

ANTIVIRAL ACTIVITY OF ENZYMATICALLY OXIDIZED CAFFEIC ACID AGAINST HERPESVIRUS HOMINIS TYPE 1 AND TYPE 2

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Summary. — The cytotoxicity and antiviral activity of the caffeic acid oxidation product (KOP), a higher molecular polyphenolic compound of strong antiviral activity against herpesvirus hominis type 1 and type 2 (HVH 1, HVH 2), were tested in 6 cell cultures: rabbit kidney primary cells (RKP), rabbit testis primary cells (RTP), primary human embryo lung fibroblasts (LF), calf testis primary cells (CTP), FL- and HEp-2 cells. A marked inhibitory effect on the multiplication of HVH 1 and 2 has been observed in all cell systems at non-cytotoxic concentrations of 0.1–20 µg/ml KOP. The adsorption of the virus to cell surface was the most KOP-sensitive phase of herpesvirus multiplication cycle.

Key words: herpesvirus hominis type 1 and type 2; antiviral activity; polyanionic virus inhibitors; caffeic acid oxidation product

Introduction

Most of the known virus inhibitors which act upon the virus adsorption process polyanionic properties (De Clercq, 1974; Museteanu *et al.*, 1975; Takemoto and Liebhaber, 1961, 1962). When testing polyanionic virus inhibitors of natural origin, the antiviral activity of sodium and ammonium humate against HVH 1 and HVH 2 has been demonstrated *in vitro* (Klöcking and Sprössig, 1972, 1975; Klöcking *et al.*, 1976; Thiel *et al.*, 1977) and *in vivo* (Klöcking *et al.*, 1977a) as well as in preliminary clinical studies (Schiller *et al.*, 1979). These observations and the fact that phenolic compounds are basic constituents of humic acids, encouraged us to look for other virus inhibitors of phenolic astructure. According to the method described by Hampton (1970) and modified by us (Klöcking *et al.*, 1977b; Klöcking and Helbig, WP.; Klöcking *et al.*, 1979a), o-diphenolic compounds were oxidized with o-diphenoloxidase (E.C. 1.14.18.1). After isolation of the spontaneously polymerizing oxidation products, the antiviral activity was tested against HVH 1 and HVH 2. The polymerized oxidation product of caffeic acid (KOP) proved to be the most effective antiviral substance (Klöcking *et al.*, 1978a, 1978b, 1979b; Thiel *et al.*, 1976).

The aim of this study was to investigate the effect of KOP on the replication of HVH 1 and HVH 2 in more detail and to gain information about the cell toxicity of KOP.

Materials and Methods

Cells. Rabbit testis primary cells (RTP), rabbit kidney primary cells (RKP), calf testis primary cells (CTP) and chick embryo fibroblasts (CEF) were used. The techniques of preparation and subculture have been previously described (Grafe, 1967; Klöcking *et al.*, 1978b; Schweizer, 1966, 1968; Thiel *et al.*, 1976, 1977; Wutzler *et al.*, 1976, 1979). In addition, the cytotoxicity of the test substances was determined in human embryonic lung fibroblasts (LF), HEP-2, and FL cells.

Medium fluids. Cultivation of primary cells was performed in Parker's medium 199, in Eagle's minimum essential medium (MEM) mixed with lactalbumin hydrolysate and Hanks' solution in the ratio of 1 : 1 supplemented with 10–15% serum. The pH value was adjusted to 7.3 with 5% NaHCO₃ solution. All growth media contained 200 IU penicillin and 200 µg streptomycin per 1 ml.

Virus. HVH 1 (strain Kupka) and HVH 2 (strain US) were obtained from Dr. R. Benda, Prague, Czechoslovakia. Strain Kupka isolated from a patient with herpes labialis was grown for 27 passages in RK cells and for 4 passages in rabbit lung fibroblasts. Strain US of HVH 2 isolated from a genital herpetic vesicle (Schubladze and Chi-Hsiang, 1959), was passaged intracerebrally in mice, then grown for 13 passages in human thyroid cells and for 4 passages in TRP-cells. After multiplication of the virus strains in human embryo lung fibroblasts, in human thyroid cells and rabbit testis primary cells, the cell-free virus suspensions were stored at -191 °C in liquid nitrogen and were thawed immediately before use.

Plaque test was performed as previously described (Thiel *et al.*, 1976, 1981).

Testing of cytotoxicity. Morphological control of cells was carried out in a Telaval microscope (VEB Carl Zeiss Jena, Jena, G.D.R.). The tested substances were scored according to the criteria proposed by Buthala (1964), Sidwell *et al.* (1974), and Lauter *et al.* (1974), including cell alteration, granulation and vacuolization, changes of the form and size as well as of the adhesive capability of cells. The percentage of morphologically changed or shed cells was determined in relation to untreated controls. Cell counting was carried out after trypsinization, centrifugation at 60 × g, repeated washing of the cells and resuspension in Hanks' solution. Determination of the viability of the cells was proved by counting in a Bürker chamber after staining with 0.5% trypan blue.

Caffeic acid oxidation product (KOP). The enzymatic oxidation of diphenolic compounds has been previously described (Ireland and Pierpoint, 1980; Klöcking, 1977b; Klöcking and Helbig, WP.). KOP is a dark brown powder, stable at room temperature, and soluble in water and alkali. It is insoluble in apolar solvents and inorganic acids. It forms water-insoluble chelate compounds with heavy metals and complexes with cationic dyes (Klöcking *et al.*, 1979a). IR spectrographic investigations favour the existence of free carboxyl- and hydroxyl groups in the polymerisate (Klöcking *et al.*, 1978a). The molecular weight is 6 500, as determined by permeation chromatography on controlled pore glass (Electro-Nucleonics, Inc., Fairfield, N. Y., U.S.A.). A reductive cleavage of KOP with sodium amalgam results in hydrocaffeic acid in a yield of 30% (Klöcking *et al.*, 1979a).

Results

Testing of cytotoxicity

Monolayers of FL and RTP cells were incubated with KOP concentrations from 1 to 1000 µg/ml. In the first experiment the substance was in contact with the cells for 72 hr. In further experiment, the substance was washed off after 90 min. Microscopic controls showed that the cytotoxic activity depended on concentration and duration of the substance action. During the period of 72 hr a 50 per cent cell destruction was found at concentration

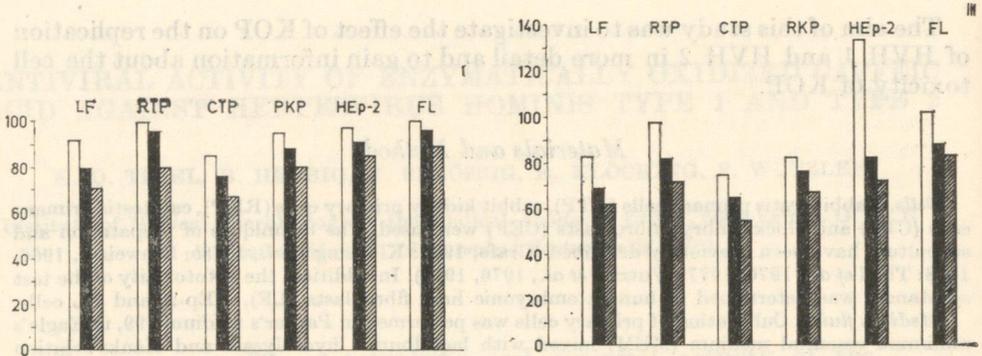


Fig. 1.

Influence of KOP on viability of various cell lines

Incubation time: 24 hr (I-I), 48 hr (I-II), and 72 hr (I-III); KOP concentration: 10 µg/ml (empty columns), 50 µg/ml (black columns) and 100 µg/ml (dashed columns).

Cells: human embryonic lung fibroblasts (LF), rabbit testis primary cells (RTP), calf testis primary cells (CTP), rabbit kidney primary cells (RKP), HEp-2 and FL.

Abscissae: µg KOP.ml⁻¹; ordinates: viability of treated cells as related to untreated ones (%).

of 200 µg/ml KOP, whereas 500 µg/ml KOP caused cell destruction after 90 min. Concentrations below 20 µg/ml completely failed to induce any morphological changes. More detailed results were obtained by determination of the survival rate of several cell lines after different duration of action. Determination of the percentage ratio of living cells after 24, 48 and 72 hr

Table 1. Influence of KOP on the adsorption of HVH 1 and 2 to RKP cells

KOP concentration (µg/ml)	Plaque formation expressed as percentage of untreated controls	
	HVH 1	HVH 2
0.01	100	100
0.05	98	79
0.1	80	37
0.5	31	18
1.0	17	12
2.0	9	6
5.0	5	3
10.0	2	1
20.0	0	0

Duration of adsorption 90 min temperature 37 °C.

Table 2. Effect of KOP on plaque formation of HVH 1 and HVH 2 in RTP cells

KOP concentration ($\mu\text{g/ml}$)	Plaque formation expressed as percentage of untreated controls					
	HVH 1			HVH 2		
	10 min*	30 min*	60 min*	10 min*	30 min*	60 min*
0.1	33	41	46	20	29	35
0.5	21	27	31	15	19	26
1.0	12	15	19	11	12	15
2.0	9	12	14	7	11	12
5	4	7	9	1	2	5
10	1	2	4	0	0	1
20	0	0	0	0	0	0

* Duration of KOP action.

showed the different sensitivities of the LF, RTP, RKP and CTP cells as well as of the continuous lines HEp-2 and FL (Fig. 1). Multiplication of LF and CTP cells was obviously limited, whereas the continuous HEp-2 and FL cells proved to be less sensitive. Simultaneous addition of KOP to the seeded cells caused decreased adhesion of the cell culture to glass vessels, indicating a possible action on the cellular membrane. The results confirmed the earlier investigations on KOP cytotoxicity carried out with the aid of the enzyme as well as by ^{51}Cr -release test (Thiel *et al.*, 1980).

Antiviral activity

The oxidation product of the caffeic acid was tested for antiviral activity during the adsorption phase at a concentration range from 0.1 to 1000 $\mu\text{g/ml}$.

In general, RKP, RTP, CTP and CEF monolayers inoculated with 2000 TCID₅₀/ml of HVH 1 or HVH 2 were incubated with KOP for 90 min at

Table 3. Effect of KOP after adsorption of HVH 2 to RTP cells

KOP concentration ($\mu\text{g/ml}$)	Plaque formation expressed as percentage of untreated controls	
	1.5 hr*	72 hr*
1	50	91
10	37	38
50	18	0
100	0	0

* Duration of KOP treatment

Table 4. Influence of preincubation of RTP cells with KOP on adsorption of HVH 1 and HVH 2

KOP concentration ($\mu\text{g/ml}$)	Plaque formation expressed as percentage of untreated controls			
	HVH 1		HVH 2	
	180 min*	300 min*	180 min*	300 min*
0.5	100	100	100	98
2	100	93	78	65
5	82	80	64	46
10	67	64	31	19
20	38	22	20	11
50	9	8	4	2
100	5	3	0	0
200	0	0	0	0

* Duration of preincubation.

37 °C followed by repeated washing with Hanks' solution and addition of methyl-cellulose. The incubation time was 48 to 72 hr. Table 1 shows the dependence of plaque formation upon KOP concentration. The average plaque values attained by untreated virus controls at an adsorption time of 90 min were taken as 100%. It is clearly shown that the two HVH strains were inhibited at KOP concentrations from 0.1 to 20 $\mu\text{g/ml}$. HVH 2 was even more sensitive, a phenomenon known from other polyanionic virus

Table 5. Influence of KOP on the preincubation* of the cell-free virus suspension of HVH 1 and HVH 2

KOP concentration ($\mu\text{g/ml}$)	Plaque formation expressed as percentage of untreated controls	
	HVH 1	HVH 2
	200	0
100	0	0
50	4	4
20	19	20
10	46	53
5	73	75
2	100	97
1	100	100
0.5	100	100
0.1	100	100

* Duration of preincubation was 120 min at 4 °C.

inhibitors (Thiel *et al.*, 1977). A 50% inhibition of plaque formation was caused for HVH type 2 by 0.08 $\mu\text{g/ml}$ and for HVH type 1 by 0.3 $\mu\text{g/ml}$ KOP.

In order to determine the time dependence of the inhibition process, the virus and virus-KOP mixtures, respectively, were adsorbed to the washed monolayers for 10, 30 and 60 min and then washed off with Hanks' solution. The results set out in Table 2 showed that, as early as 10 min after inoculation, the action of KOP was concentration dependent. The influence of KOP on the replication of HVH 1 and HVH 2 following adsorption was tested as follows: the substance was added to the monolayers for 90 min in a phosphate buffer solution or for 72 hr in the overlay, respectively. In both cases, the plaque reduction appeared dependent on the inhibitor concentration (Table 3). Beside of the duration of action, the velocity with which the substance reached the site of infection was important. For instance 1 $\mu\text{g/ml}$ KOP dissolved in phosphate buffer and given for a short time was more effective than in a viscous overlay for 72 hr.

With regard to the mechanism of action, it was of interest to elucidate the question, whether the pretreatment of cells with KOP followed by virus infection would or would not lead to a reduction of plaque numbers. For this purpose rabbit testis cells were preincubated with the substance for 3 and 5 hr, respectively, then washed and infected with HVH for 90 min. When plaque counting was done 72 hr p.i., a distinct prophylactic effect of KOP at concentrations higher than 10 $\mu\text{g/ml}$ was found (Table 4). Preincubation of the cell-free virus suspension ($10^{7.5}$ TCID₅₀/ml HVH 1, $10^{7.33}$ TCID₅₀/ml HVH 2) with various KOP concentrations was performed at 4 °C for 2 hr. This treatment led to a 100 per cent inhibition of plaque formation at concentrations higher than 50 $\mu\text{g/ml}$ (Table 5).

Discussion

To assess the possible cytotoxic action of the caffeic acid oxidation product, continuous cell lines (FL, HEp-2) as well as primary cells with fibroblastoid (LF, RTP) and epitheloid morphology (RKP, CTP) were used. LF and CTP cells proved to be especially sensitive to KOP. Concentrations of > 10 $\mu\text{g/ml}$ impaired the growth of cells. This was detectable at concentrations > 50 $\mu\text{g/ml}$ in both RKP and RTP cells. Monolayers of FL and HEp-2 cells remained relatively unchanged. It cannot be stated with certainty whether permanent cell lines are more resistant to phenolic polymerisates in general. If KOP was added during the actual cell seeding, adhesion of the cells to the glass wall was diminished, an observation which has been described by Buthala (1964) for 5-iodo-2'-deoxyuridine and by Thiel *et al.* (1977) for ammonium humate. As early as 1961 Hampton and Fulton showed the inactivating action of enzymatically oxidized phenols on phytopathogenic viruses. In subsequent years this problem has been dealt with extensively by Klöcking *et al.* (1972, 1975, 1976, 1977a, 1978a) and Thiel *et al.* (1976, 1977, 1981) for human viruses. HVH type 1 and HVH type 2 (Klöcking *et al.*, 1975, 1976, 1977a, 1978a, 1979a; Thiel *et al.*, 1977) influenza virus A and

Coxsackie virus A9 (Klößing and Sprössig, 1975) were proved to be sensitive to humate. These results could be confirmed by *in vitro* investigations with enzymatically oxidized diphenolic compounds (Klößing *et al.*, 1978a, b; 1979a, b; Thiel *et al.*, 1976). In these investigations the oxidation product of caffeic acid (KOP) proved to be highly effective against HVH 1 and HVH 2.

The results described point unambiguously to a strong inhibition of the early phase of cell-virus interaction. Simultaneous incubation of the monolayers with HVH and KOP during virus adsorption resulted in inhibition of plaque formation depending on KOP concentration within the range from 0.1 to 20 $\mu\text{g/ml}$. As the mechanism of antiviral action of KOP has not yet been finally clarified, at least 4 different sites of action must be taken into consideration. The most sensitive phase is the virus adsorption, as the average concentration of inhibition (I_{50}) was 0.1 $\mu\text{g/ml}$. Here, the inhibition was brought about by the formation of a virus-inhibitor complex. To inactivate the virus upon preincubation with the inhibitor, much higher concentrations of KOP were necessary ($I_{50} = 10 \mu\text{g/ml}$). In this case, the inhibition may be the consequence of a virus-inhibitor complex formation. The virus multiplication could be inhibited by preincubation of the host cells with KOP ($I_{50} = 7 \mu\text{g/ml}$ KOP). This inhibitory effect might be due to complex formation with the receptor protein of the cell membrane. Recently, we have found that KOP reacts with different kind of proteins by complex formation (unpublished data). Because humic acids possess antiinflammatory properties (Klößing, 1967), a "membrane stabilizing effect" described for several substances acting on the erythrocyte membrane must also be taken into account (Inglot *et al.*, 1966, 1968). Finally, KOP exerted an antiviral effect even after virus adsorption ($I_{50} = 2 \mu\text{g/ml}$). In contrast, *in vitro* studies with ammonium humate showed no effect on the post adsorption phase (Klößing and Sprössig, 1975; Thiel *et al.*, 1977).

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References

- Buthala, D. A. (1964): Cell culture studies on antiviral agents. 1. Action of cytosine arabinoside and some comparisons with 5-iodo-2-deoxyuridine. *Proc. Soc. exp. Biol. Med.* **115**, 69–77.
- De Clercq, E. (1974): Synthetic Interferon Inducers. *Top. Curr. Chem.* **52**, 173–208.
- Grafe, A. (1967): Die Verwendung von primären Kälberhodenzellen für virologische Arbeiten. *Arch. ges. Virusforsch.* **22**, 433–441.
- Hampton, R. E., and Fulton, R. W. (1961): The relation of polyphenol oxidase to instability *in vitro* of prune dwarf and sour cherry necrotic ringspot viruses. *Virology* **13**, 44–52.
- Inglot, A. D., Kochman, M., and Mastalerz, P. (1966): Activity of salicylates as inhibitors of virus replication *in vitro* and their mode of action. *Acta virol.* **10**, 185–194.
- Inglot, A. D., and Wolna, E. (1968): Reactions of non-steroidal anti-inflammatory drugs with the erythrocyte membrane. *Pharmacol.* **17**, 269–279.
- Ireland, R. J., and Pierpoint, W. S. (1980): Reaction of strains of potato virus X with chloroquinone *in vitro*, and the detection of a naturally modified form of the virus. *Physiol. Plant Pathol.* **16**, 81–92.
- Klößing, R. (1967): Untersuchungen über Stoffe vom Huminsäuretyp und ihre Metallverbindungen. Habilitationsschrift, Med. Fakultät der Univ. Rostock, Rostock, 1967.

- Klöcking, R., and Sprössig, M. (1972): Antiviral properties of humic acids. *Experientia* **28** 607—608.
- Klöcking, R., and Sprössig, M. (1975): Wirkung von Ammoniumhumat auf einige Virus-Zell-Systeme. *Z. allg. Mikrobiol.* **15**, 25—30.
- Klöcking, R., Thiel, K. D., and Sprössig, M. (1976): Antiviral activity of humic acids from peat water. In *Peat and peatlands in the natural environment protection*. Vol. 1. Poznań, p. 446—455.
- Klöcking, R., Graumann, G., Töpke, H., Blumöhr, T., Drabke, P., and Eichhorn, U. (1977a): Wirkung von Natriumhumat auf experimentellen Herpes corneae, pp. 141—143. In *Wiss. Beiträge der Martin-Luther-Universität Halle-Wittenberg*, 1977/43 (R 35).
- Klöcking, R., Helbig, B., and Drabke, P. (1977b): Eine verbesserte Methode für die Isolierung wasserlöslicher Huminsäuren und enzymatisch oxydierter o-Diphenolverbindungen. *Pharmazie* **32**, 297.
- Klöcking, R., and Helbig B. (1978): WP (DDR) Patent Nr. 129 916, C 07 G, 18. 2. 77/15. 2. 78.
- Klöcking, R., Thiel, K. D., Helbig, B., Kolbe, A., and Drabke, P. (1978a): Vergleich der chemischen, physikochemischen und antiviralen Eigenschaften von Huminsäuren und enzymatisch oxydierten o-Diphenolverbindungen. p. 141—147. In *Abh. Akad. Wiss. DDR, Abt. Math. Naturwiss. Technik* 1978, Nr. 2N. R. Franke and P. Oehme, Eds. Berlin, Akademie-Verlag.
- Klöcking, R., Thiel, K. D., Wutzler, P., Helbig, B., and Drabke, P. (1978b): Antivirale Aktivität von Phenolkörperpolymerisaten gegenüber Herpesvirus hominis Typ 1. *Pharmazie* **33**, 539.
- Klöcking, R., Helbig, B., Thiel, K. D., Blumöhr, T., Wutzler, P., Sprössig, M., and Schiller, F. (1979a): Gewinnung, Charakterisierung und antivirale Aktivität von Phenolkörperpolymerisaten. Teil 1; Gewinnung und Charakterisierung von Phenolkörperpolymerisaten. *Pharmazie* **34**, 292—293.
- Klöcking, R., Thiel, K. D., Helbig, B., Blumöhr, T., Wutzler, P., Sprössig, M., and Schiller, F. (1979b): Gewinnung, Charakterisierung und antivirale Aktivität von Phenolkörperpolymerisaten. Teil 2: Antivirale Aktivität von Phenolkörperpolymerisaten. *Pharmazie* **34**, 293—294.
- Lauter, C. L., Biley, E. J., and Lerner, A. J. (1974): Assessment of cytosine arabinoside as an antiviral agent in humans. *Antimicrob. Agents Chemother.* **6**, 598—602.
- Museteanu, C., Voss, H., and Rossner, R. (1975): Sulfatierte Glycosaminoglycane als Virusinhibitoren. 2. Mitt.: Hemmung von Glycosaminoglycanpolysulfaten auf Gelbfiebervirus 17D im Tierexperiment. *Zbl. Bakt. I. Abt. Orig.* **230**, 298—305.
- Schille, F., Klöcking, R., Wutzler, P., and Färber, I. (1979): Ergebnisse einer orientierenden klinischen Prüfung von Ammoniumhumat zur Lokalbehandlung von Herpesvirus-hominis-(HVH)-Infektionen. *Dermatol. Monatsschr.* **165**, 505—509.
- Schweizer, H. (1966): Ein Vorschlag für verbesserte Bedingungen bei der Herstellung primärer Zellkulturen. *Z. med. Labortechnik* **7**, 29—34.
- Shubladze, A. K., and Chi-Hsiang, H. (1959): Study on antigenic properties of herpes virus. *Vopr. Virusol.* **4**, 80—85.
- Sidwell, R. W., Huffman, J. H., Allen, L. B., Mayr, R. B., Shuman, D. A., Simon, L. N., and Robins, R. K. (1974): In vitro antiviral activity of 6-substituted 9- β -D-R-bifuranosylpurine 3', 5'-cyclic phosphates. *Antimicrob. Agents Chemother.* **5**, 652—657.
- Takemoto, K. K., and Liebhaber, (1961): Virus polysaccharide interactions. I. An agar polysaccharide determining plaque morphology of EMC virus. *Virology* **14**, 456—462.
- Takemoto, K. K., and Liebhaber, H. (1962): Virus polysaccharide interactions. II. Enhancement of plaque formation and the detection of variants of poliovirus with dextran sulfate. *Virology* **17**, 499—501.
- Thiel, K. D., Klöcking, R., and Helbig, B. (1976): In vitro-Untersuchungen zur antiviralen Aktivität enzymatisch oxidierten o-Diphenolverbindungen gegenüber Herpes-simplex-Virus Typ 1 und Typ 2. *Zbl. Bakt. I. Abt. Orig.* **234**, 159—169.
- Thiel, K. D., Klöcking, R., Schweizer, H., and Sprössig, M. (1977): Untersuchungen in vitro zur antiviralen Aktivität von Ammoniumhumat gegenüber Herpes simplex-Virus Typ 1 und Typ 2. *Zbl. Bakt. I. Abt. Orig.* **239**, 304—321.
- Thiel, K. D., Eichhorn, U., Schweizer, H., and Klöcking, R. (1980): Morphological and biochemical criteria for evaluating cytotoxic effects of antiviral substances, pp. 428—430. In *Further Studies in the Assessment of Toxic Actions*, Arch. Toxicol., Suppl. 4, Springer-Verlag Heidelberg, 1980.

- Thiel, K. D., Helbig, B., Klöcking, R., Wutzler, P., Sprössig, M., and Schweizer, H. (1981): Vergleich der In-vitro-Wirksamkeit von Ammoniumhumat und enzymatisch oxydierter Chlorogen- und Kaffeesäure gegenüber Herpesvirus hominis Typ 1 und 2. *Pharmazie* **36**, 50—53.
- Wutzler, P., Färber, I., Sprössig, M., Schweizer, H., Thiel, K. D., and Schneider, J. (1976): Herpesvirus-induzierte Autoantikörperbildung im Tierexperiment. *Arch. Virol.* **51**, 59—66.
- Wutzler, P., Schweizer, H., Thiel, K. D., Färber, I., and Sauerbrei, A. (1979): Typendifferenzierung von Herpesvirushominis-Isolaten aus Patientenmaterialien. *Dtsch. Gesundheitswes.* **34**, 1140—1143.