Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

The influence of stabilizers and rates of freezing on preserving of structurally different animal viruses during lyophilization and subsequent storage

H. Malenovská

Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Brno, Czech Republic

Keywords
animal virus lyophilization, animal virus preservation, freezing rate, stabilizer, virus structure.

Correspondence
Hana Malenovská, Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic.
E-mail: Malenovska@vri.cz

2014/1130: received 1 June 2014, revised 1 September 2014 and accepted 18 September 2014

doi:10.1111/jam.12654

Abstract

Aims: To make a comparative evaluation of the effects of different stabilizers and freezing rates on structurally different viruses during lyophilization and storage.
Methods and Results: Two virus strains from each of six animal virus families, including both enveloped and nonenveloped viruses, were lyophilized in (i) culture medium, (ii) with the addition of gelatine–sucrose and (iii) skim milk–sodium glutamate. All the virus suspensions were frozen (i) at −80°C or (ii) in liquid nitrogen before lyophilization. Virus titre assay after lyophilization and after 8 months storage at 4°C revealed that the efficacy of stabilizers depended on virus structure. Generally, the best protective quality for enveloped viruses was achieved with gelatine–sucrose, which best maintained their infectivity and envelope morphology. Even additive-free culture medium proved adequate for nonenveloped viruses. Differences in stabilizer efficacy were also found between virus families and were expressed immediately after lyophilization; the activity of stabilizers in the course of storage was very similar. Freezing in liquid nitrogen proved beneficial for picornaviruses.
Conclusions: The choice of an appropriate stabilizer with respect to virus type is crucial for effective lyophilization.
Significance and Impact of the Study: This study contributes to the establishment of general guidelines for animal virus lyophilization, with particular respect to differences in virus structure.

Introduction

For decades, lyophilization has been widely used for virus preservation. Lyophilized viruses are not only stable and undemanding in long-term storage but are also transportable at ambient temperatures. Lyophilization is therefore widely employed in culture collections to maintain virus stocks and in the pharmaceutical industry to preserve live viruses. Despite the use of a wide range of drying technologies and many properly developed specific methodologies for particular strains (Sarkar *et al.* 2003; Kang *et al.* 2010), more generalized guidelines for virus lyophilization, which allow for differences in virus structure and taxonomy, are still lacking.

Apart from vacuum and temperature conditions, the major factors affecting virus stability during lyophilization are chemical additives and prefreezing methods (Zhai *et al.* 2004). Rapid freezing and the addition of certain cryoprotectants before lyophilization reduce the size of ice crystals formed (Buitink *et al.* 2000; Zhai *et al.* 2004; Hansen *et al.* 2005), decreasing the level of damage to viral particles during the freezing process. Moreover, cryoprotectants such as carbohydrates and polypeptides form amorphous glass states that immobilize biomolecules and protect them during both the drying stage and storage. However, the protective effect does not appear to be directly correlated with the formation of such glass states (Carpenter *et al.* 2010). The mechanisms that may

 Journal of Applied Microbiology **117**, 1810–1819 © 2014 The Society for Applied Microbiology

be involved in the protection of viruses appear to be more complex. Some sugars are known to form hydrogen bonds to polar and charged groups as water is removed, thus preventing the damage to biomolecules that arises out of drying (Tanaka *et al.* 1991). Sodium glutamate neutralizes the carbonyl groups in the medium that are harmful to virus protein in a lyophilized state (Ferris *et al.* 1990). Furthermore, the different collapse temperatures (T_g) of the various additives may influence the structure of the cake created during lyophilization and thus affect the shelf life of the sample (Chang and Patro 2004). The stability of structurally different viruses during lyophilization and subsequent storage is evidently dissimilar (Gould 1995; Hubalek 1996). The suitability of particular stabilizers varies for each virus; the stabilizer itself induces or imposes specific stresses during the lyophilization process, notably chemical and osmotic stress and pH shift during crystallization (Peetermans 1996).

The aim of this study was to compare the effects on structurally different animal viruses of three types of stabilizers in combination with two freezing rates. Culture medium without additives was employed as the first stabilizer. Next, two stabilizer formulations based on culture medium combined with additives (gelatine, sucrose, skim milk and sodium glutamate) were tested. These organic substances appear to make a positive contribution to the lyophilization of many viruses, both separately and in various combinations (Suzuki 1970; Scott and Woodside 1976; Ferris *et al.* 1990; Hubalek 1996; Liska *et al.* 2007; Kang *et al.* 2010). To compare the differences of effect of stabilizers and freezing rates both between and within virus families, this study was carried out on six families, each represented by two selected strains: the adenoviruses (*Bovine adenovirus*, BAdV; *Canine adenovirus 1*, CAdV-1), caliciviruses (two strains of *Feline calicivirus*, FCV), coronaviruses (*Bovine coronavirus*, BCoV; *Transmissible gastroenteritis* virus, TGEV), herpesviruses (*Bovine herpesvirus*, BoHV-1; *Canid herpesvirus 1*, CaHV-1), paramyxoviruses (*Bovine parainfluenza virus 3*, BPIV-3; *Canine parainfluenza virus*, CPIV) and picornaviruses (*Porcine teschovirus*, PTV; *Bovine enterovirus*, BEV). The selected virus families included three enveloped (coronaviruses and paramyxoviruses—ss RNA, herpesviruses—ds DNA) and three non-enveloped viruses (adenoviruses—ds DNA, caliciviruses and picornaviruses—ss RNA).

Materials and methods

Viruses and cell cultures

The viruses were obtained from the Collection of Animal Pathogenic Microorganisms (CAPM) incorporated in the National Programme of Protection of Genetic Resources of Economically Significant Microorganisms and Tiny Animals of the Czech Republic. *Bovine herpesvirus* 1 (BoHV-1, strain LA, CAPM V-25), *Bovine parainfluenza virus 3* (BPIV-3, strain T6/3, CAPM V-32), *Bovine coronavirus* (BCoV, strain C-197, CAPM V-326), *Bovine adenovirus* (BAdV, strain HD-Ad-3, CAPM V-295) and *Bovine enterovirus* (BEV, strain PSU-83, CAPM V-235) were grown in MDBK cell line (ATCC, CCL-22). *Canid herpesvirus 1* (CaHV-1, strain F 205, CAPM V-479), *Canine parainfluenza virus* (CPIV, strain 78-238, CAPM V-480) and *Canine adenovirus 1* (CAdV-1, strain Utrecht, CAPM V-478) were propagated in A-72 cell line (ATCC, CRL-1542). *Transmissible gastroenteritis virus* (TGEV, strain SH, CAPM V-66) was cultivated in PD 5 cells (CSC-CLCH, BS CL 99) and *Porcine teschovirus* (PTV, strain Talfan, CAPM V-37) in PK 15 cells (ATCC, CCL-33). *Feline calicivirus* (FCV, strain F-9, CAPM V-238 and strain 255, CAPM V-254) were propagated in CRFK cell line (ATCC, CCL-94). The cell cultures inoculated with viruses were cultivated at 37°C in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Saint Louis, UK) supplemented with 3% heat-inactivated foetal calf serum (PAA Laboratories, Pasching, Austria) until cytopathology was observed in 100% of the cells; they were then frozen at −80°C.

Stabilizers

The following solutions were used as stabilizers: 1) culture medium with no additive, 2) culture medium with 2·5% w/v gelatine (HiMedia, Mumbai, India) and 3·5% w/v sucrose (HiMedia, Mumbai, India), 3) culture medium with 10% w/v skim milk (Oxoid, Basingstoke, England) and 1% w/v sodium glutamate (Fluka, Buchs, Switzerland). Gelatine and sucrose were dissolved in distilled water separately (50 ml volume each), gelatine was autoclaved at 127°C for 150 min, then mixed with sucrose, the pH adjusted to 7·3 and then everything was autoclaved together at 121°C for 20 min. Skim milk and sodium glutamate were previously dissolved in 100 ml of distilled water; pH was adjusted to 7·3, and the stabilizers were autoclaved at 121°C for 20 min. Solutions were lyophilized according to the protocol of lyophilization for virus suspensions that appears below and stored at 4°C before use.

Freezing and lyophilization

Viruses were thawed and centrifuged at 2000 ***g*** for 15 min to remove cellular debris, and their aliquots were assayed for virus titre; selected enveloped viruses were investigated by means of transmission electron microscopy (TEM). One portion of the virus suspension was

kept for lyophilization in culture medium without additional stabilizer, and the remaining suspension was pipetted into ampoules with lyophilized stabilizers to achieve original volume and properly mixed. Aliquots of 0·5 ml of virus suspension and virus-stabilizer mixtures were pipetted into trimmed stem ampoules and frozen at two different rates: (i) at −80°C for 2 h and (ii) by immersion into liquid nitrogen (−196°C) for 30 min. Frozen samples were lyophilized in ampoules connected to sample valves on the port manifold of a Labconco FreeZone 7670530 laboratory lyophilizer at a collector temperature of −50°C and under high vacuum (≤4 Pa) for 20 h. The ampoules were flame-sealed under vacuum while connected to a valve, using a sealing torch. One ampoule of each virus combined with stabilizer and freezing rate was assayed immediately for virus titre and TEM investigated in enveloped viruses. The remaining ampoules were stored at 4°C for titre assay after 8 months.

Virus titration

Lyophilized viruses were rehydrated in sterile distilled water to original volume at room temperature. Serial 10-fold dilutions of the virus supernatant were prepared and 100 μl of each dilution inoculated to five wells of a 48-well microtitration plate with an appropriate cell culture monolayer. Eight wells were used as a negative control. The plates were incubated for 7 days at 37°C in a 5% CO_2 atmosphere, and then, the cultures were checked under a light microscope for virus cytopathic effect. The dilution of the suspension that causes cytopathology in half of the cultures (the median tissue culture infective dose, *lg* $TCID_{50}$/ml) was calculated after Spearman and Kaerber (Kaerber 1931).

Transmission electron microscopy (TEM)

Lyophilized viruses were rehydrated in sterile distilled water at room temperature. Suspensions containing skim milk were centrifuged at 8000 ***g*** for 15 min to pellet most of the casein. The virus suspension was applied onto a formvar-carbon-coated 400-mesh copper grid, allowed to settle for 1 min and drained away from the grid. The sample was stained with 2% aqueous uranyl acetate at pH 4·5 for 7 min and viewed in a Philips EM 208 transmission electron microscope at 110 000–180 000× magnification.

Scanning electron microscopy (SEM)

SEM was performed to investigate the matrix morphology of the lyophilized stabilizers. Samples for SEM were prepared according to the lyophilization protocol and included all six combinations of stabilizers and freezing methods. Pure culture medium was used instead of virus suspension. A lyophilized cake was cut off with a sharp scalpel; a cross section was sputtered with palladium–platinum to an average thickness of 10 nm and analysed with a Hitachi SU8010 SEM operated in beam deceleration mode at 1 kV and 15 μA.

Statistical analysis

The data resulting from titre reduction (Δ *lg* $TCID_{50}$) of viruses after lyophilization and storage with various combinations of stabilizer and freezing rate were analysed by means of four-factor, partly nested ANOVA followed by Tukey s *post hoc* test. The effects of the following factors were tested: stabilizer type (three levels, fixed), freezing rate (two levels, fixed), broader virus structure category: enveloped/nonenveloped (two levels, fixed), and structure category nested in enveloped/nonenveloped: virus family (six levels, fixed). Stabilizer and freezing remained orthogonal to virus family (and enveloped/nonenveloped). To evaluate the suitability of stabilizer and freezing rates for different viruses within each family, three-factor ANOVA (fixed factors: stabilizer type, freezing rate; random factor: virus strain at two levels) followed by Tukey s *post hoc* test was used. Differences in the titre of each virus were established immediately after lyophilization and evaluated by paired *t*-test after lyophilization and subsequent storage. The computations were performed in STATISTICA 64 ver. 12 software (Statsoft, Tulsa, OK).

Results

Effects of stabilizers and freezing rates on $TCID_{50}$ among all viruses tested

Reduction of virus titre was assayed for all virus stabilizer–freezing rate combinations immediately after lyophilization and after 8 months' subsequent storage at 4°C. Titre reductions Δ *lg* $TCID_{50}$ after lyophilization only, storage duration only and in total are summarized in Table 1. No significant effect (at $P \leq 0{\cdot}05$) on virus titre reduction for any of the three stabilizers and two freezing rates tested was disclosed when all the viruses were analysed together (four-factor partly nested ANOVA) in any of the periods tested.

Relationship between $TCID_{50}$ reduction and virus structure

The degree to which virus structure was related to titre reduction after lyophilization and storage with various stabilizers and freezing rates was investigated. The virus

Table 1 Effects of stabilizers and freezing rates on virus titre reduction (Δ *lg* $TCID_{50}$) after lyophilization and 8 months' storage at 4°C

		Titre reduction: After lyophilization/in storage duration only (after lyophilization and storage) (Δ *lg* $TCID_{50}$)					
		Freezing −80°C			Freezing N_2		
Virus	Titre (*lg* $TCID_{50}$)	Stabilizer 1*	Stabilizer 2†	Stabilizer 3‡	Stabilizer 1*	Stabilizer 2†	Stabilizer 3‡
Coronaviridae							
TGEV	7·5	0·6/2·2 (2·8)	0·8/1·6 (2·4)	1·4/1·4 (2·8)	1·0/2·4 (3·4)	0·4/2·0 (2·4)	1·4/1·6 (3·0)
BCoV	6·3	1·0/1·0 (2·0)	1·0/1·0 (2·0)	1·6/0·8 (2·4)	0·8/1·4 (2·2)	0·8/1·2 (2·0)	1·2/1·4 (2·6)
Herpesviridae							
BoHV-1	7·9	0·8/0·4 (1·2)	0·0/0·8 (0·8)	0·6/0·4 (1·0)	0·8/0·4 (1·2)	0·2/0·6 (0·8)	0·2/0·6 (0·8)
CaHV-1	2·9	1·0/0·2 (1·2)	0·2/0·4 (0·6)	0·4/0·2 (0·6)	1·2/0·2 (1·4)	0·2/0·2 (0·4)	0·6/0·4 (1·0)
Paramyxoviridae							
BPIV-3	8·5	1·4/0·4 (1·8)	0·8/0·4 (1·2)	0·6/1·6 (2·2)	1·8/0·2 (2·0)	0·4/0·6 (1·0)	0·8/1·4 (2·2)
CPIV	5·5	1·6/0·0 (1·6)	0·6/0·4 (1·0)	0·4/1·6 (2·0)	1·0/0·4 (1·4)	0·2/0·6 (0·8)	0·4/1·0 (1·4)
Adenoviridae							
BAdV	5·3	0·6/0·0 (0·6)	0·4/0·0 (0·4)	0·6/−0·2 (0·4)	1·0/−0·4 (0·6)	0·2/−0·2 (0·0)	0·6/−0·4 (0·2)
CAdV-1	7·5	0·8/−0·8 (0·0)	0·4/−0·2 (0·2)	0·6/−0·8 (−0·2)	0·8/−0·6 (0·2)	0·6/−0·4 (0·2)	0·8/−0·6 (0·2)
Caliciviridae							
FCV CAPM V-238	8·3	0·2/1·0 (1·2)	0·0/1·2 (1·2)	0·0/1·0 (1·0)	0·2/0·4 (0·6)	0·4/0·8 (1·2)	0·0/0·6 (0·6)
FCV CAPM V-254	8·1	0·2/0·8 (1·0)	0·2/1·0 (1·2)	0·2/0·4 (0·6)	0·4/0·8 (1·2)	0·2/1·4 (1·6)	0·4/0·6 (1·0)
Picornaviridae							
PTV	7·9	3·2/1·0 (4·2)	4·2/0·6 (4·8)	3·2/1·0 (4·2)	2·6/0·4 (3·0)	3·6/0·4 (4·0)	3·0/0·8 (3·8)
BEV	6·5	2·4/1·4 (3·8)	3·6/1·2 (4·8)	3·2/0·6 (3·8)	2·0/1·4 (3·4)	3·6/0·6 (4·2)	2·8/0·8 (3·6)

*Stabilizer 1: culture medium (DMEM, 3% foetal bovine serum) without additives.
†Stabilizer 2: culture medium, 2·5% gelatine and 3·5% sucrose.
‡Stabilizer 3: culture medium, 10% skim milk and 1% sodium glutamate.

Table 2 Relationship between $TCID_{50}$ reduction and virus structure

	Differences in titre reductions between virus families:† After lyophilization/in storage duration only (after lyophilization and storage)					
Virus family	Coronaviridae	Herpesviridae	Paramyxoviridae	Adenoviridae	Caliciviridae	Picornaviridae
Coronaviridae		*/**(**)	NS/**(**)	NS/**(**)	**/**(**)	**/**(**)
Herpesviridae	*/**(**)		NS/NS(**)	NS/**(**)	NS/NS(NS)	**/NS(**)
Paramyxoviridae	NS/**(**)	NS/NS(**)		NS/**(**)	**/NS(*)	**/NS(**)
Adenoviridae	NS/**(**)	NS/**(**)	NS/**(**)		NS/**(**)	**/**(**)
Caliciviridae	**/**(**)	NS/NS(NS)	**/NS(*)	NS/**(**)		**/NS(**)
Picornaviridae	**/**(**)	**/NS(**)	**/NS(**)	**/**(**)	**/NS(**)	

NS, nonsignificant differences.
†Differences between virus families in titre reduction after lyophilization and 8 months' storage at 4°C with different stabilizers and freezing rates. Four-factor partly nested ANOVA followed by Tukey s *post hoc* test: *, **significant differences ($P \leq 0{\cdot}05$ and $P \leq 0{\cdot}01$, respectively).

titre reduction data (Δ *lg* $TCID_{50}$) from Table 1 were used for the analyses. Virus structure categories were defined as enveloped/nonenveloped viruses (broader category), and by virus family membership (nested category). For the periods tested (lyophilization only, storage duration only and in total), virus family had a significant effect on titre reduction at $P \leq 0{\cdot}05$ (four-factor, partly nested ANOVA). Significant differences between enveloped and nonenveloped viruses were found after lyophilization and in the storage period, whereas total Δ *lg* $TCID_{50}$ was nonsignificant at $P \leq 0{\cdot}05$. The differences between individual virus families in titre reduction are summarized in terms of *P* values in Table 2 (four-factor partly nested ANOVA, Tukey s *post hoc* test).

The influence of stabilizers and freezing rates on viruses of the same family

In Table 3, titre reduction data are analysed for every family with the stabilizers and freezing rates ordered by their stabilizing efficiency within virus families; individual virus differences within the family are indicated

Table 3 Efficiency of stabilizers and freezing rates within virus families

	Lyophilization			Storage			Lyophilization and storage		
Virus family	Stabilizer efficiency†	Freezing rate efficiency†	Within-family virus difference‡	Stabilizer efficiency†	Freezing rate efficiency†	Within-family virus difference‡	Stabilizer efficiency†	Freezing rate efficiency†	Within-family virus difference‡
Coronaviridae	1=2 > 3	N_2 = −80°C	NS	1=2=3	−80 > N_2	**	1=2 > 3	N_2 = −80°C	**
Herpesviridae	2=3 > 1	N_2 = −80°C	NS	1=2=3	N_2 = −80°C	**	2=3 > 1	N_2 = −80°C	NS
Paramyxoviridae	2=3 > 1	N_2 = −80°C	NS	1=2 > 3	N_2 = −80°C	NS	2 > 1=3	N_2 = −80°C	**
Adenoviridae	2 > 1=3	N_2 = −80°C	NS	1=2=3	N_2 = −80°C	**	1=2=3	N_2 = −80°C	NS
Caliciviridae	1=2=3	N_2 = −80°C	NS	1=2=3	N_2 = −80°C	NS	1=2=3	N_2 = −80°C	NS
Picornaviridae	1 > 3 > 2	N_2 > −80°C	*	1=2=3	N_2 = −80	NS	1=3 > 2	N_2 > −80°C	NS

NS, nonsignificant differences.
†Stabilizers 1, 2 and 3 (see Material and methods and Table 1 for stabilizer composition) and freezing rates N_2 and −80°C ordered by efficiency (better than > , equal=) on the basis of three-factor ANOVA, *post hoc* Tukey s test with differences considered significant at $P \leq 0{\cdot}05$.
‡Differences in titre reduction between two viruses in each virus family evaluated on the basis of three-factor ANOVA: *, ** - significant differences ($P \leq 0{\cdot}05$ and $P \leq 0{\cdot}01$, respectively).

(three-factor ANOVA, *post hoc* Tukey's test, $P \leq 0{\cdot}05$). As protectants during lyophilization, the efficacy of the stabilizers tested showed significant differences for most of the families. Further, for all enveloped viruses, stabilizer 2 (culture medium, 2·5% gelatine and 3·5% sucrose) proved more efficient in preserving the titre at $P \leq 0{\cdot}05$ than other stabilizers. As storage protectants, all stabilizers showed similar preservative activity within nearly all the families. The exception was the paramyxoviruses, among which stabilizer 3 (culture medium, 10% skim milk and 1% sodium glutamate) demonstrated significantly inferior storage stabilizing properties. In most families, freezing rate appeared to have no influence, with the exception of the picornaviruses and coronaviruses. Picornaviruses were more stable after lyophilization and maintained better stability, even after the storage period, when they had been frozen more rapidly in liquid nitrogen. In contrast, coronaviruses showed better stability when they were frozen at −80°C, but only in the storage period. Moreover, an increase in *lg* $TCID_{50}$ after the storage period was determined for the adenoviruses. Whereas the titres of adenoviruses assayed immediately after lyophilization in comparison with those assayed after the subsequent 8 months' storage showed a significant increase, the titres of other families decreased significantly (Table 1, paired *t*-test, $P \leq 0{\cdot}05$).

Relationship between stabilizers and freezing rates and morphology of viral envelopes

The effects of stabilizers and freezing rates on the morphology of enveloped viruses during lyophilization were investigated by TEM. The numbers of particles in the intact stage and the uncoating stage (visibly disrupted envelope or totally uncoated nucleocapsid) before and after lyophilization with respect to their residual infectious titres are summarized in Table 4. Micrographs of viral particles representative of these morphological classes are shown in Fig. 1. Morphological changes in TGEV (coronavirus) were slight, with only moderately increased numbers of damaged particles observed in suspensions after lyophilization. Monitoring of naked TGEV nucleocapsids was not feasible because of their naturally disordered appearance. However, damage to BoHV-1 (herpesvirus) envelopes during lyophilization was clear. The number of BoHV-1 particles with disrupted membrane and released tegument increased with decreases in titre recovery. The highest number of damaged particles of BoHV-1 was determined for lyophilization with stabilizer 1 (culture medium without additive), together with the lowest titre recovery of 16%. The most notable effect of lyophilization was observed on the morphology of BPIV-3 (paramyxovirus) particles. The numbers of particles in the extreme uncoating stage, that is clusters of naked nucleocapsids, increased the most (more than ten times) for lyophilization with stabilizer 1 (culture medium without additive), resulting in the lowest titre recoveries (4% and 2%).

Stabilizers, freezing rates and cake matrix morphology

SEM was used to investigate the micromorphology of cross sections of the cakes of the lyophilized viruses. The micrograph in Fig. 2 shows all three stabilizers at the two freezing rates. The cake structure of stabilizer 1 (culture medium without additive) collapsed during the lyophilization process, a process that was also macroscopically visible, and its micromorphology lacked the characteristic

Table 4 The effect of lyophilization with different stabilizers and freezing rates on morphology and infectious titre of enveloped viruses

Virus	Treatment*		Intact viral particles† (%)	Visibly disrupted envelops† (%)	Naked capsids† (%)	Residual titre‡ (%)
TGEV	Fresh	–	47	53	–	100
	Lyophilized	−80°C, stabilizer 1	40	60	–	25
	Lyophilized	−80°C, stabilizer 2	38	62	–	16
	Lyophilized	−80°C, stabilizer 3	39	61	–	4
	Lyophilized	N_2, stabilizer 1	35	65	–	10
	Lyophilized	N_2, stabilizer 2	48	52	–	40
	Lyophilized	N_2, stabilizer 3	36	64	–	4
BoHV-1	Fresh	–	43	44	13	100
	Lyophilized	−80°C, stabilizer 1	15	55	30	16
	Lyophilized	−80°C, stabilizer 2	39	42	19	100
	Lyophilized	−80°C, stabilizer 3	30	50	20	25
	Lyophilized	N_2, stabilizer 1	11	55	34	16
	Lyophilized	N_2, stabilizer 2	39	43	18	63
	Lyophilized	N_2, stabilizer 3	28	54	18	63
BPIV-3	Fresh	–	86	11	3	100
	Lyophilized	−80°C, stabilizer 1	29	29	42	4
	Lyophilized	−80°C, stabilizer 2	39	39	22	16
	Lyophilized	−80°C, stabilizer 3	27	53	20	25
	Lyophilized	N_2, stabilizer 1	17	34	49	2
	Lyophilized	N_2, stabilizer 2	34	38	28	40
	Lyophilized	N_2, stabilizer 3	39	40	13	16

*Descriptions of each stabilizer composition (1, 2 and 3) appear in the Material and methods section and explanations together with Table 1.
†Results obtained from transmission electron microscopy of 200 viral particles.
‡Data transformed from $\Delta\ lg\ TCID_{50}$ as: $100/(exp_{10}(\Delta lgTCID_{50}))$.

porous aspect. The cakes of stabilizer 2 (culture medium, 2·5% gelatine and 3·5% sucrose) and stabilizer 3 (culture medium, 10% skim milk and 1% sodium glutamate) were elegant and compact. Stabilizer 2 frozen at −80°C produced ice crystals that led to pores in the matrix, average size about 50 μm, whereas when frozen in liquid nitrogen, it produced ice crystals of about 10 μm in diameter and was more irregular in shape and size. SEM of −80°C-frozen stabilizer 3 revealed pores averaging approx. 40 μm in size. The pores of stabilizer 3 frozen in liquid nitrogen were irregularly shaped and directionally arrayed; average size was about 10 μm.

Discussion

For the broad spectrum of structurally different viruses examined in this study, no significant differences arising out of the stabilizing capacity of additives or freezing rates were determined. Some favourable protective activity for gelatine with sucrose and skim milk with sodium glutamate was anticipated (Suzuki 1970; Scott and Woodside 1976; Hubalek 1996; Liska *et al.* 2007; Kang *et al.* 2010), but the stabilizing activity of culture medium with no additives had never been studied properly before. However, some contributions have already demonstrated that this has a good protective function during the process of lyophilization, particularly in the stabilization of *Foot-and-mouth disease virus* (Fellowes 1965; Ferris *et al.* 1990). As sucrose and calf serum are present in the culture medium, a stabilizing effect may occur even with no additives (Grose *et al.* 1981; Hubalek 1996). Comparison of two freezing methods, at −80°C and in liquid nitrogen, in this study showed they were equally effective when all viruses were tested as a single group. Zhai *et al.* (2004) also pointed out that faster freezing before lyophilization did not always guarantee a better titre recovery. However, freezing at −80°C is known to be fast enough fully to maintain the infectivity of certain viruses (Hansen *et al.* 2005). Nevertheless, considerable differences in the effects of stabilizers and freezing rates between structurally different viruses were detected.

Although several authors have compared the stability of viruses of the same family in the lyophilization process under identical conditions (Berge *et al.* 1971; Tannock *et al.* 1987), a broader sampling enabling a comparison between different structural groups of viruses has hitherto been lacking. The present study shows that the action of various stabilizers and freezing rates clearly depends on virus structure. The presence (or absence) of a virus envelope and family classification had significant influences on titre reduction after lyophilization and storage. The amount of the titre reduction was similar in all

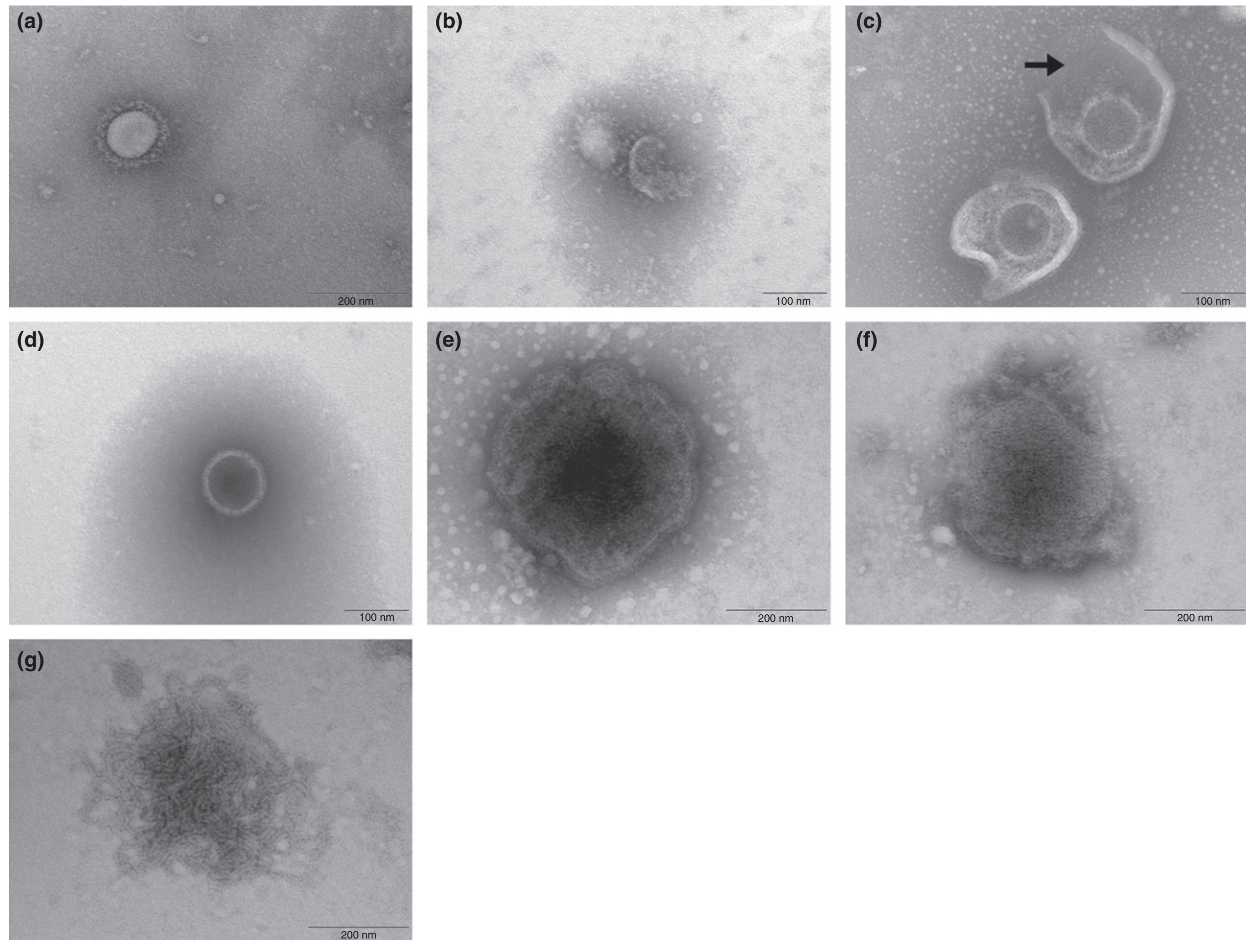

Figure 1 TEM micrographs (magnification 140 000–180 000×) of representative viral particles in the morphology classes related to the data in Table 4, summarizing morphological changes in viral envelopes after lyophilization. (a) full TGEV particle; (b) two disintegrating TGEV particles; (c) BoHV-1 enveloped particles: with (arrow) and without visible disruption in glycoprotein membrane; (d) a naked BoHV-1 nucleocapsid; (e) an intact BPIV-3 particle; (f) a particle of BPIV-3 with disrupted membrane and releasing nucleocapsid; (g) a cluster of naked BPIV-3 nucleocapsids.

enveloped viruses examined, but it varied widely within nonenveloped viruses. This would suggest that there is no general rule to encompass the sensitivity of a virus to lyophilization stress based on the presence or absence of an envelope alone. It has been recently shown that enveloped viruses are not generally more sensitive than nonenveloped ones (Tuladhar *et al.* 2012). Despite this, certain distinct, group-specific patterns emerged in the present study: the enveloped viruses were significantly better stabilized with the gelatine–sucrose additive compared to other stabilizers, while there were no differences in the effects of the various stabilizers on the nonenveloped viruses. Hence, the efficiency of stabilizers used appears to have a relationship with the presence or absence of the viral envelope. This becomes even more noticeable when the viruses are considered at family level. The responses of viruses of the same family to additives and freezing rates during lyophilization and storage were similar in the present study. Nevertheless, other studies have shown that even closely related viruses can differ in their sensitivity to identical stress, due to certain molecular differences in the proteins that make up viral particles (Coleman *et al.* 1973; Chambon *et al.* 1994; Bozkurt *et al.* 2013).

The best stabilizing activity ascertained here was for gelatine in combination with sucrose as an additive for all enveloped viruses; the same stabilizer also performed no worse than the others in the nonenveloped adenoviruses and caliciviruses. Gelatine and sucrose (in different proportions and combinations) have also been successfully used for the stabilization of enveloped viruses in other studies (Grose *et al.* 1981; Tannock *et al.* 1987; Sarkar *et al.* 2003; Liska *et al.* 2007), and thus, their combination appears to be generally suitable for this category

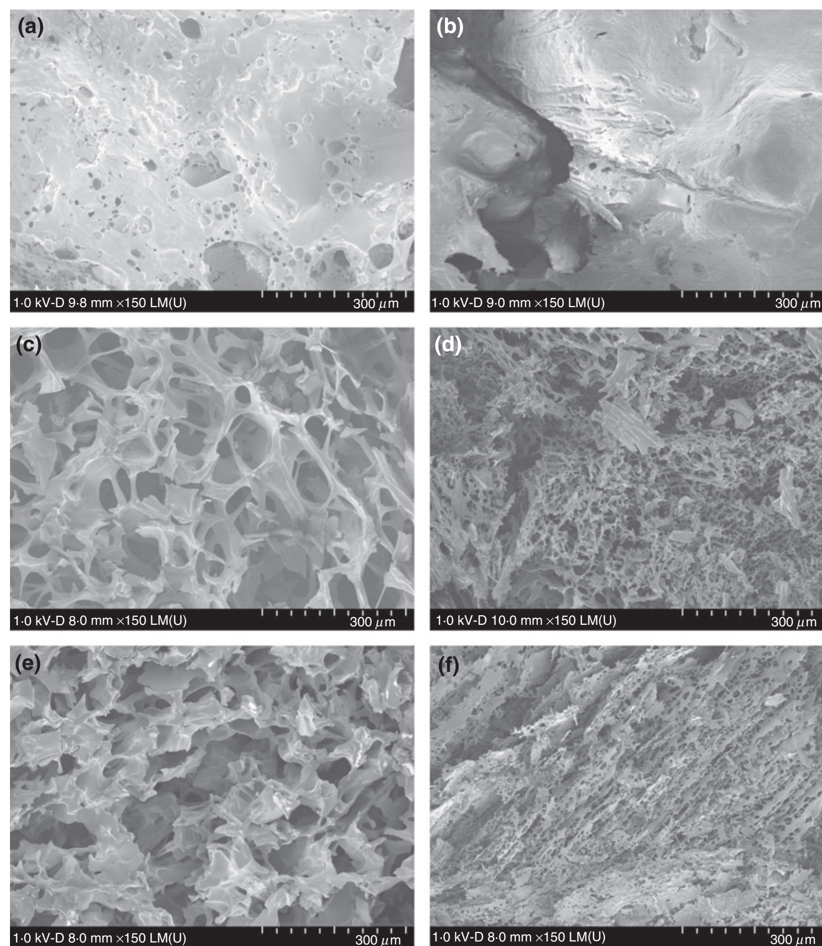

Figure 2 SEM micrographs (magnification 150×) of cross sections of lyophilized stabilizers at two freezing rates. (a, b) Freeze-dried matrix of stabilizer 1: DMEM, 3% foetal bovine serum without additive, frozen at −80°C and in liquid nitrogen, respectively; (c, d) matrix of stabilizer 2: DMEM, 3% foetal bovine serum, 2·5% gelatine and 3·5% sucrose, frozen at −80°C and in liquid nitrogen, respectively; (e, f) matrix of stabilizer 3: DMEM, 3% foetal bovine serum, 10% skim milk a 1% sodium glutamate, frozen at −80°C and in liquid nitrogen, respectively.

of viruses. However, gelatine with sucrose showed inferior protection activity for the nonenveloped picornaviruses here, although Kang *et al.* (2010) demonstrated stabilization of an attenuated *Duck hepatitis virus* (picornaviruses) vaccine using a lower concentration of gelatine (0·5%) in combination with sorbitol. The skim milk with sodium glutamate mixture did not stabilize coronaviruses and paramyxoviruses (both enveloped viruses) properly. For nonenveloped viruses, the protective activity of skim milk with sodium glutamate was not obvious, except for a slightly favourable effect in the picornaviruses. However, Suzuki (1970) successfully used protective media consisting of sodium glutamate in the poxviruses, another nonenveloped family (not examined here). Fellowes (1965) and Ferris *et al.* (1990) demonstrated that culture medium without additives provided adequate stabilizing activity for *Foot-and-mouth virus* (picornaviruses), as above, something that was also confirmed here for all nonenveloped viruses tested and also for coronaviruses. That the lyophilization of picornaviruses is generally difficult, as described by Berge *et al.* (1971) among others, was also confirmed in this study. Despite their excellent resistance to chemical stresses (Dvorakova *et al.* 2008), picornaviruses were inactivated more dramatically than other viruses in the tests, by 2–4·2 lg during lyophilization only. This observation may be related to the better effects of more rapid freezing in liquid nitrogen, which was found for only this family. Thus, only the extensive inactivation of picornaviruses could reveal an advantage of a faster freezing. In the adenoviruses, a curious increase of titre recovery occurred after the storage period in comparison with titres assayed immediately after lyophilization. A similar experimental result was found in other virus groups by Tannock *et al.* (1987) and explained in terms of the disaggregation of clumped particles after reconstitution of lyophilized material. This is a very probable explanation here as well, as adenoviruses form aggregates very readily (Galdiero 1979; Walkiewicz *et al.* 2009).

Uncoating of herpesviruses and consequent loss of infectivity during lyophilization without high concentration of protectants, especially sugars, has already been described by Grose *et al.* (1981), Hansen *et al.* (2005) and Zhai *et al.* (2004). In the present study, in the

medium without additives, obvious uncoating with corresponding titre loss due to lyophilization was observed in herpesviruses (BoHV-1) and paramyxoviruses (BPIV-3). The morphology of viruses lyophilized with additives deteriorated less; the damage was at its least for gelatine with sucrose as a stabilizer. In the coronaviruses (TGEV), no clear differences in virus envelope morphology emerged, although the titre recovery was significantly worse for the stabilizer with skim milk and sodium glutamate, whereas the medium without additives was as effective as the combination of gelatine and sucrose. This may be explained by the denaturing of certain envelope proteins without visible disruption of the membrane (Hansen *et al.* 2005) and a simultaneous loss of infectivity. The natural disordered appearance of coronavirus nucleoprotein, quite dissimilar from all the other viruses studied here (herpesviruses and paramyxoviruses), renders it possible to distinguish virus particles by TEM once they have completely lost their envelopes. This may well have distorted the counts of intact and defective particles in TGEV.

A collapsed cake structure was observed both macroscopically and microscopically in all viruses lyophilized in the culture medium without additives. Their cake microstructure lacked pores, a condition considered to lead to the degradation of the sample and adverse effects on its protective storage quality. This loss arises out of high and unevenly distributed residual moisture and weak reconstitution of lyophilized material, among other things (Chang and Patro, 2004; Puapermpoonsiri *et al.* 2010). However, the storage stability of the lyophilized viruses was the same for all the stabilizers tested, including the collapsed culture medium without additives. A single exception was detected in the paramyxoviruses, in which inferior stabilizing activity occurred in the noncollapsed skim milk with sodium glutamate. Hence, cake collapse appeared to have no impact on the stability of the viruses during storage. Despite a noticeably different micromorphology of cake arising out of different freezing rates, with approximately five times larger ice crystals in samples frozen at −80°C in comparison with crystals of samples frozen in the liquid nitrogen, both freezing rates had similar impact on virus stability during lyophilization (except in the picornaviruses). However, the rate of freezing affects ice crystal size and morphology, which in turn impact on sublimation during lyophilization and resulting residual moisture content of the lyophilized product (Dawson and Hockley 1992). Although residual moisture can have a strong influence on the storage stability of a virus (Worrall *et al.* 2000), in this study, differences in storage stability between viruses frozen less and more rapidly were minor (significant only in coronaviruses).

In conclusion, the most important option for effective lyophilization of viruses is the choice of an appropriate stabilizer, which should be selected with respect to the type of virus. The efficacy of a given stabilizer is markedly dependent on the virus family, and its suitability can be, to a large degree, generalized within the groups of enveloped and nonenveloped viruses. In enveloped viruses, a culture medium with added gelatine and sucrose provides the best protective activity for virus infectivity (among the stabilizers tested here). Gelatine and sucrose also best maintain virus envelope morphology in the herpesviruses and paramyxoviruses. Nonenveloped viruses are less stabilizer specific, and even the culture medium without additives had an adequate protective effect in all the nonenveloped families tested. The freezing rate had only a minor impact on virus stability during lyophilization, both for freezing at −80°C and in liquid nitrogen. Faster freezing is beneficial only for picornaviruses, which are generally considered the most labile viruses under lyophilization stress. The decisive impact on the efficiency of the stabilizer and freezing rate is expressed immediately after lyophilization. During the storage period, the infectivity of nearly every virus decreased similarly for all stabilizers and freezing rates in this study.

Acknowledgements

The author would like to thank Jana Prodělalová and Jiří Salát for critical reading and comments on the manuscript. Tony Long, Svinosice, kindly helped work up the English. The paper was supported by the Ministry of Agriculture of the Czech Republic (Grant No. MZe 0002716202) and the Ministry of Education, Youth and Sports, of the Czech Republic (Project no. LO1218, NPU I program).

Conflict of Interest

The author declares no conflict of interest.

References

Berge, T.O., Jewett, R.L. and Blair, W.O. (1971) Preservation of enteroviruses by freeze-drying. *Appl Microbiol* **22**, 850–853.

Bozkurt, H., D’Souza, D.H. and Davidson, P.M. (2013) Determination of the thermal inactivation kinetics of the human norovirus surrogates, murine norovirus and feline calicivirus. *J Food Prot* **76**, 79–84.

Buitink, J., van den Dries, I.J., Hoekstra, F.A., Alberda, M. and Hemminga, M.A. (2000) High critical temperature above Tg may contribute to the stability of biological systems. *Biophys J* **79**, 1119–1128.

Carpenter, J.F., Izutsu, K. and Randolph, T.W. (2010) Freezing- and drying-induced perturbations of protein structure and mechanisms of protein protection by stabilizing additives In *Freeze Drying/Lyophilisation of Pharmaceutical and Biological Products*, 3rd edn eds. Rey, L. and May, J.C., pp 167–197. London: Informa Healthcare.

Chambon, M., Bailly, J.L. and Peigue-Lafeuille, H. (1994) Comparative sensitivity of Echovirus type 25 JV-4 prototype strain and two recent isolates to glutaraldehyde at low concentrations. *Appl Environ Microbiol* **60**, 387–392.

Chang, B.S. and Patro, S.Y. (2004) Freeze-drying process development for protein pharmaceuticals In *Lyophilisation of Biopharmaceuticals* eds Costantino, H.R. and Pikal, M.J. pp. 113–138. Arlington: American Association of Pharmaceutical Scientists.

Coleman, V.R., Wilkie, J., Levinson, W.E., Stevens, T. and Jawetz, E. (1973) Inactivation of *Herpesvirus hominis* type 1 and 2 by silver nitrate in vitro and in vivo. *Antimicrob Agents Chemother* **4**, 259–262.

Dawson, P.J. and Hockley, D.J. (1992) Scanning electron microscopy of freeze-dried preparations: relationship of morphology to freeze-drying parameters. *Dev Biol Stand* **74**, 185–192.

Dvorakova, H., Prodelalova, J. and Reichelova, M. (2008) Comparative inactivation of *Aujeszky's disease virus*, *Porcine teschovirus* and *Vesicular stomatitis virus* by chemical disinfectants. *Vet Med-Czech* **53**, 236–242.

Fellowes, O.N. (1965) Freeze-drying of foot-and-mouth disease virus and storage stability of the infectivity of dried virus at 4 C. *Appl Microbiol* **13**, 496–499.

Ferris, N.P., Philpot, R.M., Oxtoby, J.M. and Armstrong, R.M. (1990) Freeze-drying foot-and-mouth disease virus antigens. I. Infectivity studies. *J Virol Methods* **29**, 43–52.

Galdiero, F. (1979) Adenovirus aggregation and preservation in extracellular environment. *Arch Virol* **59**, 99–105.

Gould, E.A. (1995) Virus cryopreservation and storage In *Methods in Molecular Biology, Vol. 38: Cryopreservation and Freeze-Drying Protocols* eds Day, J.G. and McLellan, M.R. pp. 7–20. Totowa, NJ: Humana Press.

Grose, C., Friedrichs, W.E. and Smith, K.O. (1981) Cryopreservation of varicella-zoster virions without loss of structural integrity or infectivity. *Intervirology* **15**, 154–160.

Hansen, R.K., Zhai, S., Skepper, J.N., Johnston, M.D., Oya Alpar, H. and Slater, N.K.H. (2005) Mechanisms of inactivation of HSV-2 during storage in frozen and lyophilized forms. *Biotechnol Prog* **21**, 911–917.

Hubalek, Z. (1996) Cryopreservation of particular groups of microorganisms: viruses In *Cryopreservation of Microorganisms at Ultra-Low Temperatures* ed. Hubalek, Z., pp 103–106. Prague: Academia.

Kaerber, G. (1931) Beitrag zur Kollektiven Behandlung Pharmakologischer Reihenversuche. *Arch Exp Path Pharm* **162**, 480–487.

Kang, M.S., Jang, H., Kim, M.C., Kim, M.J., Joh, S.J., Kwon, J.H. and Kwon, Y.K. (2010) Development of stabilizer for lyophilisation of an attenuated duck viral hepatitis vaccine. *Poult Sci* **89**, 1167–1170.

Liska, V., Bigert, S.A., Bennett, P.S., Olsen, D., Chang, R. and Burke, C.J. (2007) Evaluation of recombinant human gelatin as a substitute for a hydrolyzed porcine gelatin in a refrigerator-stable Oka/Merc live varicella vaccine. *J Immune Based Ther Vaccines* **5**:4, 1–6, doi:10.1186/1476-8518-5-4.

Peetermans, J. (1996) Factors affecting the stability of viral vaccines. *Dev Biol Stand* **87**, 97–101.

Puapermpoonsiri, U., Ford, S.J. and van der Walle, C.F. (2010) Stabilization of bacteriophage during freeze drying. *Int J Pharm* **389**, 168–175.

Sarkar, J., Sreenivasa, B.P., Singh, R.P., Dhar, P. and Bandyopadhyay, S.K. (2003) Comparative efficacy of various chemical stabilizers on the thermostability of a live-attenuated *peste des petits ruminants* (PPR) vaccine. *Vaccine* **21**, 4728–4735.

Scott, E.M. and Woodside, W. (1976) Stability of *Pseudorabies virus* during freeze-drying and storage: effect of suspending media. *J Clin Microbiol* **4**, 1–5.

Suzuki, M. (1970) Effects of suspending media on freeze-drying and preservation of vaccinia virus. *J Hyg (Lond)* **68**, 29–41.

Tanaka, T., Takeda, T. and Miyajama, R. (1991) Cryoprotective effect of saccharides on denaturation of catalase during freeze-drying. *Chem Pharm Bull (Tokyo)* **39**, 1091–1094.

Tannock, G.A., Hierholzer, J.C., Bryce, D.A., Chee, C.F. and Paul, J.A. (1987) Freeze-drying of respiratory syncytial viruses for transportation and storage. *J Clin Microbiol* **25**, 1769–1771.

Tuladhar, E., Bouwknegt, M., Zwietering, M.H., Koopmans, M. and Duizer, E. (2012) Thermal stability of structurally different viruses with proven or potential relevance to food safety. *J Appl Microbiol* **112**, 1050–1057.

Walkiewicz, M.P., Morral, N. and Engel, D.A. (2009) Accurate single-day titration of adenovirus vectors based on equivalence of protein VII nuclear dots and infectious particles. *J Virol Methods* **159**, 251–258.

Worrall, E.E., Litamoi, J.K., Seck, B.M. and Ayelet, G. (2000) *Xerovac*: an ultra rapid method for the dehydration and preservation of live attenuated Rinderpest and Peste des Petits ruminants vaccines. *Vaccine* **19**, 834–839.

Zhai, S., Hansen, R.K., Taylor, R., Skepper, J.N., Sanches, R. and Slater, N.K.H. (2004) Effect of freezing rates and expicients on the infectivity of a live viral vaccine during lyophilization. *Biotechnol Prog* **20**, 1113–1120.