J. gen. Virol. (1968), **2**, 357–364
With 1 *plate*
Printed in Great Britain

Characterization of Herpes Simplex Virus Strains Differing in their Effects on Social Behaviour of Infected Cells

By PILARICA M. EJERCITO, E. D. KIEFF AND B. ROIZMAN
Department of Microbiology, University of Chicago, Chicago, Ill., U.S.A.

(*Accepted* 13 *November* 1967)

SUMMARY

Established (laboratory) strains and fresh isolates of herpes simplex virus from patients with skin and genital lesions were classified into four groups depending on their effects on the social interaction among infected HEP-2 cells. The groups comprised strains causing (1) rounding of cells but no adhesion or fusion, (2) loose aggregation of rounded cells, (3) tight adhesion of rounded cells, and (4) fusion of cells into polykaryocytes. Protype strains from each group were found to differ with respect to immunologic specificity, buoyant density in CsCl solutions and stability at 40°.

INTRODUCTION

We report in this paper the properties of four strains of herpes simplex virus with different effects on social interactions among infected cells. The strains were isolated in the course of a survey of established (laboratory) and recently isolated strains for markers suitable for genetic studies. In recent years a number of investigators but notably Gray, Tokumaru & Scott (1958), Hoggan & Roizman (1959), Nii & Kamahora (1961), Hinze & Walker (1961), Schneweiss (1962*a*), Kohlhage & Siegert (1962), Munk & Donner (1963) and Wheeler (1964) have reported the isolation of strains with different effects on cells. For the most part, these strains caused fusion or rounding and clumping of infected cells. Moreover, it has been shown (Roizman & Roane, 1961, 1963; Roizman & Aurelian, 1965; Kohlhage, 1964) that strains differing in their effects on the social behaviour of infected cells also differ in some physical properties and immunological specificity. In this study it has been found that the social behaviour of cells infected with different strains ranges from little or no change, to adhesion and fusion, and that prototypes of groups causing different kinds of social behaviour differ among themselves with respect to immunological specificity, buoyant density in CsCl and stability at 40°.

METHODS

Experimental design. The plan of this study was to group laboratory strains and fresh isolates from this and other laboratories according to their effects on social interactions among infected HEP-2 cells and to select a prototype from each group for comparison of antigenic specificity, buoyant density in CsCl and stability at 40°. The viruses surveyed in this study were strain HF obtained from Dr T. Fisher, Tulane University, New Orleans, La, U.S.A.; strains MSU 3/P, RHS L/2, RHU S/2, and the Schooler strain, obtained from Dr Gordon Plummer, Wellcome Research Laboratory,

Kent, England; strain VR 3 (Macintire) obtained from Dr Walter Dowdle, Communicable Disease Laboratory, Atlanta, Georgia, U.S.A.; strains HF 378 and HF 490 obtained from Dr G. H. Silver, University of Syracuse, Syracuse, New York, U.S.A.; 4 isolates furnished by Dr Walter B. Becker, University of Cape Town Medical School, Cape, South Africa; 6 recent isolates furnished by Dr Andre Nahmias, Emory University, Atlanta, Georgia, U.S.A.; 12 strains isolated recently from patients with genital and facial lesions at the University of Chicago Hospitals; and the MP, MP, NT, Tucker and Freeman strains described previously (Hoggan & Roizman, 1959). These were classified into 4 groups, i.e. (1) strains causing rounding of cells but no adhesion or fusion, (2) strains causing loose aggregates of rounded cells, (3) strains causing very tight adhesion of rounded infected cells, and (4) strains causing polykaryocytosis. Many laboratory strains and fresh isolates from patients with skin (facial), oral, or corneal lesions comprise group 3. Several laboratory strains and fresh isolates from patients with genital lesions belong to group 2. Group 4 contained laboratory strains only. The VR 3 strain was the sole member of group 1. The prototypes of groups 1 to 4 selected for comparison were, respectively, strain VR 3, a strain designated F and isolated in HEp-2 cells from a facial lesion, a strain designated G and isolated in HEp-2 cells from a genital lesion, and strain MP.

Cells. HEp-2 cells were originally obtained from Microbiological Associates, Bethesda, Maryland, U.S.A. and subsequently maintained in this laboratory in Eagle's (1959) minimal essential medium containing 10% calf serum.

Assay. All strains tested in this study were grown and assayed in HEp-2 cells. The assay procedure was described by Roizman & Roane (1961). Briefly, HEp-2 cells grown in disposable plastic flasks (Plastics, Los Angeles, California, U.S.A.) were exposed to 1 ml. of virus diluted in 0·01 M-phosphate buffered saline pH 7·2 containing 0·2% albumin and 0·1% glucose. After 2 hr of adsorption with constant shaking at 37°, the virus inoculum was aspirated and replaced with a medium consisting of mixture 199, 1% calf serum and 0·2% pooled human γ-globulin. The function of the γ-globulin was to furnish antiviral antibody to prevent the spread of virus from cell to cell via the fluid phase (Hoggan, Roizman & Turner, 1960). After 40 hr of incubation at 37° the overlay was aspirated, the cells were fixed with methanol, stained with Giemsa stain and air-dried. Plaques were counted with the aid of a stereo microscope and a marking pen connected to an electric counter.

Antisera. Rabbit antisera were prepared as follows. An area approximately 4 × 8 in. on the back of an albino rabbit was shaved and painted with virus. Lesions covering the entire area developed between 5 and 10 days after infection and persisted for 2 to 3 weeks. Approximately 3 to 4 weeks after the lesions disappeared the rabbits were challenged subcutaneously with virus, then bled 1 week later. The sera were decomplemented by heating to 56° for 30 min. before use.

RESULTS

The nature of the changes in the social behaviour of infected cells

The plate shows the effects of VR 3, F, G and MP strains on HEp-2 cells grown in monolayer cultures. In this experiment HEp-2 cells were grown on coverslips in Leighton tubes and exposed for 2 hr at 37° to sufficient virus to infect 0·2% of the cells. The coverslips were then overlaid with a medium consisting of mixture 199 and

1% calf serum and incubated at 37°. At 18 and 40 hr after infection the coverslips were removed and Leighton tubes and the cells were fixed in methanol and stained with Giemsa stain. The characteristic features of the effects of the 4 strains on HEp-2 cells were as follows. (1) VR 3 virus caused infected cells to round and, rarely, to aggre-

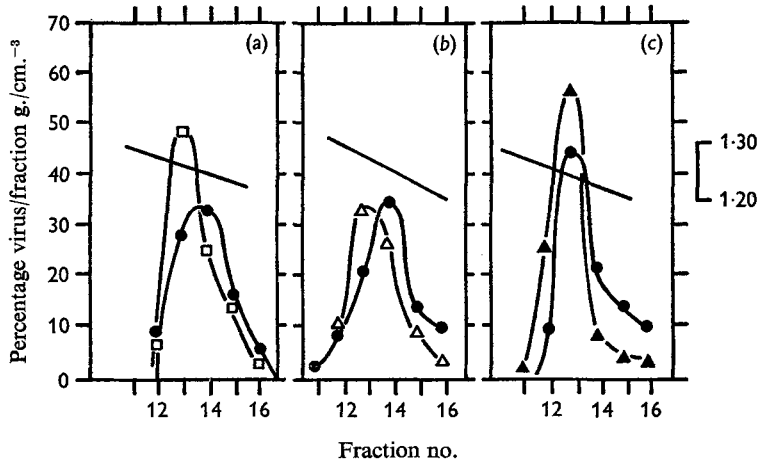


Fig. 1. The distribution of herpes simplex virus on isopycnic centrifugation in CsCl solution. The centrifugations were done with artificial mixtures of MP and one other strain. (*a*) Profile of MP and F, (*b*) profile of MP and G, (*c*) profile of MP and VR 3. ●——●, MP strain; □——□, F strain; △——△, G strain; ▲——▲, VR 3 strain.

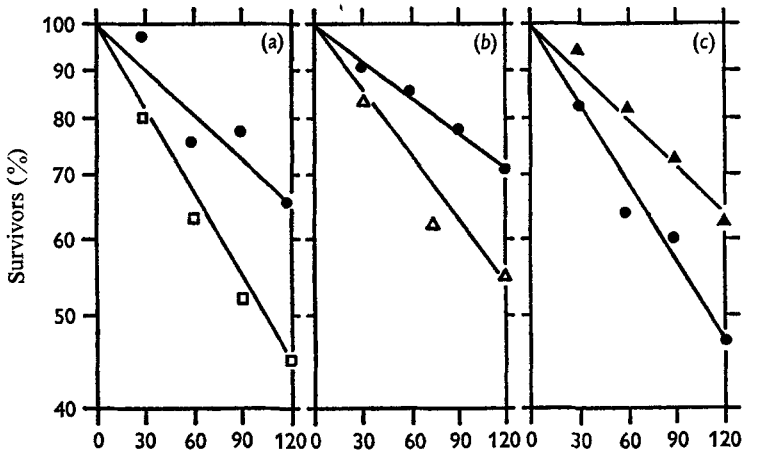


Fig. 2. Heat inactivation at 40° of VR 3, F, G, and MP strains. The inactivations were done with artificial mixtures of MP and one other strain. (*a*) MP and F, (*b*) MP and G, and (*c*) MP and VR 3. ●——●, MP strain; □——□, F strain; △——△, G strain; ▲——▲, VR 3 strain.

gate. However, the aggregated cells very rarely piled up. (2) The G strain selected as a prototype caused cells to round and to pile up into spindle-shaped clumps. Characteristically, the clumps tended to fragment. Moreover, single rounded cells abounded in the vicinity of the clumps. (3) Cells infected with the F strain rounded and formed

relatively small tight round symmetrical multilayered clumps. The cells forming the clumps were drawn from the immediate vicinity of the cell infected initially; frequently the area surrounding the clump was partially depopulated and in stained preparations appeared as a 'halo'. (4) The MP strain, as previously described (Hoggan & Roizman, 1959), caused cells to fuse into polykaryocytes.

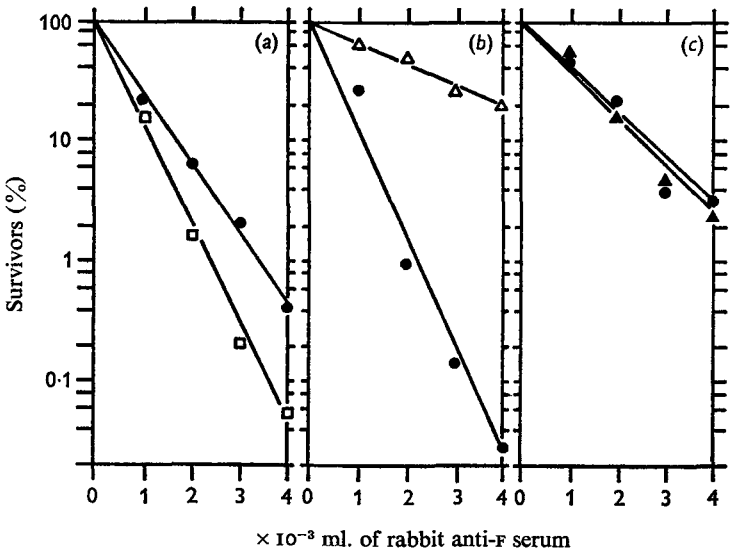


Fig. 3. Neutralization of artificial mixtures of MP and F, G or VR 3 viruses with rabbit anti-F strain sera. (*a*) MP and F, (*b*) MP and G, (*c*) MP and VR 3. ●——●, MP strain; □——□, F strain; △——△, G strain; ▲——▲, VR 3 strain.

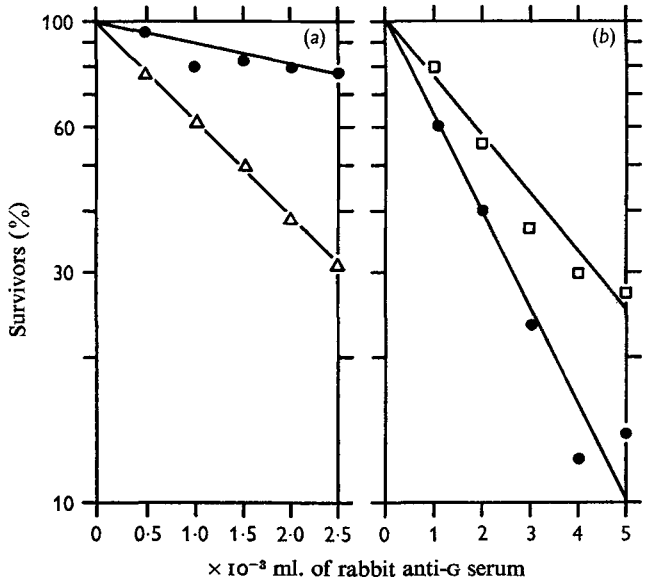


Fig. 4. Neutralizing of artificial mixtures of MP and F or G viruses with rabbit anti-G strain sera. (*a*) MP and G, (*b*) MP and F.

The lesions produced by the VR 3, F, G and MP strains in coverslip cultures did not differ significantly from those produced in cultures overlayed with medium containing antibody to prevent the spread of virus from cell to cell through the extracellular fluid. The plaques produced by VR 3 and G were similar in size. The G plaque frequently consisted of 2 or 3 clumps interconnected by a strain of single rounded cells. The F plaque was the smallest and consisted of a single clump. The plaques produced by the MP strain were consistently larger than those produced by VR 3, G or F strains.

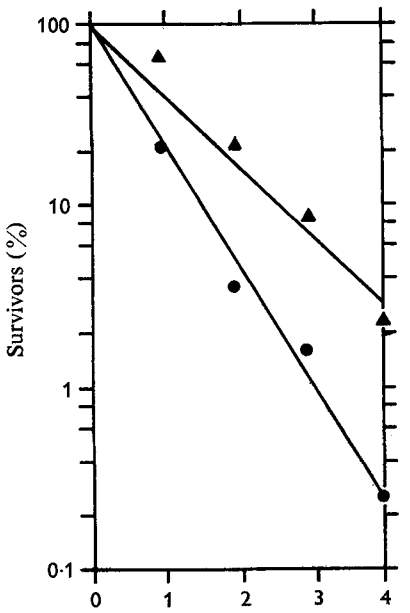


Fig. 5. Neutralization of artificial mixture of MP and VR 3 viruses with rabbit anti-MP strain serum. ●——●, MP strain; ▲——▲, VR 3 strain.

Physical and immunological differentiation of VR 3, F, G and MP strains

Elsewhere it has been shown that MP virus differs from the mP mutant and from certain host range mutants of the MP strain in surface properties, antigenic specificity, buoyant density in CsCl solutions and rates of inactivation at 40° (Roizman & Roane, 1961, 1963; Roizman & Aurelian, 1965). Accordingly, three series of experiments were performed to determine whether similar differences exist between the VR 3, F, G and MP strains. We took advantage of the observation that the plaques produced by MP strains were readily differentiated from those produced by VR 3, F and G strains. These experiments were therefore done with artificial mixtures of MP and one other strain.

In one series of experiments, artificial mixtures of MP and VR 3, G or F preparations of approximately equal infectivity were centrifuged in a solution of CsCl for 40 hr at 5° and 35,000 rev./min. in a Spinco SW 39 rotor. Five-drop fractions were then collected from the bottom of the tube and assayed in HEp-2 cells. F and G banded at a higher density than MP. VR 3 banded at an intermediate density, between F or G and MP (Fig. 1).

In another series of experiments, artificial mixtures of MP and one other strain were diluted in 0·01 M-phosphate buffered saline, pH 7·2 and heated at 40° for 2 hr. Samples taken at 30 min. intervals were assayed for infectivity. F and G strains were both more readily inactivated at 40° than the MP strain but the VR 3 strain was more stable than MP (Fig. 2).

In the third series of experiments 0·5 ml. amounts of artificial virus mixtures were added to equal volumes of buffered saline containing varying concentrations of antisera. After 1 hr of incubation with constant shaking in a 37° water bath the neutralization mixtures were diluted at least 500-fold and plated. Anti-F antibody differentiated between F, G and MP but not between MP and VR 3 (Fig. 3). The anti-F antibody reacted preferentially with the homologous virus and less readily with G virus. In this frame of reference, MP virus occupied a position intermediate between F and G. This conclusion was confirmed in reciprocal tests with anti-G serum (Fig. 4). The VR 3 and MP strains were readily differentiated by anti-MP serum (Fig. 5). The four strains are therefore related but not identical. Moreover the data presented in Figs 4 and 5 agree with the results of Schneweiss (1962*b*) and particularly with those of Dowdle *et al.* (1967) showing that herpes strains isolated from genital infections differed from those recovered from skin or eye infections.

DISCUSSION

We have previously reported differences between MP and mP strains and between MP and some of its host range mutants (Roizman & Roane, 1961, 1963; Roizman & Aurelian, 1965). The effect of these viruses on the social behaviour of cells, however, is restricted; they cause either adhesion or fusion of the infected cells. In this paper we report the properties of 4 strains covering a more extensive range of social interactions among infected cells and in addition differing with respect to antigenic specificity and physical properties. It seems desirable to focus attention on two aspects of these findings.

The range of social interactions among infected cells

It is convenient to differentiate between the effect of herpes virus cells on morphological appearance of dispersed mononucleated cells and on the interaction among infected cells in relatively crowded cultures. With possibly one exception, that of the GCF strain of Nii & Kamahora (1961) which causes some cells to become spindle-shaped, most strains cause dispersed single cells to round. In crowded cultures, the interaction among infected cells varies considerably, from little or none (VR 3), through variable degrees of adhesions (G and F strains), to fusion (MP). The virus strains in our laboratory collection are readily classified on the basis of their effects on the social behaviour of cells. These properties of the virus are stable and very useful for rapid classification. It should be noted, however, that the classification of herpes simplex virus strains into 4 groups is arbitrary, and moreover the physical properties of the strains selected as prototypes may not be representative of the entire group in which the strains are classified. A number of viruses comprising group 4 share in common the capacity to induce fusion but differ not only in the size and structure of the polykaryocyte they induce (Kohlhage & Siegert, 1962; Wheeler, 1964; Roizman & Aurelian, 1965) but also in their physical characteristics (Kohlhage, 1964; Roizman & Aurelian, 1965).

The relationship between the physical properties of the herpes virion and the social behaviour of infected cells

In this and previous reports (Roizman & Roane, 1963; Roizman & Aurelian, 1965) it has been shown that alteration in the social behaviour of infected cells invariably coincides with changes in surface properties of the herpes virions. Attempts to explain the apparent relationship between the structure of the virion and the behaviour of the infected cells must take in consideration the following.

First, available data suggest that infectivity is a preferential (if not an exclusive) property of the enveloped virions. This conclusion is based on the observations that (1) both infectivity and envelope are lost following treatment of virus with lipid solvents (Roizman & Roane, 1963), lipases and phospholipases (lecithinases) (Spring & Roizman, unpublished) and (2) in DK cells abortively infected with *dk*$^-$ mutants envelopment fails and the nucleocapsids produced in these cells are non-infectious (Spring & Roizman, 1967; Spring, Roizman & Schwartz, in preparation).

The second point concerns the structure of the envelope. Current data suggest that the envelope consists of viral antigenic determinants and components specified by the cells. Thus the virus is enveloped by the inner layer of the nuclear membrane; the structure of the viral envelope is morphologically similar to that of the cellular membranes (Epstein, 1962); cellular antigenic determinants in the envelope have been reported by Watson & Wildy (1963); and the buoyant density of the infectious virion is determined not only by the genotype of the virus but also by the host in which it is grown (Spear & Roizman, 1967). However, the envelope is probably not merely a piece of cellular membrane wrapped around the virion; more likely the membrane has been modified to the extent that it contains viral antigens. This conclusion is based on the finding that herpes simplex virus strains differ with respect to antigenic specificity.

The last point concerns the infected cells. It has been shown previously that cells infected with herpes simplex virus acquire a new antigenic specificity measured in a cytolytic test (Roane & Roizman, 1964). The new antigen is tentatively identified as a component of the envelope. This conclusion is based on three observations. (1) Serum absorbed with partially purified virus loses its reactivity with infected cells (Roane & Roizman, 1964). (2) Antisera produced against various antigenic fractions obtained from infected cells show an excellent correlation between virus neutralizing and cytolytic titres (Roizman & Spring, 1967). (3) In DK cells abortively infected with *dk*$^-$ virus, viral envelopes are not produced and the social behaviour is unaltered. Rabbits injected with extracts of infected DK cells produce relatively little neutralizing and cytolytic antibody (Roizman & Spring, 1967).

Based on these considerations the hypothesis that accounts best for the apparent correlation between the social behaviour of infected cells and selected physical properties of the virus is that cellular membranes must be modified by products specified by the virus in order for the virion to become enveloped; the amount of cellular membranes altered by the viral product exceeds that required for enveloping virus; and the product binding to the membranes is responsible for the immunological specificity of the virus and of the infected cell and for the alteration in the cytoplasmic membrane resulting in an altered social behaviour of the infected cells.

We are indebted to Drs Walter R. Dowdle and Andre Nahmias for drawing our attention to the immunological differences between the strains isolated from genital infections and other organs of the body and to Dr Nancy Furey for aid in obtaining fresh isolates. This investigation was supported by grants from the Whitehall Foundation, the National Cancer Institute (CA 08494) and the American Cancer Society (E 314).

REFERENCES

DOWDLE, W. R., NAHMIAS, A. J., HARWELL, R. W. & PAULS, F. P. (1967). Association of antigenic type of herpesvirus hominis with site of viral recovery. *J. Immun.* (in the Press.)

EAGLE, H. (1959). Amino acid metabolism in mammalian cell cultures. *Science, N.Y.* **130**, 432.

EPSTEIN, M. A. (1962). Observations on the mode of release of herpes virus from infected HeLa cells. *J. Cell Biol.* **12**, 589.

GRAY, A., TOKUMARU, T. & SCOTT, T. F. (1958). Different cytopathogenic effects observed in HeLa cells infected with herpes simplex virus. *Arch. ges. Virusforsch.* **8**, 60.

HINZE, H. C. & WALKER, D. L. (1961). Variation of herpes simplex virus in persistently infected tissue cultures. *J. Bact.* **82**, 498.

HOGGAN, M. D. & ROIZMAN, B. (1959). The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. *Am. J. Hyg.* **70**, 208.

HOGGAN, M. D., ROIZMAN, B. & TURNER, T. B. (1960). The effect of temperature of incubation on the spread of herpes simplex virus in an immune environment in cell culture. *J. Immun.* **84**, 152.

KOHLHAGE, H. (1964). Differenzierung von Plaquevarianten des Herpes simplex Virus durch Gradientenzentrifugation und Säulenchromatographie. *Arch. ges. Virusforsch.* **14**, 358.

KOHLHAGE, H. & SIEGERT, R. (1962). Zwei genetisch determinierte Varianten eines Herpes Simplex Stammes. *Arch. ges. Virusforsch.* **12**, 273.

MUNK, K. & DONNER, D. (1963). Cytopathischer Effekt und Plaque-morphologie verschiedener Herpes Simplex Virus Stämme. *Arch. ges. Virusforsch.* **13**, 529.

NII, S. & KAMAHORA, J. (1961). Cytopathic changes induced by herpes simplex virus. *Bikens J.* **4**, 255.

PLUMMER, G. (1964). Serological comparison of the herpes viruses. *Br. J. exp. Path.* **45**, 135.

ROANE, P. R. & ROIZMAN, B. (1964). Studies of determinant antigens of viable cells. II. Demonstration of altered antigenic reactivity of HEp-2 cells infected with herpes simplex virus. *Virology* **22**, 1.

ROIZMAN, B. & AURELIAN, L. (1965). Abortive infection of canine cells by herpes simplex virus. I. Characterization of viral progeny from cooperative infection with mutants differing in ability to multiply in canine cells. *J. molec. Biol.* **11**, 528.

ROIZMAN, B. & ROANE, P. R., JUN. (1961). A physical difference between two strains of herpes simplex virus apparent on sedimentation in cesium chloride. *Virology* **15**, 75.

ROIZMAN, B. & ROANE, P. R., JUN. (1963). Demonstration of a surface difference between virions of two strains of herpes simplex virus. *Virology* **19**, 198.

ROIZMAN, B. & SPRING, S. B. (1967). Alteration in immunologic specificity of cells infected with cytolytic viruses. *Transplantation* (in the Press.)

SCHNEWEISS, E. K. (1962*a*). Der cytopathische Effekt des herpes simplex virus. *Zbl. Bakt. ParasitKde. (Orig.)* **186**, 467.

SCHNEWEISS, E. K. (1962*b*). Serologische Untersuchungen zur Typendifferenzierung des Herpes virus Hominis. *Z. ImmunForsch. exp. Ther.* **124**, 24.

SPEAR, P. G. & ROIZMAN, B. (1967). The buoyant density of herpes simplex virus in CsCl solutions. *Nature, Lond.* **214**, 713.

SPRING, S. B. & ROIZMAN, B. (1967). Studies of herpes simplex virus products in productive and abortive infection. I. Stabilization of formaldehyde and preliminary analyses by isopycnic centrifugation in CsCl. *J. Virol.* **1**, 294.

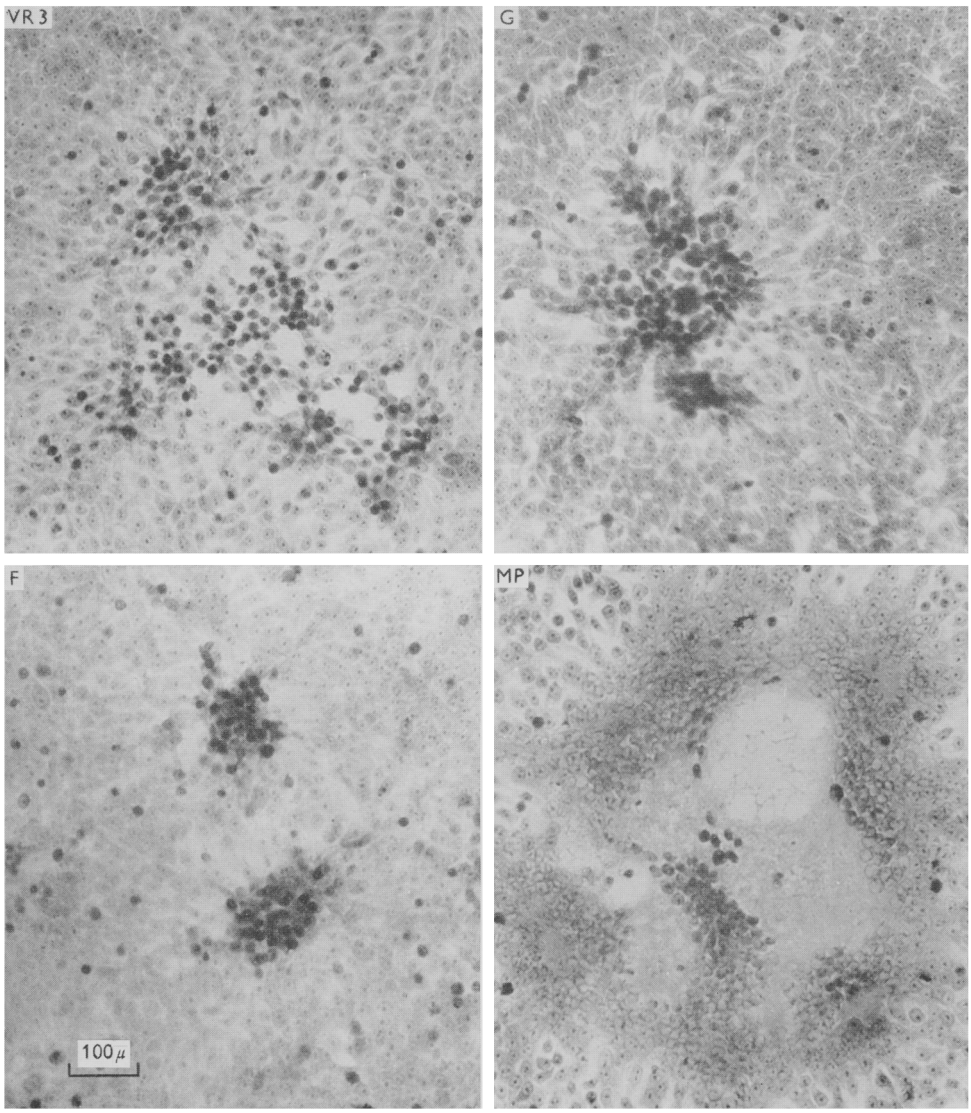
WATSON, D. H. & WILDY, P. (1963). Some serological properties of herpes virus particles studied with electron microscope. *Virology* **21**, 100.

WHEELER, C. E. (1964). Biologic comparison of a syncytial and a small giant cell-forming strain of herpes simplex. *J. Immun.* **93**, 749.

EXPLANATION OF PLATE

The social behaviour of cells infected with herpes simplex virus. HEP-2 cells grown on coverslips in Leighton tubes were infected at a multiplicity of 0·002 p.f.u./cell and incubated for 40 hr at 37° befo staining with Giemsa's stain.

(*Received* 6 *September* 1967)



P. M. EJERCITO, E. D. KIEFF AND B. ROIZMAN

(*Facing p.* 364)