Acta virol. **26**: 444—452, 1982

EFFECTS OF STREPTOVIRUDIN ON INFLUENZA VIRUSES TYPE A AND B: INHIBITION OF THE LIPID-LINKED OLIGOSACCHARIDE SYNTHESIS OF FOWL PLAGUE VIRUS

E. TONEW, M. TONEW, *M. K. INDULEN, *D. R. DZEGUZE

Central Institute of Microbiology and Experimental Therapy, Research Centre for Molecular Biology and Medicine, Academy of Sciences of the German Democratic Republic, DDR 6900 Jena; and *August Kirchenstein Institute of Microbiology, Academy of Sciences of the Latvian S.S.R., 226067 Riga, U.S.S.R.

Received May 18, 1981; revised April 2, 1982

Summary. — Antibiotics of the streptovirudin complex (SV) inhibited the growth of influenza A and B viruses such as influenza A/fowl plague virus (FPV), strain Weybridge (Hav1 Neq1), influenza A/England 42/72 (H3N2), influenza A/Port Chalmers 1/73 (H3N2), influenza B/Leningrad 235/74, influenza B/Tokyo 7/66, and influenza B/Jamagata in chick embryo cell (CEC) cultures, in permanent canine kidney cells (MDCK), and in suspended fragments of chick embryo chorioallantoic membranes (CAM). As revealed by spectrophotometric turbidity measurements, SV completely inhibited the FPV-induced cytopathic effect (CPE). A 99.99% reduction of infectious virus yield was obtained in one-step growth cycle experiments and in the plaque reduction test. The haemagglutination inhibition titres of influenza viruses in suspended CAM fragment cultures in the presence of SV drugs were also substantially reduced. The incorporation assays indicated that SV exhibited no effect on virus-induced RNA synthesis, but influenced virus maturation by inhibition of lipid-linked oligosaccharide synthesis. A partial protection from infection was found in influenza virus A/England infected mice.

Key words: influenza viruses A and B; streptovirudin; oligosaccharide synthesis inhibition

Introduction

SV are new antibiotics produced by *Streptomyces griseoflavus*. Their production, isolation and physico-chemical properties were described by Eckardt *et al.* (1973*a*, *b*, 1975) and Thrum *et al.* (1975). The antibiotic complex consists of different related compounds each of which possesses antiviral activity. The antiviral action against a great number of RNA and DNA

Table 1. Suppression of reproduction of influenza viruses A/England 42/72, A/Port Chlamers 1/73, B/Tokyo 7/66, B/Jamagata 1/73 and B/Leningrad 235/74 in CAM of chick embryos

Influenza virus strain	SV concentration	Titre decrease	Per cent inhibition
A/Port Chalmers 1/73	25*	6.67**	> 99.99
	10	6.67	> 99.99
	5	6.67	> 99.99
	1	6.67	> 99.99
A/England 42/72	1	4.0	99.99
B/Tokyo 7/66	1	4.0	99.99
B/Jamagata 1/73	0.5	5.5	> 99.99
	0.25	4.5	> 99.99
	0.1	2.83	99.85
B/Leningrad 235/74	0.5	4.17	> 99.99
	0.25	3.67	99.98
	0.1	2.67	99.8

* in μg/ml.
** log ID_{50} in the absence of SV minus log ID_{50} in the presence of SV.

viruses in vitro was described by Tonew and Eckardt (1974), Tonew *et al.* (1975) and Kobus *et al.* (1977). The present paper extends the effect of SV on influenza viruses in vitro (CAM, CEC, MDCK cells) and in vivo.

Materials and Methods

Viruses. Fowl plaque virus (FPV) strain Weybridge (Hav1Neq1) was obtained from Ivanovsky Institute of Virology, Moscow, U.S.S.R., and passaged in CEC. Influenza viruses A/England 42/72 (H3N2), A/Port Chalmers 1/73 (H3N2), B/Tokyo 7/66, B/Jamagata 1/73 and B/Leningrad 235/74 from All-Union Institute of Influenza, Leningrad, U.S.S.R., were passaged in chick embryos.

Cell cultures. CEC were prepared and cultures as described elsewhere by Tonew and Tonew (1969). MDCK cell suspension cultures obtained from All-Union Institute of Influenza, Leningrad U.S.S.R., were propagated under the same conditions as published by Gaush and Smith (1968). CAM-inhibition test was performed according to the method of Horwath (1954).

Plaques assay and one-step growth cycle experiments. The technique was the same as previously described (Tonew and Tonew, 1969, 1971).

CPE assay. The assays were conducted using a sensitive specrophotometer (UFD 100 Vitatron, The Netherlands). Suspension cultures of MDCK cells were prepared in siliconized tubes providing 5×10^5 cells/ml and infected at multiplicity of infection (m.o.i.) of 50 PFU/cell. Extinction was measured daily over a period of 60 hr in passing light at 650 nm wavelength. Both infected and uninfected cells were treated with SV in doses of 5 and 2.6 μg/ml, respectively. Observation of the differences in optical density between the infected and treated cell suspensions was carried out according to Tonew *et al.* (1973) and Augsten and Tonew (1975); statistical calculations were made by means of variance analysis.

Reversibility of the antiviral activity. Tube cultures of CEC infected at m.o.i. of 20 PFU/cell were incubated with 1 μg/ml SV for selected time intervals at 37° C. Then, the antibiotic was washed off with prewarmed medium, the cell cultures were refed with fresh medium and incubated for 24 hr. Afterwards, the haemagglutination titre was estimated according to Takatzuki *et al.* (1965) using 1% suspension of chick erythrocytes.

^{3}H-uridine incorporation. The ^{3}H-uridine uptake was followed in SV-treated and untreated CEC infected with FPV at m.o.i. of 20 PFU/cells. Cumulative labelling with ^{3}H-uridine (20 kBq/ml) of cultures was carried out for 8 hr after infection. At intervals of 2 hr the acid precipitable radioactivity was determined under one-step replication cycle conditions. Similarly infected cultures were incubated with actinomycin D (2 μg/ml) added immediately after infection. For

Table 2. Spectrophotometric turbidity measurements of MDCK cell suspensions infected with FPV

Hr after infection	Extinction at 650 nm				Virus titre*	
	I	II	III	IV	A	B
0	0.281	0.297	0.295	0.289	3.33	3.0
10	0.289	0.286	0.304	0.341	n.t.	n.t.
20	0.296	0.299	0.307	0.373	n.t.	n.t.
40	0.303	0.306	0.311	0.467	n.t.	n.t.
60	0.311	0.314	0.327	0.495	7.22	3.44

I. Uninfected and untreated cell suspension
II. Infected and treated with 2.5 μg/ml SV
III. Infected and treated with 6 μg/ml SV
IV. Infected but untreated
A = Virus adsorbed for 30 min and removed by 2-fold washing (no drug).
B = Virus adsorbed for 30 min, 5 μg/ml SV.
*$\log_{10}$ $TCID_{50}$/0.2 ml.
n.t. = not tested.

testing the action of SV on the transfer of glucose from sugar-nucleotides into lipid-linked monosaccharides, ^{14}C-glucose (20 kBq/ml) was used according to the same shedule. The radioactivity was estimated as described by Tonew and Tonew (1974).

In vivo experiments. Outbred white mice weighing 18—20 g were infected intranasally with a predetermined virus dose (ID = 10 LD_{50}). The drug was administered orally 3 times daily during 4 days. Ten animals per group and dose were used. The survival rates were calculated on the 14th day after infection according to Reed and Muench.

Chemicals and isotopes. SV was produced and kindly provided by Dr. K. Eckardt (Central Inatitute for Microbiology and Experimental Therapy, Jena, G. D. R.), actinomycin D was from Serva, Heidelberg, F.R.G.; sodium deoxycholate (DOC) from VEB Chemisches Werk, Berlin-Grünau, G.D.R.; filter discs of chromatography paper FN 7 were from Spezialpapierfabrik, Niederschlag/Erzgebirge, G.D.R.; ^{3}H-5-uridine was from Radio-Chemical Centre, Amersham, England (specific activity 902,8 GBq/mM). PPO and dimethyl POPOP were from Packard Instrument International SA, Zürich, Switzerland (for toluene scintillator fluid). ^{14}C-6-glucose was obtained from Vshesoyuznoe Obyedinenie "Isotop", Leningrad (specific activity 1850 GBq/mM).

Results

In vitro experiments

Replication of influenza A/England 42/72 and A/Port Chalmers 1/73 viruses in CAM fragment cultures indicated a complete inhibition by SV at concentration of 1 μg/ml. The influenza B/Jamagata and influenza B/Leningrad viruses were inhibited at concentration of 0.5 μg/ml by more than 4 log LD_{50} as compared to controls, i. e. virus reproduction was reduced by about 99.99% (Table 1). The maximum tolerated dose of SV was 100 μg/ml.

The CPE by FPV of suspension MDCK cultures could be prevented by addition of SV in doses 5 and 2.5 μg/ml of medium, respectively, as revealed by spectrophotometric turbidity measurements (Table 2). A restricted reproduction of MDCK cells took place in maintenance medium during 60 hr. The enhancement of the optical density as a sequence of the virus-induced CPE destruction of the cells was controlled at the beginning and at the end

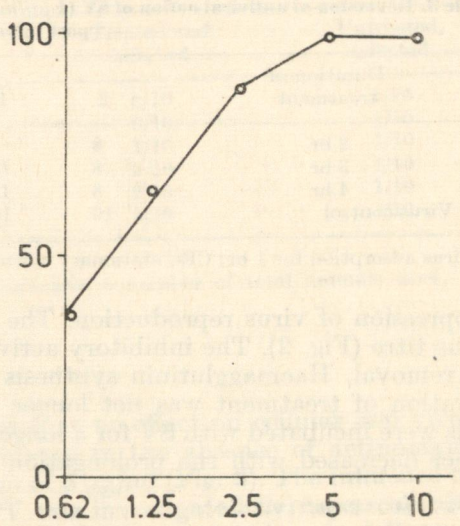

Fig. 1.

Plaque reduction of FPV by SV
CEC monolayers were infected with FPV in Demeter flakss. After 1 hr adsorption the virus was replaced with 6 ml overlay; three flasks for each concentration of the compound and untreated virus controls were used. The plaque reduction was expressed as per cent of the average plaque number in control flasks.
Abscissa: Concentration of SV in μg/ml; ordinate: plaque reduction per cent.

of the experiments by means of titration of the infectious virus yield. The quantitative antiviral action of SV against FPV was determined by plaque reduction test which showed a high sensitivity of FPV to SV. Concentrations of 20, 10 and 5 μg/ml led to a complete plaque reduction. More than 35 per cent inhibition as compared to the control was demonstrated also with concentrations lower than 1 μg/ml (Fig. 1).

The complete inhibition of the virus yield was demonstrated in one-step growth cycle experiments, when SV had been added immediately after virus adsorption. Application of SV up to 4 hr after infection also led to complete

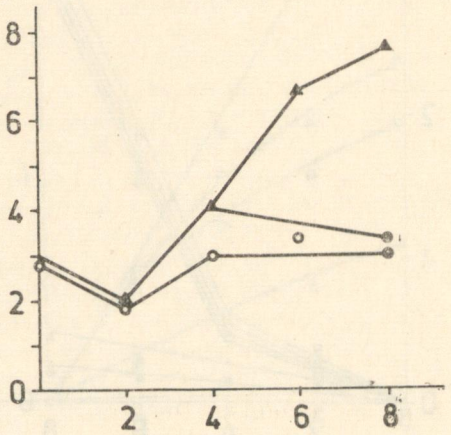

Fig. 2.

One-step replication of FPV in CEC treated with SV
The antibiotic complex (5 μg/ml) was added at 1 and 4 hr after virus adsorption; input multiplicity 20 PFU/cell. By 8 hr after virus infection the infectious virus yield was determined.
▲——▲ Titre of control virus.
○——○ Titre in infected cultures treated with 5 μg/ml SV since 1 hr after infection.
●——● The titre of the infected cultures treated with 5 μg/ml SV since 4 hr after infection.
Abscissa: hr after infection; ordinate: virus titre in $\log_{10}$ TCD_{50}/0.2 ml.

Table 3. Reversion of antiviral action of SV (1 μg/ml) after removal of the antibiotic at 2, 3 and 4 hr post infection*

Duration of treatment	Haemagglutinin units/ml at hr					
	2	3	4	8	10	24
2 hr	8	8	16	64	64	256
3 hr	8	16	16	32	32	64
4 hr	8	16	16	16	16	32
Virus control	16	16	32	64	128	512

* Virus adsorption for 1 hr; CEC stationary cultures infected with FPV.

suppression of virus reproduction. The virus yield was as low as the initial virus titre (Fig. 2). The inhibitory activity of the drug could be reversed by its removal. Haemagglutinin synthesis began by a few hr delay when the duration of treatment was not longer than two hours. When the infected cells were incubated with SV for a longer period, the reversibility of the drug effect decreased with the prolongation of treatment (Table 3).

In vivo experiments

The efficacy of SV against influneza A/England in white mice showed of 23% protection when administered in a dose od 0.025 mg/kg by oral route. However, SV exhibited a toxic effect when given orally at higher doses (Table 4).

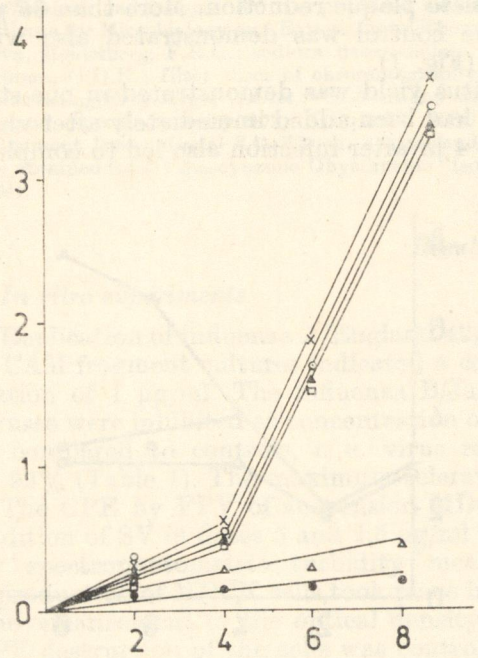

Fig. 3.

Incorporation of ^{3}H-uridine into cellular and viral RNA

One-step growth cycle of FPV infected CEC by as indicated by cumulative labelling, in the absence and presence of SV and or actinomycin D.

×——× Incorporation rate into cellular RNA.

○——○ Incorporation rate into cellular RNA in the presence of 5 μg/ml SV.

●——● Incorporation rate into cellular RNA in the presence of 2 μg/ml actinomycin D.

▲——▲ Incorporation rate into FPV-infected CEC

□——□ Incorporation rate into FPV--infected CEC in the presence of 5 μg/ml SV.

△——△ Incorporation rate into FPV--infected CEC in the presence of 2 μg/ml actinomycin D.

Abscissa: hr after infection; ordinate: ^{3}H-uridine (cpm × 10^{-3}).

Table 4. Action of SV in mice intranasally infected with influenza virus A/England 42/72

Dose of SV	Uninfected SV treated	Treated and infected	Untreated, infected
15*	0/10**	0/10	1/10
1.5	6/10	0/10	1/10
1.0	2/10	1/10	1/10
0.5	10/10	3/10	1/10
0.025	'10/10	2/10	1/10
0.005	10/10	1/10	1/10

* mg/kg body weight daily.
** Numerator = number of survivors; denominator = number of total animals used. For details see Materials and Methods.

Radiobiological assays

The radiobiological assays showed that no effect on cellular and virus-induced RNA synthesis could be achieved in the absence of actinomycin D when SV was added in concentration of 5 μg/ml (Fig. 3). The influence of SV on the glycosylation process of FPV was investigated by means of labelled ^{14}C-glucose incorporated into lipid-linked oligosaccharides. As follows from the results obtained, SV in concentration of 10 μg/ml decreased by 50% the cellular protein synthesis; the incorporation rate lower than 50%

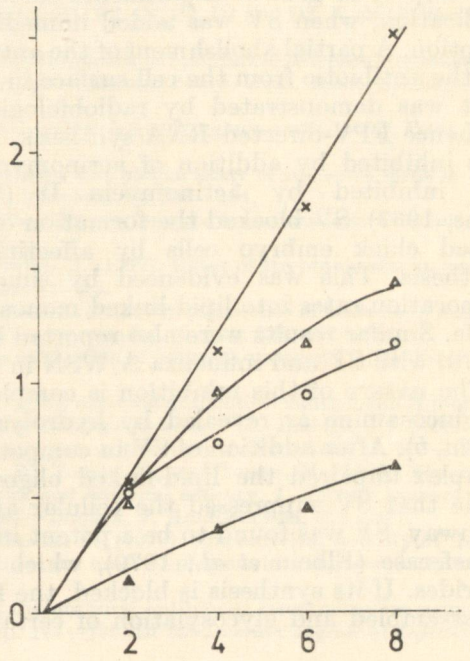

Fig. 4.
Incorporation of ^{14}C-glucose into lipid-linked oligosaccharides
One-step growth curve of FPV-infected CEC as determined by cumulative labelling.
×——× Incorporation rate of ^{14}C-glucose into lipid-linked oligosaccharides (control).
○——○ Incorporation rate of ^{14}C-glucose into lipid-linked oligosaccharides in the precense of 10 μg/ml SV.
△——△ Incorporation rate of ^{14}C-glucose into lipid-linked oligosaccharides of FPV infected CEC.
▲——▲ Incorporation rate of ^{14}C-glucose into lipid-linked oligosaccharides of FPV-infected CEC treated with 10 μg/ml SV.
Abscissa: hr after infection; ordinate: ^{14}C-glucose (cpm × 10^{-3}).

was due to the influence on the virus-directed lipid-linked oligosaccharide synthesis (Fig. 4).

At present it is not known whether SV plays a role in the formation or activation of cellular polyribosomes.

Discussion

Antibiotics of the SV complex were shown to inhibit a great number of RNA and DNA viruses belonging to different families (Tonew and Eckardt, 1974; Tonew *et al.*, 1975; Kobus *et al.*, 1977; Elbein *et al.*, 1979; Pan *et al.*, 1979). The results of the present study demonstrate that SV possess a significant antiviral effect in vitro against six serotypes of influenza viruses, such as influenza A/FPV, A/England 42/72, A/Port Chalmers 1/73, B/Tokyo 7/66, B/Jamagaty 1/73 and B/Leningrad 235/74. The data described here support our premise that SV is potentially a useful substance in molecular biology as an inhibitor of viral or cellular glycosylation. In the presence of 20—5 μg/ml SV (maximally tolerated dose for CEC and MDCK was 100 μg/ml), the plaque reduction of FPV was nearly 100%. In suspended CAM fragments the decrease of infectious yield of influenza A/England 42/72, A/Port Chalmers 1/73, B/Tokyo 1/73, B/Jamagata 1/73 and B/Leningrad 235/74 viruses amounted more than 4 log ID_{50} units, i. e. 99.99%: in the presence of 0.5—1.0 μglml drug. The inhibition of virus-induced CPE in MDCK cells estimated by spectrophotometric turbidity measurements also showed the complete prevention of cell destruction. The investigation of infectious virus yield by one-step cycle growth experiments showed the complete inhibition of virus replication, when SV was added immediately or up to 4 hr after virus adsorption. A partial abolishment of the antivital action was possible by washing off the antibiotic from the cell surface in CEC-infected with FPV.

It was demonstrated by radiobiological assays that the SV could not influence FPV-directed RNA synthesis. At the same time influenza A/FPV was inhibited by addition of actinomycin D. Influenza A/WSN and FPV are inhibited by actinomycin D (Barry, 1964; Bukrinskaja, 1967; Pons, 1967). SV blocked the formation of mature virus particles in FPV infected chick embryo cells by affecting the lipid-linked oligosaccharide synthesis. This was evidenced by time dependent lower ^{14}C-glucose incorporation rates into lipid-linked monosaccharides in one-step virus growth cycle. Similar results were also reported by Elbein *et al.* (1979) an Pan *et al.* (1979) with SV and influenza A/WSN in canine kidney cells.

The nature of this inhibition is complex resulting from secondary effects of glucosamine as revealed by hydrolysation experiments (Eckardt *et al.*, 1973*a*, *b*). After addition of SV in compatible quantities, the SV-glucosamine complex impaired the lipid-linked oligosaccharide synthesis. One can conclude that SV suppressed the cellular and the virus-directed glycosylation pathway. SV was found to be a potent inhibitor of N-acetylglucosamine-1-P transferase (Elbein *et al.*, 1979), which deals with processing of oligosacharides. If its synthesis is blocked, the lipid-linked oligosaccharides cannot be assembled and glycosylation of certain proteins is inhibited.

Further experiments were aimed to determine the minimal concentration of SV effective in mouse. The assays showed that concentrations of 0.5 and 0.025 mg/kg had a slight influence on the survival rates. However, SV cannot be used by oral route against influenza A/England in mice, although SV contained glucosamine in its molecule. D-glucosamine only is known to increase the survival of mice inoculated with human influenza virus (Floc'h and Werner, 1976).

Acknowledgement. The authors are grateful to Dr. V. Bubovic of the August Kirchenstein Institute of Microbiology, Latvian S.S.R. Academy of Sciences, for helpful discussion of the results.

References

Augsten, K., and Tonew, W. (1975): Eine nephelometrische Methode für die in vitro-Testung antiviraler Substanzen. *Acta histochem.* (*Jena*) **53**, 97—102.

Barry, R. D. (1964). The effect of actinomycin D and ultraviolet irradiation on the production of fowl plaque virus. *Virology* **24**, 563—569.

Bukrinskaja, A. G. (1968): Effect of actinomycin D on the reproduction of Newcastle disease and fowl plaque virus. *Rev. roum. Inframicrobiol.* **4**, 9—14.

Eckardt, K., Thrum, H., Bradler, G., Fügner, R., Tonew, E., and Tonew, M. (1973*a*): Verfahren zur Gewinnung eines Antibioticums. DDR Patent 102 162/5. 12.

Eckardt, K., Thrum, H., Bradler, G., Fügner, R., Tonew, E., Tonew, M., and Tkocz, H. (1973*b*): Streptovirudine, neue antibiotica mit antiviraler Wirksamkeit. *Z. allg. Mikrobiol.* **13**, 625—627.

Eckardt, K., Thrum, H., Bradler, G., Tonew, E., and Tonew, M. (1975): Streptovirudins — new antibiotics with antibacterial and antiviral activity. II. Isolation, chemical characterization and biological activity of streptovirudins A1, A2, B1, B2, C1, C2, D1 and D2. *J. Antibiotics* **28**, 274—279.

Elbein, A. D., Gafford, J., and Kang, M. S. (1979): Inhibition of lipid-linked saccharide synthesis: Comparison of tunicamycin, streptovirudin and antibiotic 24010. *Arch. Biochem. Biophys.* **169**, 311—318.

Floc'h, F., and Werner, G. H. (1976): In vivo antiviral activity of D-glucosamine. *Arch. Virol.* **52**, 169—173.

Gausch, C. R., and Smith, T. F. (1968): Replication and plaque assay of influenza virus in an established line of canine kidney cells. *Appl. Microbiol.* **16**, 588—594.

Horwath, A. (1954): A new sensitive method of the rolling drum type for influenza virus titration. *Acta microbiol. Acad. Sci. hung.* **1.**, 481—494.

Kobus, M., Korbecki, M., Szmigielski, S., and Rytko, J. (1977): Effect of streptovirudin on parainfluenza 3 virus multiplication. *Acta virol.* **21**, 31—35.

Pan, Y. T., Schmitt, J. W., Sandford, B., and Elbein, A. D. (1979): Adherence of bacteria to mammalian cells. Inhibition by tunicamyxin, and streptovirudin. *J. Bact.* **139**, 507—514.

Pons, M. (1967): Effect of actinomycin D on the replication of influenza virus and RNA. *Virology* **33**, 150—154.

Takatzuki, A., Tamura, C., and Arima, K. (1968): New antiviral antibiotics: xanthocillin X mono- and dimethylether, and methoxyxanthocillin X dimethylether. II. Biological aspects of antiviral activity. (Studies on antiviral and antitumor antibiotics). *J. Antibiotics* **21**, 667—680.

Thrum, H., Eckardt, K., Bradler, G., Fügner, R., Tonew, E., and Tonew, M. (1975): Streptovirudins — new antibiotics with antibacterial and antiviral activity. I. Culture taxonomy, fermentation and production of streptovirudine complex. *J. Antibiotics* **28**, 514—521.

Tonew, E., Augsten, K., Gumpert, B., and Ulbricht, H. (1973): The antiviral activity of 1-p'-(methylnitrosamino)-benzyl-idenamino-adamantane on fowl plague virus in tissue cultures. *Acta microbiol. pol.* **3—4**, 221—223.

Tonew, E., and Eckardt, K. (1974): Action of the antiviral antibiotics streptovirudins against DNA-containing viruses in tissue cultures, pp. 187—192. In *6th Intern. Congress* of *Infectious and Parasitic Diseases*, Warsaw, 23—27.9.

Tonew, E., and Tonew, M. (1969): Die Wirkung von Cycloheximid auf Abortus-Virus der Schafe, Sindbis-Virus and andere Viren in Screening-Untersuchungen. *Zbl. Bakt. I. Orig.* **211**, 437—444.

Tonew, E., Tonew, M., Eckardt, K., Thrum, H., and Gumpert, B. (1975): Streptovirudins — new antibiotics with antiviral activity. The antiviral spectrum and the inhibition of Newcastle disease virus in cell cultures. *Acta virol.* **19**, 311—317.

Tonew, M., and Tonew, E. (1971): Antivirale Wirkung von Imidazolderivaten. I. Die Hemmung der Vermehrung von Mengovirus in FL Zellen. *Arch. ges. Virusforsch.* **33**, 319—329.

Tonew, M., and Tonew, E. (1974): Effects of some isatinisothiosemicarbazones on cellular and viral ribonucleic acid synthesis in mengovirus-infected FL cells. *Antimicrob. Ag. Chemother.* **5**, 393—397.