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# LIQUID-PHASE AND SOLID-PHASE RADIOIMMUNOASSAY WITH HERPES SIMLEX VIRUS TYPE 1 NUCLEOCAPSIDS

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*Summary.* — Liquid-phase radioimmunoassay (LPRIA) and solid-phase radioimmunoassay (SPRIA) are described utilizing either $^{125}$I-labelled or immobilized nucleocapsids (NC) of herpes simplex virus type 1 (HSV-1). These techniques appeared sensitive and specific for quantification of HSV-NC antigens and corresponding antibodies.

*Key words: herpes simplex virus type 1; nucleocapsids; liquid-phase radioimmunoassay; solid-phase radioimmunoassay*

## *Introduction*

Radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA) (Enlander *et al.*, 1976; Vestergaard *et al.*, 1977; Colombatti and Hilgers, 1979; Dreesman *et al.*, 1979; Kimmel *et al.*, 1982) are rapid and sensitive methods for detection of a variety of viral antigens and antibodies. Both liquid-phase RIA (LPRIA) and solid-phase RIA (SPRIA) have been adapted for measurements of herpes virus nucleocapsid (NC) proteins and anti-NC antibodies (Kalimo *et al.* 1977*a*, *b*, Heilman *et al.* 1979). In the present study NC of HSV-1 were isolated and purified. Then LPRIA was performed using $^{125}$I-labelled NC and compared to SPRIA using immobilized NC. SPRIA seems to be useful for the quantification of small amounts of NC antigens and antibodies, in contrast to LPRIA, which revealed satisfactory sensitivity only for the detection of anti-NC antibodies.

## *Materials and Methods*

*Virus and cells.* The Kupka strain of HSV-1 (isolated by Dr. R. Benda, Prague) was used throughout. Virus was grown in SIRC or VERO cells, which were maintained in the Eagle's basal medium (BEM) containing 5% inactivated calf serum, glutamine and antibiotics in 1200 ml Roux bottles. Viral material was prepared by inoculation of the cell monolayer with 0.5—1 PFU per cell. When showing cytopathic effect, the cells were washed with phosphate buffered saline (PBS) pH 7.2, scrapped off in a minimal amount of 0.005 mol/l Tris-HCl buffer pH 7.0 and stored at —70 °C.

*Purification of NC.* NC were purified according to Matis *et al.* (1976). Briefly, after three cycles of freezing and thawing, the cell suspension was subjected to 2 cycles of differential centrifugation (3000 g, 15 min; 60 000 g, 60 min). The resulting suspension was sonicated and further purified through a discontinuous Ficoll gradient prepared by overlaying equal volumes of 40, 30, 20 and

10% w/w Ficoll solutions (Beckman Model SW39L rotor, 25 000 rev/min, 45 min). The bands situated at the interfaces between 10 and 20% as well as 20 and 30% of Ficoll solutions were collected, diluted, sedimented and subjected to equilibrium centrifugation in a discontinuous sucrose gradient (5—55% w/w) prepared in $D_2O$ (SW39L rotor, 36 000 rev/min, 6 hr). Fractions corresponding to pure NC (bottom peak) were pooled and rebanded under the same conditions.

*Sedimentation analysis of purified $^{125}I$-labelled NC* was performed in a discontinuous sucrose-$D_2O$ gradient (5—55% w/w) centrifuged to equilibrium as described above. The fractions (cca 50 µl) were collected by puncturing the bottom of the tube and counted in a gamma counter.

*Antisera.* Rabbit antisera against purified NC were prepared and evaluated as described in Results.

*Radioiodination.* Purified NC and protein A (Pharmacia, Uppsalla) were iodinated by the chloramine T method (Greenwood *et al.* 1963) in a modification described previously for influenza virus haemagglutinin (Russ *et al.* 1978). For NC iodination, however, gelatine has been used as carrier protein (instead of bovine serum albumin) and free $^{125}I$ was separated from $^{125}I$-NC by intensive dialysis. With both iodinated preparations more than 90% radioactivity was precipitable with trichloroacetic acid. The specific activity of $^{125}I$-NC was 74 kBq/µg and for $^{125}I$-protein A 500 kBq/µg, respectively. Both preparations were used up to 4 weeks after labelling.

*SDS-polyacrylamide gel electrophoresis (SDS/PAGE)* was carried out in 10% polyacrylamide slab gels in discontinuous buffer system as described by Spear and Roizman (1972). The proteins were visualized by staining with 0.05% Coomassie brilliant blue R-250, thereafter the slabs were dried. Autoradiographs were taken on a X-ray film (Medix-Rapid, Czechoslovakia) at —70 °C, using intensifying screens.

*Liquid-phase RIA.* For precipitation of $^{125}I$-NC, the reaction mixture in a vol of 250 µl contained 0.1% bovine serum albumin (BSA), 0.5% Triton X-100 (TX-100), 5 mmol/l EDTA in PBS pH 7.2 (RIA buffer), about 20—30 000 cpm of $^{125}I$-NC and various dilutions of immune rabbit serum. The mixture was incubated for 1 hr at 37 °C and then overnight at 4 °C. Thereafter 5 µl of normal rabbit serum and 0.1 ml of swine anti-rabbit Ig were added. After further incubation for 1 hr at 37 °C and overnight at 4 °C, the reaction mixture was diluted 10 times with RIA buffer. The immunoprecipitates were sedimented and counted in a gamma counter. Further details are described in the Results.

*For competitive-inhibition RIA* the reaction mixture contained $^{125}I$-NC (20—30 000 cpm per tube), various concentrations of competing antigen (0.5 ng—25.0 µg of unlabelled NC) and immune rabbit serum capable of binding approximately 50% of labelled antigen. The reaction mixture was incubated for 1 hr at 37 °C and then overnight at 4 °C. Then 5 µl of normal rabbit serum and 0.1 ml of swine anti-rabbit Ig were added and the assay proceeded similarly as for precipitation of $^{125}I$-NC with immune serum. Sonication of immune precipitates was performed by Raytheon, Modell DF 101, supply 115 V, 250 W, 10 kc . $s^{-1}$.

*Solid-phase RIA.* At room temperature 25 µl of purified NC in PBS pH 7.2 was adsorbed overnight to individual wells of polystyrene microtiter plates (KOOH-I-NOOR, Czechoslovakia). Nonspecific sticking to the plastic was prevented by addition of 200 µl of 10 mg/ml BSA for 2 hr. Then immobilized NC was incubated with various dilutions of immune sera for 90 min, 3 times washed (PBS pH 7.2 containing 1% foetal calf serum and 0.1% sodium azide), incubated with $^{125}I$-labelled protein A (30 000 cpm per well) and again 3 times washed. Radioactivity bound to each well was eluted with hot 2 mol/l NaOH (cca 100 µl per well) and counted in gamma counter. In the competitive-inhibition assay various concentrations of NC were incubated in vials with limiting amount of antibody for 90 min. Thereafter, 25 µl aliquots were transferred into wells with adsorbed NC and the assay was continued as described for the antibody titration procedure.

## *Results*

### *Characterization of HSV-1 NC and preparation of specific anti-NC sera*

NC obtained from HSV-1 infected SIRC or VERO cells were tested for their purity. NC preparations formed one narrow band in sedimentation analysis. Particles of this band examined by electron microscopy were found to be free of contaminating material (results not shown). NC were further analysed by

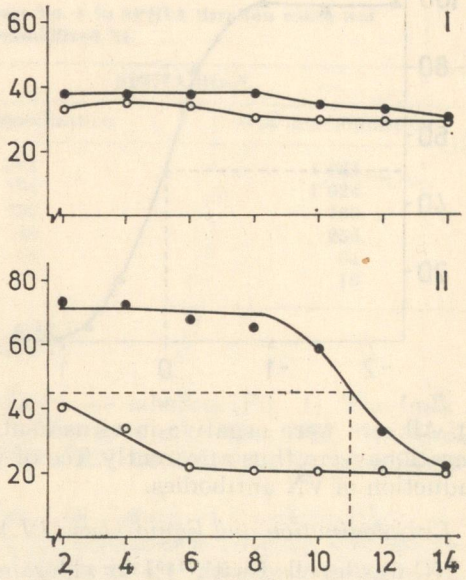


**Fig. 2.**
Liquid-phase RIA with $^{125}$I-NC
Effect of washing procedures on binding of immune No. 1 (●———●) and nonimmune (○———○) sera to $^{125}$I-NC. The assay proceeded either:
I. in the presence of 0.5% TX-100 when immune precipitates were washed once with RIA buffer,
II. in the absence of TX-100 and immune precipitates were washed twice in RIA buffer without TX-100 and sonicated for 1 min. Dotted line determines RIA titre (as defined in Table 1).
Abscissae: reciprocals of serum dilution (in $\log_2$)
Ordinate: % of $^{125}$I-NC bound

SDS-PAGE (Fig. 1). Several major zones were found in the gel at positions corresponding to the HSV-1 NC structural proteins $M_r$ 150K, 50K, 40K, 25K, 12K described in previous studies (Gibson and Roizman 1972, Cohen *et al.* 1980). Such NC preparations were used for immunization.

Rabbit sera were obtained after 3 intramuscular injections of NC (15, 75, 150 μg, respectively) emulsified in Freund's complete adjuvans. The intervals between antigen applications were 21 days. Blood was taken 10 days after the last antigen administration. Antibodies specific to NC were demonstrated by anti-complement immunofluorescence (ACIF) SPRIA and LPRIA (Table.

**Table 1. Comparison of anti-NC serum titres determined by VN, ACIF and RIA**

| Immune serum | Serum titre | | | |
|---|---|---|---|---|
| | VN[a] | ACIF[b] | LPRIA[c] | SPRIA[d] |
| anti-NC No. 1 | 2 | 40 | 100 000 | 1 024 |
| anti-NC No. 2 | 2 | 80 | 100 000 | 512 |
| anti-NC No. 3 | 2 | 40 | 50 000 | 374 |

[a]performed as described by Libíková et al. (1979);
[b]performed as described by Matis and Rajčáni (1980);
[c]LPRIA titre was expressed as reciprocal value of serum dilution precipitating 50 % $^{125}$I-NC present in reaction mixture;
[d]SPRIA titre was expressed as reciprocal value of serum dilution giving 50 % decrease of bound radioactivity.

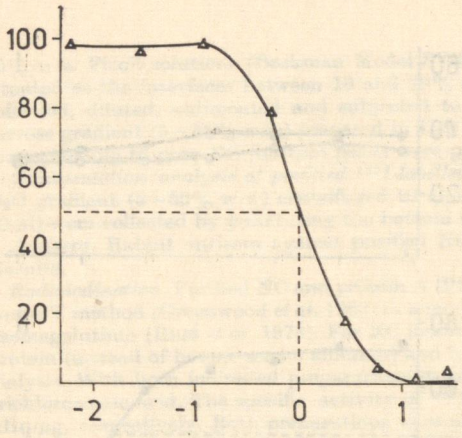


**Fig. 3.**

Liquid-phase competitive-inhibition RIA with $^{125}$I-NC

Immune serum No. 1 was used at a dilution binding approximately 50% of the $^{125}$I-NC. Dotted line determines the amount of competing antigen (1000 ng) decreasing $^{125}$I-NC binding by 50%.

Ordinate: $^{125}$I-NC bound in the presence of various concentrations of competing antigen relative to $^{125}$I-NC bound in the absence of competing antigen. The values are corrected for nonspecific binding (nonimmune serum).

Abscissa: Amount of competing unlabelled NC present in the reaction mixture ($\mu$g as $\log_{10}$).

1). All sera were negative in virus neutralization (VN) test. Purified NC preparations were thus apparently free of viral envelope antigens responsible for induction of VN antibodies.

*Radioiodination and liquid-phase RIA*

NC was labelled with $^{125}$I by chloramine T procedure as described in Materials and Methods and used for further radioimmunoprecipitation experiments. Sedimentation analysis of $^{125}$I-NC (specific radioactivity 74 kB q/$\mu$g) showed that labelled particles maintained their structural integrity (results nol shown). SDS-PAGE analysis of $^{125}$I-NC revealed that out of many structurat

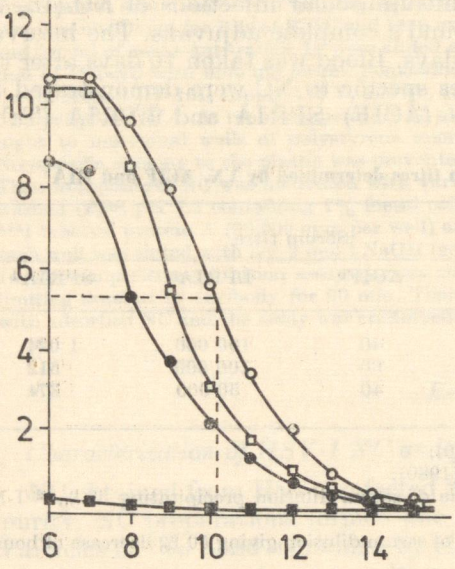


**Fig. 4.**

Solid-phase RIA titration of anti-NC immune sera

Titration of immune sera No. 1 (○——○) No. 2 (□——□), No. 3 (●——●) and nonimmune serum (■——■) by solid-phase RIA with immobilized NC was performed as described in Materials and Methods. Dotted line determines the RIA titre of immune serum No. 1 (compare Table 1).

Abscissa: reciprocals of serum dilution as $\log_2$.

Ordinate: amount of $^{125}$I-SPA bound in cpm . $10^{-3}$.

**Table 2. Comparison of anti-NC serum No. 1 in SPRIA titration using wet and dry immobilized NC**

| NC proteins concentration for immobilization in μg/ml[a] | SPRIA titre[b] | |
|---|---|---|
| | Dry immobilization | Wet immobilization |
| 20 | 384 | 1 024 |
| 10 | 192 | 1 024 |
| 5 | 192 | 768 |
| 2.5 | 48 | 256 |
| 1.25 | 16 | 64 |
| 0.65 | 16 | 16 |

[a]25 μl of stock solution were applied into each well;
[b]SPRIA titre were determined as defined in Table 1.

proteins only three (40K, 25K and 12K) were labelled (Fig. 1). The bulk of radioactivity, however, was seen in the front of migration (Fig. 1). Similar pattern has been obtained using IODO-GEN for NC labelling (results not shown).

Immunoprecipitation of $^{125}$I-NC tested by means of standard RIA procedures (e.g. Russ *et al.*, 1978) using nonionic detergent in reaction mixture gave poor results (Fig. 2-I), as we were unable to detect significant difference between the amount of $^{125}$I-NC precipitated with immune and nonimmune sera. Moreover, sedimentation analysis of $^{125}$I-NC treated with 0.5 % Triton X-100 showed a partial disintegration of the viral subunits (results not shown). For these reasons we omitted Triton X-100 from the reaction mixture and we sonicated the immunoprecipitates in addition to their intensive washing (Fig. 2-II) to decrease the binding of nonimmune sera to the $^{125}$I-NC. Using this modified procedure, we significantly improved the specificity of the assay so that it was possible to titrate the antibodies in antisera specific to NC at a sensitivity of at least 1000 times higher than by ACIF (Tab. 1).

The competitive-inhibition test was used for quantification of NC proteins. In this assay unlabelled NC were tested for the ability to compete with $^{125}$I-NC (5—10 ng $^{125}$I-NC per tube) for the binding to a limiting amount of antibody (Fig. 3). Amount of competing unlabelled NC proteins — about 1000 ng — resulting in 50 % decrease of $^{125}$I-NC binding was relatively high. This reflects a weak interaction between $^{125}$I-NC and corresponding antibody, either due to the damage of antigenicity as a consequence of iodination procedure or a strong nonspecific binding of immunoglobulins to $^{125}$I-NC.

*Solid-phase RIA with immobilized NC*

Different concentrations of the purified NC were immobilized on polystyrene microtitre plates either by overnight incubation of the plates at 37 °C without cover which allowed the samples to dry (dry immobilization) or by overnight incubation of tightly covered plates at room temperature to prevent evaporation of protein solution (wet immobilization). Thereafter the immunoadsorbents were saturated with BSA, incubated with diluted antiserum, washed and finally incubated with $^{125}$I-labelled protein A. Based on the results shown in Table 2

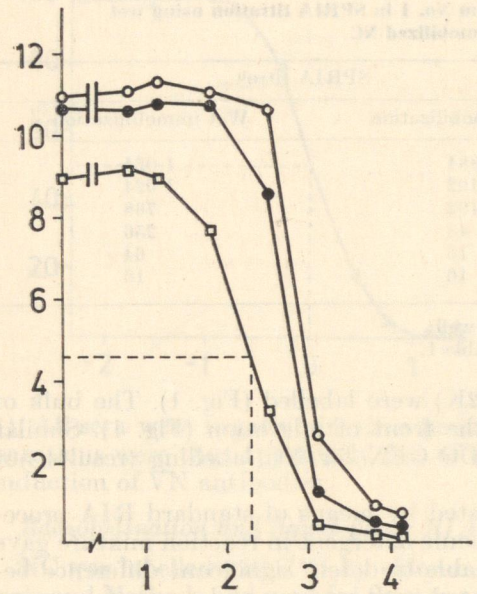


**Fig. 5.**
Solid-phase RIA competitive-inhibition test
Competitive-inhibition test was performed with immobilized NC (125 ng/well) as described in Materials and Methods. Anti-NC serum No. 1 was used at dilutions 1:128 (○——○), 1:256 (●——●), 1:512 (□——□). Dotted line determines the amount of competing antigen (200 ng) decreasing the binding of $^{125}$ I-SPA by 50%, when antiserum was used at dilution 1:512.
Abscissa: the amount (ng) of competing antigen present in the reaction mixture ($\log_{10}$ values).
Ordinate: amount of $^{125}$I-SPA bound in cpm . $10^{-3}$.

we have chosen for further experiments a wet immobilization of 125 ng of NC proteins per well.

The representative titrations of anti-NC sera are illustrated in Fig. 4 and the results are summarized in Table 1. Under the conditions employed, the titres were lower than those obtained by liquid-phase RIA using $^{125}$I-NC. The binding of nonimmune sera to immobilized NC in this case, however, was negligible. Therefore, solid-phase RIA with immobilized NC seems to be more suitable for determination of low levels of specific antibodies than liquid-phase RIA with $^{125}$I-NC.

In competitive-inhibition assay the NC in solution competed very efficiently with immobilized NC (125 ng per well) for a limiting amount of antibody. A 50 % competition was reached by adding of 200 ng NC proteins in solution (Fig. 5). The competitive-inhibition test proved suitable for quantification of small amounts of NC proteins.

## *Discussion*

The purpose of this work was to develop liquid-phase and solid-phase RIAs using purified NC of HSV-1 for detection of viral antibodies and antigens and to compare both methods. We decided to use purified NC instead whole virions for two reasons: 1. NC could be purified to a higher degree of purity than virions, 2. antibodies against host antigens (oligosaccharide side chains, glycolipids, etc.) which might bind to whole virions and cause false positivity, do not bind to purified NC.

The development of a sensitive and reliable liquid-phase RIA was complicated by two facts. First of all, the iodination of purified NC, using standard procedure (e.g. chloramine T) had been poor and the resulting specific radioactivity of our preparation was rather low (74 kBq/$\mu$g). HSV-1 NC consist of six major polypeptides ranging from 12K to 155K. We have found, however, that only polypeptides 40K, 25K and 12K were iodinated. Moreover, in front of 12K polypeptide the bulk of radioactivity, probably corresponding to degradation products, has been detected in SDS-PAGE. Because all radioactivity cosediment with NC particles, we assume, that these degradation products remained bound to the labelled particles. The reason for poor iodination of most NC polypeptides is not clear. It might be due either to low tyrosine content of corresponding polypeptides, or to inaccessibility of relevant amino acid residues, or to both. The preferential labelling of 40K dalton polypeptide with $^{125}$I might explain the development of sensitive LPRIA for this particular protein (Heilman *et al.*, 1979).

Using standard LPRIA procedures (nonionic detergent in reaction mixtures, (e.g. Russ *et al.*, 1978) we faced another problem, namely the low difference between binding of immune and nonimmune serum to $^{125}$I-NC. We succeded to increase this difference (i.e. to improve the specificity of the assay) by omitting the nonionic detergent from the reaction mixture and by intensive washing of immunoprecipitates combined with sonication. These modifications of the standard LPRIA conditions allowed us to detect anti-NC antibodies with sensitivity 1000 times higher than by means of ACIF. In contrast, sensitivity of estimation of NC proteins by competitive-inhibition RIA test was low (minimum 1000 ng) probably due to partial damage of important antigenic sites after radioactive labelling with $^{125}$I. In conclusion, for further development of liquid-phase RIA seems to be crucial to apply more efficient as well as more gentle methods (e.g. Bolton-Hunter reagent) for NC iodination.

The second alternative of RIA, with unlabelled NC in solid-phase, gave very low binding of nonimmune sera to immunoadsorbent. This allowed us to evaluate also sera with rather low levels of specific anti-NC antibodies, although the final titres determined in solid-phase RIA have been generally lower as those from liquid-phase assay. Competitive-inhibition test was sensitive and suitable for estimation of small doses of NC proteins (down to approximately 100 ng).

The direct quantitative comparison between liquid and solid phase RIAs is difficult because some antigenic determinants on immobilized NC might not be accessible for interaction with antibodies and because the exact quantity of immobilized NC is not known (Cantarero *et al.* 1980, Kennel 1983).

At present SPRIA seems to be a method of choice. Generally, RIA with immobilized antigen is rapid, simple and reproducible (Kalimo *et al.* 1977*a*, *b*, Colombatti and Hilgers 1979, Dreesman *et al.* 1979). For practical applications in diagnostic virology and basic research (screening of sera and monoclonal antibodies) it is most important that assay with immobilized NC is particularly suitable for examination of great many samples. Moreover, radioactive detection of bound antibodies can be easily replaced by immunoenzymatic and the assay can be transformed to ELISA.

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## References

Cantarero L. A., Butler J. E. and Osborne J. W. (1980): The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase immunoassays. *Anal. Bioch.* **105,** 375—382.

Cohen G. M., Ponce de Leon M., Diggelman H., Lawrence W. C., Vernon S. K. and Eisenberg R. J. (1980): Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. *J. Virol.* **34,** 521—531.

Colombatti A. and Hilgers J. (1979): A radioimmunoassay for virus antibody using binding of $^{125}$I-labelling protein A. *J. gen. Virol.* **43,** 395—401.

Dreesman G. R., Watson D. O., Courtney R. J., Adam E. and Melnick J. L. (1979): Detection of herpes virus type — specific antibody by a microsolid phase radioimmunometric assay. *Intervirology* **12,** 115—119.

Enlander D., Dos Remedios L. V., Weber P. M. and Drew L. (1976): Radioimmunoassay for herpes simplex virus. *J. immunol. Meth.* **10,** 357—362.

Gibson W. and Roizman B. (1972): Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. *J. Virol.* **10,** 1044 to 1052.

Greenwood F. C., Hunter W. M., and Glover J. S. (1963): The preparation of $^{131}$I-labelled human growth hormone of high specific activity. *Biochem. J.* **89,** 114—123.

Heilman C. J., Zweig M., Stephenson J. R., and Hampar B. (1979): Isolation of a nucleocapsid polypeptide of herpes simplex virus type 1 and 2 possessing immunologically type-specific and cross-reactive determinants. *J. Virol.* **29,** 34—42.

Kalimo K. O. K., Marttila R. J., Granfors K. and Viljanen M. K. (1977*a*): Solid-phase radioimmunoassay of human immunoglobulin M and immunoglobulin G antibodies against herpes simplex virus type 1 capsid, envelope and excreted antigens. *Infect. Immun.* **15,** 883—889.

Kalimo K. O. K., Ziola B. R., Viljanen M. K. and Toivanen P. (1977*b*): Solid-phase radioimmunoassay of herpes simplex virus IgG and IgM antibodies. *J. immunol. Meth.* **14,** 183—195.

Kennel S. J. (1983): Binding of monoclonal antibody to protein antigen in fluid-phase or bound to solid supports. *J. immunol. Meth.* **55,** 1—12.

Kimmel N., Friedman M. G. and Sarov I. (1982): Enzyme-linked immunosorbent assay (ELISA) for detection of herpes simplex virus-specific IgM antibodies. *J. virol. Meth.* **4,** 219—227.

Libíková H., Pogády J., Wiederman V. and Breier Š. (1976): Prítomnosť protilátok proti vírusu herpes simplex typu 1 u osôb s psychiatrickými poruchami a v kriminálnej populácii. *Čsl. Psychiatrie* **72,** 206—210.

Matis J., Leššo J., Mucha V. and Matisová E. (1975): Purification and separation of enveloped and unenveloped herpes simplex virus particles. *Acta virol.* **19,** 273—280.

Matis J., and Rajčáni J. (1980): Preparation of immune serum to immediate early and early polypeptides specified by herpes simplex virus type 1. *Acta virol.* **24,** 105—113.

Russ G., Styk B. and Poláková K. (1978): Radioimmunoassay of influenza A virus haemagglutinin I. Preparation and properties of radioactivite $^{125}$I-labelled bromelain-released haemagglutinin. *Acta virol.* **22,** 1—10.

Spear P. G. and Roizman B. (1972): Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. *J. Virol.* **9,** 143—159.

Vestergaard B. F., Grauballe P. C. and Spanggaard H. (1977): Titration of herpes simplex virus antibodies in human sera by the enzyme-linked immunosorbent assay (ELISA). *Acta path. microbiol. scand. Sect. B.* **85,** 466—468.

*Explanation of Figure (Plate XXIV):*

*Fig. 1.* SDS-PAGE of HSV-1 NC structural polypeptides.
Left lane: $^{125}$I-labelled NC structural polypeptides detected by autoradiography. The arrow indicates the presence of low-molecular weight degradation products.
Right lane: NC polypeptides detected by staining with Coomassie Brilliant Blue R-250. Major NC structural polypeptides are indicated with corresponding molecular weights ($M_r \cdot 10^{-3}$).