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Enzymatic Iodination of Sindbis Virus Proteins

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Sindbis virus was iodinated by using the enzyme lactoperoxidase, an iodination technique which labels only surface proteins. By this technique, the two viral glycoproteins are labeled, and the internal viral protein is not. The two glycoproteins are iodinated to strikingly different extents. This difference in susceptibility to iodination apparently is due to the position or conformation of the glycoproteins in the envelope spikes of the virion and not to differing contents of tyrosine, the amino acid substrate of lactoperoxidase. Both viral glycoproteins are iodinated by lactoperoxidase on the surface of Sindbis-infected chicken cells. Here, as in the virion, the glycoproteins are iodinated unequally, with the smaller glycoprotein again being preferentially iodinated. Another virus-specific protein found in large amounts in infected cells, and from which the preferentially iodinated virion glycoprotein is produced by a proteolytic cleavage, is not iodinated by lactoperoxidase. Thus it appears that the viral glycoproteins are present on the cell surface and that the precursor protein is not.

Sindbis virus, a group A arbovirus, contains three proteins. The two largest proteins, E_1 and E_2, are glycoproteins, have apparent molecular weights of approximately 50,000, and are associated with the viral envelope. The smallest of the proteins, C, is not glycosylated, has an apparent molecular weight of 30,000, and is associated with the viral ribonucleic acid (18, 20). Information about the localization of the viral proteins relative to the lipid bilayer of the viral envelope has come from two observations. Compans has shown that the protease bromelin selectively degrades both glycoproteins of the virus but leaves the internal protein intact (2). Furthermore, he has shown that spikes present on the surface of the virus are removed during this digestion with bromelin, but that the lipid bilayer of the viral envelope remains morphologically intact. Thus, he has concluded that the spikes of the virion contain the viral glycoproteins, that both glycoproteins are accessible to digestion by bromelin, and that the glycoproteins do not play a major role in maintaining the structure of the lipid bilayer. Proteolytic removal of the glycoprotein spikes of influenza virus, another enveloped virus, also does not affect the physical characteristics of the viral lipids (8).

The work of Harrison et al. suggests that the electron density of the lipid bilayer of Sindbis virus is that of pure lipid (5). They argue from this observation that no viral proteins pass through the lipid bilayer. These observations taken together suggest that the internal protein of the virus is located wholly inside the lipid bilayer and that the glycoproteins are located in spikes and are wholly outside the lipid bilayer.

We chose to investigate the precise location of the glycoproteins of Sindbis virus by using iodination by the enzyme lactoperoxidase. This technique has been shown to label proteins which have a portion of their polypeptide chain exposed to the external environment. It has been used successfully to label only the external proteins of influenza virus (19), lymphocytes (9), and red blood cells (6, 13). The specificity of the procedure for external proteins is thought to be due to the inability of lactoperoxidase, a protein of 80,000 mol wt, to cross a biological membrane.

MATERIALS AND METHODS

Preparation and infection of chicken embryo cells. Primary cultures of chicken embryo cells were prepared as described by Rein and Rubin (15). Secondary cultures were used for infection. Trypsinized primary cells were seeded at a density of 2×10^6 cells per 35-mm plastic petri dish (Falcon) in Eagle medium containing 1% heat-inactivated calf serum and incubated at 36 C for 18 h. For infection, the medium was removed, and 100 PFU of wild-type Sindbis virus per cell was added to the dish in 0.3 ml of growth medium. Adsorption was allowed to proceed for 60 min at 36 C with occasional shaking. Eagle medium (2.2 ml) containing 1% fetal calf serum was then added to the dish, and incubation at 36 C was continued.

When the monolayer was to be labeled with ^{14}C-amino acids, 1% heat-inactivated calf serum replaced the fetal calf serum, 1 μg of actinomycin D (Mann) per ml was included in the medium, and 3.5 h after infection the complete medium was replaced with 1.5 ml of amino acid-free Eagle medium containing 20 μCi of a mixture of ^{14}C-amino acids (Amersham Searle), 1% heat-inactivated calf serum, 2 mmol glutamine, and 1 μg of actinomycin D per ml. Incubation was continued for 90 min at 36 C, at which time the cells were iodinated.

Virus purification. Virus was purified from large volumes of medium by the procedure of David (3) which involves concentration of the virus with polyethylene glycol and a single velocity sucrose gradient centrifugation.

Virus was purified from small volumes of medium by dilution to low ionic strength. It has been observed that Sindbis virus precipitates from solutions of low ionic strength in the cold and can be redissolved with little loss of infectivity (11). Thus, virus solutions containing at least 40 μg of virus were diluted with 10 vol of H_2O, kept at 2 C for 16 h, and collected by centrifugation at 18,000 × *g* for 15 min. As was observed by Pfefferkorn and Clifford, recovery of virus was nearly complete. To examine whether the mobility of the viral proteins in acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was affected by this procedure, virus concentrated by water precipitation was mixed with virus taken directly from a sucrose gradient, and the mixture was subjected to electrophoresis. As can be seen in Fig. 1, this procedure had no effect on the mobility of the viral proteins under these conditions of electrophoresis.

Iodination. Lactoperoxidase was purified from raw milk by the procedure of Morrison and Hultquist (10). Intact virus was iodinated in a 0.5-ml solution containing 180 μg of Sindbis virus, 3 μg of lactoperoxidase, 1 nmol of NaI, 50 μCi of carrier-free Na ^{125}I (New England Nuclear), 0.1 M NaCl, 0.05 M Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 22% sucrose. Hydrogen peroxide (5 μmol) was added to initiate the reaction, and an additional 5 μmol of H_2O_2 was added at 5 min. The reaction was carried out at 25 C for 10 min.

To iodinate disrupted virus, 360 μg of virus was dissolved in 0.1 ml of a 1% SDS solution in 0.05 M sodium phosphate buffer (pH 7.1), which was then diluted with 0.90 ml of H_2O. Lactoperoxidase (3 μg), sodium iodide (1 nmol), and Na ^{125}I (100 μCi) were added to the solution. Iodination was carried out in a final reaction volume of 1.01 ml for 10 min at 25 C. The reaction was initiated by the addition of 50 μmol of H_2O_2, and an additional 50 μmol of H_2O_2 was added at 5 min.

Sindbis-infected cells were washed twice with warm calcium-magnesium-free phosphate-buffered saline prior to iodination. Iodination was carried out at 25 C in 2.0 ml of phosphate-buffered saline containing sodium iodide (5 nmol), lactoperoxidase (15 μg), glucose (10 μmol), glucose oxidase (0.025 units, Worthington; 1 μmol of glucose oxidized per min at 25 C per unit), and Na ^{125}I (20 μCi [Fig. 5] or 100 μCi [Fig. 6]). The reaction was allowed to proceed for 10 min, at which time the monolayer was washed three times with ice-cold phosphate-buffered saline. The cells were then prepared for electrophoresis.

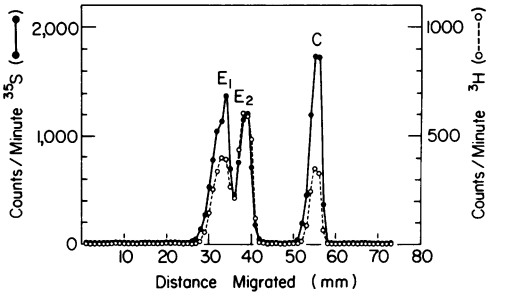

FIG. 1. *Comparison of Sindbis virus concentrated by dilution with untreated virus. ^{35}S-methionine-labeled virus was precipitated by dilution, collected by centrifugation, and mixed with virus labeled with ^{3}H-amino acids. The mixture was disrupted with sodium dodecyl sulfate and 2-mercaptoethanol and subjected to electrophoresis. Migration here and in subsequent figures was from left to right.*

Gel electrophoresis: disc gels. Polyacrylamide disc gel electrophoresis was performed as described by Laemmli (7) with two modifications; the ratio of acrylamide to *N'*-*N'*-methylenebisacrylamide was 30:1.6, and the concentrations of Tris-hydrochloride in the stacking and resolving gels were 0.062 and 0.188 M, respectively. All resolving gels were 7.5% acrylamide. Virus samples were dissolved in 1% SDS, 1 to 5% 2-mercaptoethanol, 5 mM sodium phosphate buffer (pH 7.1), 4 to 5 mM phenylmethane sulfonyl fluoride, and 10 to 15% glycerol and heated for 60 s at 100 C. Cell monolayers were dissolved in a hot solution (0.5 ml) of 2% SDS and 4 mM phenylmethane sulfonyl fluoride. The petri dish was washed with 0.25 ml of 1.5 M Tris-hydrocholoride (pH 8.8). 2-Mercaptoethanol was added to a concentration of 1%, and the solution was heated for 60 s at 100 C. Iodoacetamide then was added to a concentration of 0.3 M, and the solution was incubated at 25 C for 20 min. The sample then was dialyzed against 0.1% SDS, 5 mM sodium phosphate (pH 7.1), and 15% sucrose for 16 h at 25 C.

Samples were subjected to electrophoresis at 100 to 150 V for 3 to 5 h. The gels were frozen and fractionated into 1-mm slices with stacked razor blades. The slices were shaken with 1.0 ml of 0.1% SDS for 16 h at 25 C, mixed with 10 ml of a toluene-Triton X-100 scintaillation fluid, and counted in a Beckman scintillation counter.

Slab gels. Cell monolayers were harvested by scraping with a rubber policeman. The cells were suspended in phosphate-buffered saline solution and centrifuged. The cell pellet was dissolved by boiling in a solution (0.8 ml) consisting of 1% SDS, 1% mercaptoethanol, 10% glycerol, and 0.05 M Tris-hydrochloride (pH 6.8). Suitable samples were subjected to electrophoresis at 150 V for 2 to 3 h on 10% polyacrylamide slab gels (a modification of the system of Reid and Bieleski [14]). The gel system used was that described above, except that the ratio of acrylamide

to N'-N'-methylenebisacrylamide was 30:0.8, and the stacking gels were 5% acrylamide.

After electrophoresis, the slab gels were dried (4), and the location of the labeled proteins was determined by autoradiography.

RESULTS

Location of the viral proteins in the virion. Sindbis virus, purified by polyethylene glycol precipitation and velocity sucrose gradient centrifugation, was iodinated with lactoperoxidase. The iodinated virus was mixed with virus labeled with ^{14}C-amino acids, and the mixture was disrupted with SDS and subjected to electrophoresis through a 7.5% polyacrylamide gel in the presence of SDS. Both glycoproteins, E_1 and E_2, were iodinated, but to markedly different extents (Fig. 2). Three and one-half times as much ^{125}I was incorporated into E_2 as into E_1. Very little ^{125}I was transferred to internal protein C by the lactoperoxidase. The small amount of core protein that was iodinated was most probably due to a small population of disrupted virions in the virus preparation. Two explanations can account for the unequal iodination of the two glycoproteins. E_1 could be an inherently poorer substrate for lactoperoxidase because of a lower tyrosine content, the principle site of iodination (6). Alternatively, the position of E_1 in the virus could make its tyrosines poorly accessible to lactoperoxidase.

Two experiments were performed to test these possibilities. First, purified virus was disrupted in 1% SDS, diluted 10-fold, iodinated with lactoperoxidase, and subjected to electrophoresis. The distribution of ^{125}I in the viral proteins can be seen in Fig. 3. After disruption by SDS, the glycoproteins were iodinated equally, and the previously inaccessible core protein was iodinated.

To more directly estimate the relative tyrosine content of the viral proteins, Sindbis virus was grown in the presence of ^{14}C-tyrosine. It has been shown that little, if any, exogenous tyrosine is converted to other amino acids by Sindbis-infected chicken cells (12). After purification and electrophoresis, the distribution of tyrosine in the viral proteins could be seen (Fig. 4). Although we could not calculate the absolute tyrosine content of each protein from this experiment, it appeared that the two glycoproteins contain approximately equal amounts of tyrosine and that the internal protein contains significantly less tyrosine than do the glycoproteins. The patterns were very similar (Fig. 3 and 4), suggesting that in the presence of 0.1% SDS the degree to which the proteins were iodinated by lactoperoxidase was proportional

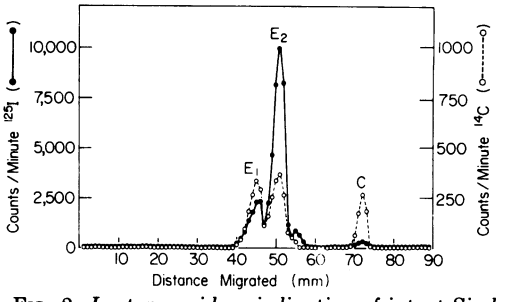

FIG. 2. *Lactoperoxidase iodination of intact Sindbis virus. Purified Sindbis virus was iodinated with lactoperoxidase, concentrated by dilution and centrifugation, and prepared for electrophoresis as described in Materials and Methods. Virus labeled with a mixture of ^{14}C-amino acids was included as a marker.*

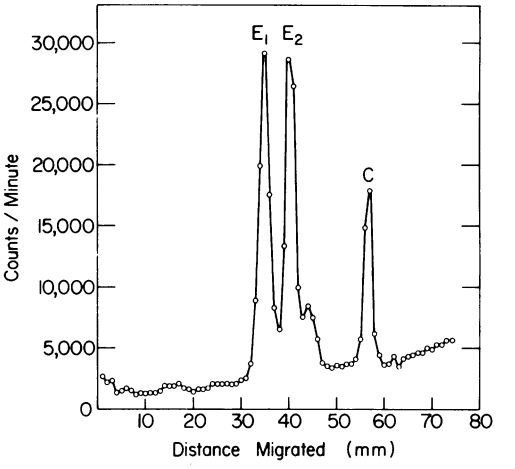

FIG. 3. *Lactoperoxidase iodination of sodium dodecyl sulfate (SDS)-disrupted Sindbis virus. Virus, disrupted with SDS as described in Materials and Methods, was iodinated and subjected to electrophoresis.*

to the tyrosine content of the proteins. Thus, we conclude that the position of E_1 in the envelope spikes of Sindbis virus makes it relatively inaccessible to lactoperoxidase.

Location of the viral proteins in infected chicken cells. Because lactoperoxidase-catalyzed iodination has been shown to specifically label proteins on the outer surface of red blood cells (6, 13) and lymphocytes (9) without injury to the labeled cells, Sindbis-infected chicken embryo cells, growing in a monolayer, were iodinated to examine whether virus-specific proteins could be detected on the cell surface. After disruption of the labeled cells with SDS and electrophoresis, the pattern (II) shown in Fig. 5 was obtained. Purified ^{35}S-methionine-labeled virus (III) and iodinated, uninfected chicken cells (I) also were subjected to electro-

phoresis so that viral proteins could be distinguished from serum and chicken cell proteins. Both viral glycoproteins were detected on the surface of the infected cells. The core protein was not labeled. Again, E_2 was more extensively labeled than was E_1. Except for the two viral glycoproteins, no differences were apparent in the iodination patterns of the infected and uninfected chicken cell surfaces.

Protein E_2 has been shown to be derived from a somewhat larger virus-specific glycoprotein by proteolytic cleavage (17). This precursor to E_2, designated PE_2, is normally found in large amounts in Sindbis-infected cells (17, 18). PE_2 migrates, under these conditions of electrophoresis, slightly slower than protein E_1. No protein migrating with the mobility of PE_2 was iodinated in the infected chicken cells (Fig. 5). Because PE_2 is the precursor to the more heavily iodinated viral protein, intrinsically it should be a good substrate for lactoperoxidase iodination. Therefore, the fact that it is not iodinated by lactoperoxidase in infected cells suggests that it is not located on the cell surface.

However, because it was impossible to prove that PE_2 was indeed present in normal amounts in the infected cells (Fig. 5), a monolayer of Sindbis-infected chicken cells, prelabeled with ^{14}C-amino acids, was iodinated with lactoperoxidase and subjected to disc gel electrophoresis.

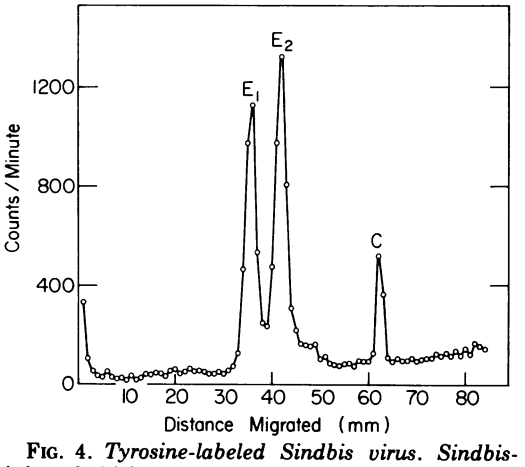

FIG. 4. *Tyrosine-labeled Sindbis virus. Sindbis-infected chicken cells were labeled with ^{14}C-tyrosine (400 mCi/mmol, New England NuclearCorp.) from 4 to 7 h after infection in amino acid-free Eagle medium containing 2% tryptose phosphate broth, 1% heat-inactivated calf serum, 2 mM glutamine, and 3.5 μCi of ^{14}C-tyrosine per ml. The medium was collected, cell debris was removed by centrifugation at 10,000 × g for 5 min, 40 μg of unlabeled virus was added as carrier, and the mixture was concentrated and prepared for electrophoresis as described in Materials and Methods.*

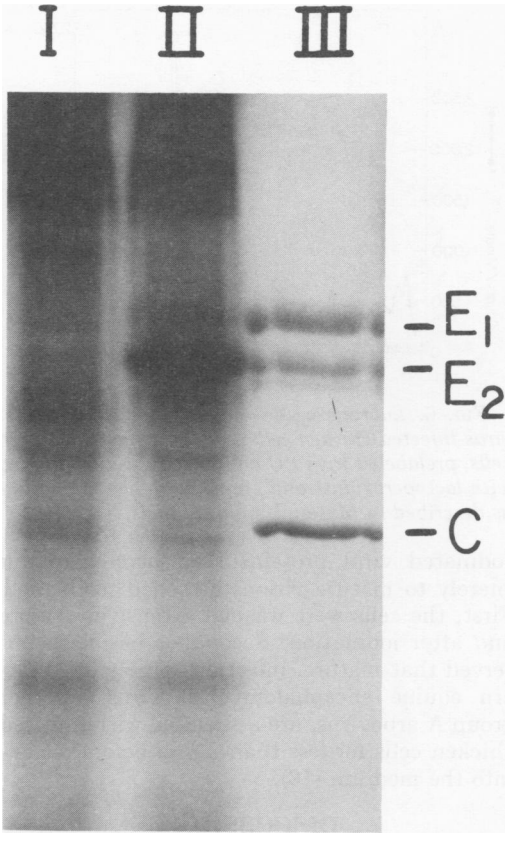

FIG. 5. *Lactoperoxidase iodination of Sindbis-virus-infected chicken cells. A culture of Sindbis-infected chicken cells 8 h after infection (II) and an uninfected sister culture (I) were iodinated with lactoperoxidase and prepared for slab gel electrophoresis as described in Materials and Methods. I, Uninfected chicken cells; II, Sindbis-infected chicken cells; III, ^{35}S-methionine-labeled Sindbis virus.*

When the gel was sliced, the pattern in Fig. 6 was obtained. Although approximately equal amounts of PE_2 and E_2 were present in the cells, PE_2 was not iodinated and E_2 was heavily iodinated. The small amount of ^{125}I-label migrating in the PE_2 position was due to serum or normal chicken cell surface protein (Fig. 5). Thus, as measured by accessibility to lactoperoxidase, PE_2 is not present on the cell surface.

Strikingly, as in intact virions, approximately 3.5 times as much iodine label was bound to E_2 as to E_1 on the surface of infected cells. Some label was found to comigrate with internal protein C of the virus. However, this label is due to two normal chicken cell polypeptides which are iodinated by lactoperoxidase even in uninfected cells (Fig. 5).

It seems unlikely for two reasons that the

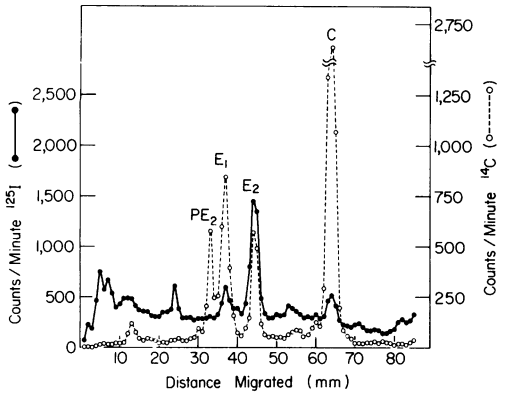

FIG. 6. *Lactoperoxidase iodination of Sindbis-virus-infected chicken cells. Sindbis-infected chicken cells, prelabeled with ^{14}C-amino acids were iodinated with lactoperoxidase and prepared for electrophoresis as described in Materials and Methods.*

iodinated viral proteins seen here were due merely to mature virions attached to the cell. First, the cells were washed extensively before and after iodination. Second, it has been observed that mature, infectious virions of Western equine encephalomyelitis virus, another group A arbovirus, are associated with infected chicken cells for less than 2 min before release into the medium (16).

DISCUSSION

From these results, it appears that glycoprotein E_1 of Sindbis virus is located in the envelope spike of the virion in such a way that it is significantly less accessible to iodination by lactoperoxidase than is the other glycoprotein, E_2. The work of Harrison et al. indicates that it is unlikely that E_1 is buried in the lipid bilayer of the envelope (5). The inaccessibility of E_1 could be due to a superficial interaction with the lipids, an interaction with the other glycoprotein, or to a lack of accessible tyrosine residues in the native conformation. We have no evidence to allow us to choose between these alternatives. A similar, though less pronounced, observation of restricted iodination of the surface proteins of an enveloped virus by lactoperoxidase was noted by Stanley and Haslam. They observed that the ratio of iodine transferred to the two subunits of the hemagglutinin of the A-Bel strain of influenza virus was altered by prior disruption of the virus with SDS (19).

Our results also allow several conclusions to be drawn about the location of the Sindbis glycoproteins in infected chicken cells. The presence of the Sindbis hemagglutinin on the surface of infected chicken cells has been previously demonstrated by hemadsorption (1). Although the identity of the hemagglutinin is not known, we assume it to be one or both of the glycoproteins. Our results demonstrate that both viral glycoproteins can be detected by lactoperoxidase on the surface of the infected chicken cell. Indeed, 5 h after infection, the Sindbis glycoprotein E_2 is the major iodinated protein of the chicken cell and represents 10% of the iodine bound to the cell.

It is interesting to note that the ratio of the iodine label bound to the two viral glycoproteins is the same in mature virus and in infected cells, 3.5:1. This suggests that the organization of the glycoproteins that is responsible for the unequal iodination in mature virions is already present in the virally modified cell membrane. Furthermore, the pattern of lactoperoxidase-iodinated chicken cell surface proteins is unchanged, except for the viral glycoproteins, by Sindbis infection. This suggests that within the limits of this technique, marked topological changes in the cell surface do not accompany Sindbis infection.

What is perhaps most striking about the location of the Sindbis-specific proteins in the infected cell is that PE_2, the immediate precursor to the heavily iodinated viral protein, is not accessible to lactoperoxidase. It is tempting to hypothesize that prior to cleavage, PE_2 is located on the inner surface of the cellular plasma membrane and appears on the outer surface of the cell only following cleavage. We have, however, no direct evidence that the intracellular location of PE_2 is the inner surface of the plasma membrane. It is worth noting that we have found that completion of the glycosylation of E_2 accompanies the cleavage of PE_2 to E_2 and the displacement of E_2 to the cell surface (B.M. Sefton and B.W. Burge, manuscript in preparation).

Because specific cell surface changes due to Sindbis infection can be detected easily by this technique, iodination by lactoperoxidase should prove useful in examining cell surface modifications by other animal viruses.

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LITERATURE CITED

1. Burge, B. W., and E. R. Pfefferkorn. 1967. Temperature-sensitive mutants of Sindbis virus: biochemical correlates of complementation. J. Virol. 1:956–962.

2. Compans, R. W. 1971. Location of the glycoprotein in the membrane of Sindbis virus. Nature N. Biol. **229**:114-116.
3. David, A. 1971. Lipid composition of Sindbis virus. Virology **46**:711-720.
4. Fairbanks, G., C. Levinthal, and R. H. Reeder. 1965. Analysis of ^{14}C-labeled proteins by disc electrophoresis. Biochem. Biophys. Res. Commun. **20**:393-399.
5. Harrison, S. C., A. David, J. Jumblatt, and J. E. Darnell. 1971. Lipid and protein organization in Sindbis virus. J. Mol. Biol. **60**:523-528.
6. Hubbard, A. L., and Z. A. Cohn. 1972. The enzymatic iodination of the red cell membrane. J. Cell Biol. **55**:390-405.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
8. Landsberger, F. R., J. Lenard, J. Paxton, and R. W. Compans. 1971. Spin label electron spin resonance study of the lipid-containing membrane of influenza virus. Proc. Nat. Acad. Sci. U.S.A. **68**:2579-2583.
9. Marchalonis, J. J., R. E. Cone, and V. Santer. 1971. Iodination, a probe for accessible surface proteins of normal and neoplastic lymphocytes. Biochem. J. **124**:921-927.
10. Morrison, M., and D. E. Hultquist. 1963. Lactoperoxidase. II. Isolation. J. Biol. Chem. **238**:2847-2849.
11. Pfefferkorn, E. R., and R. L. Clifford. 1963. Precipitation and recovery of Sindbis virus from solutions of low ionic strength. Virology **21**:273.
12. Pfefferkorn, E. R., and R. L. Clifford. 1964. The origin of the protein of Sindbis virus. Virology **23**:217-233.
13. Phillips, D. R., and M. Morrison. 1970. The arrangement of the proteins in the human erythrocyte membrane. Biochem. Biophys. Res. Commun. **40**:284-289.
14. Reid, M. S., and R. L. Bieleski. 1968. A simple apparatus for vertical flat-sheet polyacrylamide gel electrophoresis. Anal. Biochem. **22**:374-381.
15. Rein, A., and H. Rubin. 1968. Effects of local cell concentration upon the growth of chick embryo cells in tissue culture. Exp. Cell Res. **49**:666-678.
16. Rubin, H., M. Baluda, and J. E. Hotchkin. 1955. The maturation of Western equine encephalomyelitis virus and its release from chick embryo cells in suspension. J. Exp. Med. **101**:205-212.
17. Schlesinger, S., and M. J. Schlesinger. 1972. Formation of Sindbis virus proteins. Identification of a precursor for one of the envelope proteins. J. Virol. **10**:925-932.
18. Schlesinger, M. J., S. Schlesinger, and B. W. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. Virology **47**:539-541.
19. Stanley, P., and E. A. Haslam. 1971. The polypeptides of influenza virus. V. Localization of polypeptides in the virion by iodination techniques. Virology **46**:764-773.
20. Strauss, J. H., B. W. Burge, and J. E. Darnell. 1969. Sindbis virus infection of chick and hamster cells: synthesis of virus-specific proteins. Virology **37**:367-376.