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With West Nile virus in the news, viral inactivation is under public scrutiny. Yet, the culture media, the therapeutic product, and the potential viral contaminants can all affect the inactivation method chosen during production. Viral inactivation techniques — those tried or used in manufacturing or in final biopharmaceuticals, vaccines, and media products — have been gathered from recent scientific literature and organized in this article series as an addition to your process development toolbox.

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Virus Inactivation in the 1990s — and into the 21st Century

Part 4, Culture Media, Biotechnology Products, and Vaccines

Regulations and safety concerns mandate viral clearance in biopharmaceuticals. Clearance is achieved by removal and/or inactivation. Different types of viruses require different inactivation methods. This latest installment to the article series on inactivation methods published during the last decade of the 20th century and into 2001 focuses on inactivation in culture media, biotechnology products, and vaccines. Previous articles in this series were Part 1, which discussed inactivation of viruses in skin, bone, and cells other than platelets and red blood cells (1); Part 2, which reviewed the literature on inactivation of viruses in red blood cells and platelets (2); and Parts 3a and 3b, which addressed plasma and plasma products (3,4). (The full names of all virus abbreviations used in this article and whether or not those viruses are enveloped can be found in the "Virus Abbreviation" sidebar.)

Cell and Tissue Culture Media

Inactivation methods for viruses in cell and tissue culture media include heat and pasteurization, irradiation, iodine, ozone, ethylenimine, ultraviolet (UV) and photoactive compounds, surfactin, and high hydrostatic pressure.

Heat and pasteurization. Cell-free BIV and BIV in infected fetal bovine lung cells in DMEM containing 10% fetal calf serum (FCS) and antibiotics were inactivated by heat treatment at 47 °C or 62.8 °C for 30 minutes. High-temperature, short-time (HTST, 72 °C for 15 seconds) treatment was also effective. Additionally, suspension of BIV-infected cells in milk did not protect the virus during heat treatment (5).

HIV-1 and HIV-2, Rous sarcoma virus, FeSV, and SSV were all inactivated by

heating to 60 °C in cell culture media containing 5% (v/v) FCS. HIV-1 and HIV-2 were considerably more labile than the three animal type C retroviruses (6). Heating at 60 °C in cell culture medium inactivated the retroviruses HIV-1, HIV-2, FeSV, and SSV within 30 minutes. Rous sarcoma virus, however, required 60 minutes (7).

Heating rabbit serum at 56 °C for 30 minutes provided a log₁₀ reduction of only 2.6 for BVDV. PRV was more effectively inactivated. The authors state that PRV is usually resistant to heat in the presence of protein stabilizers (8).

HTST inactivation of MMV in culture media and in a trypsin solution was evaluated. Microwave and electroheating systems were used. Temperatures ≥98 °C reduced MMV by ≥5.4 log₁₀. Growth of VERO cells in HTST-treated DMEM was unaffected, but HTST-treated DMEM plus 5% fetal bovine serum (FBS) had a detrimental effect on cell growth. For trypsin, electroheating was unsuitable as an HTST method, but the microwave system was acceptable (9).

Irradiation. Gamma radiation between 30 and 40 kGy is used to inactivate viruses in bovine sera. Canine ADV in FBS was not completely inactivated at 30 kGy, but no virus was present after treatment with 35 kGy. For some cell lines, however, the use of 35 kGy destroys growth-promoting properties. For PPV, a six log₁₀ reduction can be obtained. However, the amount of gamma radiation can vary from 30 kGy to 100 kGy, depending upon the laboratory where the work was performed and the starting titer (10). In a 1999 review, it was noted that data for the effectiveness of gamma irradiation in inactivation of viruses potentially found in FCS are incomplete. Although most viruses were inactivated in

the range of 20 to 25 kGy, inactivation was incomplete for some. BVDV, IBR, PI-3, and BTV were reduced by about four log₁₀. Reo was inactivated less quickly, and PPV was not inactivated (8).

The effective doses of gamma radiation required to inactivate the viruses of Akabane disease, Aino, bovine ephemeral fever, swine vesicular disease, hog cholera, BTV, and MMV suspended in bovine serum and irradiated at -68 °C were determined. The viruses were selected because of their presence in the Eastern Hemisphere and the desire to import Australian FBS to the United States (11).

Gamma radiation of 30 or 45 kGy may not inactivate porcine circovirus in FBS. This finding was reported at a conference in 2001 (12). However, it was noted during the discussion that with a higher titer virus and a quantitative stem cell assay, PCV was inactivated to the same extent as HAV. The latter was presented in a discussion session.

Electron beam irradiation in frozen or liquid samples with 11 and 46 kGy was evaluated for inactivation of PPV, BVDV, and PEV in bovine serum. The rate of inactivation was almost twice as great in the liquid samples. The authors concluded that the penetrating capacity of the electron beam irradiation through biological material should ensure a safe inactivation, irrespective of the possible masking of virus particles by serum components. However, they state that large samples may be a problem because the penetration is limited. Extrapolated data indicated a six log₁₀ reduction of PPV with a dose of 35 kGy for liquid samples or 55 kGy for frozen samples (13).

Iodine. Depth filters containing PVPP-iodine provided log₁₀ reduction of > five for HIV-1, >7.3 for VSV and > seven for HSV either with or without FCS at room temperature at a filtration speed of about 500 L/h × m². BVDV was reduced by four log₁₀ at 40 °C in DMEM culture medium containing FCS at a filtration speed of about 100 L/h × m². HSV-1 was reduced by seven log₁₀ in EMEM with FCS at 40 °C (14).

Ozone. Ozone treatment of culture media effectively inactivated HIV. Either serum or culture media were treated with ozone before the addition of the virus. It is believed that the virus inactivation is a result of secondary reaction products of the ozone in serum and media containing 20% serum (15).

Virus Abbreviations

Virus	Enveloped	Not Enveloped
ADV Adenovirus		√
BIV Bovine immunodeficiency virus	√	
BLV Bovine leukemia virus	√	
BPV Bovine parvovirus		√
BTV Blue tongue virus		√
BVDV Bovine viral diarrhea	√	
CMV Cytomegalovirus	√	
FeSV Feline sarcoma virus	√	
FMD Foot and mouth disease virus		√
FrMuLV Murine leukemia virus, friend strain	√	
HAV Hepatitis A virus		√
HIV Human immunodeficiency virus	√	
HSV Herpes simplex virus	√	
IBR Infectious bovine rhinotracheitis		√
MCF Mink cell focus virus	√	
MMV Murine minute virus		√
MV Measles virus	√	
PCV Porcine circovirus		√
PEV Porcine enterovirus		√
PI-3 Parainfluenza virus	√	
PPV Porcine parvovirus		√
PRV Pseudorabies virus	√	
RSV Respiratory syncytial	√	
SFV Semliki Forest virus	√	
SIN Sindbis virus	√	
SIV Simian immunodeficiency virus	√	
SSV Simian sarcoma virus	√	
VSV Vesicular stomatitis virus	√	
XMuLV Xenotropic murine leukemia virus	√	

Ethyleneimine. Sterile bovine serum was spiked with PPV, PEV, and BVDV. The time for a four log₁₀ inactivation of PPV was nine hours for a five mM ethyleneimine solution and three hours for a 10-mM solution, both at 37 °C. BVDV was inactivated by four log₁₀ in one hour, 44 minutes, by a five mM solution. PEV was inactivated by four log₁₀ in 24 minutes with a five mM solution and in 17 minutes with a 10 mM solution. This work was performed to ensure worker safety when transferring serum samples between laboratories. Ethyleneimine causes alkylation of free sulfhydryl and amino groups. The authors state that this method has been found to be gentler to proteins than formaldehyde and glutaraldehyde treatments (13).

Although not in serum, the inactivation capability of ethyleneimines was assessed for MMV and SFV. With trimeric ethyleneimine (TEI), the inactivation kinetics for MMV were different than those found with other viruses. A higher inactivation rate was found with higher pH. The mechanism of viral inactivation by TEI is thought to result from a modification of the viral surface proteins (16).

UV and photoactive compounds. BPV and BVDV in FCS were completely inactivated by UV irradiation, but Reo and IBR were reduced by only about four log₁₀ (8).

UVC (220–280 nm) provided high clearance rates for BPV, PI-3, BVDV, and FMD, with no adverse effects on the growth

Table 1. pH inactivation of XMuLV after 30 minutes for monoclonal antibodies A–H

	A		B		C		D		E		F		G		H	
pH	3.14	3.53	3.31	3.52	3.29	3.39	3.37	3.50	3.57	3.60	3.62	3.46	3.46	3.46	3.46	3.46
Log ₁₀ reduction	5.18	4.81	≥6.59	≥5.11	≥5.38	≥5.73	6.67	≥5.91	≥3.77	4.70	≥6.06	≥5.75	≥6.25	≥6.25	≥6.25	≥6.25

promoting properties of either 1% or 10% FCS. The log₁₀ reduction factors were BVDV: 8; PI-3: 7; Reo-3: 4; IBR: 6; FMD: 8; BPV: 8; and PPV: 5.5 (17).

Hematoporphyrin derivatives and other photosensitive dyes were evaluated for their effectiveness in inactivating HSV in culture media. Hematoporphyrin derivative, dihematoporphyrin ether; some benzoporphyrin derivatives; and a sapphyrin provided a log₁₀ reduction ≥5. The ability of these dyes to inactivate HIV, CMV, and SIV in culture media was also investigated (18).

Benzoporphyrin derivative and light treatment effectively inactivated HIV in tissue culture media. Cultures were examined by ELISA for the HIV-1 p24 antigen (19).

Surfactin is a cyclic lipopeptide antibiotic and surfactant produced by *Bacillus subtilis*. It was found to inactivate lipid-enveloped viruses in 5% FCS when present at a concentration of 25 μM. At concentrations up to 80 μM, HIV was inactivated by >4.4 log₁₀ in 15 minutes, and SIV and VSV were inactivated in 60 minutes. The inactivation rate increased linearly with incubation temperature and logarithmically with concentration (20).

High hydrostatic pressure. Application of pressure was shown to inactivate HIV. When a pressure of more than 400 MPa was applied to HIV-1 suspended in RPMI-1640 medium with 10% FCS for 10 minutes at 25 °C, the log₁₀ reduction was >five (21).

Biotechnology Products

Although not nearly as much information is published on viral inactivation in biotechnology products when compared to that in plasma products, the amount of information available in the public domain is increasing. Modern analytical techniques such as electrospray ionization mass spectrometry are being used to assess protein damage during virus inactivation (22).

pH. Low pH has been shown to inactivate XMuLV. In one study, pH 3.5–4.0 was found to be effective at 18–26 °C, and very

little difference was seen in inactivation kinetics for pH 3.7 up to pH 4.1. At 2–8 °C, however, pH 4.1 inactivation was slower and required up to one hour, compared to about 30 minutes for pH 3.7. Variability was observed for different proteins. An inactivation time for one product was 60 minutes. For another it was 120 minutes, and in one product XMuLV was not completely inactivated even after 120 minutes. Protein concentration also affected the inactivation kinetics. In buffer only, XMuLV was inactivated in 120 minutes; addition of protein prevented complete inactivation with the same pH, temperature, and exposure time. The ionic strength of the inactivating solution appeared to mitigate the effect of increasing protein concentration (23).

In another study, low pH was investigated for inactivation of XMuLV and PRV in several monoclonal antibodies (MAbs) produced in either Sp2/0 or NS0 mouse cell lines (data are summarized in Tables 1 and 2). These data are being used to support generic virus inactivation approaches (24).

A different study of 13 products, mostly but not all MAbs, illustrated the ability of pH 3.6–4.0 to inactivate several different viruses in five to 60 minutes (25).

Caprylate has been found to inactivate lipid-enveloped viruses in MAb production processes. Cell culture harvest broth containing a *Pseudomonas* exotoxin A–monoclonal antibody conjugate and a *Pseudomonas* monoclonal IgM were each spiked with viruses. HSV-1 and VSV were completely inactivated at 20 °C in less than 60 minutes. However, at 5 °C only partial inactivation of VSV was shown after 120 minutes. A nonionized form of the caprylate is maintained over a broad pH range, and the nonionized form is effective in viral inactivation at concentrations between 0.001 and 0.07 weight %. VSV and vaccinia were inactivated slower than HSV-1 or SIN at pH 6.3 (26).

Detergents. Triton X-100 (0.5%, 4 °C) completely inactivated RSV and FrMuLV

within four hours without influencing the binding capacity of a number of MAbs. Log₁₀ reduction values were ≥3.8 for FrMuLV and ≥5.4 for RSV (7). Other data have shown that MuLV was not inactivated by 0.1 to 1% Tween (27).

Solvent/detergent (S/D). Commonly used for plasma proteins, S/D is also used for inactivation of enveloped viruses during production of recombinant proteins and MAbs. During the production of B-domain deleted recombinant factor VIII, S/D is used for virus inactivation. Although no viruses are associated with the CHO cell line used for production, S/D was added after a cation exchange step. Concentrations of 0.3% TNBP and 1% Triton X-100 were targeted and treated for at least 30 minutes (7). S/D treatment completely and rapidly inactivated all the enveloped viruses tested, which included PI-3, XMuLV, IBR, and MCF (22).

Beta-propiolactone has been proposed for viral inactivation in naked DNA vaccines. It was found that for a 16-hour treatment at 4 °C, the initial concentration of β-propiolactone should not exceed 0.25% to prevent loss of gene expression (28).

Vaccines

Viral inactivation treatments in vaccines include anilino-naphthalene sulfonate compounds, urea, and pressure; heat and pasteurization; detergent and S/D; formaldehyde; psoralens; aziridines; β-propiolactone; sodium periodate; and UV.

Anilino-naphthalene sulfonate compounds, urea, and pressure. The compound Bis-(8-anilino-naphthalene-1-sulfonate) (bis-ANS) has been shown to inactivate VSV at micromolar concentrations. Butyl-ANS and ANS required higher concentrations. The mechanism of inactivation by bis-ANS appears to be similar to that produced by pressure. When VSV was inactivated by 10 μM bis-ANS and 2.5 Kbar hydrostatic pressure, a high titer of neutralizing antibodies was obtained. Very little viral inactivation was found by

Table 2. pH inactivation of PRV after 30 minutes for monoclonal antibodies A–D

	A	B	C	D			
pH	3.39	3.32	3.51	3.48	3.36	3.47	3.51
Log ₁₀ reduction	3.78	5.93	5.77	≥5.60	≥5.60	≥6.01	≥6.01

treatment with ≥ 2 M urea at atmospheric pressure, but 2 M urea at a pressure of 2.5 Kbar caused inactivation (29).

Rotavirus treated with pressure resulted in noninfectious particles with previously occult epitopes exposed. Urea produced more dramatic changes than pressure, when evaluated by fluorescence spectroscopy (30).

Pressure was used for inactivation of SIV. The inactivation was found to be independent of virus concentration. The extent of inactivation depended on the time of compression. At 150 MPa at room temperature, five log₁₀ were inactivated. The authors suggest that this method would be useful for sterilization of biological preparations such as vaccines (31).

Heat and pasteurization. Heat treatment of Sabin (www.sabin.org) strains of poliovirus vaccine at 42–45 °C caused inactivation, loss of native antigen, and release of viral RNA. Adding pirodavin protected the vaccine against the loss of antigenicity even after infectivity was lost (32). The kinetics of thermal inactivation of different MV strains at 45 °C was evaluated, and it was concluded that development of a thermostable measles vaccine does not require selection of a thermoresistant variant if certain conditions for virus harvest are fulfilled (33).

Baculovirus contamination of a recombinant vaccine for PPV was addressed by evaluating different baculovirus inactivation methods that would enable maintenance of the integrity and immunogenicity of the vaccine. Pasteurization, detergent treatment, and alkylation with binary ethylenimine were evaluated. Pasteurization (60 °C, one hour, with 20% sucrose as stabilizer) inactivated the virus, but disrupted the viral particles (34).

Detergent and S/D. Triton X-100 inactivation of baculovirus was found to be suitable for the recombinant PPV vaccine (as described in the previous paragraph). The vaccine

maintained its structural and immunogenic properties. S/D was also evaluated. Although S/D was also able to inactivate the baculovirus while maintaining vaccine structure and function, Triton X-100 was selected for inactivation of baculovirus because of the speed of treatment and its simplicity and safety (34).

Formaldehyde. Formalin inactivation of VSV prevented infection in vitro. Time and formalin concentration were shown to be critical parameters. Inactivation within 30 minutes required a 1% formalin concentration. Inactivation for 18 hours required a concentration of 0.0635%. In both cases, the temperature was 4 °C. This inactivation method did not impair B-cell immunogenicity (35). Purified dengue vaccine made in Vero cells was inactivated with 0.05% formalin at 22 °C. The virus retained its antigenicity after this treatment (36).

Inactivation of MV by formaldehyde, however, may create an imbalance in the immune system (37). The same observation has been made for formaldehyde-inactivated RSV (38). Formaldehyde (0.02%) was incorporated into a multistep inactivation treatment for an experimental HIV vaccine (39).

Formaldehyde has also been used for inactivation of a purified HAV vaccine. The treatment was with a formaldehyde concentration of 0.2 mg/mL for five days at 32 °C, and the vaccine was found to be safe and immunogenic in experimental models (40). Inactivation of FMD vaccines by formaldehyde is discussed in a 1991 paper. It was observed that formaldehyde concentration, pH, and medium composition were critical to inactivation. A doubled concentration of formaldehyde at 26 °C, pH 8.4 for 48 hours was sufficient for inactivation (41).

Inactivation by formaldehyde is not a linear or a first-order reaction. Outbreaks of foot-and-mouth disease were, for the most part, associated with vaccines that had been

formaldehyde-inactivated (42). In a review of accidents caused by incomplete inactivation of vaccines, significant doubt was expressed about the safety of formaldehyde-inactivated vaccines. It is now believed that outbreaks of Venezuelan equine encephalitis were a result of incomplete inactivation of the vaccines by formaldehyde (43).

Psoralens in the presence of long wave UV appear to leave protein antigens and other surface components relatively unmodified. The psoralen method is reported to be a better inactivation method than formalin treatment for retaining antigenicity and immunogenicity in experimental inactivated vaccines. Some of those experimental vaccines include those for Lassa fever, influenza, HIV, and Pichinde (44). Aminomethyltrimethyl-psoralen (AMT) has been used to completely inactivate rotavirus while retaining antigenically intact particles (45).

Aziridines. Acetylenimine (AEI) has been shown to inactivate FMD. At 37 °C, a concentration of 0.05% was effective in less than two hours. AEI, however, is unstable at ambient temperatures, and BEI (binary ethylenimine) inactivation is now a preferred method (42). The use of AEI, ethyleneimine (EI), and propyleneimine (PI) for inactivation of FMD vaccines was compared to formaldehyde inactivation. The use of bromoethylamine hydrobromide was recommended because it is less toxic than AEI, EI, or PI (41).

BEI inactivates viruses in a first-order reaction and is reported to be superior in both safety and antigenicity to formalin. Important variables for inactivation include pH, osmolarity of the virus suspension, and agitation rate. A temperature of 37 °C is commonly used. Viruses that have been inactivated with BEI include African swine fever, BLV, bovine rhinovirus, BTV, BVDV, Eastern equine encephalomyelitis, FMD, IBR, Newcastle disease, PPV, PRV, rabies, and VSV (46).

BEI treatment (10 mM) is part of the protocol for inactivation of an experimental HIV vaccine (39). BEI was also used to inactivate African horse sickness vaccine. There was complete inactivation at BEI concentrations of 0.004 M for 18 hours, 0.003 M for 48 hours, and 0.002 M for 84 hours.

BEI was evaluated for inactivation of baculovirus in a recombinant PPV vaccine. Complete inactivation was observed after 48 hours at 35 °C, and no change in the vaccine function was found. However, because of its carcinogenicity, it was not chosen as the inactivation method (34).

Beta-propiolactone (0.2%) treatment was combined with three other inactivation steps for an experimental HIV vaccine. The other steps included 10 mM BEI, 0.05% sodium cholate, and 0.02% formaldehyde (39).

Sodium periodate. A human CMV vaccine candidate and ADV (Ad5) can be inactivated by sodium periodate. The inactivation is dependent on the concentration of periodate, and it was not determined whether the inactivated CMV could be integrated into cellular DNA (47). The work on ADV demonstrated that oxidized ADV could adsorb and penetrate the host cell, but viral replication was partially impaired. The average inactivation of infectious virions was seven log₁₀ (48).

UV. The antigenicity of HAV was shown to be almost unaltered after treatment with UV in a study intended to determine the suitability of UV inactivation for vaccine preparation. UV doses up to 920 J/m² were applied. The ability of the UV treatment to preserve antigenicity was compared to treatment with formalin at 250 µg/ml, which requires up to 15 days (49).

Looking Ahead

The next installment in this literature survey will cover disinfection. Disinfection is included as a stand-alone topic in the series because of the importance of making equipment and components, such as chromatography resins that might come into contact with viruses, safe for subsequent use. This point has been repeatedly emphasized by regulatory authorities, and it is also critical for ensuring worker safety when dealing with high risk materials. A disinfectant has been defined as “an agent that frees from infection; usually a chemical agent that destroys disease germs or other harmful microorganisms or inactivates virus (50).” In that article, disinfectants targeted to specific viruses will be addressed, followed by specific approaches that have been reported for disinfection in plasma and culture media. **BPI**

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