Virus challenge biopharmaceutical manufacturers, and different viruses require different inactivation methods. This article series summarizes viral inactivation methods published during the last decade of the 20th century and into the year 2001. Part 1 discussed inactivation of viruses in skin, bone, and cells (1). Part 2 discussed red blood cells and platelets (2). Part 3, on plasma and plasma products, was divided into two separate articles: Part 3a on heat and solvent/detergent (S/D) inactivation methods (3), and this (part 3b) includes treatments other than heat and S/D.

**Methylene Blue Phototreatment**

Methylene blue phototreatment (fluorescent light for one hour at 60,000 lux) has been shown to inactivate cell-free, lipid-enveloped viruses in frozen plasma. It has also been shown to inactivate some nonenveloped viruses (such as ADV). Cell-free HIV in frozen plasma was inactivated by >6.7 log_{10}.

Filtration was used to remove cell-associated HIV infectivity (4).

Cell-associated viruses are first removed by freezing and thawing or by filtration. Dye is added first so that it associates with the viral membrane components or the viral nucleic acid, then fluorescent light at 45,000 lux is used to activate the dye. When exposed to light in the presence of oxygen, the virus nucleic acid or membrane components can be damaged by the singlet oxygen created (5). No virus inactivation was observed for EMC, polio, HAV, or PPV.

Methylene blue (1 μM) photodynamically inactivated viruses in fresh, frozen plasma by illuminating the single units of plasma in their plastic containers with fluorescent tubes. Table 1 shows these data and the specific illumination times required to achieve viral inactivation (5,6).

HIV-1 was spiked into fresh, frozen plasma and illuminated with visible light in the presence of 1 μM of methylene blue. After five minutes, the infective titer was reduced by 4.3 log_{10} and after 10 minutes, it was reduced below the limit of detection. Methylene blue without illumination, and illumination alone, both had minimal effect on HIV-1 inactivation (7).

HCV and HIV were spiked into fresh plasma, which was then treated with 1 μM of methylene blue. After one hour in the dark at 4°C, fluorescent lighting was applied. The effects of the treatment on the RNA of HCV and HIV-1 were studied by reverse transcriptase polymerase chain reaction (RT-PCR) analysis. A few minutes of illumination in the presence of methylene blue strongly reduced the detection of viral RNA. However for HIV, the effect of photosensitization on PCR signals was much smaller than on infectivity. The same was found to be true for HCV, but HCV was slightly more sensitive (8).

The inactivation of HSV-1 and SHV-1 by methylene blue and light was suppressed by the addition of plasma and by the presence of a singlet oxygen quencher (imidazole). The data indicated that singlet molecular oxygen is involved in virus inactivation by methylene blue and light (9). The target structures for HIV-1 inactivation by methylene blue and light are the envelope and core proteins and the inner core structures (10).

Methylene blue and light inactivation of enveloped viruses spiked into human plasma is time-dependent. SFV was inactivated in five to 10 minutes, but VSV took one hour for complete inactivation. HIV infectivity was removed within 10 minutes. DHBV could be inactivated, but required high doses of light. A methylene blue concentration of 0.3 μM was sufficient to reduce SFV infectivity below the detection limit in less than 15 minutes. For VSV inactivation, a concentration of 0.8–1.0 μM methylene blue was necessary with 45–60 minutes of illumination. A minimal light strength of 45,000 lux by fluorescent light was recommended for inactivation of viruses in fresh plasma. The light strength required is dependent on the light source. Most of the nonenveloped viruses, however, were resistant to methylene blue and light treatment. SV-40, calicivirus, and ADV were somewhat susceptible to inactivation. EMC, MEV, HAV, and PPV were not reduced after one hour. Polio was not reduced after two hours (11).

Inactivation of VSV in plasma by methylene blue with and without illumination was studied. In the absence of dye, illumination for four hours led to only marginal inactivation. Low concentrations of methylene blue in the dark also had minimal effect. More than six log_{10} VSV were inactivated with 1 μM of methylene blue in combination with light. Influenza virus required a five- to 10-fold higher methylene blue concentration than did VSV or HSV (12).

The efficacy of virus inactivation by methylene blue depends on light irradiance, duration of irradiation, bag volume, and methylene blue concentration (13). Fresh, frozen plasma treated with methylene blue was taken off the market in Germany after it was found that incomplete removal of methylene blue could result in genetic toxicity. However, methylene blue is still used in several applications (14).

Three bags of plasma can be illuminated together after adding a dry methylene blue pill that results (after it is dissolved) in a concentration of 1 μM (15).

**Chlorin-Type Photosensitizer**

The ability of a chlorin-type molecule to photoinactivate a series of HIV-1 strains in buffer, plasma, and whole blood was investigated. Complete inactivation of HIV-1 in plasma was obtained with a reasonable light dose (661 nm) (16).

3,3'-[1,4-Naphthylidene] Dipropionate

Inactivation of HSV-1 and SHV-1 by singlet oxygen produced by 3,3'-[1,4-naphthylidene] dipropionate (NDPO₂) provided good inactivation in phosphate-buffered saline (PBS).
The presence of 80% v/v human plasma, however, diminished the amount of virus killed, and a higher concentration of NDPO2 was required. Using 30 mM NDPO2, inactivation of three log_{10} of SHV-1 was obtained (9).

**Psoralens**

Photochemical inactivation of viruses in plasma and plasma fractions by 8-methoxypsoralen (8-MOP) and UVA reduced HCV by four log_{10} and HBV in factor 8 (FVIII) concentrate by 4.5 log_{10}. BTV in plasma was inactivated by aminomethyl triethylpsoralen (AMT). VSV and FeLV in FVIII concentrate were inactivated by 8-MOP (17).

**Beta-Propiolactone**

Cryosupernatant produced during plasma fractionation can be treated with 0.25% β-propiolactone at 4°C for five hours at pH 7.2 to inactivate both enveloped and nonenveloped viruses during the production of immunoglobulin, albumin, and factor 9 (FIX) (18). FVIII, however, is unstable in the presence of β-propiolactone. For immunoglobulin G (IgG) solutions, 0.1% β-propiolactone, at pH 8.1 and 20–22°C for eight hours, provides inactivation factors about 10% higher than those found in plasma. FCV inactivation was 7.2 log_{10} in plasma and 8.12 log_{10} in IgG (19).

The capability of β-propiolactone (0.25% at 4°C) to inactivate viruses was affected differently by the presence of cryoprecipitate-poor (cryo-poor) plasma and IgG. In the presence of IgG or cryo-poor plasma, SFV, VSV, BVDV, and MEV were effectively inactivated. In IgG, SIV, BHV, BVDV, and FCV were also sufficiently inactivated. In plasma, however, SIV, HIV-2, BHV-1, SV-40, FCV, and PPV were not sufficiently inactivated by this treatment. Viral reduction factors were:

<table>
<thead>
<tr>
<th>Virus</th>
<th>log_{10} Inactivation</th>
<th>log_{10} Inactivation</th>
<th>Illumination Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADV</td>
<td>4.00</td>
<td>4</td>
<td>120</td>
</tr>
<tr>
<td>BHV</td>
<td>≥8.11</td>
<td>&gt;4.86</td>
<td>30</td>
</tr>
<tr>
<td>BVDV</td>
<td>&gt;5.63, &gt;6.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calici</td>
<td>&gt;3.90</td>
<td>&gt;3.9</td>
<td>5</td>
</tr>
<tr>
<td>Classical swine fever</td>
<td>&gt;3.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMC</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>HAV</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1</td>
<td>≥6.32</td>
<td>&gt;4.00</td>
<td>10</td>
</tr>
<tr>
<td>HIV-2</td>
<td>≥3.81</td>
<td>&gt;1.76</td>
<td>10</td>
</tr>
<tr>
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<td>&gt;5.5</td>
<td>60</td>
</tr>
<tr>
<td>Influenza</td>
<td>5.10</td>
<td>5.1</td>
<td>60</td>
</tr>
<tr>
<td>Polio</td>
<td>0</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>PPV</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>Reo-3</td>
<td>3.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semliki forest</td>
<td>&gt;8.77</td>
<td>&gt;7.00</td>
<td>10</td>
</tr>
<tr>
<td>SIN</td>
<td>&gt;9.73</td>
<td>&gt;9.73</td>
<td>5</td>
</tr>
<tr>
<td>SIV</td>
<td>≥6.26</td>
<td>&gt;3.29</td>
<td>15</td>
</tr>
<tr>
<td>SV-40</td>
<td>≥4.43</td>
<td>&gt;4.42</td>
<td>60</td>
</tr>
<tr>
<td>VSV</td>
<td>≥4.89</td>
<td>&gt;4.89</td>
<td>60</td>
</tr>
<tr>
<td>West Nile</td>
<td>≥4.39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reference 5 (Mohr 2000)

Reference 6 (Mohr 1993)

In minutes.

**Table 1. Photodynamic inactivation of various viruses in fresh plasma using methylene blue (1 μM) with different times of illumination to achieve inactivation (6)**

**Table 2. References that discussed virus inactivation performed in plasma and plasma products using β-propiolactone**

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCV</td>
<td>19</td>
</tr>
<tr>
<td>BHV, BVDV, FCV, HIV-2, MEV, PPV, SFV, SHV, SIV, SV-40, VSV</td>
<td>20</td>
</tr>
<tr>
<td>HIV-1</td>
<td>21</td>
</tr>
<tr>
<td>BHV, BVDV, calicivirus, EAV, HIV-1, MEV, PRV, PPV, SFV, SHV, SIV, SV-40, VSV</td>
<td>18</td>
</tr>
<tr>
<td>HBV*, HCV*, HIV*</td>
<td>22</td>
</tr>
</tbody>
</table>

*Plus UV

**Hydrostatic Pressure**

Hydrostatic pressure cycling was used to inactivate virus in human plasma. Lambda phage (λ phage) was used as a model virus. When performed at near 0°C, λ phage titers were reduced by approximately six log_{10} after 10 to 20 minutes (25).

**Iodine**

Iodine/ SephadeX (Amersham Biosciences, www.apbiotech.com) has been shown to deliver iodine to IVIG in a slow, controlled way that inactivates >4.2 log_{10} PPV, >4.7 log_{10} BVDV, >4.2 log_{10} PRV, and >8.1 log_{10} EMC (26).

Viruses spiked into an antithrombin III complex containing up to 0.1% human albumin were inactivated by liquid iodine. Log_{10} reduction values were greater than six for SIN, EMC, and VSV, greater than four for PRV, and greater than three for HIV. With the exception of SIN, inactivation was complete (27).
Cross-linked starch–iodine was shown to be an effective inactivation method for both lipid-enveloped and nonenveloped viruses in plasma. An iodine concentration of 1.05 mg/mL and a 60-minute incubation time inactivated more than nine log_{10} VSV and more than seven log_{10} of EMC (28).

VSV spiked into cryo-poor plasma was treated with cross-linked povidone iodine at concentrations from four to 10 mg/mL for up to 120 minutes at 4°C. At 24°C, the 10 mg/mL treatment inactivated more than seven log_{10} of VSV within five minutes (29).

A polyvinylpolypyrrolidone (PVPP) and iodine complex in a depth filter was used for viral inactivation of VSV in 2% IgG. The log_{10} reduction was 7.3. Filtration was at room temperature, at pH 7.4, with a filtration speed of about 500 Lm²/h. Inactivation of several different species of parvovirus was between four and six log_{10} in 2–5% IgG, FIX solution (0.44 mg/mL), or buffered saline, pH 7.0. Reo-3 was inactivated by 4.9 log_{10} (the available starting titer) in a 2% IgG solution. For parvovirus and Reo-3, the filtration speed was 100–500 Lm²/h and the temperature was either 40°C or room temperature (30).

**Caprylate**

Caprylic acid (99% octanoic acid) can inactivate lipid-enveloped viruses in plasma products. In albumin, VSV was inactivated in 60 minutes or less (31). Caprylate has also been used with amino acids and heat as a virus elimination method in the production of FVIII. At a recent conference, Bayer Corporation (www.bayer.com) discussed the use of caprylate as a very robust inactivation method for an IVIG (32). Twelve mM of caprylate (0.2% at 25°C, pH 5.1) provided a log_{10} reduction of ≥4.4 for BVDV, which has been described as the most caprylate-resistant enveloped virus associated with the plasma fractionation processes. Caprylate at a 40 mM concentration, at pH 5.4, 40°C, also inactivated BVDV in an albumin solution. The presenter also found that the rate of inactivation of BVDV by 19 mM caprylate in a fraction II + III solution was 20 to 60-fold faster than inactivation by solvent/detergent (S/D) in filtrate III (32).

**UVC Irradiation**

A fibrinogen solution (containing rutin to protect the fibrinogen) was irradiated with UVC (254 nm, 0.1 J/cm²). Nonlipid viruses inactivated were PPV (>5.5 log_{10}), EMC (>6.5 log_{10}), and HAV (>6.5 log_{10}). Inactivated lipid-enveloped viruses included HIV (>5.7 log_{10}) and VSV (>5.7 log_{10}) in filtrate III (33).

UVC irradiation was also used for virus inactivation in plasma and in a FVIII concentrate. UVC light targets nucleic acids and was shown to inactivate EMC, HAV, VSV, and PPV. In the FVIII concentrate, all four viruses were inactivated to undetectable levels. Log_{10} reductions were >4.7 for EMC, >6.3 for HAV, >4.6 for VSV, and >4.8 for PPV. In plasma, all four viruses were completely killed (34).

UVC was effective in inactivating polio-2, SFV, HSV, and vaccinia in albumin and IVIG preparations. UVC was found to be more effective than UVB (280–320 nm). Virus inactivation occurred at or before five minutes and provided log_{10} reductions of 3.4 for SFV, ≥6.6 for vaccinia, >6.4 for polio, and >5.4 for HSV (35).

UVC light was used to inactivate parvovirus B19 in coagulation factor concentrates. B19 was inactivated by three log_{10} at 750 J/cm² and was undetectable after 1,000 or 2,000 J/cm². Rutin or catechins were required to maintain biological activity. With epigallocatechin gallate, FVIII activity was retained almost 100% and B19 was decreased to undetectable levels (>3.9 log_{10}) (36).

**Pulsed Light**

Pulsed power and broad spectrum light has been shown to cause dimer formation and DNA and RNA strand breakage at low energy levels that do not appear to destroy proteins. The lights tested were UVA, UVB, UVC (200–400 nm), visible (400–780 nm), and infrared (780 to >100 nm). The optical properties of the solution are important. Alpha-1 (α-1) proteinase inhibitor, IgG, and monoclonal antibodies have been tested. Over 26 different viruses have been shown to be inactivated by this technology (37).

**Gamma Irradiation**

Gamma irradiation in the presence of fibrinogen, FVIII, and α-1 proteinase inhibitor at doses of 23, 28, and 30 kGy, respectively, was able to inactivate four log_{10} of PPV. BVDV was completely inactivated by 20–30 kGy in these products, but gamma irradiation was less effective in inactivating viruses in freeze-dried immunoglobulin (26).

Inactivation of HIV by gamma irradiation was studied in both frozen and liquid plasma. The dose required to inactivate five to six log_{10} of HIV was 50–100 kGy at −80°C and 25 kGy at 15°C. At those doses, however, less than 85% of the biological activity of plasma products was retained. The tolerable dose for plasma irradiated at −80°C should not exceed 14 kGy. For lyophilized FVIII concentrate, the dose should not be more than two and four kGy at 15°C and −80°C, respectively. Hemstra et al. concluded that at those doses, HIV is not

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**Table 3. The effect of variables on pH and pepsin inactivation tests on IgG**

<table>
<thead>
<tr>
<th>pH</th>
<th>Pepsin</th>
<th>Other Variables</th>
<th>Temperature °C</th>
<th>VSV</th>
<th>BVDV</th>
<th>SFV</th>
<th>PRV</th>
<th>HIV</th>
<th>HSV</th>
<th>CMV</th>
<th>HCV</th>
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</thead>
<tbody>
<tr>
<td>4.0</td>
<td>+</td>
<td>37</td>
<td>+, 5 min</td>
<td>slow</td>
<td>N⁴</td>
<td>slow</td>
<td>slow</td>
<td>slow</td>
<td>slow</td>
<td>N⁴</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>+</td>
<td>4</td>
<td>N⁴</td>
<td>N⁴</td>
<td>slow</td>
<td>N⁴</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>7.0</td>
<td>−</td>
<td>37 or 4</td>
<td>N⁴</td>
<td>slow</td>
<td>N⁴</td>
<td>slow</td>
<td>N⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>+ sucrose</td>
<td>37</td>
<td>N⁴</td>
<td>N⁴</td>
<td>slow</td>
<td>N⁴</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4.0</td>
<td>+ NaCl</td>
<td>37</td>
<td>N⁴</td>
<td>N⁴</td>
<td>slow</td>
<td>N⁴</td>
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</tr>
<tr>
<td>4.0</td>
<td>+ IgG 1%</td>
<td>37</td>
<td>N⁴</td>
<td>N⁴</td>
<td>slow</td>
<td>N⁴</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4.0</td>
<td>+ IgG 10%</td>
<td>37</td>
<td>N⁴</td>
<td>N⁴</td>
<td>slow</td>
<td>N⁴</td>
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<td>N⁴</td>
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<td>N⁴</td>
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<tr>
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<td>+</td>
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</tr>
</tbody>
</table>

⁴Negligible or no effect
adequately inactivated and that gamma irradiation should not be used for sterilization of plasma and plasma products (38).

**Pepsin and Low pH**

Virus inactivation of IgG preparations by treating them with pH 4 and pepsin has been used as part of the manufacturing process for IgG for intravenous use. Factors found to influence the inactivation kinetics include temperature and content of the IgG solution. At 37°C, inactivation of BVDV and SFV was rapid and complete to the limit of detection within five minutes of incubation. Complete inactivation of PRV and HIV, however, was slower and required about 30 minutes. Log$_{10}$ reduction factors were $>-4.4$, $>-6.8$, $>-5.6$, and $>-5.0$ for BVDV, SFV, PRV, and HIV, respectively.

**Temperature, sucrose, NaCl, and IgG concentration.** A marked decrease in inactivation rates for all viruses was found at temperatures below 37°C, with the lowest rates at 4°C. At neutral pH, where no pepsin activity was detectable, virus inactivation was negligible at both 37°C and 4°C. Increasing the sucrose concentration in the IgG solutions inhibited the rate of inactivation of PRV but not the inactivation rate of SFV. Increasing the NaCl concentration decreased the rate of inactivation of SFV, but the effects on PRV inactivation were inconsistent. Increases in IgG concentrations had no clear effect on PRV inactivation, but decreased the rate of SFV inactivation (39).

The effects of time, temperature, pH, and stabilizers (sucrose and NaCl) on inactivation of lipid-enveloped model viruses, SFV and VSV, in the IVIG production was investigated. Lowering the pH, raising the temperature, and increasing the incubation time improved inactivation. Small changes in pH and stabilizer concentrations failed to influence the results. Protein concentration had an effect. VSV was inactivated several logs faster in IVIG (6% protein) than in immunoglobulin intramuscular (IGIM), which was 16% protein. Complete inactivation required incubation of 20 hours or more. Freeze-drying in the presence of ethanol or storage in a liquid state at pH 7 only partially inactivated SFV and VSV (40). After fractionation of immunoglobulins using the Kistler–Nitschmann method, SFV, VSV, CMV, HSV-1, and HIV were spiked into the immunoglobulin preparations and treated for 16 hours at 37°C. The pH was adjusted to either seven or four. All viruses, except VSV, were totally inactivated after incubation at pH 4 with or without pepsin. Incubation in IgG solutions at pH 7 resulted in total inactivation of HSV and CMV, which may be because neutralizing antibodies were present. Log$_{10}$ reduction factors at pH 4 were greater than six for SFV, HSV-1, CMV, and HIV. For VSV, log$_{10}$ reductions were 3.9 without pepsin and 5.7 with pepsin. Studies on SFV showed that temperature alone had little effect, but the amount of virus spike did seem to affect the survival of virus. At higher virus concentrations, the viral particles appeared to exhibit a self-protecting effect that could have been a result of aggregation (41).

IgG solutions were spiked with HIV at more than six log$_{10}$. At 37°C and pH 4, HIV was totally inactivated in two hours, with or without pepsin (42). Table 3 highlights the effect of variables on inactivation using pH and pepsin in IgG.

**Low pH**

Inactivation of HCV by low pH was investigated in an IVIG final solution. At pH 4.25 for 21 days at 21°C, the model virus (BVDV) titer decreased 10,000-fold. After seven days, PCR assays showed a 10-fold decrease, and that decrease remained constant. Complete inactivation was also found for 1,000 chimpanzee infectious doses of HCV (43). Viral inactivation by low pH will be discussed further in a future article of this series, which will summarize inactivation methods in biotechnology products.

**References**

Part 3b: Plasma and Plasma Products (Other than Heat and Solvent/Detergent)


(18) Dichtelmuller, H. et al., “Validation of Virus Inactivation and Removal for the Manufacturing Procedure of Two Immunoglobulins and a 5% Serum Protein Solution Treated with Beta-Propiolactone,” Biologicals 21(3), 259–268 (September 1993).


