A and B) from both gradients showed that, whereas radiolabeled VSV was localized in one major band from the velocity-sedimented gradient, two peaks of radioactivity, the virus-containing band at a density of 1.16 to 1.18 g/cm^3 and a less dense band, were detectable from the equilibrium density gradient. The less dense peak of radiolabel was detected in other VSV preparations from a variety of cell lines

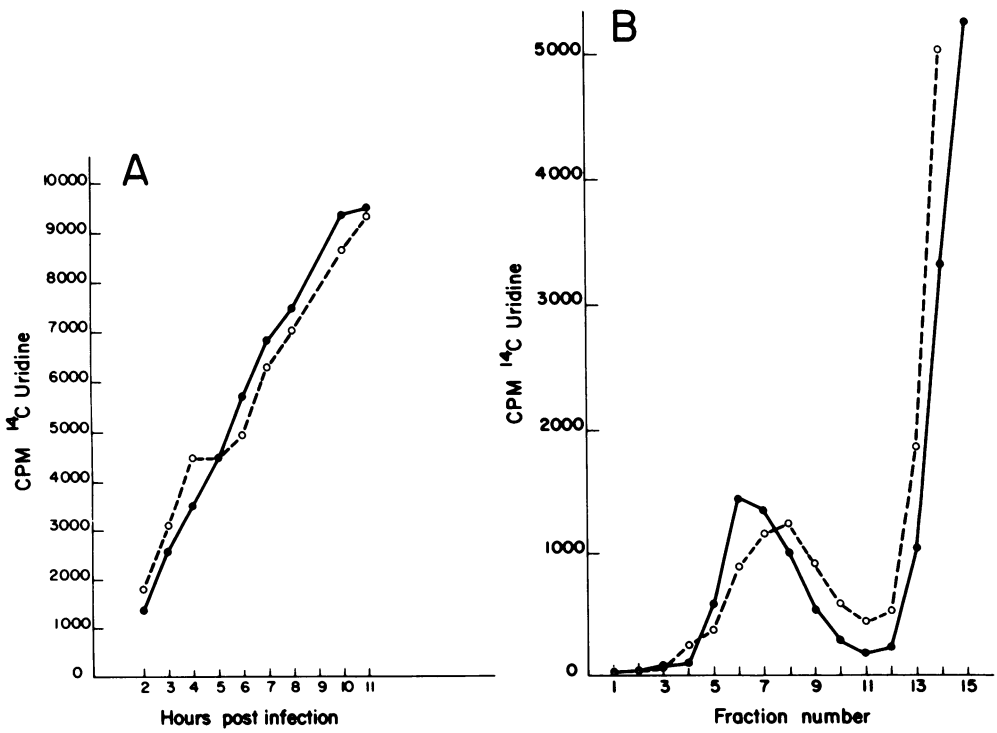

FIG. 3. *Infection of L cells with anti-H-2^k-coated VSV. (A) Incorporation of [^{14}C]uridine into trichloroacetic acid-precipitable material in L cells infected with antibody-coated VSV. (B) Equilibrium density sucrose gradient of virus produced in L cells infected with antibody-coated VSV. Virus was centrifuged in a Spinco SW41 rotor for 12 h at 35,000 rpm. The radiolabels of fractions of both gradients were plotted on the same graph. Symbols: ●, VSV reacted with anti-H-2^k serum; ○, VSV reacted with normal mouse serum.*

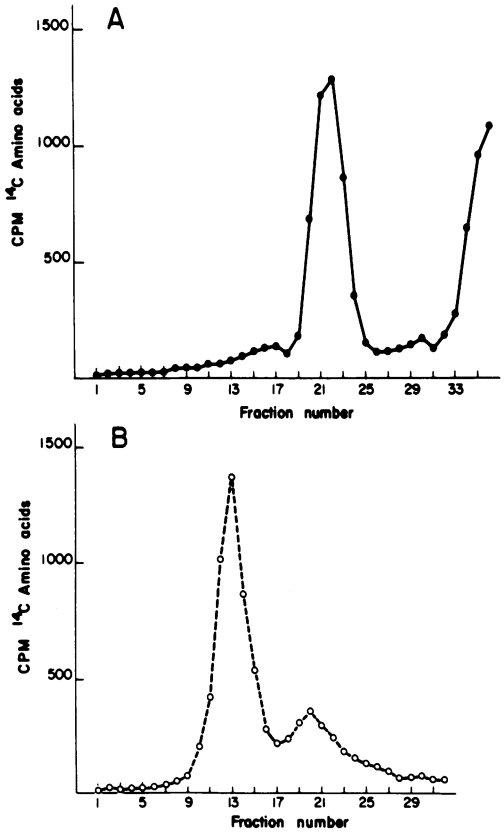

FIG. 4. *(A) Velocity sedimentation of VSV grown in L cells. VSV was centrifuged on a linear 5 to 20% (wt/wt) sucrose gradient in a Spinco SW27 rotor at 18,000 rpm for 40 min, gradient fractions were collected, and 10-μl samples were analyzed for radioactivity. (B) Equilibrium density centrifugation of VSV grown in L cells. VSV was centrifuged in a Spinco SW27 rotor at 26,000 rpm for 16 h, gradient fractions were collected, and 10-μl samples were analyzed for radioactivity.*

under these conditions of centrifugation, but the structure of the particles in this band is unknown. The three radiolabeled peaks were collected and assayed for viral plaques and H-2 activity. The preparations were also subjected to 7.5% polyacrylamide-sodium dodecyl sulfate gel electrophoresis.

If the H-2 antigenic activity of the virus preparation was merely due to cellular contamination, velocity-sedimented virus would have less H-2 activity compared with equilibrium-banded virus, since VSV would sediment only partially through the gradient whereas whole membranes and membrane fragments containing H-2 would sediment to a pellet and closed membrane vesicles would remain at the top of the gradient (P. H. Atkinson, personal communication). However, the "velocity VSV" preparation showed somewhat greater H-2 activity than "equilibrium VSV" (Fig. 5). H-2 antigen, which therefore either is a part of the virion itself or else is tenaciously attached to VSV, is enriched in the case of velocity VSV since it is less diluted by other non-H-2 host proteins.

Gel electrophoresis patterns of the three viral preparations showed all the VSV structural proteins (Fig. 6A, B, and C). Because the viral proteins G and M are probably synthesized at sites associated with or near the cell plasma membrane and virus nucleocapsid protein N is synthesized in the membrane-free cytoplasm (7, 48, 49), plasma membrane fragments from infected cells should have large amounts of inserted proteins G and M. VSV preparations contaminated with host plasma membrane fragments and debris, subjected to electrophoresis, would have larger ratios of radiolabeled proteins G/N than highly purified VSV preparations. The radioactivity of each protein peak was totaled, and it was seen that the ratios of proteins G/N of velocity VSV and of equilibrium VSV were nearly identical at 0.69 and 0.70, respectively. Therefore, no appreciable cell membrane material carrying large amounts of incorporated protein G was present in VSV preparations prepared by isopycnic banding in sucrose. The "equilibrium light fraction" showed a lower protein G/N ratio of 0.58 (Fig. 6C). It is possible that this preparation, although containing all the major VSV structural proteins, lacked RNA. The nucleic acid content of this fraction was not determined, but a peak of radioactivity at this density was never

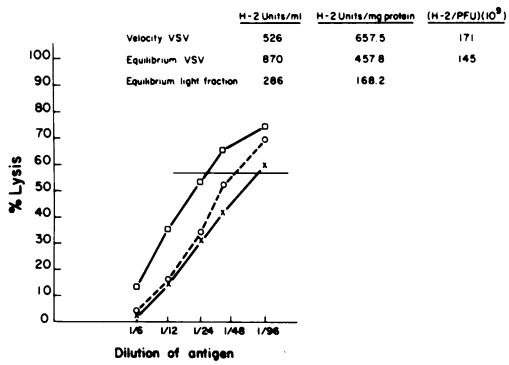

FIG. 5. *H-2^k antigenic activity of VSV preparations purified by velocity sedimentation or equilibrium density centrifugation in sucrose. Gradient peaks from Fig. 4 were assayed for H-2^k antigenic activity after centrifugation to a pellet of appropriate fractions and resuspension in 0.5 ml of ET buffer. Symbols: ○, velocity VSV; ×, equilibrium VSF; □, equilibrium light fraction. The horizontal line is the adjusted 50% lysis level.*

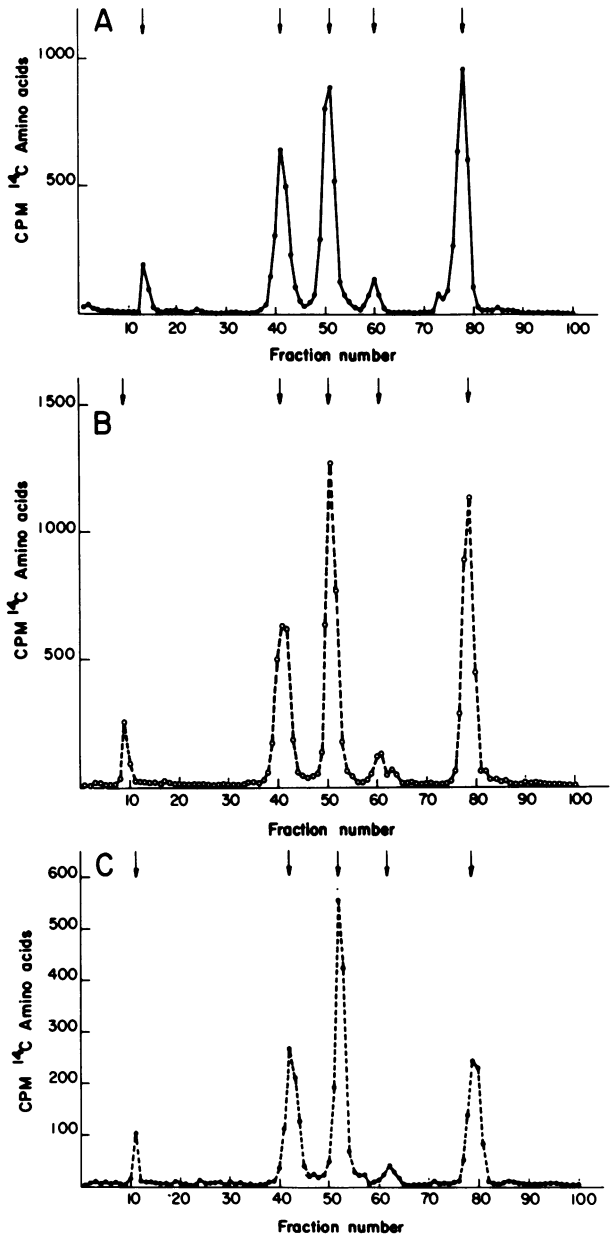

FIG. 6. *¹⁴C-amino acid-radiolabled VSV of purified viruses and equilibrium light fraction. Twenty-five microliters of each virus preparation and 150 μl of the equilibrium light fraction of Fig. 5 were added to 0.2 ml of 0.01 M phosphate buffer containing 2% sodium dodecyl sulfate and 1% 2-mercaptoethanol and subjected to 7.5% polyacrylamide-sodium dodeyl sulfate gel electrophoresis. The arrows represent the position of [³H]VSV marker proteins. (A) Velocity VSV; (B) equilibrium VSV; (C) equilibrium light fraction.*

seen when VSV was radiolabeled with uridine. It was of interest that this fraction, although yielding no infectious viral plaques, did contain H-2 antigenic activity (Fig. 5).

Stability of H-2 activity in VSV preparations during sequential purification steps. To determine whether H-2 activity remained stably associated with VSV during various centrifugation procedures, VSV grown in L cells and radiolabeled with [³H]uridine was sequentially purified by equilibrium density centrifugation, by velocity sedimentation, and finally, by isopycnic banding in potassium tartrate. Samples of virus were removed at each step of purifica-

tion to assay for H-2 activity, infectivity on L cell monolayers, and uridine radiolabel.

Virus subjected to one, two, or three centrifugation procedures was compared for extent of virus purification, measured by increasing ratios of total viral particles ([^{3}H]uridine)/milligram of protein and infectious virus (PFU)/milligram of protein, and for enrichment of infectious B particles, measured by a decreasing ratio of total virus particles/infectious particles ([^{3}H]uridine/PFU), as well as for H-2 activity (Table 5).

The velocity VSV preparation clearly showed a greater degree of purification than the equilibrium VSV of the previous step. There was also a concomitant enrichment of H-2 activity with VSV purification (Table 5). These data again suggest that either H-2 antigen is a structural component of VSV grown in L cells or the binding of cell membrane H-2 antigen with virus is so tight that it cannot be easily separated from the virus once bound. The strength of binding is demonstrated by the fact that both velocity VSV and equilibrium VSV preparations had almost equal particle ([^{3}H]uridine)/H-2 units ratios.

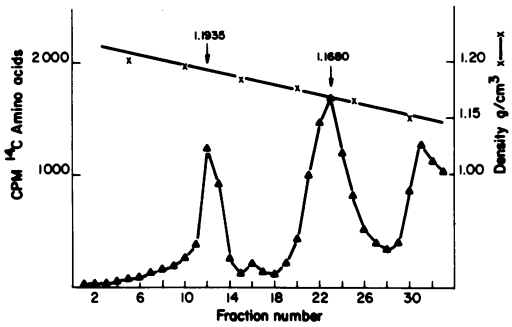

FIG. 7. *Potassium tartrate density centrifugation of VSV grown in L cells. L cells infected with VSV in the presence of actinomycin D were radiolabeled with ^{14}C-amino acids from 4 to 24 h p.i., when virus was collected and purified on a 20 to 30% (wt/wt) potassium tartrate gradient in a Spinco SW27 rotor for 2.5 h at 27,000 rpm at 0°C. Gradient fractions were collected and analyzed for radioactivity.*

The third step of purification on potassium tartrate gradients resulted in an increased virus particle purification ([^{3}H]uridine/milligram of protein) but no further enrichment of infectious virus particles (PFU/milligram of protein). In addition, H-2 activity (H-2 units/milligram of protein) of this VSV preparation decreased. Isopycnic banding of VSV in tartrate resulted in two major radiolabeled peaks, a heavy band at a density of 1.194 and a lighter band at a density of 1.168. Other investigators have described the denser of the two viral bands as consisting of VSV that may have been stripped of part of its envelope lipids (24, 29). Although both bands were shown in a preliminary experiment to contain all of the VSV structural proteins, only the lighter band was collected and assayed in these experiments. It is possible that interactions of tartrate with VSV resulted in denaturation of H-2 activity still associated with the virion particle, or that this final centrifugation step resulted in a purification of H-2 antigen away from VSV. A third explanation is that tartrate may cause an aggregation of VSV particles and thus cause an apparent decrease in H-2 activity, as detected by the immune cytotoxicity inhibition assay. It is not known precisely what effect tartrate has on the integrity of VSV virions.

When H-2 activity was measured as H-2 units per PFU, there was a steady decrease in antigenic activity during these three sequential purification steps. It is possible that there was some decrease in the yield of H-2 activity during repeated sonication and centrifugation steps, whereas the infectivity of VSV (PFU) under these conditions was fairly stable.

DISCUSSION

Using VSV as a prototype of membrane-maturing viruses, we have shown that the antigenic activity of a specific plasma membrane constituent, the histocompatibility antigen H-2, of several murine cell lines decreased during virus infection to about 24 to 38% of the antigenic activity of uninfected cells.

TABLE 5. *Sequential purification of VSV from L cells*

VSV prepn[a]	[^{3}H]uridine (cpm)/mg of protein ($\times 10^6$)	PFU/mg of protein ($\times 10^{10}$)	[^{3}H]uridine (cpm)/PFU ($\times 10^{-5}$)	H-2 units/mg of protein	[^{3}H]uridine (cpm)/H-2 units ($\times 10^3$)
Equilibrium VSV	3.47	0.69	5.0	83	4.2
Velocity VSV	4.41	1.19	3.7	97	4.5
Tartrate VSV	5.07	1.04	4.9	70	7.2

[a] 1.6×10^9 L cells in 160 ml were infected with VSV. Actinomycin D and 10 μCi of [^{3}H]uridine per ml was added at 30 min and 3 h p.i., respectively. Extracellular virus was collected and concentrated at 22 h p.i. Conditions for gradient centrifugation steps are described in Materials and Methods.

The loss of H-2 antigenic activity during VSV infection has been demonstrated in cells of the H-2^k haplotype (15) and H-2^d haplotype (Fig. 1, Table 1), and in cells that express two H-2 haplotypes independently as a result of somatic cell hybridization (Table 2). The phenomenon of antigenic loss occurs in cells adapted for culture in vitro as well as in tumor cells grown in mice and infected with VSV in vitro. Infected cells showed decreases in activity of several individual H-2 antigenic determinants specified by the H-2D or H-2K gene (Fig. 2A and B). Since each gene specifies a separate molecular product (10), the decrease in antigenic activity of at least two glycoprotein membrane molecules might indicate that all plasma membrane glycoproteins are affected in a similar manner during VSV infection. There is no reason to believe that H-2 glycoproteins, which make up approximately 1% of the plasma membrane, represent a unique population of membrane glycoprotein molecules.

It is possible that the loss of H-2 antigenic activity during virus infection is due to the extrusion of host membrane antigens during the insertion of newly synthesized viral proteins into the host cell surface. Reeve and coworkers (39) have suggested that the inability to detect an increase in membrane concanavalin A binding sites in paramyxovirus-infected cells may be due to a loss of host cell lectin binding sites concomitant with an increase in new viral lectin binding sites during virus infection.

We have shown that the production of large amounts of infectious viral particles is not necessary to demonstrate a decrease of H-2 activity. L5178Y cells produce 1 log less infectious virus titer than do L cells, and yet the loss of H-2 activity in both these cell lines is similar. In addition, Newcastle disease virus-infected L cells also show a loss of H-2 activity even though only few infectious virus particles are produced (16). At this time we do not know whether the loss of H-2 activity is correlated with the total number of virus particles produced by the various host cells. However, it is possible that completion of the virus maturation process by budding may not be necessary for H-2 activity to decrease. Polyoma virus, a DNA-containing virus, which causes cell transformation of mouse cells, has been shown to cause decreases in host H-2 antigen (47). This virus does not contain a viral envelope or mature at the host cell surface, but it does induce the synthesis and insertion into the host plasma membrane of a tumor-specific cell surface antigen. The amount of tumor-specific cell surface antigen induced correlated inversely with the amount of H-2 remaining on the host surface after transformation by polyoma virus (47).

Similar results were also reported by Cikes et al. (6), who showed, using several different techniques in various murine lymphoma cell lines, that there was a reciprocal relationship between the expression of H-2 antigens and the concentration of Moloney leukemia virus-induced cell surface antigen.

The question of whether virus budding is necessary for the loss of H-2 antigenic activity to occur might be resolved by the use of temperature-sensitive mutants of VSV, which is blocked at a maturation step or in the synthesis of one of the viral envelope proteins at the nonpermissive temperature.

Another possibility is that H-2 antigenic activity decreases in the cell membrane during VSV maturation as a result of the inclusion of the antigen into the viral envelope. Aoki and Takahashi (1) have demonstrated by electron microscopy the presence of H-2^k, but not H-2^b, on approximately one-fourth of the extracellular as well as budding gross murine leukemia virus particles from murine leukemia cell lines.

We have found that, upon purification, VSV grown in L cells contained about 75% of the H-2^k activity of the uninfected host cell (Table 4). However, experiments in which VSV containing H-2 activity was reacted with anti-H-2 serum showed that the presence of these antibodies in no way affected a subsequent normal course of infection with this virus (Fig. 3A and B).

One explanation for this finding is that incorporated H-2 antigen is unrelated topographically to the VSV-specified G protein, which is the surface neutralization antigen and required for VSV infectivity. Anti-H-2 antibody combined with VSV might not sterically hinder virus from attaching to a host cell receptor. It is yet to be determined whether H-2 antigen and inserted G protein can co-cap on the surface of VSV-infected L cells.

A second explanation of the data is that the binding of anti-H-2 antibodies to VSV may form infectious virus-antibody complexes. Similar infectious complexes have been described in mice infected in vivo with lactic dehydrogenase virus (37). More recently, Schlesinger demonstrated that antibody produced against the major glycoprotein of Rous sarcoma virus, gp85, a protein necessary for viral infectivity, will not cause neutralization of the virus but will bind to the intact virion to form a virus-antibody complex that is infectious (41).

A third possibility is that the actual number of anti-H-2 antibody molecules is small in serum used for the cytotoxicity inhibition assay compared with the number of H-2 molecules associated with VSV. A low level of neutralization of VSV caused by a reaction of virus and this antiserum might not be readily apparent.

Simple adsorption or co-purification of cellular H-2 with VSV was ruled out by mixing experiments that demonstrated that the association of H-2 activity with virus is specific for a particular haplotype (Table 3). If only nonspecific adsorption of H-2 antigen to virus occurred, one would expect VSV grown in any cell type to show equal levels of antigenic activity when mixed with homogenates containing equal amounts of H-2 antigen.

The specificity of the association may be explained by an incorporation of H-2 glycoprotein into the virus envelope during the exit of the virus from a cell containing H-2 antigen. This incorporated antigen might then act as a center around which additional H-2 antigen on cellular fragments in the homogenate, or from the original infected cell culture, would aggregate. The basis of the specificity of association would have to reside in the protein and not the carbohydrate portion of the H-2 glycoprotein, since the carbohydrate composition of different H-2 antigens is fairly similar (33, 35).

Whether the association of H-2 antigen and VSV occurs as a result of H-2 antigen becoming a structural component of the virion or by H-2 antigen being specifically and tenaciously bound to the virion, the H-2 antigenic activity and VSV particles cannot be separated by velocity sedimentation in sucrose. In fact, a greater H-2 specific activity of velocity VSV compared with equilibrium VSV was observed (Fig. 5) even though the former preparation contained less than one-half the total amount of protein of the latter preparation. This indicates that large amounts of non-H-2 proteins that might have been host cell contamination were removed from the virus preparations during velocity sedimentation but were not removed during equilibrium density centrifugation. However, VSV-specified proteins, which were not assembled into mature virions and were therefore either in cell membrane fragments or in the cytoplasm, were probably removed to the same extent during both centrifugation procedures, since polyacrylamide gel electrophoresis patterns of the virus preparations obtained after purification by these methods showed the same glycoprotein-to-nucleocapsid protein ratios (Fig. 6A and B).

During sequential VSV purification steps in which virus was first subjected to equilibrium density centrifugation followed by velocity sedimentation, infectious virus (PFU)/milligram of protein as well as total number of virion particles ([^{3}H]uridine)/milligram of protein was purified 1.7- and 1.3-fold, respectively. The concomitant increase of H-2 units/milligram of protein with this virus purification again suggests that either H-2 antigen is actually a structural component of VSV as a result of virus maturation or the binding of cell membrane H-2 antigen and VSV that occurred in the culture medium after viral maturation is of such a tenacity that two sucrose gradient centrifugation steps cannot separate these components.

When the virus preparation from velocity sedimentation gradients was then subjected to centrifugation on a potassium tartrate gradient to its isopycnic density, only then was there a decrease in the H-2 activity of the virus preparation to 72% of that of virus from the previous step. Tartrate gradient centrifugation is a more "harsh" procedure for VSV purification than is sucrose gradient centrifugation, since VSV was physically separated into two different isopycnic density peaks, possibly due to alterations of the lipid-containing viral envelope. In addition, although the total number of [^{3}H]uridine-containing virion particles was enriched by this method relative to the amount of protein in the virus sample, the amount of infectious virus relative to the amount of protein decreased from the previous purification step (Table 5; compare [^{3}H]uridine/milligram of protein and PFU/milligram of protein ratios). It is not known whether the loss of H-2 antigenic activity in "tartrate VSV" was due to inactivation of H-2 antigen still associated with the virus or a physical separation of part of the H-2 antigenic activity from VSV, or both a separation and inactivation.

When H-2 antigenic activity of virus preparations was expressed as H-2 units/PFU, as contrasted to H-2 units/milligram of protein, a steady decrease of antigenic activity was observed during the course of VSV purification. The explanation might be that, compared to the stability of infectious VSV particles, the antigenic activity of H-2 is relatively labile during the purification steps. Thus, whereas the yield of H-2 antigen decreased somewhat and large amounts of non-H-2 protein were removed from VSV preparations during these procedures, the original amount of infectious virus remained relatively constant.

Two models consistent with the experimental data are proposed. (i) During infection with and assembly of enveloped viruses, the virus enve-

lope proteins are synthesized and inserted into the host plasma membrane adjacent to host membrane proteins and glycoproteins (e.g., H-2 antigens). During the budding process, some of the adjacent cell surface antigen is incorporated into the bud site and becomes a component of the intact virion. Gooding and Edidin (14) have recently demonstrated that a cell surface tumor antigen possibly induced by a virus co-caps with H-2 antigen, implying a physical association of these two antigens on the cell surface. Because there is no replacement of H-2 antigen by the infected cell, the incorporation of H-2 antigen into virus accounts for both the loss of cell surface H-2 activity and the H-2 activity of virus preparations. Elimination of a portion of the H-2 activity during tartrate gradient centrifugation (Table 5) does not necessarily rule out this model. Although H-2 is a structural component of the membranes of murine cells, the antigenic activity can be largely removed from these cells without physically disrupting the cells (42). Thus, the bonds that associate H-2 with cells and possibly with virus are more susceptible to denaturation than other cell surface bonds. Tartrate may act to break these labile bonds. (ii) During the infectious process of enveloped viruses, virus-specific membrane proteins are inserted into the host cell membrane. To maintain the total amount of protein of the cell membrane at a constant level so as not to require a large increase in cell volume or surface, some of the host-specified proteins and/or glycoproteins, possibly as membrane fragments, are expelled into the culture. When virus appears extracellularly, there is an interaction of H-2 antigen in the medium with mature virions in such a manner as to form a specific stable attachment, which is resistent to dissociation by sucrose gradient centrifugation procedures.

Although at this time it is not possible to determine which of these models reflects the mechanism of virus maturation, the use of temperature-sensitive mutants of VSV and other viruses, inhibitors of viral maturation, and also other membrane markers might be useful in distinguishing between these possibilities.

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