JOURNAL OF VIROLOGY, Mar. 1985, p. 867–870
0022-538X/85/030867-04$02.00/0

Vol. 53, No. 3

# Properties of the Deoxycholate-Solubilized HeLa Cell Plasma Membrane Receptor for Binding Group B Coxsackieviruses

DAVID L. KRAH† AND RICHARD L. CROWELL*

*Department of Microbiology and Immunology, Hahnemann University, Philadelphia, Pennsylvania 19102*

Received 20 July 1984/Accepted 20 November 1984

**Physical and chemical properties of deoxycholate-solubilized HeLa cell plasma membrane receptors for binding group B coxsackieviruses were determined. Receptors eluted from Sepharose 4B with an apparent molecular weight of 275,000 and sedimented with an S value of between 14.7 and 4.9 and a buoyant density of 1.06 to 1.10 g/cm$^3$. Virus-binding activity was destroyed after treatment with proteases, glycosidases, and periodate but was unaffected by lipases or reducing or alkylating agents. Additionally, lectins, including concanavalin A, adsorbed receptors and inhibited virus attachment. The composite data suggested that glycoprotein is an integral part of the receptors for binding virus.**

The role of specific cell surface receptors in determining the tissue tropism or cell susceptibility to infection with picornaviruses has been well documented (2, 15). Within this family, the receptors for the various human enteroviruses have been most extensively studied, and some general properties have been identified. They are integral components (4, 17, 19, 22; N. H. Levitt, Ph.D. thesis, Hahnemann Medical College, Philadelphia, 1968) of the outer surface of the plasma membrane (17, 23) and are present at ca. $10^5$ copies per cell (1).

The six serotypes of the group B coxsackieviruses bind to a common receptor on HeLa cells which is distinct from that binding the polioviruses, group A coxsackieviruses, and echoviruses (14). The receptors on intact HeLa cells are inactivated by chymotrypsin (22), and their regeneration requires host cell protein and mRNA synthesis (13). The development of a sodium deoxycholate (DOC) extraction procedure to solubilize biologically active, native receptors from HeLa cells (4, 12) provided an impetus to further characterize them.

The results presented herein represent the first characterization of soluble host cell receptors for binding group B coxsackieviruses. An essential glycoprotein component was revealed from the sensitivity of DOC-solubilized receptors to protease and glycosidase treatment and to periodate oxidation, their affinity for specific lectins, and their stability regardless of extremes of pH and temperature. Treatment of extracts with lipases revealed that lipid was not required for virus binding, but an association with lipid was implicated from the buoyant density of solubilized receptors and their interaction with heparin. The data will be compared with previous reports of insoluble receptors.

## MATERIALS AND METHODS

**Cells.** The Mandel (3) and JJH (6) strains of HeLa cells and the MCN strain of mouse L cells (7) were grown as previously described.

**Viruses and virus purification.** The origins of the strains of coxsackievirus B1 (Conn-5), B3 (Nancy), and poliovirus T1 (Mahoney) were described elsewhere (5, 6). Viruses were grown in suspension cultures of Mandel HeLa cells and purified on two successive CsCl gradients (3). The PFU of infectious virus were determined on JJH HeLa cells (6).

**Protein determination.** Amounts of protein were estimated in a modified Lowry method (16) or from the absorbance at 280 nm, with bovine serum albumin (crystallized and lyophilized) as a standard.

**Preparation and solubilization of cell membranes.** Plasma membranes were isolated in 5 to 50% discontinuous sucrose gradients (21), solubilized in phosphate-buffered saline (PBS; devoid of $Ca^{2+}$ and $Mg^{2+}$) containing 0.2% DOC (4), and stored in the vapor phase of a liquid $N_2$ refrigerator.

**Cell radiolabeling.** Mandel HeLa cells ($10^6$ cells per ml) were metabolically labeled for 4 h with 100 μCi of L-[4,5-$^3$H]leucine (76 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml, in double-strength Eagle medium devoid of leucine and containing only 1% calf serum. Membrane fractions and DOC extracts were prepared as described for unlabeled cells. Specific activities of $4.8 \times 10^3$ cpm/μg of protein were obtained in soluble extracts.

**Radioactivity determinations.** Polyethylene solvent-saver vials (Kimble Glass Co., Division of Owens-Illinois, Toledo, Ohio) received 500 μl of aqueous sample and 5 ml of ACS II aqueous scintillation cocktail (Amersham). Tubes were counted for 5 min in the isoset windows of a Beckman LS-133 liquid scintillation spectrometer.

**Receptor assay.** Virus-binding activity was determined in a solid-phase assay, which provides a sensitive and specific measure of receptors for binding group B coxsackieviruses and polioviruses (12). Briefly, serial two- or three-fold dilutions of receptor were made in PBS in 96-well polystyrene micro-enzyme-linked immunosorbent assay substrate plates (plate M129A, Cooke Engineering Co. [Dynatech Corp.], Alexandria, Va.). Receptors were immobilized by passive adsorption (16 h, 6°C) and incubated for 24 to 48 h at 6°C with $10^6$ PFU of coxsackievirus B3 (CB3) in 50 μl of virus attachment buffer (50 mM phosphate- and citrate-buffered saline, pH 4.5, containing 0.5% bovine serum albumin). The amount of unattached virus was reduced by washing (seven times in PBS at 6°C), and attached virus was recovered in 200 μl of 50 mM glycine-HCl, pH 1.5. The pH was adjusted to neutrality by 10-fold dilution in balanced salt solution containing 3% calf serum and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.2. Virus was quantitated in plaque assay on JJH HeLa cells. The receptor titer (receptor units [RU] per ml) was desig-

* Corresponding author.
† Present address: Rockefeller University, New York, NY 10021.

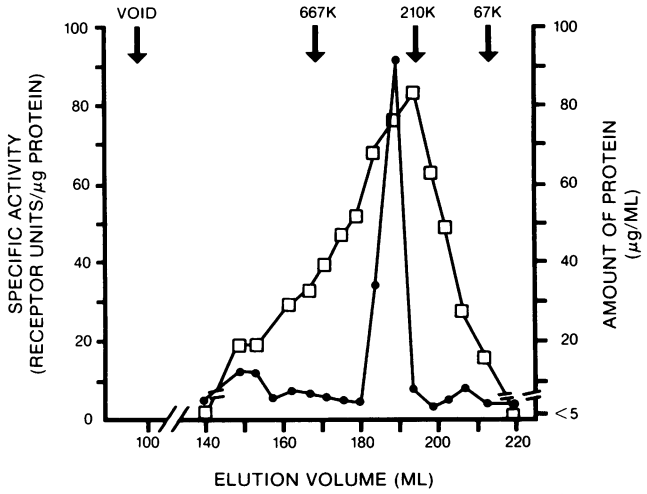


FIG. 1. Recovery of receptor activity for CB3 from solubilized HeLa cell plasma membranes after chromatography on Sepharose 4B. Membrane extract (1 ml) was loaded on a column of Sepharose 4B (40 by 2.5 cm) and eluted with PBS–0.1% DOC. Amounts of protein (optical density at 280 nm [□]) and receptor (●) in fractions (4.5 ml) were determined as described in the text. The column was calibrated with Blue dextran 2000 (molecular weight, $2 \times 10^6$ [2000K]), bovine thyroglobulin (667K), catalase (210K), and bovine serum albumin (67K) (vertical arrows), and with ribonuclease (13K), and phenol red (354) (not shown).

nated as the reciprocal of the highest dilution of sample which bound fivefold more virus than background (uncoated wells or wells coated with L cell extracts) or, alternatively, as the amount of virus specifically bound to wells coated with a single dilution (≦20 μg of protein per ml) of extract.

**Chemicals.** Reagents and chemicals were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Mo., unless otherwise indicated.

## RESULTS

**Gel filtration.** Receptors for binding CB3 eluted from Sepharose 4B as a single peak between 180 and 192 ml (Fig. 1). This peak corresponded to a molecular weight of ca. 275,000 and closely matched the extract protein elution peak.

**Sedimentation.** Receptor activity was not sedimented after centrifugation of solubilized membranes in 0.1% DOC for 6 h at 208,000 × *g* (S < 14.7) but was pelleted (30% recovery) within 16 h (S ≧ 4.9). Equilibrium sedimentation of extracts in sucrose revealed peaks of receptor-specific activity (≈0.13 RU/cpm in original extract) at 13.9 (1.058 g/cm$^3$) and 22.5% (1.095 g/cm$^3$) sucrose (0.53 and 0.95 RU/cpm, respectively) in DOC-containing gradients and at 15% (1.063 g/cm$^3$) sucrose (0.51 RU/cpm) in gradients containing octyl glucoside (Fig. 2).

**Association with lipid.** Solubilized membranes were incubated in 10 mM $MnCl_2$–0.01% heparin buffered with 10 mM HEPES, pH 7.0 (3 h, 6°C). The resulting precipitate was collected by centrifugation (3,700 × *g*, 20 min) and suspended in PBS–0.1% DOC. Receptor activity was recovered in the pellet after heparin treatment (810 RU/ml of pellet; 30 RU/ml of supernatant), whereas receptors remained in the supernatant (810 RU/ml of supernatant; 270 RU/ml of pellet) in the absence of heparin.

The capacity of different lipases to inactivate receptors was examined. Receptor titers were not reduced after treatment (1 h, 37°C) of HeLa extract (5 μl) with phospholipase $A_2$ (50 U from *Naja naja* venom), phospholipase C (14 U from *Bacillus cereus*), or lipase (150 U from *Candida cylindracea*).

**Association with protein.** The effects of different protein-modifying agents on receptor activity were determined (Table 1). Receptors were inactivated by the proteases trypsin and chymotrypsin. In a control experiment with intact HeLa cells (22), trypsin inactivated only the receptors for poliovirus T1 and not those for CB3, whereas chymotrypsin inactivated the receptors for CB3 but not those for poliovirus T1 (data not shown).

Sulfhydryl groups were not required for binding of virus to solubilized receptors, as reducing (dithiothreitol and 2-mercaptoethanol) or alkylating (iodoacetamide) agents had no effect on receptor activity (Table 1).

**Association with carbohydrate.** Mandel HeLa cells ($2.0 \times 10^5$ cells per ml) were grown for 48 h in suspension culture (to $6.4 \times 10^5$ cells per ml) in the presence of 1 μg of tunicamycin per ml, an inhibitor of protein *N*-glycosylation (11). This concentration of tunicamycin permitted optimum (50 to 60%) inhibition of [$^{14}$C]fucose incorporation (trichloroacetic acid-precipitable counts) into cell proteins, without appreciably affecting synthesis of cell protein (17% reduc-

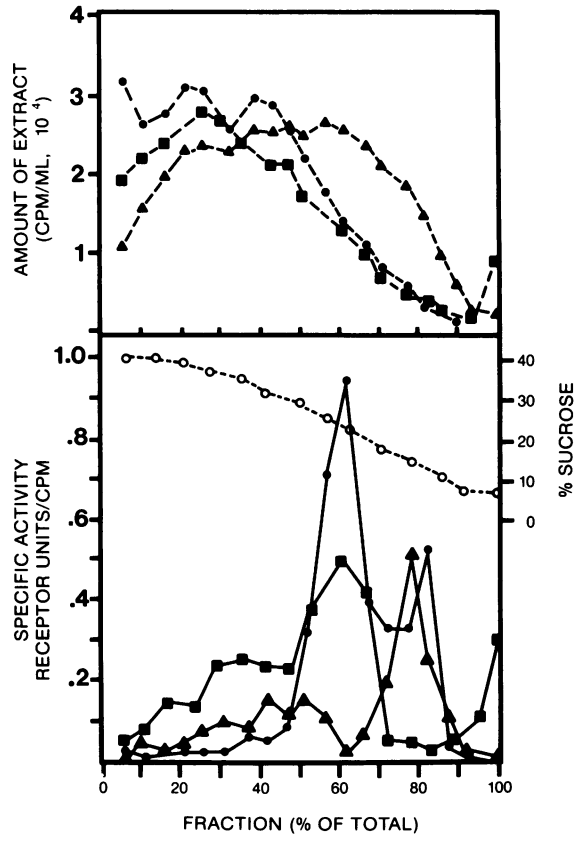


FIG. 2. Recovery of receptor activity for CB3 from solubilized HeLa cell plasma membranes after sucrose gradient centrifugation. DOC-solubilized membranes (200 μl, [$^3$H]leucine labeled) were centrifuged to equilibrium (190,000 × *g*, 24 h) in 10 to 40% gradients of sucrose in PBS containing 0.1% DOC (●), 0.5% octyl glucoside (▲), or no detergent (■). Receptor specific activity (——) and amounts of extract ($^3$H cpm, – – –) were determined as described in the text. Sucrose concentrations (○) were determined from the refractive index (Bausch & Lomb Abbe refractometer).

tion in incorporation of $^3$H-amino acid mix) or of DNA (20% reduction in incorporation of [$^3$H]thymidine) or cell growth. Even though a twofold reduction in receptor titer would have been detected, no decrease in the activity of DOC extracts from tunicamycin-treated cells was observed.

Results presented in Table 1 show that periodate treatment resulted in a threefold reduction in receptor activity, implicating carbohydrate as an integral part of receptors. Similar results were obtained after treatment of receptors adsorbed on the assay plate (67% inactivation in 30 min, 81% in 90 min, 93% in 180 min).

A subsequent evaluation of an association with carbohydrate relied on the capacity of different lectins to bind to solubilized receptors. Receptors were bound to immobilized lectins with an affinity for residues of glucosyl (concanavalin A [ConA], wheat germ, lentil, pea) and *N*-acetylgalactosyl (soybean), but not α-D-galactosyl (peanut) (Table 2). However, the capacity of only ConA, pea, and lentil lectins to reversibly inhibit virus attachment (Table 2) indicated an association of glucosyl or mannosyl residues near the virus-binding site. Furthermore, preliminary results showed that receptor activity was reduced >90% after treatment (1 h, 37°C) of the extract (5 μl) with α-glucosidase (5 U from type VI brewer's yeast), β-glucosidase (5 U from type II almonds), or α-mannosidase (0.9 U from jack beans), but only 50% after treatment with α-galactosidase (0.1 U from green coffee beans).

**Thermal stability.** Samples of solubilized HeLa cell plasma membranes, in PBS–0.1% DOC, were incubated at different temperatures and rapidly chilled to 0°C. Remaining receptor activity was determined. Receptor titers were reduced 25 and 60 to 81% after incubation for 10 and 30 min, respectively, at 56°C and 93 and 98% after incubation at 100°C for 1 and 5 min, respectively. Receptors were stable for at least 20 days at 6°C, 8 h at 25°C, and 2 h at 37°C. Activity was reduced 3-fold after 24 h of incubation at 25°C and >27-fold within 8 h at 37°C.

TABLE 1. Effect of protein- and carbohydrate-modifying treatments on receptor activity

| Treatment[a] | Receptor inactivation (%)[b] |
|---|---|
| Periodate[c] | 67 |
| Protease[d] | |
| Chymotrypsin | 82 |
| Chymotrypsin-TPCK | <1 |
| Chymotrypsin-TLCK | 80 |
| Trypsin | 75 |
| Trypsin-TPCK | 89 |
| Trypsin-TLCK | <1 |
| Sulfhydryl modifying[e] | |
| Dithiothreitol | <1 |
| 2-Mercaptoethanol | <1 |
| Iodoacetamide | <1 |

[a] Membrane extract (20 μl) was added to 200 μl of 0.1% DOC in 20 mM HEPES-buffered saline, pH 8.0 (protease treatments), or 20 mM PBS, pH 7.0, containing the indicated compound.

[b] Normalized to receptor activity in buffer alone (mock treatment).

[c] Periodate treatment, 30 min at 37°C; final concentration, 10 mM sodium *m*-periodate. The reaction was stopped by addition of glycerol to 10% (vol/vol).

[d] Protease treatment, 60 min at 37°C; final concentration, 500 μg/ml of protease and 62.5 μg/ml of L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK) or sodium-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). Tubes were chilled on ice and assayed immediately.

[e] Sulfhydryl-modifying treatment, 30 min at 37°C; final concentrations, 20 mM dithiothreitol, 20 mM iodoacetamide, and 10 mM 2-mercaptoethanol.

TABLE 2. Interaction of lectins with solubilized receptors

| Lectin[a] | Sugar specificity[b] | RU of receptor unadsorbed per ml (% mock) | Virus attachment (%)[c] |
|---|---|---|---|
| ConA | α-D-glucose or α-D-mannose | 90 (4) | 42 |
| Lentil | α-D-glucose or α-D-mannose | 813 (33) | 55 |
| Garden pea | α-D-glucose or α-D-mannose | 813 (33) | 55 |
| Wheat germ | *N*-acetyl-D-glucose | 813 (33) | 104 |
| Peanut | D-galactose | 2,427 (100) | 86 |
| Soybean | *N*-acetyl-D-galactosamine | 813 (33) | 92 |
| None | | 2,427 (100) | 100 |

[a] Lectin (400-μl samples) immobilized on beaded agarose or Sepharose 4B was washed with PBS–0.05% DOC and incubated with 20 μl of membrane extract in a total volume of 550 μl. A tube containing no agarose served as a control for the total amount of extract applied. Tubes were incubated with shaking for 3.5 h at 6°C. The beads were allowed to settle, and the receptor activity in the supernatants was determined.

[b] From Polysciences Catalog, 1982–83 ed., p. 140–143 (Polysciences, Inc., Warrington, Pa.).

[c] Wells of a micro-enzyme-linked immunosorbent assay receptor assay plate were coated with 50 μl of a 1:50 dilution of HeLa extract (16 h, 6°C) and incubated with 50 μg of lectin in PBS–0.05% DOC (16 h, 6°C). ConA and wheat germ lectins were used in the succinylated form. Wells were washed (three times in PBS) and incubated with virus, as described in the text. Receptor activity was expressed as the amount of virus attached, relative to a mock lectin treatment (average of three experiments). The inhibition of virus attachment by ConA, pea, and lentil lectins was abolished by including 100 mM 3-*O*-methyl-D-glucose in the lectin solution. No reversal of the inhibition was obtained with D-galactose. No binding of virus to lectins was detected.

## DISCUSSION

Receptors for the enteroviruses have not been extensively characterized. Results obtained after enzymatic treatment of cells did not account for possible secondary membrane changes (10). Furthermore, previous attempts to study receptors extracted from cells used receptor preparations which were insoluble (17; N. H. Levitt, Ph.D. thesis, Hahnemann Medical College, Philadelphia, 1968), inactive (9), active only for hemagglutinating strains of virus (18, 19), or extracted from erythrocytes, which do not support virus replication (19). The present study used a detergent extraction procedure (4) to solubilize receptors for the group B coxsackieviruses from HeLa cells and permit their characterization.

The capacity of proteases to inactivate soluble or membrane (9, 18–20, 22, 23; N. H. Levitt, Ph.D. thesis, Hahnemann Medical College, Philadelphia, 1968) receptors for CB3 indicated an essential role for protein in receptor activity. Receptor sulfhydryl groups were not required, since virus-binding activity was unaffected by reducing or alkylating agents. An evaluation of the effect of a panel of site-specific proteases on receptor activity should reveal the specific region(s) of receptors involved in binding virus.

The role of carbohydrate in receptor activity has been equivocal. In the present study, a carbohydrate requirement was suggested from the inactivation of solubilized receptors after periodate oxidation. In contrast, membrane receptors for these viruses were periodate insensitive (8, 19, 20). However, the stability of receptors to pH extremes (pH 3 to 9 [9, 24; unpublished data]) and elevated temperatures (20, 24) is consistent with a glycoprotein involvement (2).

Perhaps the best evidence for a carbohydrate (including glucosyl and mannosyl residues) requirement in receptors

comes from the capacity of lectins to reversibly inhibit virus attachment and the sensitivity of receptors to glycosidase treatment. These residues may be *O*-linked to receptor protein, since tunicamycin did not reduce receptor titers. It is unlikely that the resistance of virus-binding activity to tunicamycin treatment represented a carry-over of *N*-linked receptor carbohydrate from the inoculum cells, since a threefold reduction in receptor number, resulting from the threefold increase in cell number during treatment, would have been detected.

Receptors were resistant to treatment with assorted lipases, indicating that lipid was not required for virus binding. However, an association of lipid or detergent was consistent with the receptor buoyant density and the capacity of heparin to precipitate receptors from DOC-solubilized preparations. It will be of interest to reconstitute receptors with different lipids and determine whether lipid modulates receptor activity at stages subsequent to virus binding.

The composite data suggest that the virus-binding activity of DOC-solubilized HeLa cell plasma membranes involves glycoprotein with tightly associated lipid. Similarly, others (17, 19) showed that partially purified receptor preparations contained protein (53%), lipid (32%), and carbohydrate (5%).

The data are being interpreted to develop a rationale for a receptor purification protocol. The partially purified receptor will then be analyzed to further define its composition. Additionally, it will be possible to determine whether isolated, purified receptors can process (eclipse) virus. The information obtained should lead to a better understanding of virus-cell interactions and may reveal the role that virus receptors play in the host cell.

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-03771 from the National Institute of Allergy and Infectious Diseases.

## LITERATURE CITED

1. **Crowell, R. L.** 1966. Specific cell-surface alteration by enteroviruses as reflected by viral-attachment interference. J. Bacteriol. **91**:198–204.
2. **Crowell, R. L., and B. J. Landau.** 1983. Receptors in the initiation of picornavirus infections, p. 1–42. *In* H. Fraenkel-Conrat and R. W. Wagner (ed.), Comprehensive virology: virus-host interactions: receptors, persistence, and neurological diseases, vol 18. Plenum Publishing Corp., New York.
3. **Crowell, R. L., and L. Philipson.** 1971. Specific alterations of coxsackievirus B3 eluted from HeLa cells. J. Virol. **8**:509–515.
4. **Crowell, R. L., and J.-S. Siak.** 1978. Receptor for group B coxsackieviruses: characterization and extraction from HeLa cell plasma membranes, p. 39–53. *In* M. Pollard (ed.), Perspectives in virology, vol. 10. Raven Press, New York.
5. **Crowell, R. L., and J. T. Syverton.** 1955. The viral range *in vitro* of a malignant, human epithelial cell (strain HeLa, GEY). IV. The pathogenicity of C viruses. J. Immunol. **74**:169–177.
6. **Crowell, R. L., and J. T. Syverton.** 1961. The mammalian cell-virus relationship. VI. Sustained infection of HeLa cells by coxsackie B3 virus and effect on superinfection. J. Exp. Med. **113**:419–435.
7. **Goldberg, R. J., M. Gravell, and R. L. Crowell.** 1969. Evidence for the susceptibility of primary fetal mouse cells in culture to coxsackievirus A13. Proc. Soc. Exp. Biol. Med. **132**:743–748.
8. **Holland, J. J., and L. C. McLaren.** 1959. The mammalian cell-virus relationship. II. Adsorption, reception, and eclipse of poliovirus by HeLa cells. J. Exp. Med. **109**:487–504.
9. **Holland, J. J., and L. C. McLaren.** 1961. The location and nature of enterovirus receptors in susceptible cells. J. Exp. Med. **114**:161-171.
10. **Holmes, K. V.** 1981. The biology and biochemistry of cellular receptors for enveloped viruses, p. 85–115. *In* K. Lonberg-Holm and L. Philipson (ed.), Receptors and recognition, series B, vol. 8. Chapman and Hall, London.
11. **King, I. A., and A. Tabiowo.** 1981. Effect of tunicamycin on epidermal glycoprotein and glycosaminoglycan synthesis *in vitro*. Biochem. J. **198**:331–338.
12. **Krah, D. L., and R. L. Crowell.** 1982. A solid-phase assay of solubilized HeLa cell plasma membrane receptors for binding group B coxsackieviruses and polioviruses. Virology **118**: 148–156.
13. **Levitt, N. H., and R. L. Crowell.** 1967. Comparative studies of the regeneration of HeLa cell receptors for poliovirus T1 and coxsackievirus B3. J. Virol. **1**:693–700.
14. **Lonberg-Holm, K., R. L. Crowell, and L. Philipson.** 1976. Unrelated animal viruses share receptors. Nature (London) **259**:679–681.
15. **Lonberg-Holm, K., and L. Philipson (ed.).** 1981. Receptors and recognition, series B, vol. 8. Chapman and Hall, London.
16. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
17. **McLaren, L. C., J. V. Scaletti, and C. G. James.** 1968. Isolation and properties of enterovirus receptors. Wistar Inst. Symp. Monogr. **8**:123–135.
18. **Philipson, L., and S. Bengtsson.** 1962. Interactions of enteroviruses with receptors from erythrocytes and host cells. Virology **18**:457–469.
19. **Philipson, L., S. Bengtsson, S. Brishammar, L. Svennerholm, and O. Zetterqvist.** 1964. Purification and chemical analysis of the erythrocyte receptor for hemagglutinating enteroviruses. Virology **22**:580–590.
20. **Quersin-Thiry, L., and E. Nihoul.** 1961. Interaction between cellular extracts and animal viruses. II. Evidence for the presence of different inactivators corresponding to different viruses. Acta Virol. **5**:283–293.
21. **Roesing, T. G., P. A. Toselli, and R. L. Crowell.** 1975. Elution and uncoating of coxsackievirus B3 by isolated HeLa cell plasma membranes. J. Virol. **15**:654–667.
22. **Zajac, I., and R. L. Crowell.** 1965. Effect of enzymes on the interaction of enteroviruses with living HeLa cells. J. Bacteriol. **89**:574–582.
23. **Zajac, I., and R. L. Crowell.** 1965. Location and regeneration of enterovirus receptors of HeLa cells. J. Bacteriol. **89**:1097–1100.
24. **Zajac, I., and R. L. Crowell.** 1969. Differential inhibition of attachment and eclipse activities of HeLa cells for enteroviruses. J. Virol. **3**:422–428.