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Virus Specific Antigens in Mammalian Cells Infected with Herpes Simplex Virus

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Summary. Antisera to specific proteins in herpes simplex infected cells were produced by immunization of rabbits with infected rabbit kidney cells. These antisera were highly virus specific and produced up to twelve lines in immunodiffusion tests against infected cell extracts. Acrylamide electrophoresis and immunoelectrophoresis revealed up to ten virus specific proteins of varying size.

INTRODUCTION

It is well known that cells infected with herpes simplex virus produce virus specific proteins that are distinct from complete virus particles; the overall kinetics of their formation during virus growth were described by Gold, Wildy and Watson (1963) and Russell, Gold, Keir, Omura, Watson and Wildy (1964). More recently Tokumaru (1965) has distinguished several herpes specific antigens by gel diffusion techniques.

All this work was done using antisera from herpetic subjects and it would clearly be much better to use specially prepared sera. In order to ensure that only herpes specific antibodies were made we have immunized rabbits with extracts of herpes infected rabbit kidney cells (Beale, Christofinis and Furminger, 1963) grown in rabbit serum of appropriate allotype (Dray, Dubiski, Kelus, Lennox and Oudin, 1962) to avoid anti-allotypic response. The resulting antisera reacted strongly and specifically with a number of herpes antigens of small size. In this paper we describe some properties of these antigens.

MATERIALS AND METHODS

Tissue culture media. Two media were used, both being supplemented Eagle's medium (Vantsis and Wildy, 1962), containing 10 per cent tryptose phosphate broth. One contained 10 per cent of serum of rabbits of allotypic specificity As 1/4 and is referred to as ETR. The second contained 10 per cent of calf serum in place of rabbit serum and is referred to as ETC.

*RK*13 *cells* (Beale *et al.*, 1963) were grown in ETR. No pleuropneumonia-like organisms could be cultured from extracts of these cells. They were shown to have the species specificity of rabbit by mixed agglutination tests (Coombs and Bedford, 1955).

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BHK cells were baby hamster kidney cells (BHK21 C13) (Macpherson and Stoker, 1962) grown in ETC. No pleuropneumonia-like organisms could be detected in these cells either by culture or by the cytological method of Shedden and Cole (1966).

Cell culture. The work required the production of large numbers of cells. These were grown in rotating 80-oz Winchester bottles as previously described (Russell *et al.*, 1964; House and Wildy, 1965). These bottles were inoculated with cells from a stem line maintained in antibiotic free medium (Shedden, Wildy and Watson, 1966). Fresh stem lines were started at frequent intervals from a large pool of stored cells, so that genetic differences between batches of cells were minimized. The stored cells, originally divided into 5-ml aliquots, had been held at −70° in a medium containing Eagle's medium 70 per cent, calf serum 20 per cent, glycerine 10 per cent.

Herpes virus was strain HFEM of herpes simplex virus. Seed virus was propagated as previously described (Holmes and Watson, 1963). It was assayed in BHK or RK13 cells by the method of Russell (1962).

Preparation of immunizing extracts of herpes virus-infected cells. Twenty millilitres of seed virus grown in BHK cells were inoculated into cultures of RK13 cells in 80-oz bottles, each containing about 3×10^8 cells, using approximately one plaque forming unit (pfu) (assayed in RK13 cells) per cell. After the cultures had been rotated at 37° for 4 hours, 200 ml ETR were added. Cells were harvested after rotation for a further 48 hours at 37°, washed in phosphate buffered saline (PBS) (Dulbecco and Vogt, 1954), resuspended in distilled water at a concentration of 10^8 cells/ml, and disrupted by ultrasonic vibration in an 'Electrosonic' bath (Surgical Instruments, Ltd). The resulting virus was used as the inoculum for a further cycle of growth in RK13 cells. These second cycle cells were grown, infected, harvested and sonically disrupted as in the first cycle. The suspension was freeze-dried and used as immunizing antigen. In some cases the virus in the antigen preparation was inactivated before freeze-drying by adding formalin to a concentration of 0·015 per cent formaldehyde and incubating overnight at 37°. This reduced the infectivity to less than 10 pfu/ml as assayed in BHK cells.

Preparation of antiserum to extracts of herpes-infected cells. Young adult rabbits of allotypic specificities As 1,3/4 or As 1,3/4,5 were inoculated intramuscularly with two doses of 200 mg freeze-dried formolized infected cell extract in Freund's incomplete adjuvant, 4 weeks being allowed between each dose. They were then inoculated with three doses of 200 mg unformolized extract in incomplete adjuvant at intervals of 4 weeks, and then with one dose of 200 mg unformolized extract in complete adjuvant 2 weeks later. Rabbits were bled before immunization and 10 days after each injection. In this work we have used sera from blood taken after the rabbits had received at least three doses of unformolized extract.

Immunization with freeze-dried uninfected BHK cells. Monolayers of BHK cells growing in ETC in 80-oz Winchesters were washed in PBS and the medium replaced with ETR. Two days later the cells were harvested and freeze-dried. Rabbits of allotype As 1,3/4 or As 1,3/4,5 were inoculated with five doses of 160 mg extract in incomplete adjuvant at monthly intervals. Rabbits were bled before immunization and 10 days after each injection. Only sera taken after at least four injections were used.

Immunization with freeze-dried uninfected RK13 cells. Rabbits were inoculated in a similar manner with extracts prepared from RK13 cells. Some batches of these cells were grown in ETR and some in similar media where the allotype of the rabbit (serum) was unknown. Only those sera taken after six injections were used.

Preparation of extracts of infected BHK cells. Approximately 10^{10} BHK cells were infected in suspension with 10^{11} pfu herpes virus in a total volume of 250 ml ETC. After 40 minutes adsorption the cells were washed twice in ETC and plated out in ten 80-oz Winchester bottles each containing 200 ml ETC. An aliquot was removed from the bulk suspension for counting and infective centre assay (Vantsis and Wildy, 1962). The cultures were rotated at 37° for 12 hours. The cells were then harvested, washed and disrupted in distilled water as described above. The crude suspension was spun at 30,000 ***g*** for 45 minutes in a Christ Omega II ultracentrifuge. A little liquid paraffin B.P. was layered on top of each tube. Supernatant lipid adhered to this layer and was consequently more easily separated. The top and bottom halves of the supernatants of this centrifugation were removed and centrifuged in separate tubes at 100,000 ***g*** for 30 minutes. The bottom third of the supernatant from the tubes containing the lower supernatant from the first run was rejected. The remaining supernatants from the second centrifugation were pooled and then homogenized at 4° with fluorcarbon 'Arcton' 113 (Imperial Chemical Industries, Ltd) in a homogenizer (M.S.E., Ltd) at top speed. Homogenization was stopped as soon as frothing occurred. The layers were separated by centrifugation at 1500 ***g*** for 5 minutes. The resultant protein solution had a concentration of 3–7 mg/ml, measured spectrophotometrically by the Folin–phenol method of Lowry, Roseborough, Farr and Randall (1951). The solution was sometimes concentrated to about 20–30 mg/ml by dialysis against 0·002 M phosphate buffer pH 7 followed by pervaporation.

Preparation of extracts of uninfected BHK cells was carried out in a similar manner.

Agar gel immunodiffusion tests were carried out in 3 mm layers of 1 per cent 'Ionagar' No. 2 (supplied by Oxoid Ltd) in a solution of 0·2 M phosphate buffer pH 7, containing 0·85 per cent sodium chloride, 0·1 per cent sodium azide and 1 per cent glycerol. The pattern of wells used consisted of one central well 8 mm in diameter with six peripheral wells 5 mm in diameter with a diffusion distance of 3·5 mm. Sometimes a reversed pattern with six larger holes surrounding a smaller hole was used. 0·15 ml of antigen and 0·05 ml of serum were used, the serum always being in the smaller hole. The gels were prepared in covered moist chambers and used immediately. Diffusion was continued for 48–72 hours at 20°.

Acrylamide gel electrophoresis was performed in 5 mm diameter cylinders of 7 per cent acrylamide gel at pH 8·5 in 0·375 M Tris–chloride buffer as described by Davis (1964). A 4·5 cm column of 7 per cent gel was used surmounted by a 1·5 cm column of 3·5 per cent 'stacking' gel in 0·06 M Tris–phosphate buffer pH 6·7 containing 10 per cent sucrose. The 7 per cent gels were polymerized with 0·0625 per cent dimethylaminopropionitrile and 0·0375 per cent ammonium persulphate. For the stacking gels the proportion of dimethylaminopropionitrile was doubled. The sample, containing about 300 μg of protein, was layered on top of the stacking gel in 0·06 M Tris–phosphate buffer pH 6·7 containing 10 per cent sucrose and 0·01 ml phenol red in a total sample volume of 0·5 ml. Six tubes were electrophoresed simultaneously between two electrode tanks containing Tris–glycine buffer pH 8·3, 0·025 M with respect to Tris. During concentration a current of 2·5 mA per tube was used. This was increased to 5 mA during separation. Electrophoresis continued for about 45 minutes after the samples entered the separation gel. In this time the phenol red marker had migrated about 2·5–3 cm from the inter-gel boundary. Gels were stained for 1 hour in 1 per cent amido-black in 5 per cent acetic acid. Excess stain was removed electrophoretically.

Acrylamide immunoelectrophoresis required larger amounts of protein than the above

analytical method. Five to 10 mg protein were used and this was incorporated in a sample gel of 3·5 per cent acrylamide layered on top of the spacer gel as described by Davis (1964). This gel was polymerized using twice as much dimethylaminopropionitrile and ammonium persulphate as in the 'stacking' gel above. After electrophoresis as before the gels were embedded in a 4 mm layer of agar in which antiserum troughs were cast at 3 mm distances from either side of the acrylamide cylinder (cf. Crowle, 1956; Seto and Hokama, 1964), and one filled with antiserum to uninfected BHK cells and the other with antiserum to infected RK cells. The gels were stored in moist chambers at 20°. Some precipitin bands formed after 1 day but observation was continued for at least 1 week.

Other acrylamide gels prepared similarly were sliced into five pieces. The pieces were disrupted by extrusion through a plastic 1-ml syringe 5 mm in diameter and stirred gently with 0·4 ml electrode buffer for 42 hours at 4°. The protein fractions thus obtained were tested by agar gel immunodiffusion and re-run in analytical acrylamide electrophoresis. The use of acrylamide electrophoresis techniques in the study of these antigens will be described in more detail elsewhere (Watson, Shedden and Wildy, to be published).

RESULTS

GEL IMMUNODIFFUSION TESTS

Gel immunodiffusion tests showed no reaction between RK13 extracts and serum from rabbits that had received an extensive series of inoculations with such extracts. This result was obtained from cells grown in rabbit serum of both homologous and heterologous allotypes.

Fig. 1 shows typical immunodiffusion plates in which an extract of infected BHK cells at protein concentrations of 26 and 13 mg/ml is tested against undiluted, 1 : 2 and 1 : 4

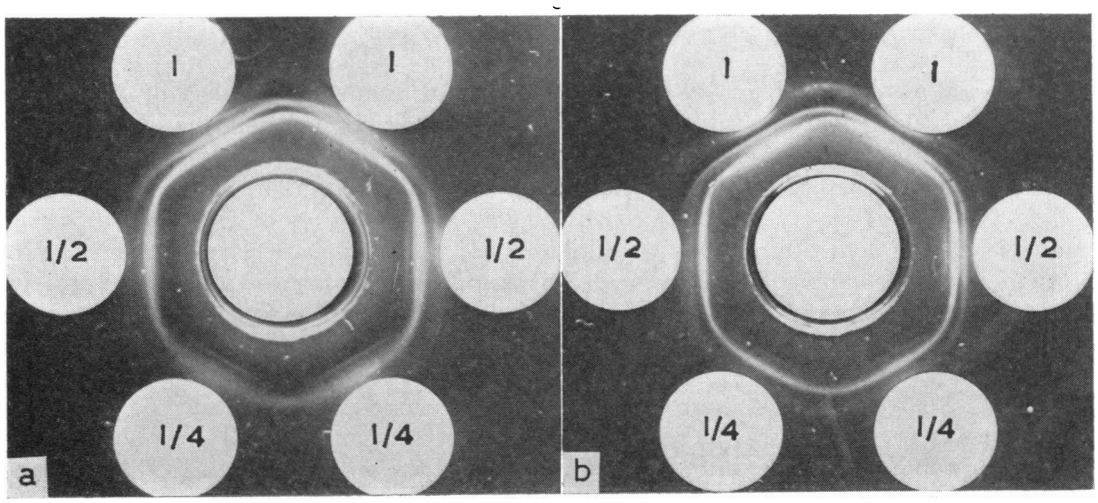

FIG. 1. Agar gel immunodiffusion tests of herpes simplex infected BHK cell antigens at protein concentrations of: (a) 26 mg/ml, and (b) 13 mg/ml in the central well and, in the peripheral wells, two different antisera to infected RK13 cell antigens used undiluted (1), diluted 1 : 2 ($\frac{1}{2}$) and diluted 1 : 4 ($\frac{1}{4}$).

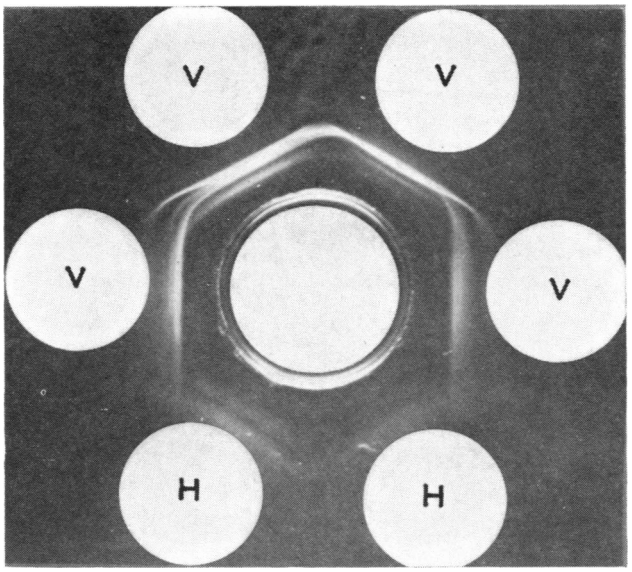

FIG. 2. Agar gel immunodiffusion test of herpes simplex infected BHK cell antigen (26 mg/ml) in the central well against antisera to herpes infected RK13 cells (V) and to uninfected BHK cells (H). There are no reactions of identity between the two antisera.

dilutions of antiserum to infected RK13 cells. Fig. 2 shows a similar extract tested against antisera to infected RK13 cells (V) and uninfected BHK cells (H); there is no cross-reaction between the V and H antisera, all the lines produced against infected cell antiserum thus being virus specific. This conclusion was confirmed by absorption tests. No absorption of virus specific antigen could be detected after incubation of infected cell extract with five-fold concentrated antiserum to uninfected cells for 24 hours at 4°. Further, there was no loss of virus specific precipitins when antiserum to RK13 cells was absorbed with uninfected BHK extract under the same conditions. Finally, uninfected BHK cell extract at a protein concentration of 20 mg/ml produced no reaction in immunodiffusion tests with antiserum to infected RK13 cells.

It is impossible to estimate accurately the total number of virus specific antigens detected in these tests. In some plates eight to twelve lines were distinguished. Some of these antigens were detected in extracts made as early as 3 hours after infection but were not demonstrated earlier. Further details will be published later.

ACRYLAMIDE ELECTROPHORESIS

Extracts of infected and uninfected cells showed differences in the intensity of some of the fourteen to eighteen bands visible in the stained acrylamide gels. Observation of these differences requires, of course, equivalent protein loads in the electrophoresis of the two preparations. In addition, there is an optimum load for this differentiation. This point is discussed more fully elsewhere (Watson, Shedden and Wildy, to be published); most, but not all, of the differences are observed when a load of 300 μg is used. Fig. 3 shows the patterns obtained in electrophoresis of extracts of infected and uninfected BHK cells, and for comparison a similar gel on which 200 μg of protein from human serum had been run.

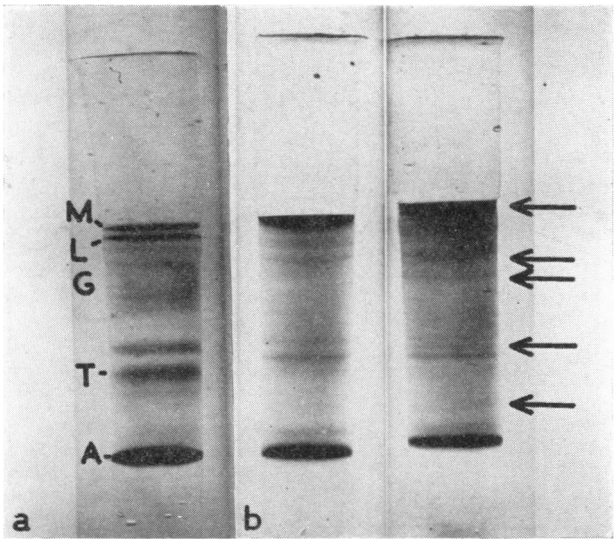

FIG. 3. Stained acrylamide gel columns of: (a) human serum (L, β-lipoprotein; M, α-macroglobulin; G, γ-globulin region; T, transferrin; A, albumin); and (b) on the left extract of uninfected BHK cells and on the right extract of infected BHK cells. Arrowed bands are more intense in the infected cell preparation.

The arrows indicate bands which were more intense in the sample from infected cells than in the sample from the uninfected cells. The relative mobilities (albumin = 100) of these bands were 5, 23, 32, 59 and 85. When only 100 μg of protein was run the top band split into two, of relative mobilities 3 and 6, each of which was more intense in the infected cell pattern. Table 1 lists the bands obtained with two different samples of infected cell antigens and indicates which of them were more intense than the corresponding bands of uninfected cell antigens.

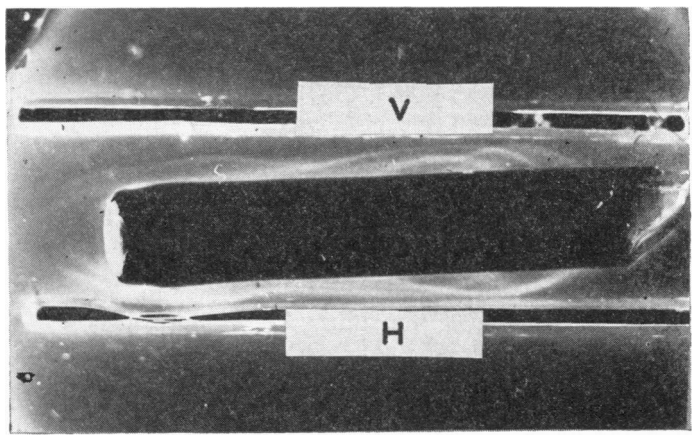

FIG. 4. Acrylamide immunoelectrophoresis of extract of infected BHK cells. The direction of electrophoresis was from left to right. V, Antiserum to infected RK13 cells; H, antiserum to uninfected BHK cells.

TABLE 1

RELATIVE MOBILITIES (ALBUMIN = 100) OF PROTEINS DETECTED IN ANALYTICAL AND IMMUNO-ELECTROPHORESIS OF EXTRACTS OF HERPES SIMPLEX-INFECTED BHK CELLS

Experiment 1		Experiment 2	
Analytical electrophoresis*	Immuno-electrophoresis†	Analytical electrophoresis*	Immuno-electrophoresis†
3	0 (2 bands)	**2**	0 (2 bands)
	4	5	
6		**6**	9
9			
11		11	
		15	
16		16	
19		21	
23	25	**24**	24
25			
28		27	
32	31	**31**	
38			34
41			
44		48	49
55		58	
59		60	
63	65	**64**	62
		67	
70	78	71	
85	82 (2 bands)		81
94			85
100	100	100	100
110		110	

* Bands recorded in bold type were those which were more intense with extracts of infected than of uninfected cells.

† Bracketed bands were linked.

ACRYLAMIDE IMMUNOELECTROPHORESIS

Fig. 4 shows the results of acrylamide immunoelectrophoresis of 6 mg of protein from virus-infected BHK cells. At least ten virus specific precipitin lines were detectable on the original plate. The relative mobilities (albumin = 100) of the virus specific proteins observed with two different samples of infected cell antigens are shown in Table 1. The albumin band, and several others, were seen as refractile bands in the unstained acrylamide gel cylinder when this quantity of protein was electrophoresed. This made it possible to locate the fastest protein forming a precipitin line in the albumin region. Correlation of the mobilities of the other proteins with those listed for serum proteins by Ornstein (1964) suggested that there were two or three in the post-albumin region, one or two in the β-globulin region, two in the γ-globulin region and three in the α-macroglobulin–β-lipoprotein region. Two of the proteins (relative mobilities 4 or 9 and 24 or 25) were shown to be antigenically related by the fusion of the corresponding precipitin lines. It should be noted that in electrophoresis using 7 per cent gels the albumin region also contains smaller proteins. Thus, ribonuclease and bovine serum albumin are barely separated under these conditions.

Fig. 5 shows a gel diffusion test on the protein fractions eluted from a segmented gel through which 12 mg of infected cell protein had been electrophoresed. Analytical

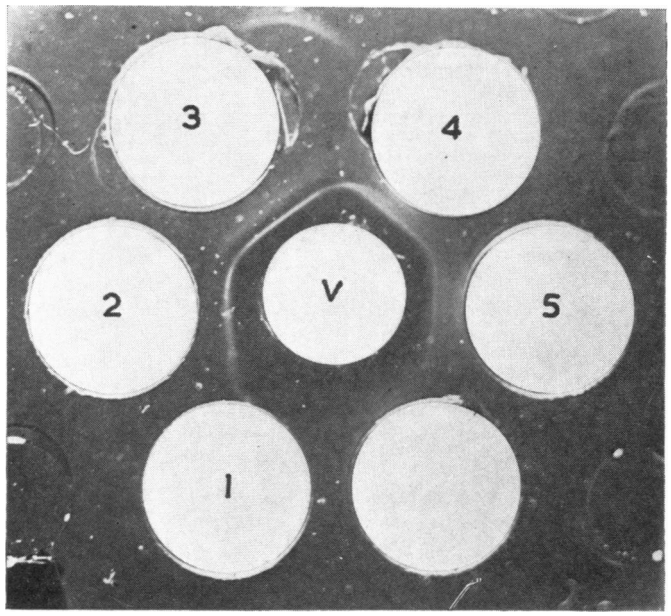

FIG. 5. Agar gel immunodiffusion test of five protein fractions eluted from a segmented acrylamide gel after electrophoresis of extract of infected BHK cells. The fractions are numbered from the lower end of the gel, fraction 1 containing the phenol red marker band and fraction 2 the albumin band.

acrylamide electrophoresis on the fractions showed that each fraction ran true although there was a certain amount of band overlap, and some albumin occurred in each fraction. The gel plate showed six to eight different lines.

DISCUSSION

Our results generally confirm and extend the observations of Tokumaru (1965) on the multiple antigens in cells infected with herpes simplex virus. Rabbit antisera against herpes-infected RK 13 cells reacted with extracts of herpes infected cells, whether RK13 or BHK, and not at all with extracts of uninfected cells. In gel diffusion tests we were able to distinguish up to twelve lines; of these, at least some were certainly separate and did not merge, indicating several differing antigenic determinant groups.

The difference between the staining patterns in acrylamide electrophoretograms of infected and uninfected cells is by itself unconvincing in view of the multitude of bands in cell extracts. Nevertheless, Table 1 shows that proteins detected in this way corresponded fairly well with the immunoelectrophoretic precipitin lines. (We follow Poulik (1964), in our use of the term immunoelectrophoresis.) These results showed that we were separating proteins of varying size. The straightforward interpretation is that we were dealing with herpes virus specific proteins of these sizes. Unfortunately, this is not necessarily so and we may have been concerned with small herpes virus proteins that became associated

with other molecules of undetermined nature. Indeed this might explain why two of the relatively slow moving proteins shared antigenic determinants and also why electrophoresis of eluted bands showed proteins of the size of the albumin molecule or smaller in each fraction. However, most of the eluted protein ran in a second electrophoresis in the position expected from the first electrophoresis. The most probable explanation is that we demonstrated about ten different herpes virus specific proteins, and it follows that these proteins must have been present in the material used to immunize the rabbits. We should expect this material to consist of a mixture of proteins destined for inclusion in new virus particles, the structure units and capsomeres (*cf.* Wildy, Russell and Horne, 1960) and newly specified enzymes such as DNA polymerase and deoxyribonuclease-I (Keir and Gold, 1963). It is probable that structure units and capsomeres would share some antigenic determinants.

We suppose that these ten or so proteins are only a small proportion, perhaps 10 per cent, of those specified by the virus, whose nucleic acid is of about 7×10^7 molecular weight (Russell and Crawford, 1963). We therefore intend to examine much larger quantities of cell extracts, applying preparative electrophoresis and other forms of fractionation in an effort to identify and characterize the antigens functionally.

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