Herpes Simplex Virus-specific Polypeptides Studied by Polyacrylamide Gel Electrophoresis of Immune Precipitates

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SUMMARY

Antisera raised in rabbits against RK13 cells infected with herpes simplex virus type 1 were capable of specifically precipitating proteins synthesized after infection of BHK-21 cells with the virus. Analysis of these immune precipitates by polyacrylamide gel electrophoresis demonstrated ≥ 15 polypeptides with mol. wt. from 25 to 100,000. A number of these polypeptides were not detected in purified preparations of virus particles.

Precipitates formed with two ‘monoprecipitin’ antisera were also analysed. Antiserum to the structural antigen Band II precipitated a major polypeptide of mol. wt. 47,000, which was glycosylated, and corresponded in mobility to a minor component polypeptide of the herpes virus particle. The other monoprecipitin antiserum, to the herpes-specified thymidine kinase, precipitated a polypeptide with a mol. wt. of 44,000. The thymidine kinase polypeptide was not glycosylated.

INTRODUCTION

Multiple virus specific antigens have been recognized in cells infected with herpes simplex virus (Tokumaru, 1965; Watson et al. 1966; Honess et al. 1974). Some of these represent antigenic activities which are also present as components of the virus particle (e.g. Band II antigen; Watson & Wildy, 1969; Sim & Watson, 1973) while others may correspond to virus specific enzymes, such as thymidine kinase (Klemperer et al. 1967; Buchan, Luff & Wallis, 1970a), or other non-structural antigens. We wished to obtain other evidence for the existence of non-structural components in the infected cell and to identify the polypeptides bearing particular enzymic and antigenic activities.

Previous studies (Watson et al. 1966) had demonstrated that ‘general’ antisera (obtained from rabbits immunized with RK13 cells infected with herpes simplex type 1) reacted exclusively with virus specific components. As well as containing type specific and cross neutralizing antibody against infectious virus particles (Sim & Watson, 1973) these sera also contained antibodies to a number of virus specific enzyme activities (Keir et al. 1966; Klemperer et al. 1967; Morrison & Keir, 1967; Buchan & Watson, 1969; Buchan et al. 1970a, b).

We have, therefore, compared the structural polypeptides observed by SDS polyacrylamide gel electrophoresis of purified virus particles with the polypeptides precipitated by

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general antisera from extracts of radioactively labelled infected cells. Using this approach we were able to detect the existence of non-congruent components. Using sera with restricted specificity, we have identified the polypeptide bearing a particular non-structural function (thymidine kinase) and the major polypeptide bearing 'Band II' antigenicity.

METHODS

Cells. BHK-21 cells were grown as previously described (Watson et al. 1966).

Virus. Herpes simplex, strain HFEM, serologically type-I (Geder & Skinner, 1971) was routinely employed as 'wild-type'. Thymidine kinase deficient virus mutant was the BUdR resistant mutant, B 2006 of Dubbs & Kit (1964). Both it and its parental virus are serologically of type-I, and the parental virus cannot be distinguished from HFEM in immuno-diffusion tests on infected cell extracts. The mutant virus is referred to throughout the text as MDK.

Stock virus inocula were prepared by infection of BHK-21 cell monolayers at low multiplicity, as described by Holmes & Watson (1963). Infectivity assays were performed by the suspension method of Russell (1962) and particle counts by the 'loop drop' method (Watson, 1962).

Immune sera. Antisera to virus infected RK 13 and to uninfected BHK-21 and HEp-2 cells were prepared as previously described (Watson et al. 1966; Watson & Wildy, 1969). Antiserum to Band II antigen was prepared as described by Watson & Wildy (1969). Antisera specific for the virus thymidine kinase were prepared by absorption of antisera to wild-type-I (HFEM) infected RK 13 cells with extracts of cells infected with the kinase-less mutant virus, as described by Buchan et al. (1970a).

The following abbreviations are used: anti-I (general antiserum to type I virus, HFEM, infected rabbit kidney cells); anti-MDK-I (antiserum to thymidine kinase mutant virus infected rabbit kidney cells); anti-BHK (antiserum to uninfected BHK-21 cells); anti-HEp-2 (antiserum to uninfected HEp-2 cells); anti-Band II (antiserum to Band II antigen); anti-cas (antiserum to calf serum); anti-RlgG (sheep antiserum to rabbit γ-globulin); and anti-TK (antiserum to thymidine kinase prepared by absorption as above). The anti-RlgG was a gift from Dr D. Catty.

Preparation of radioactively labelled virus and soluble antigen. BHK-21 cells were infected in suspension (5 × 10^5 cells/ml) with virus to give an input multiplicity of 20 p.f.u./cell. After 1 h at 37 °C in a magnetically stirred vessel, cells were thoroughly washed by sedimentation from Eagle's medium without added amino acids (amino acid-free Eagle's), three successive 50 ml washes being given for 5 × 10^6 cells. Washed cells were then resuspended in amino acid-free Eagle's medium and 5 × 10^8 cells dispensed into 80 oz bottles with labelling medium (see below). After an incubation period of 18 h at 37 °C, cells were removed from the glass, washed in phosphate-buffered saline (PBS), and disrupted by ultrasonic vibration (MSE, Crawley, Sussex) at a concentration of 10^8 cells/ml. This homogenate was then treated with fluorocarbon ('Arcton' I.C.L), and the aqueous layer separated after low speed sedimentation (3500 rev/min for 10 min) sedimented, first at 15000 rev/min for 45 min and the supernatant fluid centrifuged at 35000 rev/min for 60 min. The supernatant fluid from this latter sedimentation is referred to as 'soluble antigen' or 'high-speed supernatant'. Uninfected cell soluble antigen was prepared in the same manner. Virus for purification according to the procedure of Robinson & Watson (1971) was recovered by resuspension of the pellet from the first high speed sedimentation (15000 rev/min for 45 min).
The labelling conditions described by Robinson & Watson (1971) were modified by restoration of normal levels of amino acids, excepting isoleucine and cystine and those employed for labelling (leucine, lysine, phenylalanine and valine). The composition of the medium was: 100 ml of amino acid-free Eagle's (containing the normal level of glutamine), with arginine, histidine, threonine, tyrosine, tryptophan and methionine restored, 3·0 ml of normal Eagle's/20% calf serum, and the isotopically labelled amino acids leucine, lysine, phenylalanine and valine. For $^3$H labelling 60 to 100 µCi of each amino acid, and for $^{14}$C labelling 10 or 15 µCi of each amino acid, were employed for 5 x 10^6 cells. Labelled compounds (all obtained from the Radiochemical Centre, Amersham, Bucks.) were: [4,5-3H]-L-leucine (19·7 to 23 Ci/m-mol), [3H]-L-lysine (0·25 and 18·8 Ci/m-mol), [3H]-L-3-phenylalanine (1·00 Ci/m-mol), [2,3-3H]-L-valine (1·5, 19·9 and 31·6 Ci/m-mol), [14C]-L-leucine (0·33 Ci/m-mol), [14C]-L-lysine (0·31 Ci/m-mol), [14C]-L-3-phenylalanine (0·48 Ci/m-mol), and [14C]-L-valine (0·26 Ci/m-mol).

For glucosamine labelling, 5 x 10^6 cells were labelled with 100 µCi of [1-3H]-D-glucosamine (2·3 Ci/m-mol) and [14C]-amino acids as above except that the calf serum was dialysed against amino acid-free Eagle's before use.

**Virus purification.** Preparations of naked virus particles from BHK-21 cells were purified by sucrose velocity gradient sedimentation followed by calcium phosphate chromatography, essentially as described by Robinson & Watson (1971). The only modifications were: omission of unlabelled low multiplicity infected cell 'carrier', reduction of fluorocarbon homogenization time to 5 s, and the addition of a 45% (w/v) layer to the sucrose gradient such that the velocity gradient was 5 to 45% (w/v). The product of the purification was a preparation of > 98% of morphologically naked nucleocapsids with 2 to 6 x 10^-16 g protein per particle. These properties and the polypeptide composition of such preparations were in good agreement with those reported by Robinson & Watson (1971); however, the particle/infectivity ratios of preparations obtained by us (normally > 5 x 10^3 particles/p.f.u.) were higher than those reported by these authors.

Purified preparations of enveloped particles from HEp-2 cells were kindly donated by Dr K. L. Powell and details of their purification and properties are to be reported.

**Agar-gel immunodiffusion tests** were performed as described previously (Watson, 1969).

**Radio-immune precipitation reactions.** Immune-precipitation reactions were performed by incubating an appropriate range of serum vol. with a constant vol. of isotopically labelled antigen in highly tapered (10 ml) glass centrifuge tubes. A constant total vol. was maintained by the addition of phosphate-buffered saline. Mixtures were incubated at 4°C for 24 to 48 h and then centrifuged at 3500 rev/min for 15 min. The supernatant fluids were then removed and the coherent pellets rinsed with 6 x 10^-9 ml vol. of ice-cold phosphate-buffered saline. The washed pellets were then drained of excess fluid and resuspended by ultrasonic vibration ('Electrosonic' bath) in a small vol. of either 1% SDS (if protein was to be estimated) or 1% SDS, 0·5 M-urea, and 0·1% dithiothreitol (for electrophoretic analysis) and sampled for estimation of radioactivity.

**Polyacrylamide gel electrophoresis.** The electrophoretic system employed was the discontinuous buffer system of Ornstein (1964) and Davis (1964) modified as described by Dimmock & Watson (1969) for use with SDS in the analysis of dissociated polypeptides. In each experiment, purified proteins of known mol. wt. were electrophoresed in parallel with experimental gels for estimations of mol. wt. of unknowns. The log mol. wt. of separated marker proteins was inversely proportional to their electrophoretic mobility for the range of gel strengths employed in this buffer system. The polypeptide mol. wt. and appropriate text abbreviations of these marker proteins were as follows: phosphorylase-a
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(phos-a, 95,000); bovine serum albumin (BSA, 66 to 69,000); catalase (cat, 59,000); heavy chains of rabbit gamma globulin (heavy, 50 to 53,000); fumarase (fum, 48 to 49,000); ovalbumin (oval, 43 to 45,000); pepsin (pep, 35,500); light chains of rabbit gamma globulin (light, 20 to 23,000); and egg white lysozyme (lys, 14,500).

Bovine serum albumin and rabbit gamma-globulin (heavy and light chains) for use as internal markers by co-electrophoresis were labelled in vitro with $^{[131]}I$ by the chloramine-T method as described by McConahey & Dixon (1966).

Procedures for staining of gels with Coomassie brilliant blue and fractionation of gels on which radioactive samples were separated have been described previously (Dimmock & Watson, 1969; Robinson & Watson, 1971).

Other methods. Protein was estimated by the method of Lowry et al. (1951) employing bovine serum albumin as calibrating standard.

Thymidine kinase activity was assayed as described by Klemperer et al. (1967).

RESULTS

Immune precipitation with polyprecipitin antisera to infected heterologous (RK 13) cells and homologous uninfected cells

Quantitative immune precipitation

In order to precipitate all possible antigens and to ensure maximum specificity we determined the serum/antigen ratio of the mixture giving maximal precipitation of radioactively labelled antigen with each serum/antigen combination. In an experiment of this type, constant vol. of an artificial mixture of soluble antigen from infected HEp-2 cells labelled with $[^{14}C]$-amino acids and infected BHK-21 cells labelled with $[^{3}H]$-amino acids were incubated with a range of vol. of either anti-1, anti-BHK or anti-HEp-2 antisera. Increasing vol. of antisera precipitated an increasing percentage of radioactivity until a stable maximum was obtained. The lowest serum/antigen ratio of a mixture leading to maximal precipitation of labelled antigen was regarded as the ‘equivalence’ ratio, and precipitates for subsequent analyses were formed with serum/antigen mixtures in slight serum excess. Precipitates were not soluble in serum excess, and the protein content did not change significantly with further serum additions after the attainment of the plateau for precipitated radioactivity.

Serum-excess anti-1 immune precipitates typically contained approximately 20% of input radioactivity and anti-BHK immune precipitates 5 to 10% of the input radioactivity. There was a 20-fold difference in the ratios of counts precipitated from the artificial mixture of labelled homologous and heterologous infected cells by anti-sera to BHK-21 and to HEp-2 cells.

The contribution of non-specific co-precipitation of labelled components to radioactivity in immune precipitates was estimated in two ways. Firstly, we compared the radioactivity precipitated in a range of anti-1 immune precipitates (formed at the same optimal serum antigen ratio but with increasing absolute vol. of each reactant) with the radioactivity co-precipitated from the same infected cell antigen by a range of calf-serum/anti-calf serum immune precipitates containing a comparable range of precipitated protein. Both specifically (anti-1) and non-specifically (calf serum/anti-calf serum) precipitated radioactivities were proportional to the amounts of protein precipitated, but the non-specifically precipitated radioactivity was only 0.075% of specifically precipitated radioactivity when identical amounts of precipitated protein were compared. Secondly, non-specific co-precipitation
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Fig. 1. Polypeptides revealed by polyacrylamide gel electrophoresis of total labelled antigen preparations and of the derived immune precipitates.

(a) Polypeptides of a total infected BHK-21 cell homogenate, labelled with $^{14}$C-amino acids from 1 to 18 h after high multiplicity infection with HFEM (—) are compared with those of the derived ‘soluble antigen’ fraction (—) electrophoresed on a parallel gel.

(b) Polypeptides represented in an anti-$t$ immune precipitate formed against the labelled infected cell soluble antigen fraction shown in (a), (—) are compared with those in an analogous immune precipitate formed against unlabelled infected cell antigen in the presence of an equivalent quantity of labelled protein present as labelled uninfected cell antigen (—).

(c) Comparison of labelled polypeptides in anti-BHK immune precipitates of labelled infected (—) and unlabelled uninfected cells (—) electrophoresed on companion gels.

Annotations at the head of each part of the figure refer to positions of marker polypeptides electrophoresed on companion gels. RB = refractile boundary, PR = phenol red dye marker.

was also shown to represent a minor contribution to specifically precipitated radioactivity when the radioactivities precipitated by anti-$t$ antisera from artificial mixtures of labelled infected and unlabelled uninfected and of labelled uninfected and unlabelled infected cells were compared. When such mixtures were composed so as to give equal amounts of input radioactivity and infected and uninfected cell antigen, equivalent amounts of protein were precipitated but the precipitate from labelled infected cell antigen contained 20-fold more radioactivity than that of unlabelled infected cell antigen formed in the presence of labelled uninfected cell antigen.

The polypeptide composition of immune precipitates formed with poly precipitin antisera

Analyses of the labelled polypeptide profiles of immune precipitates formed with poly precipitin antisera, and their relationship to the profiles of a total homogenate of infected BHK-21 cells and the derived soluble antigen fraction employed for immune precipitation are shown in Fig. 1 (a, b, c).

In Fig. 1 (a) the upper profile (solid line) is a sample of a total infected-cell homogenate and the lower profile (broken line) is the soluble antigen or ‘high-speed supernatant’ fraction derived from it, electrophoresed on a companion gel. A feature of both profiles is the presence of a large number of ill-resolved components, giving a high background.
between recognized peaks, and a relatively high proportion of the radioactivity associated
with species of less than ~ 20000 mol. wt. which run at the refractile-boundary (Dimmock
& Watson, 1969) at the gel strengths shown in this Fig. The sedimentation step leading to
the preparation of soluble antigen leads to preferential losses in components of > 70000 mol.
wt. The polypeptide profile of the anti-I immune precipitate formed against this labelled
soluble antigen sample is shown in Fig. 1(b) (solid line), which in contradistinction to the
soluble antigen has relatively fewer (about 15 polypeptides on a rather low background,
although not all of these are resolved in Fig. 1(b)). These polypeptides range from about
100000 (polypeptide no. 1) to about 25000 (polypeptide no. 15) mol. wt. and in general
they represent the efficient precipitation of a restricted number of the ‘available’ labelled
polypeptides from the soluble antigen sample. We shall term these polypeptides IP (immune
precipitate) 1 to 15. The precipitation discriminates markedly against the lower mol. wt.
components (< 20000 mol. wt.; compare the ratio of the radioactivity in the region of IP-1
to that at the refractile boundary for soluble fraction and the immune precipitate). The other
profile of Fig. 1(b) (dashed line) is a companion gel on which we electrophoresed the
precipitate formed by reacting unlabelled infected cell antigen with anti-I antiserum in
the presence of labelled uninfected cell antigen. The profiles of Fig. 1(b) are plotted for
precipitates of comparable protein content and they demonstrate that qualitatively as well
as quantitatively the contribution of co-precipitation is negligible, in that the non-specifically
precipitated radioactivity is constituted of trace amounts of a large number of components.

The other gels presented as Fig. 1(c) are analyses of anti-BHK immune precipitates of
labelled-infected and labelled ‘mock’ infected cells, analysed on companion gels. The
differences between the two profiles are of doubtful significance, each being constituted of
small amounts of a large number of polypeptides not resolved by slicing; however, they
contrast markedly with the profile of the anti-I immune precipitate.

Evidence for non-structural polypeptides in immune precipitates

Comparisons in parallel and co-electrophoresis of polypeptides of immune precipitates
with those of ‘naked’ virus particles (‘capsids’), purified by the method of Robinson & Watson
(1971), and with those of enveloped virus particles, purified by K. L. Powell, established that
there were polypeptides represented in anti-I immune precipitates which were not recog-
nized in virus particles. These experiments have recently been repeated and extended,
taking advantage of the increased resolution of autoradiographic techniques (R. W. Honess
& B. Roizman, personal communication), therefore we shall only summarize our original
results. We observed polypeptides in immune precipitates which co-electrophoresed with
structural polypeptides and others which were not recognized in virus particles. For ex-
ample, IP-1 of Fig. 1(a) was composed of at least two polypeptides, the larger of which co-
electrophoresed with the major ‘capsid’ polypeptide ‘A’ of Robinson & Watson (1971),
whereas the smaller component did not have a recognized counterpart in virus particles.
Also, at least one polypeptide in the peak numbered IP-10 in Fig. 1(a) is not a structural
polypeptide. We also noted some structural polypeptides which were not detected in
immune precipitates (e.g. polypeptide ‘F’ of Robinson & Watson, 1971).

Polypeptides precipitated by ‘monoprecipitin’ antisera

Polypeptides precipitated by antisera to Band II antigen

Precipitates with antisera to Band II antigen were taken from serum/antigen mixtures
at or in ‘serum-excess’ to the ‘equivalence-ratio’ of the reactants. This ratio was defined
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Fig. 2. Polyacrylamide gel electrophoresis of an anti-Band II immune precipitate (○—○) which was co-electrophoresed with [131I]-labelled BSA and rabbit γ-globulin 'heavy' and 'light' chains as internal markers (●●●).

as the ratio of serum to antigen which gave a minimum precipitin line width in agar gel radial double-diffusion tests. Such anti-Band II immune precipitates normally contained 3 to 6% of the radioactivity precipitable by polyprecipitin, anti-1 antisera. Control anti-Band II immune precipitates with mixtures of unlabelled infected cell antigen and labelled uninfected cells contained about 15% of the specifically precipitated radioactivity of a comparable precipitate formed with labelled infected cell antigen.

Fig. 2 shows an electrophoretic analysis of an anti-Band II immune precipitate, formed at equivalence with an infected cell antigen preparation from cells labelled with [3H]-amino acids throughout infection. Most of the radioactivity (67%) migrated as a single component slightly smaller than the 'heavy' chains of the [131I]-labelled rabbit γ-globulin marker, but with minor components at higher (62000 mol. wt., about 8.5% of recovered radioactivity) and lower (33000 mol. wt., about 17% of recovered radioactivity) mol. wt. These components were designated regions IIa, IIb, and IIc and the mol. wt. for these regions (based on 17 estimations) were: IIa = 61.7 ± 2.6 × 10^3; IIb = 47 ± 2.5 × 10^3; and IIc = 32.9 ± 4.4 × 10^3. Both minor component regions IIa and IIb were heterogeneous and with low applied loads only the major polypeptide, IIb, was clearly apparent.

Electrophoretic analyses of control immune precipitates (anti-Band II with unlabelled infected cell antigen in the presence of labelled uninfected cell antigen or γ-globulin/anti-γ-globulin immune precipitates in the presence of labelled infected cells) showed that although components of less than 30000 mol. wt. were equally represented in control and specific precipitates (i.e. counts migrating beyond fraction 76 of Fig. 2 could be entirely due
Fig. 3. (a) Anti-Band II immune precipitate labelled with $[^{14}C]$-amino acids (○—○) was co-electrophoresed with purified naked virus particles labelled with $[^{3}H]$-amino acids (●—●). (b) Anti-Band II immune precipitate labelled with $[^{3}H]$-amino acids (○—○) was co-electrophoresed with polypeptides of enveloped virus particles labelled with $[^{14}C]$-amino acids (●—●).

to non-specific co-precipitation) this could not account for the presence of polypeptides IIa nor all of the components of IIc. Further, the contribution from minor components IIa and IIc relative to IIb could be manipulated by varying the serum/antigen ratio used in the immune precipitation reaction. This behaviour was not consistent with the origin of minor components solely as alternative molecular forms of Band II antigen but would be predicted if these components arose from trace levels of antibody to other antigens present in antisera to Band II.
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Table 1. *Immune precipitation with mixtures of wild-type and mutant virus infected cell antigen*

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Radioactivity in immune precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Wild-type [14C] ct/min</td>
</tr>
<tr>
<td>1 Anti-I</td>
<td>Wild type and mutant</td>
</tr>
<tr>
<td>2 Anti-TK</td>
<td>Wild type and mutant</td>
</tr>
<tr>
<td>3 Anti-MDK-t</td>
<td>Wild type and mutant</td>
</tr>
<tr>
<td>4 Anti-t</td>
<td>Supernatant fluid from 3</td>
</tr>
</tbody>
</table>

* 19.5% of input [14C], ct/min.
† 15.5% of input [3H], ct/min.
‡ The ratio of [14C]/[3H] for anti-BHK precipitate was 1.1 to 1.2.

Fig. 3 illustrates the co-electrophoresis of anti-Band II immune precipitates formed against infected cell antigen labelled with either [14C]-amino acids (Fig. 3a) or [3H]-amino acids (Fig. 3b) with a [3H]-amino acids labelled preparation of morphologically 'naked' virus particles or a sample of enveloped particles labelled with [14C]-amino acids. The Band II polypeptide IIb co-electrophoreses with a trace component of naked particles (which corresponds to the anodal side of polypeptide 'D' of Robinson & Watson, 1971) and with a minor polypeptide of the enveloped virus particle.

**Identification of the polypeptide bearing herpes virus thymidine kinase activity**

Monoprecipitin anti-TK antisera, which were capable of neutralizing this enzyme activity, were capable of precipitating at least 90% of the thymidine kinase activity of wild-type infected cell extracts. Neither anti-Band II nor anti-MDK-1 had any significant enzyme neutralizing or precipitating activity (< 1%), this despite the fact that the immune precipitate with polyprecipitin anti-MDK-1 precipitated approx. 10-fold more radioactivity from labelled infected cell antigen than anti-TK and a comparable level of radioactivity to that of unabsorbed anti-t which was likewise capable of precipitating all enzyme activity. Thus, anti-TK antisera were capable of reacting with essentially all the wild-type enzyme activity and the activity behaved independently in immune precipitation, i.e. it was not co-precipitated with any component to which there was antibody in anti-MDK-1 antisera. Moreover, dependent on the serum/antigen ratio of the mixture, all precipitated enzyme activity was not necessarily neutralized. By choice of the minimum ratio giving efficient precipitation of the original enzyme activity a high proportion (~ 40 to 50%) of the original activity was demonstrated within the immune precipitate. Consequently it was valid to examine the virus-specific polypeptide content of such immune precipitates in order to identify the polypeptide bearing the virus-specific thymidine kinase activity.

In these experiments we used an artificial mixture of differentially labelled mutant and wild-type infected cell extracts to provide an internal control for immune precipitation. Cells infected with wild-type or mutant virus were labelled with [14C]-amino acids or [3H]-amino acids, respectively, to approx. equivalent specific activities. Mixtures containing equivalent activities of soluble antigens from cells infected with mutant or wild-type virus were incubated with sufficient anti-t, anti-MDK-1 or anti-TK to give maximal precipitation. The overall contributions from mutant and wild-type antigens to these precipitates are summarized in Table 1. They show a slight advantage in precipitation of wild-type components with anti-t, not seen with anti-MDK-1 (mixtures 1 and 3). After absorption
Fig. 4. Polyacrylamide gel electrophoresis of immune precipitates formed with artificial mixtures of wild type (HFEM) labelled with [14C]-amino acids and mutant (MDK) virus infected cell soluble antigens labelled with [3H]-amino acids and (a) anti-TK (reaction mixture 2 of Table 1) or (b) anti-I antiserum (reaction mixture 4 of Table 1). O—O, wild-type antigen labelled with [14C]-amino acids; ●—●, mutant antigen labelled with [3H]-amino acids.

the absolute number of wild-type counts precipitated was reduced to about 10 % of the value with polyprecipitin serum, and the decrease was accompanied by selection for wild-type components (mixtures 1 and 2). An alternative procedure for selective precipitation of thymidine kinase, the precipitation of antigens remaining in the supernatant fluid after an anti-MDK-I precipitation with antiserum to wild-type virus infected cells (anti-I), was performed in reactions 3 and 4 of Table 1. The selective effect of this procedure was less
Table 2. Immune precipitation with antigen labelled with \([^3\text{H}]\)-glucosamine\([^{14}\text{C}]\)-amino acids

<table>
<thead>
<tr>
<th>Serum</th>
<th>([^3\text{H}])-glucosamine</th>
<th>([^{14}\text{C}])-amino acids</th>
<th>([^3\text{H}])/([^{14}\text{C}])*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Anti-(\text{I})</td>
<td>22,400</td>
<td>515,400</td>
<td>0.044</td>
</tr>
<tr>
<td>2 Anti-BHK</td>
<td>9,100</td>
<td>154,300</td>
<td>0.059</td>
</tr>
<tr>
<td>3 Anti-Band II</td>
<td>4610</td>
<td>14,330</td>
<td>0.320</td>
</tr>
<tr>
<td>4 Anti-TK</td>
<td>4510</td>
<td>63,000</td>
<td>0.070</td>
</tr>
</tbody>
</table>

* Ratio of \([^3\text{H}]\)/\([^{14}\text{C}]\) for input antigen was 0.23.
† 18\% of input \([^{14}\text{C}]\)-radioactivity.

than that of the anti-TK serum (compare \([^{14}\text{C}]\)/[^3\text{H}]\) ratios for precipitates of mixtures 2 and 4).

The precipitates from the above experiments were analysed electrophoretically. Fig. 4(a) shows the results of electrophoresing the precipitate formed with anti-TK (reaction mixture 2), and Fig. 4(b) the product of the anti-\(\text{I}\) precipitation formed from the supernatant fluid of a serum-excess anti-MDK-\(\text{I}\) precipitation (reaction mixture 4). A single prominent wild-type polypeptide is seen in the product of both reactions. The ratio of wild-type/mutant activity over this region (fractions 33 to 44 in Fig. 4(a)) was 14/1 whereas over the remainder of the separation it was 1/1, notably this was also the case at the refractile boundary. Since these antigen preparations had specific activities in the ratio of 5.5/6.9 this gives a ‘molar selectivity’ for wild-type over mutant polypeptides of \(\sim 17/1\) over the region of the specifically precipitated polypeptide.

The product of the second type of reaction, i.e. precipitation with anti-\(\text{I}\) after anti-MDK-\(\text{I}\), had a very much higher ‘background’ of ‘mutant’ (and wild-type) polypeptides as would have been anticipated from the ratio in the immune precipitate, but this also contained a wild-type ‘excess’ polypeptide of the same mol. wt. as that seen in Fig. 4(a).

Electrophoresis of a number of precipitates such as those in Fig. 4(a) on 12 and 14% gels, in which the components from the refractile boundary were separated, has uniformly shown coincidence of specific (wild-type) and ‘non-specific’ (mutant or counts in rabbit serum/antirabbit serum precipitate) components in this region. The nature and amount of these components was dependent on washing efficiency but in all experiments they were composed of a heterogeneous collection of small mol. wt. (12,000 to 15,000) polypeptides which invariably represented a small percentage of total radioactivity.

The same single wild-type polypeptide was observed in 15 different analyses, employing a number of different anti-\(\text{I}\) sera, mutant and wild-type antigen preparations. The polypeptide had an estimated mean mol. wt. of 44,000.

Glucosamine content of Band II and thymidine kinase polypeptides

Cells infected with wild-type virus were labelled with \([^3\text{H}]\)-d-glucosamine and \([^{14}\text{C}]\)-amino acids and the derived soluble fraction subjected to immune precipitation reactions with poly- and monoprecipitin sera. The results are summarized in Table 2.

A feature of the results was the low efficiency of precipitation of \([^3\text{H}]\)-glucosamine relative to \([^{14}\text{C}]\)-amino acids for all polyprecipitin reactions. Notably this was true for both anti-\(\text{I}\) and anti-BHK precipitates. However, the monoprecipitin anti-Band II immune precipitate had a 7 to 8 times higher ratio of \([^3\text{H}]\)/\([^{14}\text{C}]\) than did the optimal anti-\(\text{I}\) precipitation (compare lines 3 and 1). This was not merely a consequence of a higher
contribution of non-specific contamination since not only was the ratio for the anti-Band II immune precipitate higher than that for the input antigen (0.23), it was very significantly higher than that for monoprecipitin 'thymidine kinase' precipitates (compare lines 3 and 4). This indication that the polypeptides precipitated by antiserum to Band II were glycosylated was amply confirmed by the electrophoretic analyses of these immune precipitates (Fig. 5). There was no detectable glucosamine associated with the thymidine kinase polypeptide (Fig. 5a) whereas the polypeptides of the anti-Band II immune precipitate were glycosylated. The ratio of glucosamine/amino acids for the Band II polypeptide IIb was at least 80-fold higher than for the thymidine kinase polypeptide, and we may therefore consider Band II as a glycoprotein and thymidine kinase as a non-glycoprotein.
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We have confirmed that $[^{14}N]D$-glucosamine was incorporated as such (or as an acid labile derivate) by showing that $> 90\%$ of the incorporated radioactivity released by acid hydrolysis of labelled samples co-chromatographed with an authentic glucosamine marker.

**DISCUSSION**

The polypeptides precipitated by general antisera to type 1 virus represented a highly selected subpopulation of the available polypeptides incorporating isotope after infection. Only a proportion of the surviving radioactivity could be attributed to incorporation into 'host' species. This allows us to make the necessary, albeit obvious, point that precipitation by anti-'virus' sera can only be employed as a positive criterion and the failure to precipitate a given component does not argue against its virus specificity. Such a failure may indicate merely that the particular serum employed does not contain antibody to this component, as an example of this effect we have cited our failure to recognize the analogue of the virus structural polypeptide 'F' (Robinson & Watson, 1971) in immune precipitates. Its application in a positive sense provides, however, a criterion for the virus specificity of a number of polypeptides not recognized as components of the herpes virus particle. Accordingly, these components must be considered either as unrecognized trace components of the virus particle or as precursors or alternative stable forms of recognized structural polypeptides, or as non-structural products (e.g. thymidine kinase).

The major polypeptide of Band II immune precipitates, a glycosylated polypeptide with a mol. wt. of 47000, seems likely to form the major antigenic component of the type common precipitin arc by which Band II antigen was originally characterized. However, the reproducible occurrence of a number of minor components (regions II$_a$ and II$_c$) is still not explained fully. Since we were able to manipulate the proportions of these minor components relative to polypeptide II$_b$ by altering the serum/antigen ratio, it seems necessary to propose that they were precipitated by different antibodies from those precipitating polypeptide II$_b$. We have noted elsewhere that antiserum to Band II agglutinates enveloped as well as naked particles, that it can form a second precipitin line (Honess et al. 1974) and possesses both type common and type specific neutralizing activities (Sim & Watson, 1973). The minor polypeptides may well relate to some of these other activities.

These complications make it difficult to realize one of the original objectives of our present study, namely to identify and to locate the 'Band II' antigenic activity on the herpes virus particle. We have shown in this paper that polypeptide II$_b$ migrates with a minor polypeptide of the enveloped particle which is glycosylated (K. L. Powell & D. H. Watson, personal communication), and with a trace component of naked particles. It is therefore possible that polypeptide II$_b$ itself consists of two unresolved components, one glycosylated and corresponding to an envelope glycoprotein and the other corresponding to the non-glycosylated naked particle polypeptide. The simplest interpretation of these results is that a proportion, if not the exclusive form, of the polypeptide interacting with type common neutralizing antibody is present as a glycosylated polypeptide of 47000 mol. wt. exposed at the surface of the enveloped particle. However, the significance of the naked particle polypeptide and the possibility that component II$_b$ is itself heterogeneous are subjects requiring further experimentation.

In contrast, the identification of the thymidine kinase polypeptide seems quite clear. Immune precipitates formed with thymidine kinase specific antiserum contained a single, non-glycosylated, virus-specific polypeptide of 44000 mol. wt. Our present identification of
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the thymidine kinase polypeptide represents the only instance where a non-structural herpes virus-specific polypeptide has been associated with a known function.

The work we have reported here, although not without complications, we believe illustrates the utility of immunological techniques both for obtaining novel results of interest to the biochemical virologist and also for achieving those correlations between antigenic and other properties and functions of virus polypeptides which are essential for the rational basis of diagnostic procedures exploiting these antigenic properties.

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Immune precipitation of herpes virus proteins


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