THE LOCATION AND NATURE OF ENTEROVIRUS RECEPTORS IN SUSCEPTIBLE CELLS*

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It has been demonstrated previously that susceptible primate cells possess "receptor substance" capable of binding poliovirus (1), and that the presence or absence of such receptors determines cell susceptibility or resistance to a number of enteroviruses (2–4). Receptor is demonstrated in disrupted cells by ability to neutralize virus infectivity (1). It has recently been shown with Coxsackie A9 that this neutralization has the same ionic cofactor requirements as does virus adsorption by intact cells, and that it is probably due to firm binding of virus by receptor, since it is completely reversed at very low pH, or by chelation of cation cofactor (4). The present report indicates that poliovirus and Coxsackie virus receptors are present chiefly in the microsome fraction of cell homogenates, that more receptor is present in disrupted cells than is exposed at the surface of cells, and that the receptor contains a labile protein as an essential component.

Materials and Methods

Viruses.—Type 1 poliovirus (Mahoney) was employed as pooled fluid of 9th, 10th, or 11th HeLa culture passage of virus received from Connaught Medical Laboratories, Toronto; Coxsackie B_1 (Conn. 5) virus was purchased from The American Type Culture Collection, and used after 6 or 7 HeLa passages.

Virus Assay.—Virus infectivity was assayed in screw-cap bottles by a plaque technique described previously (5) in which the soft agar overlayer is poured out after several days of incubation and the cells stained with crystal violet for enumeration of plaques.

Cell Cultures and Methods.—Methods and media used for routine cultivation of HeLa cells, L cells, cottontail rabbit epithelial cells, human amnion cells, and monkey kidney cells have been described previously (2). The medium used routinely contained 20 per cent serum and 0.1 per cent yeast extract in Hanks' balanced salt solution (BSS).

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EXPERIMENTAL

Location of Enterovirus Receptors in Subcellular Fractions.—Preliminary studies on disrupted HeLa cells (1) showed that poliovirus receptor is sedimented in a high centrifugal field, is destroyed by exposure to organic solvents, or by heat or trypsin; but is not destroyed by periodate oxidation or by treatment with receptor-destroying enzyme of *Vibrio cholera.*

These findings suggested that the receptor is a labile protein or lipoprotein associated with the insoluble membrane lipoproteins of the cell. Thus it is of interest to determine the location of poliovirus (and other enterovirus) receptors in subcellular fractions of cell homogenates.

Subcellular fractions of HeLa cells, L cells, cottontail rabbit epithelial cells, primary human amnion cells, and monkey kidney cells were prepared according to a modification of the methods reviewed by Schneider (6) (using 0.25 M sucrose as the suspending medium). Thoroughly washed cells were suspended in 0.25 M sucrose to a concentration of approximately 1×10^7 cells per ml, chilled to 0°C in an ice-alcohol bath, disrupted by grinding in a Potter-Elvejhem grinder with a teflon shaft, and immediately fractionated by centrifugation at 0°–4°C. All procedures between disruption of cells and assay for receptor activity were carried out at 0°–4°C to prevent thermal inactivation of receptor. The nuclear fraction was sedimented at 800 *g* for 10 minutes, washed and sedimented twice more in 0.25 M sucrose, and the final pellet suspended in 0.15 M NaCl for receptor assay. The supernatant from the first 800 *g* centrifugation was sedimented at 7000 *g* for 10 minutes to deposit the mitochondrial fraction. The "fluffy layer" over the mitochondria was withdrawn with the supernatant fluid, and the mitochondrial sediment was washed once with 0.25 M sucrose, and following another sedimentation at 7000 *g* was resuspended in 0.15 M NaCl for receptor assay. The microsomal fraction was then prepared by sedimenting the first mitochondrial supernatant fluid at 38,000 *g* for 30 minutes, washing once in 0.25 M sucrose, and resuspending in 0.15 M NaCl for receptor assay. The post-microsomal fraction was prepared by sedimenting the microsomal supernate at 105,000 *g* for 2 hours and resuspending the pellet in 0.15 M NaCl. The final supernatant fluid (cell sap) was made 0.15 M with respect to NaCl by addition of 5 M NaCl before assay of receptor activity.

After each fraction was resuspended in 0.15 M NaCl to original volume (to a concentration corresponding to about 10^7 intact cells/ml) concentrated phosphate buffer was added to a final concentration of 0.01 M to adjust the pH to 7.2. Receptor activity of each fraction was assayed by adding poliovirus or Coxsackie virus to an aliquot of each fraction and incubating at 25°C with frequent agitation. Samples were withdrawn at intervals, diluted 100-fold in BSS to stop adsorption, and plated on HeLa monolayers to determine the number of plaque-forming units inactivated by attachment to receptor. Residual infective virus was allowed to adsorb to the assay monolayers for 1 hour at 25°C, then unattached virus was removed by 3 BSS washes and the monolayers were overlaid with semisolid agar (5) and incubated at 37°C for plaque development. In this way the kinetics of virus adsorption by each fraction was determined in order to ascertain the relative amounts of receptor activity in each.

Figs. 1 and 2 show results obtained with type 1 poliovirus, and Coxsackie B_1, respectively. It can be seen that with both viruses the greatest amount of virus-binding material was found in the microsome fraction. Considerably less receptor was present in other particulate fractions of the cell (mitochondria,

nuclei, and post-microsomal particles), and very little or no receptor activity remained in the cell sap. Thus enterovirus receptors are associated with the insoluble lipoprotein membranous structures of the cell, and are not found at all

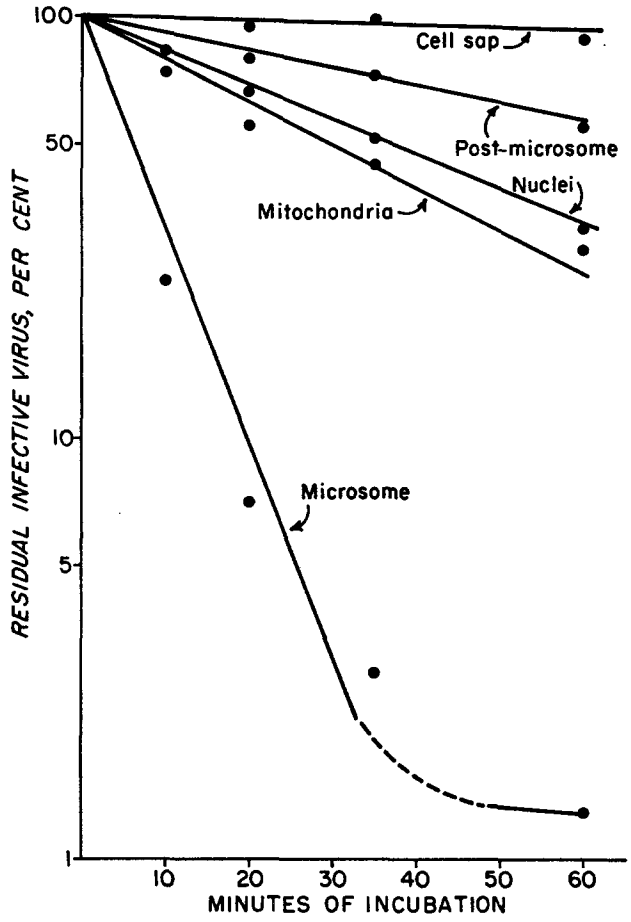

FIG. 1. Kinetics of binding of type 1 poliovirus by subcellular fractions of HeLa cells. All cell fractions were prepared from a single lot of HeLa cells, and each was restored to original concentration corresponding to about 10^7 intact cells per ml before incubation with virus at 25°C.

in (or are present in insignificant amounts in) solution in the cytoplasm. Similar results were obtained using subcellular fractions from primary monkey kidney cell cultures and primary human amnion cell cultures. In every instance the bulk of the receptor activity was associated with the microsomes. However, when microsomal fractions and other subcellular fractions of the enterovirus-

resistant L strain mouse fibroblast and cottontail rabbit epithelial cells were tested it was found that none of the fractions adsorbed significant amounts of poliovirus under the same conditions (Fig. 3 and Table I). Since it has been shown previously that L cells and other non-primate cells lack receptor for

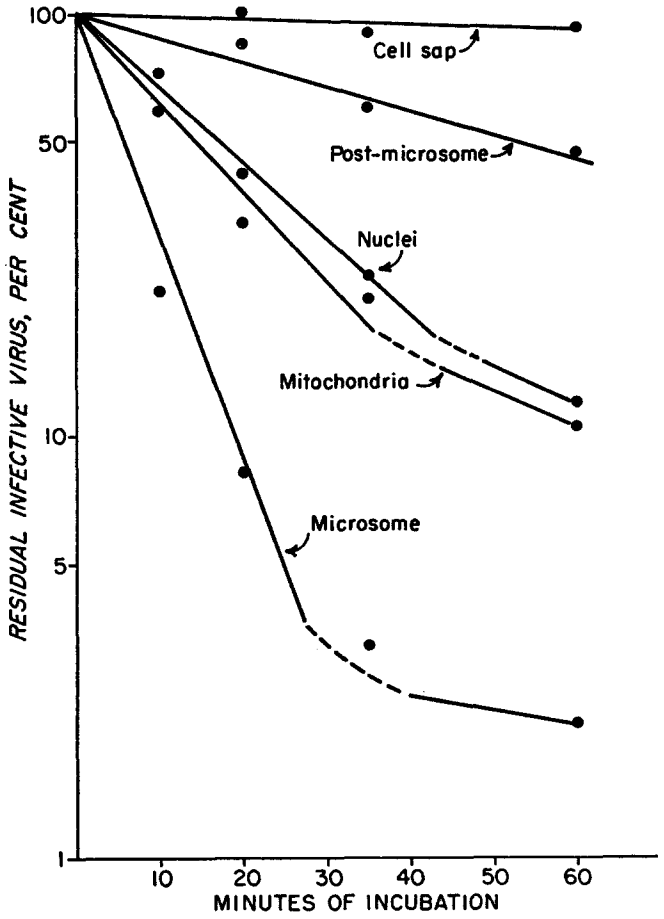

FIG. 2. Kinetics of binding of Coxsackie B_1 virus by subcellular fractions of HeLa cells.

poliovirus detectable in whole cell homogenates (1), fractions from these cells provide a control which indicates that the virus loss observed in Figs. 1 and 2 is not due to non-specific binding of virus by cell particulates.

Comparison of Receptor Activities of Intact Cells and Disrupted Cells.—In the whole cell, only those receptors which are at the cell surface (*i.e.*, exposed to the liquid menstruum) would be capable of adsorbing virus. Nevertheless, it was of interest to determine whether enterovirus receptor material is found ex-

TABLE I

Presence of Poliovirus and Coxsackie Virus Receptors in Microsome Fractions of Susceptible (Primate) and Insusceptible (Non-Primate) Cells

Source of microsomal fraction	Virus inactivated by incubation* with microsomal fraction	
	Type 1 poliovirus	Coxsackie B_1 virus
	per cent	*per cent*
HeLa cell cultures	>90	>90
Primary human amnion cell cultures	>90	>90
Primary monkey kidney cell cultures	>90	>90
L strain mouse fibroblasts	<10	<10
Cottontail rabbit epithelial cells	<10	<10

* Virus and microsome fractions were incubated together for 1 hour at 25°C in 0.15 M NaCl buffered to pH 7.2 with 0.01 M phosphate. Then the mixtures were diluted in BSS and plated on HeLa cell monolayers to detect residual infective virus plaque-forming units.

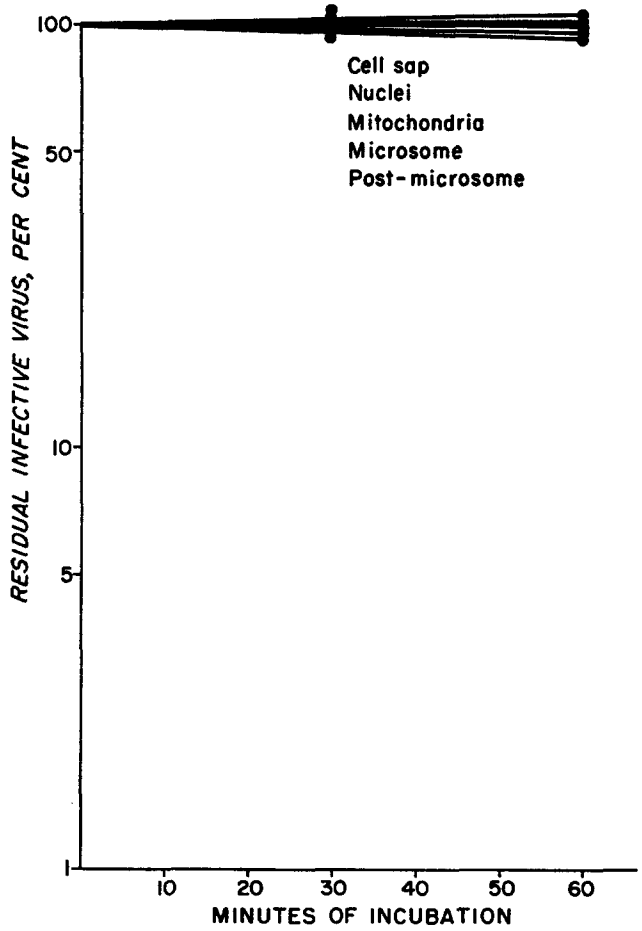

FIG. 3. Failure of subcellular fractions of L strain mouse fibroblasts to bind detectable amounts of type 1 poliovirus under the same conditions employed for HeLa cell fractions seen in Fig. 1.

clusively at the surface of the cell or whether it is found intracellularly as well. Certainly the membranes of the microsome fraction are not derived from the plasma membrane only, but include endoplasmic reticulum, etc. (7). If receptor

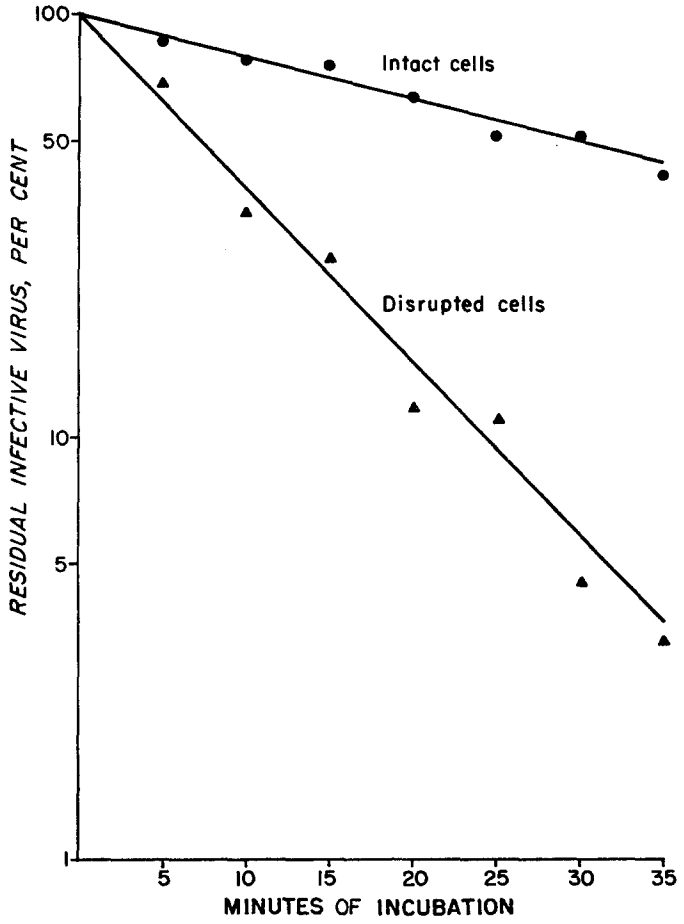

FIG. 4. Kinetics of binding of type 1 poliovirus by a suspension of 4×10^6 intact HeLa cells per ml compared to kinetics of binding by an equal aliquot of the same HeLa cell suspension which had been disrupted before incubation with virus at 25°C.

material were present in larger amounts within the cell than on the surface it should be possible to demonstrate greater virus-binding activity in cell homogenates than is found in intact cells.

To test this possibility 4×10^6 thoroughly washed HeLa cells were suspended in 0.1 M NaCl adjusted to pH 7.2 with phosphate buffer (final concentration 0.01 M), and divided into two 2 ml aliquots. One aliquot of cells was disrupted by homogenizing in a Potter-Elvejhem grinder at 0°C, and the other was left intact as a control. The kinetics of virus adsorption by

intact cells was then compared to virus inactivation by disrupted cells. Virus-cell and virus-homogenate mixtures were allowed to incubate at 25°C, with frequent agitation to keep the intact cells suspended, and samples were removed at intervals, diluted 1/100 to stop attachment, and assayed to detect residual unadsorbed virus. After dilution of cell-virus samples, cells were removed by centrifugation for 5 minutes at 2000 *g* before assaying for free virus.

TABLE II

Attempts to Release Enterovirus Receptor Activity from Insoluble (Sedimentable) Lipoproteins of HeLa Cells

Treatment of HeLa cell microsomes	Virus inactivated by incubation with sedimenting and non-sedimenting fractions of treated microsomes*			
	Type 1 poliovirus incubated with		Coxsackie B_1 virus incubated with	
	Sediment	Supernatant	Sediment	Supernatant
	per cent	*per cent*	*per cent*	*per cent*
None (washed microsome control)	>90	<10	>90	<10
Sonic oscillation, 15 min., 0°C	>90	<10	>90	<10
Extracted with 1 M NaCl, 10 min., 25°C	>90	<10	>90	<10
Held at pH 4.0, 10 min., 25°C	>90	<10	>90	<10
Held at pH 9.0, 10 min., 25°C	>90	<10	>90	<10
Extracted with 0.1 N HCl, 10 min., 25°C	<10	<10	<10	<10
Extracted with 0.1 N NaOH, 10 min., 25°C	<10	<10	<10	<10
Extracted with 0.5 per cent sodium desoxycholate‡	<10	<10	<10	<10
Extracted with 1 per cent SDS§	<10	<10	<10	<10
Digested with 0.5 per cent trypsin‖	<10	<10	<10	<10
Digested with 0.5 per cent papain‖	<10	<10	<10	<10
Extracted with *N*-butanol, 1 min., 0°C¶	<10	<10	<10	<10

* Following each indicated treatment the microsomes were centrifuged at 105,000 *g* for 10 minutes, and both supernatant and sediment were diluted in 0.15 M NaCl to a concentration corresponding to 5 X 10^6 HeLa cells per ml, buffered to pH 7.2, and incubated with either poliovirus or Coxsackie virus for 1 hour at 25°C to detect receptor activity.

‡ After 10 minutes at 25°C in sodium desoxycholate or sodium dodecyl sulfate sediment was removed by centrifugation at 105,000 g and the supernatant was dialyzed at 0°C against 0.15 M NaCl.

§ SDS, sodium dodecyl sulfate.

‖ Enzyme digestion was carried out at 37°C for 1 hour at pH 7.2 in 0.1 M NaCl. Papain was reduced with cysteine.

¶ *N*-butanol was added to an equal volume of microsomal suspension at 0°C and was shaken gently for 1 minute. The aqueous phase and interphase precipitate were collected and dialyzed at 0°C, centrifuged, and tested for activity.

Results of a typical experiment are shown in Fig. 4. It can be seen that a large increase in poliovirus receptor activity occurred when HeLa cells were disrupted. In this case the rate of adsorption was increased by about fourfold. The attachment rate constant increased from 4.7×10^{-9} cm^3 min^{-1} $cell^{-1}$ with intact cells to 1.9×10^{-8} cm^3 min^{-1} $cell^{-1}$ with disrupted cells. Similar results

were obtained when binding of Coxsackie B_1 was tested. This indicates that all the receptor in the cell is not exposed at the surface of the cell. It is, however, possible that the increased rate of attachment is simply due to fragmentation of the plasma membrane into very small particles with increased collision probability, and/or to exposure of greater plasma membrane surface by cell disruption.

TABLE III

Effect of Protein Denaturants and Non-Ionic Surface-Active Agents on Enterovirus Receptor Activity

Treatment of HeLa cell microsomes*	Virus inactivated by incubation with sedimenting and non-sedimenting fractions of treated microsomes*			
	Type 1 poliovirus incubated with		Coxsackie B_1 virus incubated with	
	Sediment	Supernatant	Sediment	Supernatant
	per cent	*per cent*	*per cent*	*per cent*
None (washed microsome control)	>90	<10	>90	<10
5 per cent phenol	<10	<10	<10	<10
5 per cent formaldehyde	<10	<10	<10	<10
8 M urea	<10	<10	<10	<10
1 M urea	>90	<10	>90	<10
5 M guanidine·HCl	<10	<10	<10	<10
0.1 M guanidine·HCl	>90	<10	>90	<10
1 per cent tween 20	>90	<10	>90	<10
1 per cent tween 80	>90	<10	>90	<10

* All treatments were carried out at pH 7.2 for 15 minutes at 25°C. After treatment the reaction mixture was diluted 10-fold and dialyzed overnight at 0°C against 0.15 M NaCl. The microsome fraction was then centrifuged at 105,000 *g* for 10 minutes, and the supernatant and sediment were diluted in 0.15 M NaCl to a concentration corresponding to 5×10^6 intact HeLa cells per ml, and each tested for ability to bind poliovirus and Coxsackie B_1 virus.

Attempts to Solubilize Enterovirus Receptor Substance.—It was demonstrated above that enterovirus receptor is found mainly in particulate cellular constituents. Since it would be very desirable to obtain receptor in soluble form in order to purify and identify it, a number of attempts have been made to free it from the insoluble lipoproteins. Microsomes from HeLa cells were washed 5 times at 0°C with 0.15 M NaCl and resedimented at 50,000 *g* each time to eliminate soluble proteins. The sediment still retained full receptor activity after 5 washings. Aliquots of this washed microsomal material were then subjected to various treatments in an effort to solubilize the receptor. Table II shows the results of a number of procedures employed. After each treatment of the microsome fraction, it was sedimented at 105,000 *g* for 10 minutes, and both the supernatant and sediment were tested for receptor activity. Sonic disruption

when carried out at 0°C did not inactivate receptor although prolonged sonic oscillation without adequate temperature control does destroy receptor (1). Nevertheless, extensive sonic oscillation failed to solubilize receptor—all activity was still sedimented. Neither did extraction with 1 M NaCl, nor with high pH (9.0) or low pH (4.0) solutions inactivate or liberate receptor activity of the microsomes. Stronger acid or alkaline solutions (0.1 N HCl or 0.1 N NaOH) destroyed receptor activity, as did extraction with *N*-butanol. Sodium desoxycholate and sodium dodecyl sulfate destroyed receptor. Desoxycholate is useful for "solubilizing" microsomal lipoproteins (8), but apparently both these detergents irreversibly denatured receptor. Finally, proteolytic enzymes were tested. It was shown previously that following trypsin treatment of receptor, high speed sediments had lost activity. Table II also shows that receptor activity was not rendered soluble by proteolysis but apparently was destroyed by crystalline trypsin or papain.

Effect of Other Agents on Receptor.—The destruction of receptor by ether, chloroform, and proteolytic enzymes described previously (1) as well as by detergents, butanol, and dilute acid and alkaline solutions seen above suggests that it is protein and probably lipoprotein. This probability was further explored by testing the effect of other agents on receptor activity. Table III shows that 5 per cent solutions of phenol or formaldehyde destroyed receptor. Urea or guanidinium salt also rendered receptor inactive at high concentrations. That this inactivation was attributable to hydrogen bond disruption is shown by the lack of effect of urea or guanidine at lower concentrations. Finally, mild, nonionic surface-active agents were tested. It can be seen that tween 20 and tween 80 neither destroyed receptor activity nor solubilized it, indicating that loosely bound lipids probably are not important to virus-binding ability.

DISCUSSION

It appears that the material in cell homogenates which binds enteroviruses is the same receptor which enables the intact cell to adsorb these viruses, since receptor activity of microsomes or other subcellular fractions was obtained only from susceptible cells which were capable of adsorption when intact (Table I and Fig. 3). Furthermore, the ionic cofactor requirements for receptor activity in disrupted cells reflects the cofactor requirements for virus adsorption to whole cells (4). (See Note Added in Proof).

The above results suggest that enterovirus receptor activity resides in the insoluble lipoproteins of the cell membranes, mainly in the microsome fraction. It seems probable that the weak virus-binding ability of nuclei and other subcellular fractions represents contamination of these fractions with microsomal lipoproteins. In fact, most preparations of nuclei contain obvious adherent membrane fragments, and removal of all of the "fluffy layer" above the mitochondria is clearly not possible.

The data in Fig. 4 which demonstrate a large increment in receptor activity

upon disruption of HeLa cells suggest that receptor is present on the intracellular membranes as well as being at the cell surface. This interpretation is not certain however, since little is known about the fate of the plasma membrane (or other membranes) after homogenization. Quantitative evaluation would require knowledge of the average number, size, and surface area after folding of the membrane fragments following homogenization. It has recently been reported that rat liver cell membranes can be isolated by gentle homogenization and sedimentation in a low centrifugal field (1500 *g*) (9). If the cell membranes in the present study were also sedimenting at low speeds then the receptor activity of the nuclear and mitochondrial fractions may represent plasma membrane receptor, while the slower sedimenting majority of receptor may derive from intracellular membranes. However, the slower sedimenting receptor could be on very small fragments of plasma membrane, and the increase in receptor activity upon disruption of cells may simply be due to exposure of large areas of membrane which are not exposed at the surface of intact cells. It would not be surprising if the intracellular membranes exhibited the same virus affinities as the plasma membrane, since electron micrographs (10) show continuity between membranes of the endoplasmic reticulum and the plasma membrane.

The data in Tables II and III show clearly that enterovirus receptor activity resides in protein, and that the active protein cannot easily be dissociated from insoluble cellular lipoproteins. Precise characterization of this protein would require "solubilizing" and purification procedures. Efforts to free receptor from other membrane components of the cell are continuing, but it is possible that enterovirus receptor activity depends upon interaction between a number of protein species in a large insoluble complex.

The failure of tween 20 and tween 80 to destroy receptor activity indicates that if lipids are essential for receptor function such lipids are not loosely bound at the surface of the membranes. Evidence is accumulating that the lipids of cell membranes are not exposed at the surface but occupy an internal position in the membrane structure, sheathed by proteins (11). It is not possible from the data presented above to determine whether lipid plays any role in enterovirus receptor activity.

It can be seen in Table I and II that receptor activity for type 1 poliovirus always paralleled that for Coxsackie B_1. It appears possible that HeLa cell receptors for poliovirus may also bind the Coxsackie group B viruses, but it is obvious that not all enteroviruses have the same receptor affinities since group A Coxsackie virus receptors and adsorption cofactor requirements are not the same as for poliovirus (4).

SUMMARY

It is shown that enterovirus receptors are found mainly in the microsomal fraction of disrupted primate cells. Greater virus adsorption was exhibited by

disrupted cells than by intact cells, indicating that enterovirus receptor may be present on intracellular membranes as well as on the surface of the cell. Poliovirus receptor is an integral part of, or is firmly attached to, the insoluble lipoproteins of the cell. All attempts to solubilize receptor have either destroyed virus-adsorbing activity, or have failed to separate it from sedimentable lipoproteins. The destruction of poliovirus receptor activity by proteolytic enzymes, surface active agents, organic solvents, concentrated urea solutions, phenol, formaldehyde, etc., all strongly indicate that this receptor function depends upon integrity of a protein portion of the membrane lipoproteins.

Note Added in Proof.—Quersin-Thiry recently confirmed specificity of enterovirus inactivation by cell extracts, and extended these findings to other virus groups. (Quersin-Thiry, L., Interaction between cellular extracts and animal viruses. I. Kinetic studies and some notes on the specificity of the interaction, *Acta Virol.*, 1961, **5**, 141.)

BIBLIOGRAPHY

1. Holland, J. J., and McLaren, L. C., The mammalian cell-virus relationship. II. Adsorption, reception, and eclipse of poliovirus by HeLa Cells, *J. Exp. Med.*, **1959, 109,** 487.
2. McLaren, L. C., Holland, J. J., and Syverton, J. T., The mammalian cell-virus relationship. I. Attachment of poliovirus to cultivated cells of primate and nonprimate origin, *J. Exp. Med.*, 1959, **109,** 475.
3. Holland, J. J., McLaren, L. C., and Syverton, J. T., The mammalian cell-virus relationship. IV. Infection of naturally insusceptible cells with enterovirus ribonucleic acid, *J. Exp. Med.*, 1959, **110,** 65.
4. McLaren, L. C., Holland, J. J., and Syverton, J. T., The mammalian cell-virus relationship. V. Susceptibility and resistance of cells *in vitro* to infection by Coxsackie A9 virus, *J. Exp. Med.*, 1960, **112,** 581.
5. Holland, J. J., and McLaren, L. C., Improved method for staining cell monolayers for virus plaque counts, *J. Bact.*, 1959, **78,** 596.
6. Schneider, W. C., Methods for isolation of particulate components of the cell, *in* Manometric Techniques, (W. W. Umbreit, R. H. Burris, and J. F. Stauffer, editors), Minneapolis, Burgess, 1957, 188.
7. Palade, G. E., and Siekevitz, P., Liver microsomes. An integrated morphological and biochemical study, *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 171.
8. Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C., Studies on cytoplasmic ribonucleoprotein particles from the liver of the rat, *J. Biol. Chem.*, 1955, **217,** 111.
9. Neville, D. M., Jr., The isolation of a cell membrane fraction from rat liver, *J. Biophysic. and Biochem. Cytol.*, 1960, **8,** 413.
10. Palade, G. E., The endoplasmic reticulum, *J. Biophysic. and Biochem. Cytol.*, 1956, suppl. 2, 85.
11. Few, A. V., Gilby, A. R., and Seaman, G. V. T., An electrophoretic study on structural components of *Micrococcus lysodeikticus, Biochim. et Biophysica Acta*, 1960, **38,** 130.