2.1 Introduction to Vaccines Based on Inactivated Viruses

The first report of “virus” inactivation for vaccine purposes was described in 1886 when Daniel Elmer Salmon and Theobald Smith immunized pigeons with what they thought was a heat-killed hog cholera “virus” (Salmon and Smith 1886). Although in reality it was a cholera-like bacterium, it seeded the scientific community with evidence that immunization with inactivated pathogens can provide protection against infectious disease. Research continued for at least 15 years when at the beginning of the twentieth century the first killed (bacterial) vaccines for humans were developed for typhoid fever, cholera, and plague (Wright and Semple 1897; Haffkine 1899). The foundations of immunization with inactivated virus preparations were also laid at the end of the nineteenth century with Pasteur’s partially inactivated rabies virus (Pasteur et al. 1885), which was cultured in rabbit spinal cords. However, inactivated viral vaccine development was only truly launched with the discovery of cell culture procedures that supported the replication of viral pathogens in vitro, outside the host organism, thus allowing the large scale production of viruses as a source for whole inactivated vaccines. This breakthrough was attributed to Enders, Weller, and Robbins who received the Nobel Prize in 1954 for their discovery on how to cultivate poliovirus in fibroblasts in vitro (Enders et al. 1949; Weller et al. 1949).

In general, all inactivated viral vaccines follow a similar production course in which the pathogen is first cultivated on a substrate to produce large quantities of antigen. Historically, vaccine manufacturers have been using primary cells, tissues,
fertilized eggs, and even whole organisms as substrates for virus propagation (Hess et al. 2012; Barrett et al. 2009). Today, vaccine manufacturers are increasingly shifting toward virus growth on continuous cell lines. This brings certain advantages such as reduced production costs, increased vaccine safety, and relatively straightforward upscaling (Barrett et al. 2009). Once the virus has been propagated, it is often purified and concentrated prior to inactivation. Inactivation can be performed using chemical or physical methods or a combination of the two. A wide range of well-established and novel inactivation agents or methods have been described to successfully inactivate viruses for vaccine purposes. Examples are ascorbic acid (Madhusudana et al. 2004), ethylenimine derivatives (Larghi and Nebel 1980), psorlens (Maves et al. 2011), hydrogen peroxide (Amanna et al. 2012), gamma irradiation (Martin et al. 2010a; Alsharifi and Mullbacher 2010), UV treatment (Budowsky et al. 1981), heat (Nims and Plavsic 2012), and many more (Stauffer et al. 2006). Nonetheless, only formaldehyde and β-Propiolactone (BPL) are widely used for inactivation of licensed human viral vaccines for decades.

Historical events have shaped the way in which inactivated vaccines are currently developed and characterized today. The Cutter incident in 1955 was one of the worst pharmaceutical disasters in the US. history. Here, 380,000 doses of inactivated poliovirus vaccine (IPV), produced at Cutter laboratories, were administered to healthy children. However, these vaccines contained replication competent poliovirus due to inadequate purification of the viral harvest during production. The presence of cell debris in the vaccine pools prevented suitable exposure of the viral particles to formaldehyde and therefore complete inactivation (Offit 2005). As a consequence, 40,000 children who received the vaccine contracted abortive poliomyelitis, 51 were permanently paralyzed, and five died (Nathanson and Langmuir 1963). Federal requirements for vaccine manufacturers were immediately revised in reaction to the Cutter incident creating a better system of regulating vaccines. However, the legacy remains and vaccine manufacturers should always exercise utmost caution when inactivating pathogens to ensure complete inactivation.

Two inactivated vaccines have led to the development of enhanced disease, and even deaths, when the vaccinated persons encountered the pathogen. Indeed, clinical trials with a formalin-inactivated respiratory syncytial virus (RSV) in naïve infants had a disastrous outcome. Not only did the vaccine fail to prevent disease, 80% of vaccine recipients were hospitalized after encountering circulating RSV as compared to hospitalization of only 5% in the control vaccine group. Furthermore, two vaccine recipients died as a consequence of the vaccine induced enhanced disease (Kapikian et al. 1969; Kim et al. 1969; Castilow et al. 2007). The enhanced disease was later attributed to an unfavorable immune response due to a skewed Th2 response and lack of antibody affinity maturation after vaccination (Delgado et al. 2009; Johnson and Graham 2004). Furthermore, a large portion of vaccine-induced antibodies were directed to nonprotective epitopes as formalin treatment had altered the epitopes which induce functional (neutralizing and fusion inhibiting) antibodies that are assumed to be required for protection (Murphy and Walsh 1988).

A formalin-inactivated measles vaccine was licensed in 1963 and unfortunately resulted in a similar, albeit less severe, outcome. The vaccine did induce neutralizing
antibodies, however, immunity waned rapidly and recipients regained susceptibility to measles. When contracted, a more severe, atypical measles disease developed (Griffin and Pan 2009). As with RSV, the enhanced disease was associated with a lack of cytolytic T-cell response and low avidity antibodies (Polack et al. 1999, 2003), linked to the formaldehyde-induced alteration of the measles F protein (Annunziato et al. 1982). In general, it has been suggested that the carbonyl groups on vaccine antigens introduced by formaldehyde treatment induce profound effects on immunogenicity which may tip the balance between protection and adverse effects, or enhanced disease (Moghaddam et al. 2006). These unfortunate events serve as a warning to all vaccine developers; inactivation of a pathogen does not necessarily translate into a vaccine that by default elicits protective immunity, viral epitopes necessary for induction of protective immunity should be preserved after inactivation.

These past events have shaped the manufacture and regulatory control of inactivated vaccines today resulting in vaccines with extremely high safety profiles which protect millions of people against a range of pathogens. The realization that inadequately inactivated viruses could result in such tragedy instigated the need for the exertion of control over the production process. It is for this reason that today inactivation processes require vigilantly designed and validated inactivation assays to ensure pathogens are inactivated with utmost certainty. Concurrently, the successfully formulated inactivated viral vaccines on the market today have a heightened safety aspect as compared to live attenuated viral vaccines. The fact that the pathogen is completely inactivated directly negates reversion to a virulent phenotype within the vaccine recipient. Moreover, these inactivated vaccine viruses are non-transmissible as their live attenuated counterparts. Indeed, reversion and transmission are the greatest drawbacks of attenuated vaccines and have been observed in the past with the attenuated poliovirus vaccine (Henderson et al. 1964), yellow fever vaccine (Lindsey et al. 2008), and rotavirus vaccine (Patel et al. 2009). The improved safety profile of inactivated vaccines entails that they are also suitable for the rapidly increasing group of immunocompromised individuals (Ljungman 2012).

To achieve such a high degree of safety, the analyses of virus inactivation are crucial for production of an inactivated vaccine. The kinetics of inactivation (KOI) must be completely understood and to ascertain the completeness of inactivation the test for effective inactivation must be validated and well characterized with respect to sensitivity and robustness. The KOI will differ per pathogen and inactivation method, therefore, to ensure the safety of the inactivated vaccine bulk, the inactivation process should be studied extensively, where observation of a reproducible KOI is essential. Quantification of viral infectivity in either the vaccine bulk or in-process intermediates is usually achieved by an in vitro cell culture-based assay, however this can also be done in vivo. Generally, either the cell line used for virus propagation or an alternative cell line, demonstrated to be equally susceptible, is inoculated with the (inactivated) virus sample to amplify any potential infectious unit present. Presence or absence of virus in in vitro cultures can be detected by various methods; for lytic viruses this is enabled by monitoring of cytopathic effect (CPE), for nonlytic viruses methods based on genome amplification (PCR) or
antigen detection (immune-fluorescence or ELISA) can be used. In addition, a second step involving inoculation of amplified material in an appropriate in vivo model followed by monitoring for the onset of disease symptoms, can be performed.

Since testing for effective inactivation is dependent on assay sensitivity, sample volume, and the absence of interference by inactivated particles, the assay used to confirm completeness of inactivation (COI) should be designed to be easily scaled up, with high sensitivity and rigorous controls for assay sensitivity and matrix effects. Specifically, positive controls including samples spiked with a known concentration of virus to confirm susceptibility of the cell cultures. Furthermore, negative matrix controls are taken along to ensure no other components in the formulation induce cell death or interfere with assay sensitivity. When making an inference on the COI, two variables play a role. The first one relates to the sensitivity of the COI assay which must be characterized to assign a minimum number of infectious units (i.e., lower limit of detection) that can be detected using the assay. The second one relates to the sampling size; the larger the volume of the test sample, the higher the chance of detecting a potential infectious unit in the entire batch. The combination of these two variables allows the manufacturer to either specify a maximally tolerable level of outgoing infectivity (Cornfield et al. 1956) or adhere to a predefined criterion. The World Health Organization (WHO) and European Pharmacopoeia (Ph. Eur.) have stipulated guidelines for testing for effective inactivation with a minimum sample, expressed in volume or number of doses, which must be tested. Furthermore, cell types, duration of incubation, and dilution of vaccine sample prior to inoculation are parameters that can influence the sensitivity of the COI and thus must be thoroughly optimized. The testing for COI for the different vaccines will be discussed per vaccine in more detail later in this chapter.

Once inactivated, the viral bulk is typically further purified to remove contaminants, this can be achieved by utilizing various techniques, examples are: ultrafiltration, size-exclusion chromatography (SEC), and sucrose gradient centrifugation. Furthermore, tests are required to assess the purity of the vaccine product, such as testing for the absence of contaminants arising from the production process such as host cell protein and DNA. Additionally, to ensure no modification of epitopes occurs during inactivation, as was observed with RSV and measles in the past, the immunogenic potency of the inactivated virus particle must be measured. This can be achieved by measuring the immune response before and after immunization in vivo, for example the Rat potency assay which is used to measure poliovirus neutralizing antibodies after immunization of rats with an inactivated poliovirus vaccine (van Steenis et al. 1981). In vivo immunogenicity testing can also been correlated to in vitro cell-based potency assays; such as the D-antigen ELISA which quantifies the antigenic content of inactivated poliovirus particles and is consequently used for dosing of the vaccine (Beale 1961). Different in vitro quality control systems to monitor antigenic integrity of an inactivated vaccine have been developed for rabies (Rooijakkers et al. 1996a, b), influenza (Di Trani et al. 2003), poliovirus (Morgeaux et al. 2005), and hepatitis A virus (Poirier et al. 2010). Naturally, the
development of such assays requires knowledge of the neutralizing epitopes necessary for an adequate immune response, and consequently, protection.

Not only do inactivated vaccines possess a higher safety profile as compared to live vaccines, they are also generally less reactogenic, relatively straightforward, and technically feasible to produce with fewer regulatory hurdles for licensure (Zepp 2011). However, inactivated vaccines are typically associated with a lower immunogenicity which can imply the necessity of multiple doses or adjuvant addition which consequently raises the costs of goods and vaccine pricing. Therefore, choosing an inactivated vaccine approach is in general a trade-off with on one hand increased safety (if inactivation is of course complete) and a fast pathway to regulatory approval, but on the other hand the risk of reduced antigenicity of the immunogen which often requires adjuvant addition and/or multiple doses which not only raises production costs but also the complexity of formulation and administration.

Today, there are six licensed viral vaccines that are inactivated with either formaldehyde or BPL. Formaldehyde is used for the inactivation of Poliovirus (PV), Hepatitis A Virus (HAV), Japanese Encephalitis Virus (JEV), and Tick Borne Encephalitis Virus (TBEV) to generate vaccines. BPL is used for the inactivation of Rabies and Influenza virus vaccines, however, there are also licensed vaccines against these infections that use formaldehyde as inactivating agent. This chapter will focus on the background, inactivation procedures, and analyses of inactivation of the six currently licensed inactivated viral vaccines categorized per inactivation method. Furthermore, attention will be paid to new inactivated viral vaccines in development.

2.2 Inactivated Vaccines Based on Formaldehyde Inactivation

Formaldehyde is the most widely used inactivating agent for vaccine purposes and many pathogens have been subjected to the irreversible modifications formaldehyde inflicts by cross-linking of various amino acids. Here, we describe the history, kinetics, and mechanism of formaldehyde inactivation, and subsequently the vaccines which currently use this inactivation agent.

For the sake of clarity and ease of referencing, nomenclature referring to either formaldehyde or formalin will reflect the terminology used by the original authors. For reference, 37 % w/v formaldehyde (13.3 M) equals 100 % formalin and a 1/4,000 dilution of formalin is thus identical to 0.009 % formaldehyde (i.e., 3.3 mM or 100 µg/ml formaldehyde).

Formaldehyde, with chemical formula CH2O, is the simplest member of the aldehydes, a group of organic compounds containing a carbon double bonded to hydrogen and a varying side chain. Formaldehyde exerts its effect by a great diversity of modifications (methylol groups, Schiff bases, and methylene bridges)
and the precise mechanisms are subject of investigation in several recent studies (Metz et al. 2004, 2006; Thaysen-Andersen et al. 2007). These modifications culminate in inactivation, stabilization, or immobilization of proteins with consequent loss of viral infectivity.

The first successful use of formaldehyde treatment was in the field of toxin inactivation. Here, Glenny and Hopkins accidentally discovered (Brown 1995) that residual traces of formalin—used for cleaning—rendered batches of diphtheria toxin non-toxic in guinea pigs (Glenny and Hopkins 1923). At the same time Ramon demonstrated that diphtheria toxoid, prepared by incubation with formalin at 37 °C for several weeks, retained its antigenic qualities (Rappuoli 1994; Wassilak et al. 2004). A similarly developed tetanus toxoid by Ramon and Zoeller was used to vaccinate humans against tetanus in 1926 (Wassilak et al. 2004). The form of the toxin which results from denaturing with formaldehyde is called an anatoxin, "toxoids", or "formalin-toxoids", and is used in vaccinations worldwide. Following the discovery of diphtheria and tetanus toxoids, formaldehyde was applied in the 1930s for the preparation of whole inactivated viral vaccines by inactivation of Russian Autumnal Encephalitis (Halstead and Tsai 2004), (a synonym for JEV), and TBEV (Smorodintsev and Ilyenko 1960).

The development of a formalin-IPV in the 1950s by Salk followed the previous attempts undertaken in the 1930s which had resulted in paralysis and several deaths due to incomplete inactivation of active virus (Brodie and Park 1936). At the time, it was largely believed that long lasting immunity required the administration of a live vaccine for successful immunization and protection, as was observed with the live attenuated small pox and yellow fever vaccines. However, Salk’s prior experience with formalin-inactivated influenza (Salk and Francis 1946) prompted him to believe that inactivated vaccines were possible and stimulated his research on PV inactivation using formalin. Aware of the disastrous events that came to pass with the first generation of inactivated PV vaccines, Salk vigilantly investigated formalin inactivation to guarantee safety of the vaccine preparations and established the principle of inactivation which started with the description of PV inactivation in 1953. At low temperatures the activity of formaldehyde is relatively low and might, thereby, be more selective in destroying infectivity without impairing too greatly the antigenic activity of the virus. Salk therefore inactivated PV using a 1/250 dilution of 37 % formaldehyde (corresponding to a final concentration of 0.4 % formalin) at the temperature of melting ice (Salk 1953). In the meantime, virus cultivation methods improved (Farrell et al. 1955) and higher titers of virus required up to 3 weeks of inactivation (Salk et al. 1954). To be able to inactivate also more potent batches of virus, Jonas Salk modified his inactivation process, now using a higher temperature (35–37 °C) and a lower concentration of formalin (1/4,000–1/8,000). The resulting process demonstrated linear inactivation kinetics and was able to inactivate also more potent batches of PV in a comparable timeframe (Salk et al. 1954; Salk and Gori 1960). Figure 2.1 is a graphical representation of an inactivation kinetics curve and illustrates Salk’s determination of the minimum required inactivation time.

A different approach was advocated by Swedish scientist, Sven Gard. In his experience, the thermal inactivation of PV at 37 °C was in the order of magnitude of
1–2 log units in 3 days ($\frac{1}{3} - \frac{2}{3}$ log per day). With the aim of eliminating the thermal component from the PV inactivation, Gard selected 25 °C where the thermal inactivation was only 0.3 log units in 5 days (0.06 log per day), together with 0.006 M formaldehyde (Gard 1957a, b). However, using these conditions a significant deviation from Salk’s linear relationship was observed (Gard 1957a, b; Gard and Lycke 1957; Gard et al. 1957; Lycke et al. 1957). To date, these observations are referenced to as evidence that the kinetics of formaldehyde inactivation is not linear or first-order (Bahnemann 1990; Brown 1993). In fact, whereas the kinetics of inactivation did not follow a first-order inactivation when performed at 4 or 25 °C, no deviation from first-order was observed at 37 °C (Salk and Gori 1960). Similarly, the inactivation of HAV—a process based on the principles of Salk—for the VAQTA vaccine showed no evidence of departure from linearity through 7 logs of inactivation (Armstrong et al. 1993).

A key consideration of Gard for selecting a lower inactivation temperature than Salk was the prevention of thermal inactivation of PV. In 1957, Charney et al. (1957), studied both formalin and thermal inactivation at 37 °C. Their results indicated that, contrary to the previously perceived thermal inactivation of 0.3–0.6 log per day (Gard 1957a, b), the thermal inactivation could be limited to 0.2 log per day and the addition of cations further improved thermal stability of PV
(Melnick 1991). Specifically, the addition of 1 M of MgCl₂ prevented thermal inactivation without an effect on the rate of formalin inactivation (Ozaki and Melnick 1963).

Having established the conditions for PV inactivation, Salk formulated the principles of inactivation of PV (Salk et al. 1954) which are still used as the basis for the development of inactivated vaccines (for a comprehensive overview see Pittman and Plotkin 2004). Briefly, the rate of destruction of infectivity in the bulk is followed by measuring for example the viral titer over time, which is measured by observing the (lack) of cytopathic effect in cells inoculated with the sample. When taking samples during inactivation, it must be taken into account that the formaldehyde present in the in-process sample must be neutralized prior to titration, for example, by addition of sodium bisulfite. Once viral titers during inactivation are obtained a straight line is drawn through these points and extrapolated to the point that indicates complete absence of infectivity. By taking into account the volume of the bulk that is being inactivated and a safety margin, the inactivation time can be defined as a total period equal to three times the interval required for interception of baseline (Salk et al. 1954). This principle is still used to define the minimum time that is required for complete inactivation (Armstrong et al. 1993; WHO 2002; Plotkin and Vidor 2004). However, studies investigating the effect of prolonged inactivation—treatment with formaldehyde beyond the time that is required for complete destruction of infectivity—showed that after a certain time, antigenicity of the material will decline (Salk and Gori 1960). Specifically, overtreatment of PV is defined as a period equal to more than fivefold the time required to reduce infectivity beyond the point at which it cannot be measured (Salk 1955). In summary, the inactivation time for PV can be defined as at least 3X but not more than 5X.

The use of formaldehyde for the inactivation of PV has one particular phenomenon that was discovered after Dulbecco demonstrated in 1954 that the number of plaques obtained for the three poliomyelitis viruses using monolayer tissue of monkey kidney and monkey testis was proportional to the concentration of virus (Dulbecco and Vogt 1954). Using this technique, Schultz et al. and Böttiger et al., demonstrated in 1957 and 1958, that formalin treatment alone produces a delay in initiating infection of tissue cultures as evidenced by the rate of appearance of plaque formation. Since the delay is progressively greater with extended formalin treatment, it becomes more and more important to prolong the observation period as complete inactivation by formalin is approached (Schultz et al. 1957; Bottiger et al. 1958). Consequently, large scale tissue culture safety tests include extended incubations—up to 35 days (Beardmore et al. 1957) for the detection of replication competent virus. Noteworthy here is that fully active PV could be detected within 14 days whereas false negatives occurred with formalin treated virus if not for the 21- and 28-days subcultures (Beardmore et al. 1957). Consequently, design of methods for demonstrating completeness of inactivation of formalin-inactivated virus should include verification and challenging of incubation times not only with fully active virus but also with formalin treated virus. In conclusion, current guidelines recommend to continue cultures for the detection of residual replication competent virus for as long as technically feasible (WHO 2002).
After completion of the inactivation period, it is common practice to demonstrate that the inactivating agent is active and therefore not rate limiting at the end of inactivation. One way of demonstrating inactivating capacity at the end of the incubation period is by spiking a sample with a known concentration of infectious virus and showing an additional reduction over time in titers of replication competent virus. Alternatively, one can estimate the concentration of residual free formaldehyde. There are a number of methods available for the determination of residual free formaldehyde in inactivated vaccines which are based on Hantzsch, MBTH, Phenylhydrazine, Tryptophan, Chromotropic acid, and Schiff reagents (Frerichs and Chandler 1980; Chandler and Frerichs 1980). Whereas the different methods give comparable results with non-neutralized formaldehyde, only the MBTH, Phenylhydrazine, and Schiff reagent method were accurate with vaccines in which formaldehyde was neutralized with sodium bisulphate (Chandler and Frerichs 1980) and of these three methods, the MBTH method is currently prescribed by the Ph. Eur. (2011a) for sodium bisulfite neutralized samples.

2.3 Examples of Formaldehyde Inactivated Vaccines

2.3.1 Inactivated Picornavirus Vaccines: IPV and HAV

Today, two licensed vaccines that are directed at PV and HAV, both members of the picornaviridae family, are based on formaldehyde inactivated whole viruses. The IPV was first licensed in 1955 while it took another 40 years before a HAV vaccine became available on the market. This delay was primarily due to the inability to propagate HAV to high titers in cell culture. Nonetheless, these two picornavirus vaccines have a comparable production method, where the well-established inactivation and testing of IPV has been used as a benchmark for the HAV vaccine inactivation and testing thereof.

Salk’s extrapolation of the linear regression of viral titers to ascertain completeness of inactivation to undetectable levels of infectivity resulted in a minimum inactivation time defined as 3X (Fig. 2.1), which included an incorporated safety margin for complete vaccine safety (Salk et al. 1954), as described earlier. Today, the WHO requires a similar extrapolation of inactivation kinetic curves to ensure sufficient inactivation time, as Salk proposed in the 1950s. The WHO stipulates a thoroughly studied inactivation curve where the inactivation time used for manufacture must exceed the time taken to reduce the virus titer to undetectable levels by at least a factor of 2 (WHO 2002). After inactivation the bulk must also be tested for completeness of inactivation, which must be done with sufficient rigor (Bodian 1958) as administration of incompletely inactivated vaccines is an unacceptable consequence for any manufacturer or regulatory body.

Testing for completeness of inactivation necessitates fixing a maximally tolerable level of outgoing infectivity, but this level need not necessarily ever be realized (Cornfield et al. 1956). The test strategy is based on detection of very low
concentrations of virus; at low concentrations where it is evident that an aliquot representing only a small percentage of the overall bulk may not contain infectious virus (USFDA 1998). The probability \( p \) that a sample does not contain virus can be expressed as:

\[
p = \left( \frac{V - v}{V} \right)^n
\]

where \( n \) is the absolute number of virus particles distributed in the overall volume \( V \) of the sample and \( v \) is the volume of the aliquot taken for residual virus testing. The above equation can be approximated by the Poisson distribution when a small aliquot of the overall sample is taken \( V \gg v \):

\[
p = e^{-cv}
\]

\[
c = \frac{(\ln p)}{-v}
\]

where \( c \) is the concentration of infectious virus particles per liter. Together with assumption on viral dispersion in the vaccine bulk, sensitivity of the detection system, and the viral concentration capable of inducing infection in man, one can determine an acceptable volume to test for a satisfactory probability of complete inactivation (Cornfield et al. 1956; Meier 1957). Therefore, the probability of detection of infectious virus at various virus concentrations per liter can be calculated, and upon assigning values for \( c \) and \( p \) one can calculate the testing volume necessary to measure virus at low levels with high probability of true detection. For example, a report published in 1956 by the US Public Health Service (Cornfield et al. 1956) recommended that 1,500 mL be tested from each of the single strain bulks on the basis of an “acceptable” level of infectivity set at an arbitrary 5 particulates per liter (White 1955). This means that with a bulk containing 5 or more particulates per liter, sampling 1,500 mL of this bulk should result in a probability of \( \geq99.9\% \) that the sample will contain \( \geq1 \) replication competent virus which should be unequivocally positive in the detection system.

Today, WHO and Ph. Eur. continue to adhere to the statistical sampling described in the 1950s, and consequently the testing for completeness of inactivation of IPV requires two samples that are equivalent to 1,500 adult doses to be tested in vitro on a susceptible cell type. Absence of PV infection in cell tissue cultures inoculated with these doses for at least 3 weeks is required before the monovalent bulk can be released for vaccine formulation (Ph. Eur. 2011d; WHO 2002).

The decades of research and experience with IPV has lead to its role as benchmark for the formalin inactivation of other pathogens in the vaccine field. The lessons learned in the beginning and middle of the last century had paved the way for the formulation of the Hepatitis A picornavirus vaccine. As with IPV, the manufacturability of a Hepatitis A vaccine required the successful propagation of the virus in a suitable substrate. The breakthrough of HAV growth in MRC-5 and WI-38 cell culture was achieved at the end of the 1970s by Maurice Hilleman et al.
(Provost and Hilleman 1979). Not only were these strains adapted to cell culture to allow large scale vaccine production, they had also lost their virulence during cell culture adaptation and were considered attenuated. In fact, their use as live vaccines has also been investigated (Midthun et al. 1991; Provost et al. 1986a). Nonetheless, development of an inactivated vaccine was favored due to the faster route to regulatory approval, resulting in a safe and effective formalin-inactivated vaccine in 1986 (Provost et al. 1986b).

Table 2.1 gives an overview of the currently licensed Hepatitis A vaccines. Manufacturing of these vaccines follows the general flow of events for all inactivated vaccines; virus propagation, harvest and purification, inactivation, further purification, and testing for residual replication competent virus. All manufacturers use the diploid human MRC-5 cell line for the propagation of different Hepatitis A strains of which HM175 and CR326 have been shown to be highly attenuated. Purification and concentration are followed by inactivation. Specifications for inactivation conditions such as formaldehyde concentration, temperature and length of inactivation, are undefined. Instead a manufacturer validated inactivation procedure is used, with elucidation of inactivation kinetics and extrapolation of the curve where 100% inactivation is achieved and multiplication of this inactivation time by a factor 3 is required, as is the case for IPV (Ph. Eur. 2011b; WHO 1995). The inactivation kinetics of Merck’s VAQTA vaccine have been described in the literature and show linear inactivation kinetics for formaldehyde at various formaldehyde concentrations tested. Formaldehyde at a concentration of 100 μg/ml and

<table>
<thead>
<tr>
<th>Name</th>
<th>Havrix</th>
<th>Vaqta</th>
<th>Avaxim</th>
<th>Epaxal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>GSK</td>
<td>Merck</td>
<td>Sanofi Pasteur</td>
<td>Crucell Switzerland</td>
</tr>
<tr>
<td>Virus strain</td>
<td>HM175</td>
<td>CR326</td>
<td>GBM</td>
<td>RG-SB</td>
</tr>
<tr>
<td>Cell substrate</td>
<td>MRC5</td>
<td>MRC5</td>
<td>MRC5</td>
<td>MRC5</td>
</tr>
<tr>
<td>Concentration and purification</td>
<td>Sterile filtration, ultrafiltration, gel permeation chromatography</td>
<td>Precipitation in polyethylene glycol, chromatography</td>
<td>Sterile filtration, chromatography, ultrafiltration, diafiltration</td>
<td>Ultrafiltration, ultracentrifugation</td>
</tr>
<tr>
<td>Inactivation parameters</td>
<td>250 μg/ml formaldehyde 15 days at 37 °C</td>
<td>100 μg/ml formalin 20 days at 37 °C</td>
<td>Formaldehyde concentration not specified 14 days at 37 °C</td>
<td>0.25 % formalin (w/v) 10 days at 37 °C</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>Aluminum hydroxide</td>
<td>Aluminum hydrophosphate</td>
<td>Aluminum hydroxide</td>
<td>Immunopotentiating reconstituted influenza virosomes (IRIV)</td>
</tr>
<tr>
<td>Antigen per dose for adult and child doses</td>
<td>1440 ELISA units ≥19 years</td>
<td>50 HAV Antigen units ≥19 years</td>
<td>160 Antigen units &gt;15 years</td>
<td>&gt;24 IU HAV protein ≥2 years</td>
</tr>
<tr>
<td></td>
<td>720 ELISA units 2–18 years</td>
<td>25 HAV Antigen units 2–18 years</td>
<td>80 Antigen units &lt;16 years</td>
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</tbody>
</table>
at a temperature of 37 °C reduced infectious titers to 1 (TCID\textsubscript{50})/ml within 48 h (Armstrong et al. 1993). The reduction in infectivity is quantified by titration in susceptible cells (i.e., MRC5). During manufacturing of VAQTA, HAV is inactivated during 20 days, which is tenfold the time needed to reach the x-intercept. This process thus exceeds by far the threefold inactivation time specified by regulatory authorities; the authors suggest that prolonged incubation of HAV with formaldehyde does not compromise the immunogenicity of the virus particle, which is not the case for PV, as described earlier.

A manufacturer’s validated and thoroughly investigated inactivation procedure is evaluated for approval by the regulatory authorities. As with IPV, testing for effective inactivation relies on reproducible inactivation kinetics and the testing of two samples of 1,500 doses on a suitable substrate, the identical number of doses as used for the testing of inactivated PV (Ph. Eur. 2011b; WHO 1995).

In contrast to IPV, the Hepatitis A vaccine includes the addition of an adjuvant for adequate immunogenicity. Most manufacturers of Hepatitis A vaccines have utilized the well characterized and established aluminum salts as adjuvants that are thought to strengthen humoral immune responses (Gupta 1998). An exception is Epaxal, manufactured by Crucell, in which the inactivated Hepatitis A particles are adsorbed to virosomes of Influenza haemagglutinin, which are argued to stimulate both cellular and humoral immune responses (Bovier 2008; Bungener et al. 2005).

2.3.2 Inactivated Flavivirus Vaccines: JE and TBE

Two formalin-inactivated vaccines are available for protection against infection with the flaviviruses JEV and TBEV. JEV is most prevalent in Asian regions (Gupta 1998) whereas TBE can be encountered in European to Asian regions (Rendi-Wagner 2008). Due to their limited distribution, vaccines against these viruses are not produced in quantities for global immunization but instead at quantities that can supply inhabitants of endemic areas, as well as travelers and expatriates (WHO 2006b, 2011). Manufacturing of these vaccines occurs mainly in the endemic areas by local manufacturers; therefore regulations for safe vaccine production are largely left to the scrutiny of the national regulatory authority. As is the case for HAV vaccines and IPV, global regulatory authorities such as WHO and Ph. Eur. do not specify any inactivation conditions. Instead manufacturers are again expected to demonstrate extensive studies on the inactivation of JE and TBE virus by using, for example, formalin to create safe vaccine batches. The local national authority would then determine whether the vaccine inactivation procedure is sufficient to guarantee a safe, completely inactivated vaccine. The WHO does, however, give examples of methods that have been used for the inactivation of JEV and TBE vaccines; for JEV this is stated as 50–60 days at 4 °C with a formaldehyde concentration of 1 in 2000 (WHO 2007b) although for a Vero-based JEV vaccine inactivation during several months at 4 °C with a higher formalin concentration of 0.08 % was used (Sugawara et al. 2002). For TBE manufacture a formaldehyde
concentration of 0.05 % is proposed with an inactivation time of 5 days at 22 °C (WHO 1999). For WHO approval, testing for completeness of inactivation requires testing of 25 human doses for JEV (WHO 2007b) and 20 doses for TBE (WHO 1999), while the Ph. Eur. requires a minimum of 10 human doses for TBE (Ph. Eur. 2011f). The vaccine doses are to be amplified on a suitable cell substrate followed by intracerebral inoculation of mice with the resulting culture fluid.

2.3.3 Formaldehyde Vaccines Summarized

Methods for formaldehyde inactivation vary greatly between vaccines. Differences lie in formalin concentrations (from 0.08 to 0.009 % w/v), time of inactivation (from days to months), and temperature (usually 4 or 37 °C). In general, the higher the formalin concentration and temperature the faster the inactivation, but this may come at a cost on immunogenicity as thermal degradation and destruction of important epitopes upon higher formalin concentration is a known phenomenon. Therefore, an inactivation time must be sufficient to ascertain complete inactivation but not too long as to destroy immunogenicity, concurrently, a manufacturer should monitor immunogenicity of the inactivated sample during inactivation to ensure no loss of antigenic potential. When faced with the development of an inactivated viral vaccine, an in depth understanding of the particular pathogen’s inactivation kinetics curve is inevitable as well as the necessity for a robust test for completeness of inactivation. Only then a reproducible and validated inactivation procedure can be set in place to ensure that a negative result in the test is a true measure for the absence of replication competent virus in a vaccine batch.

2.4 BPL Inactivation of Viruses for Vaccine Purposes

β-Propiolactone (BPL) is the second agent that is widely used for the inactivation of viruses in order to use them as vaccines. BPL is used in the production of Influenza and Rabies vaccines but is also used for vaccines that are currently being developed. BPL, a colorless liquid with a slightly sweet odor, is part of the four-member ring lactones family. The chemical reactivity of the almost planar, energetically highly strained four-membered ring provides the organic compound with its electrophilic nature and thus the ability to react readily with nucleophiles. While being stable in concentrated liquid form, in aqueous solutions it is unstable due to rapid hydrolysis that allows it to react with hydroxyl, amino, carboxyl, sulfhydryl, and phenolic groups (Hartman and Logrippo 1957). The reactions of BPL with all solution constituents make BPL in essence a self limiting compound. The rapid hydrolysis into nontoxic, noncarcinogenic products will completely eliminate BPL levels from the reaction within 2 h at 37 °C. This gives BPL an advantage over formaldehyde inactivation where residual formalin must be removed. In addition to
its self-limiting nature, excess BPL can be neutralized by the addition of thiosulphate, for example, when sampling virus during inactivation. This method is preferred over a high temperature spike for the neutralization of BPL activity as the latter may have the unwanted side effect of thermal degradation of the virus (Lawrence 2000). All reactions with BPL are rapid and stable. Alkylation or acylation reactions with interacting nucleophiles, which are repeatedly present in large biological macromolecules such as DNA and RNA, are irreversible.

BPL was first produced in 1915 by Johannson (Hartman and Logrippo 1957) who studied the salt of β-iodopropionic acid. However, it was not until 1941 when a novel method of synthesis was introduced by Kung to form BPL from ketene and formalin, that the widespread use of this organic compound began (Hartman and Logrippo 1957). After the discovery of this novel production method, BPL was subject to extensive characterization and research leading to the chemical’s fast introduction into multiple industries with varying roles. BPL has been used as a sterilizing agent for tissue grafts and plasma, a monomer for the plastic polymerization industry, an intermediate in the synthesis of propionic compounds, and a virucidal inactivating agent for vaccine purposes (Hartman et al. 1954; Lawrence 1999). The extensive use of BPL in these industries was somewhat reduced when BPL’s carcinogenic nature was recognized. Rightfully so, the use of carcinogens in production processes is considered a safety hazard (Hueper 1963). Nonetheless, BPL has remained essential for the production of the abovementioned licensed BPL-inactivated vaccines as levels of BPL are completely hydrolyzed during vaccine production and residual levels verified.

The mechanism of action by which BPL can inactivate viruses is thought to lie primarily in its direct interaction with nucleic acids (Colburn et al. 1965; Mate et al. 1977; Roberts and Warwick 1963). The alkylation and acylation reactions between nucleotides and BPL have been extensively studied. It has been shown that BPL mainly reacts with the Nitrogen-7 atom of guanosine, and to a lesser extent with adenosine at the N1 position (Hemminki 1981). The BPL-modified guanine is then misread by the polymerase as an adenine, therefore for every alkylated guanosine a GC-AT transition mutation is incorporated (Segal et al. 1981). These multiple mutations in combination with BPL-induced DNA double helix cross-linking (Perrin and Morgeaux 1995) can render the genomes dysfunctional, ultimately making the pathogen replication incompetent, leading to its complete inactivation.

As BPL primarily interacts with DNA or RNA, it was assumed that the immunogenic epitopes of the protein would remain intact; entailing that BPL-inactivated viruses would maintain their high immunogenicity, which is not always the case for formaldehyde inactivated viruses. However, amino acids and proteins also display nucleophilic moieties with which BPL can readily interact, and so, as with formaldehyde, the interaction of BPL with viral proteins could induce conformational changes on the viral surface. This could result in the alteration of epitopes necessary for the induction of neutralizing antibodies against the pathogen. Uittenbogaard et al. have elucidated the alkylation or acylation modifications that BPL induces on amino acids. They have shown that BPL reacts with nine different amino acids (Uittenbogaard et al. 2011) which implies that BPL inactivation could result in
alteration of important immunogenic epitopes necessary for an immune response. However, BPL will react more readily with nucleotides in DNA and RNA than with amino acid moieties. BPL mediated alkylation or acylation of viral constituents induces significant modifications of these complex molecules which prevents viral replication, upon which the virus particle is considered to be inactivated (Dijkstra 1975; Logrippo 1960; Roberts and Warwick 1963). Overall, the impact of BPL on immunogenicity varies per pathogen but is lower than the impact of formalin as BPL interacts with protein moieties to a lesser extent.

Viral inactivation is directly correlated with the nature of the virus, the initial concentration of BPL, the temperature, and the composition of the solution during inactivation (Budowsky et al. 1991). For vaccine production, these parameters are generally set at 4 °C for 18–24 h with a BPL concentration of 0.1–0.25 %, however, this can differ per pathogen (Lawrence 2000). Understanding of BPL inactivation kinetics is essential to ensure complete vaccine safety which dictates a reduction of infectivity of initial virus suspension by 15–20 orders of magnitude. This is based on various factors such as minimum probabilities of viral presence in a whole batch and doses necessary for immunization (Budowsky and Zalesskaya 1991). The magnitude of 15–20 orders of inactivation cannot be determined experimentally but requires extrapolation of kinetics data obtained from the experimentally detectable parts of the inactivation kinetics curves (Budowsky and Zalesskaya 1991). Viral inactivation curves after exposure to BPL are not linear and display a phenomenon called “tailing”: a reduction in inactivation rate during BPL treatment. This is due to the decrease in BPL concentration over time that is caused by the reaction of the agent with virus, water, or medium components, which decreases the rate of inactivation over time. The kinetics of viral inactivation can be described rather accurately when the varying BPL consumption rates over the reaction are taken into account using the following formula:

$$\ln \frac{S_0}{S_t} = \frac{k}{k_1} A_0 [1 - \exp(-k_1 t)]$$ (Budowsky et al. 1991; Budowsky and Zalesskaya 1991)

where $S_0$ and $S_t$ are the numbers of infectious particles before and at time $t$ after beginning of inactivation, $A_0$ is the initial concentration of BPL, $k$ is the initial infectivity inactivation rate constant, and $k_1$ is the total rate constant for the consumption of BPL during inactivation. This description of inactivation kinetics allows, when rate constants are known, the calculation duration of inactivation necessary to reduce infectivity of the virus to any given extent, which can ensure a completely inactivated, safe vaccine (Budowsky and Zalesskaya 1991). Figure 2.2 depicts the theoretical BPL inactivation curves as calculated by the above formula, as well as experimental data. Initial concentrations of BPL and inactivation temperatures are investigated; in all reactions the tailing phenomenon is visible. In the end, the BPL mediated alkylation or acylation of viral constituents, whether nucleic acids or proteins has resulted in the organic compounds being used in virus inactivation for decades, with two, currently licensed, human BPL-inactivated vaccines on the market and multiple vaccines in development.
Fig. 2.2  Kinetics of inactivation graph BPL, theoretical curves (black lines) calculated according to equation listed above and experimental data (open diamonds) of phage MS2 inactivation at different BPL concentrations (a) and temperature (b). Taken from Budowsky and Zalesskaya 1991, with permission.
2.5 Examples of BPL-Inactivated Vaccines

2.5.1 Inactivated Influenza Vaccine

The first reports of vaccination against influenza stem from the 1930s (Stokes et al. 1937) which ultimately lead to the licensure of the first inactivated influenza vaccine in 1945 in the US (Francis et al. 1946; Salk and Francis 1946). Over the course of more than 80 years, the currently available inactivated influenza vaccines have undergone several improvements and have shown significant benefits for society (Clover et al. 1991; Edwards et al. 1994; Gruber et al. 1990; Neuzil et al. 2001; Wilde et al. 1999), however, breadth of protection and efficacy of currently available vaccines are still insufficient to diminish the current annual health burden induced by the virus. Differences in protective efficacy may result from continuing antigenic variation in the prevalent epidemic strains. Due to this variation, the composition of inactivated influenza virus vaccine, unlike that of most viral vaccines, must be kept constantly under review. Accordingly, WHO publishes recommendations concerning the strains to be included in the vaccine twice annually (WHO 2000, 2009a; Ghendon 1991).

Until recently inactivated influenza vaccines consisted of three inactivated viruses; two Influenza A strains and one B strain, however, a new pattern of influenza B circulation has rendered it troublesome to predict the global dominance of one of the two influenza B lineages (Paiva et al. 2013). Therefore, quadrivalent influenza vaccines have been developed to ensure broader protection against Type B influenza viruses as compared to the trivalent vaccines which contained only one Type B influenza strain from one lineage. The licensed quadrivalent inactivated influenza vaccines are formulated in the same way as their trivalent counterparts, however, two influenza B strains, one from the Victoria lineage and one from the Yamagata lineage, are included in the formulation.

After inactivation the vaccine strains are either formulated as virosomes (Herzog et al. 2009), whole inactivated virus (WIV), or detergent-treated “split” vaccines, where the viral envelope is disrupted after inactivation (Wood 1998; Schultz-Cherry and Jones 2010). All the US-licensed inactivated influenza vaccines are split vaccines as “splitting” of the virus is thought to reduce reactogenicity, especially in children (Verma et al. 2012; Nicholson et al. 2003). However, WIV vaccines have been reported to induce stronger immune responses in immunologically naive individuals than split-virus or subunit vaccines (Beyer et al. 1998; Nicholson et al. 1979). Budimir et al. have recently shown that only WIV influenza vaccines, and not split or subunit vaccines, are capable of inducing cross-protection against heterosubtypic challenge due to elicitation of a strong CTL response in mice as whole (BPL) inactivated vaccines are capable of endosomal fusion into the cell cytoplasm (Budimir et al. 2012). The necessity of an influenza vaccine that can elicit cell-mediated immunity and the superiority of WIV vaccines over split vaccine variants has recently been reviewed (Furuya 2012).
After harvesting the (reassorted) vaccine strains, purification steps such as filtration or sucrose gradient centrifugation may be performed before inactivation of the monovalent bulks. Currently, licensed vaccines are inactivated using either BPL or formaldehyde. Although formaldehyde inactivated vaccines are successful in eliciting protection against influenza, it has been reported that formaldehyde interferes with the fusion ability of the inactivated virus particle (Geeraedts et al. 2012) which in turn prohibits an optimal CTL response. This is not observed for BPL-inactivated influenza virus (Budimir et al. 2012). According to the Ph. Eur (2011c), the concentration of BPL and formaldehyde cannot exceed 0.1 % (v/v) and 0.02 %, respectively, during the entire inactivation procedure. Other inactivation parameters, such as time of inactivation and temperature are not specified. As with formalin-inactivated vaccines, a manufacturer-validated inactivation method must be demonstrated to the approving regulatory body. The principles of influenza inactivation have been investigated with respect to initial BPL concentration, temperature, and composition of the solution where it was shown that inactivation of influenza A at 20 °C at BPL concentrations of 0.011 or 0.055 M ranges from 35 to 170 min, depending on whether the virus sample is purified or in harvested allantoic fluid (Budowsky et al. 1991). Testing for effective inactivation is specified by the Ph. Eur. (2011c), and should be performed by inoculation of either embryonated hens’ eggs or the cell substrate that was used for vaccine production with a fixed amount of vaccine product, after which the cultures are tested for absence of hemaglutination, which is a surrogate for infectious virus activity.

After inactivation, antigen is purified to reduce nonviral contaminants and concentrated by various methods such as centrifugation through sucrose gradient, passage over chromatographic column, dialysis, or filtration. Splitting of the virus particles is achieved by addition of a solvent (an ether or detergent) during or before purification which disrupts the viral membrane. Monovalent vaccines are finally combined to form the final vaccines. Table 2.2 gives an overview of the FDA approved inactivated influenza vaccines, all of which are split vaccines.

### Table 2.2 Overview of FDA approved inactivated influenza vaccines

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Manufacturer</th>
<th>Inactivation agent</th>
<th>Splitting agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLURIA</td>
<td>CSL</td>
<td>BPL</td>
<td>Sodium taurodeoxycholate</td>
</tr>
<tr>
<td>Agriflu</td>
<td>Novartis</td>
<td>Formaldehyde</td>
<td>CTAB</td>
</tr>
<tr>
<td>Flulaval (Quadrivalent)</td>
<td>ID Biomedical Corporation of Quebec</td>
<td>UV + Formaldehyde</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>Fluarix (Quadrivalent)</td>
<td>GlaxoSmithKline</td>
<td>Formaldehyde</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>Fluvirin</td>
<td>Novartis</td>
<td>BPL</td>
<td>Nonylphenol ethoxylate</td>
</tr>
<tr>
<td>Fluzone (Quadrivalent)</td>
<td>Sanofi</td>
<td>Formaldehyde</td>
<td>Octylphenol ethoxoylate</td>
</tr>
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</table>


2.5.2 Inactivated Rabies Vaccine

The second currently licensed BPL-inactivated viral vaccine is a rabies vaccine which has an equally rich history of development. Pasteur introduced an experimental rabies vaccine in 1885 when he observed the rapid decrease of rabies virus virulence upon air drying of rabies-infected rabbit spinal cords. Serially less dried rabies-infected rabbit spinal cords containing inactivated—or at least partially inactivated—rabies viruses induced protection of dogs and later humans against challenge following inoculation (Bazin 2011; Pasteur et al. 1885). This method of vaccination, although it was considered a treatment for infected people at the time, was the foundation for rabies vaccines. However, Pasteur faced significant criticism from the scientific community as recipients were essentially inoculated with virulent virus at the end of the treatment (Burke 1996; Gelfand 2002; Wu et al. 2011). This set the incentive to chemically inactivate the rabies virus with phenol in 1908 leading to the first completely inactivated rabies vaccine, despite the disruptive action of phenol on the antigenic sites on the proteins (Fermi 1908; Semple 1911; Briggs 2012).

In the 1950s and 1960s the vaccine was further improved by using alternative substances to cultivate rabies virus, such as chicken and duck embryos (Peck et al. 1955). This due to the fact that vaccines based on adult mammalian nerve tissue were associated with effects such as encephalomyelitis and demyelination lesions in the CNS due to the presence of myelin (Bonito et al. 2004; Bahri et al. 1996). Therefore, the WHO currently does not recommend the use and production of nerve tissue vaccines (WHO 2005) and has been advocating use of cell culture or embryonated eggs as production platforms since 1983 (WHO 1984). In the US, only cell culture derived rabies vaccines are approved for commercial use, however, some African and Latin American countries continue to produce and use nerve tissue vaccines by phenol inactivation, where the vaccine production protocol resembles the methods from a century ago (Briggs 2012). Today, there are two primary avian cell lines used for rabies vaccine production; purified chick embryo cell vaccine (PCECV) and purified duck embryo rabies vaccine (PDEV) and multiple continuous cell lines such as MRC-5, Vero, and primary hamster kidney cells. However, inactivated vaccines produced on continuous cell lines are not completely free from adverse reactions. There are reports on reactogenicity in response to vaccination with the human diploid cell rabies vaccine (HDCRV) which may relate to the presence of BPL-altered human albumin, added as a stabilizer to vaccine preparations (Anderson et al. 1987; Swanson et al. 1987). Nonetheless, cell culture based vaccines are still vastly preferred over nerve tissue vaccines. Moreover, an additional advantage of the use of a cell line platform, for instance Vero cells, is that they can be cultured in large scale in fermenters on microcarriers which contributes to standardization, safety, and upscaling of the production system resulting in constant yields.

Despite the variation in vaccine cell substrates, the majority of the rabies vaccines are inactivated in a similar manner using a concentration of not more than
1:3,500 and up to 1:5,000 v/v of BPL at 2–8 °C for 24 h (WHO 2007a; Ph. Eur. 2011e). However, there are exceptions such as the use of formalin for Primary Hamster kidney cell culture vaccine (PHKCV). As with the formalin-inactivated vaccines, the inactivation curves have to be validated and approved by the regulatory body. After inactivation, different purification standards can be used such as ultrafiltration, ultracentrifugation, zonal centrifugation, or chromatography. Once formulated, the vaccine potency for all these vaccines is determined by quantifying the degrees of protection against rabies following immunizing and intracerebral challenge of mice (de Moura et al. 2009; Fitzgerald et al. 1978). Based on the results of this National Institutes of Health (NIH) test, the vaccine dosing is set at 2.5 International Units/dose. Many regulatory authorities, including the Ph. Eur. and WHO, have adopted the NIH potency test as the only assay for potency quantification of inactivated Rabies vaccines, despite the recognition of the fact that the animal test should be replaced by an antigen quantification procedure (Bruckner et al. 2003). The vaccine is further tested for complete inactivation by inoculating the cell substrate used for manufacturing with 25 human vaccine doses or more. Cultures are examined for the presence of newly produced rabies virus using immunofluorescence.

### 2.5.3 BPL-Inactivated Vaccines Summarized

As with the formaldehyde inactivated vaccines, BPL-inactivated vaccines have varying inactivation procedures, with different temperatures, BPL concentrations, and inactivation times for complete inactivation. A thoroughly studied inactivation curve of virus activity is required for validation of inactivation parameters and confident declaration of viral absence, which a regulatory agency can consequently approve for vaccine licensure. As compared to formaldehyde inactivation, BPL inactivation times are significantly shorter where minutes to hours can suffice in inactivating viral activity as compared to the days or months needed for formaldehyde inactivation. Further advantages of BPL inactivation is the lower inactivation temperature which may prevent thermal degradation of important epitopes, moreover, protein moieties are less likely to be altered by BPL due to the primary reaction of the compound with nucleic acids. Despite the evident advantages of BPL, formaldehyde is more widely used for inactivation of pathogens, perhaps due to historical use of the compound and years of experience which has paved regulatory pathways for the licensure. Nonetheless, it is evident that the advantages of BPL should be considered by vaccine manufacturers for viral inactivation.
2.6 Inactivated Vaccines in Development

The century old concept of the use of inactivating viruses to elicit protection against the virulent pathogen continues to bear fruit for humanity. Countless improvements and innovations in the field of vaccinology, such as the introduction of recombinant, DNA-based, and vectored vaccines have not stopped the use and development of inactivated vaccines. The relative straightforwardness in which an inactivated vaccine is produced and licensed, accompanied by the fact that inactivated vaccines cannot revert as their replication competent counterparts can do, explains the fact that there are new inactivated pathogens that are being evaluated as vaccine candidates. However, inactivation does not always guarantee the creation of a suitable vaccine as was observed with pathogens such as RSV and measles, therefore immunogenicity of the novel inactivated particle must always be thoroughly tested. Furthermore, new inactivation methods are also being investigated to circumvent the disadvantages of formalin and BPL such as altered immunogenicity due to epitope masking. This section will provide an overview of novel inactivated vaccines in development as well as new inactivation methods.

2.6.1 New Inactivation Methods

The increased safety associated with inactivated vaccines does not entail a spotless track record, as was described for the formalin-inactivated RSV and measles vaccines. The inadequate immune response induced with inactivated viruses is thought to be due to masking of essential epitopes. This drives the investigation of alternative inactivation methods that do not alter epitopes or skew immune responses to ensure a protective vaccine with high efficiency. Three new inactivation methods, being hydrogen peroxide treatment, zinc-finger reactive treatment, and gamma irradiation are described in more detail below. Whether these inactivation methods will be implemented in the manufacturing of vaccines remains to be determined.

A hydrogen peroxide based vaccine platform has been proposed by Amanna et al. (2012). Oxidizing agents are an essential part of the mammalian innate immune system (Valko et al. 2007) and use of such agents, like H$_2$O$_2$, as antimicrobial and antiseptic agents have been well established (Linley et al. 2012). However, the use of H$_2$O$_2$ in the inactivated vaccine industry was never considered as H$_2$O$_2$ is believed to irreversibly damage basic molecular structure of proteins (Skykes 1965). H$_2$O$_2$ inactivation of a range of DNA and RNA viruses showed minimal damage to epitopes compared to BPL and formalin. In addition, superiority of H$_2$O$_2$ inactivated vaccines was demonstrated by the elicitation of a strong neutralizing antibody response, effective T cell responses, and protection in mice (Amanna et al. 2012). Furthermore, the use of H$_2$O$_2$ requires a much shorter inactivation time in comparison to formalin and decomposes into nontoxic products (water and oxygen). Mechanism for H$_2$O$_2$ inactivation is the genomic damage induced by hydroxyl
radical attack on nucleosides resulting in single- or double-strand breaks ultimately leading to inactivation of the virus (Termini 2000). The authors propose $\text{H}_2\text{O}_2$ as a feasible, broad-spectrum, and effective inactivation platform.

Conserved zinc-finger motifs within the small, basic, nucleic acid-binding nucleocapsid proteins of retroviruses are essential for virus replication (Aldovini and Young 1990). The vital role of this motif has lead to the discovery and development of various compounds that covalently bind these motifs and abrogate infectivity of viruses (Rice et al. 1995). Despite the loss of infectivity, the inactivated virus still retains the capacity to enter target cells as well as its structural and functional integrity (Rossio et al. 1998). To that end, use of zinc-finger reactive compounds was examined for RSV inactivation with maximum preservation of the virion surface structure. RSV contains zinc-finger motifs in the M2-1 protein, necessary for processivity of the viral polymerase (Hardy and Wertz 1998). The 2,2-dithiodipyridine zinc-finger reactive compound was shown to effectively inactivate RSV and conveyed moderate immunogenicity in cotton rats, which could be raised dramatically in combination with the ribi adjuvant system (RAS), an oil-in-water emulsion. However, addition of this adjuvant also induced the enhanced disease, which was not observed with the inactivated RSV alone (Boukhvalova et al. 2010). More research would be necessary to produce a safe and immunogenic-inactivated RSV, however the authors argue the superiority of zinc-finger reactive compounds over the traditional methods with respect to an unaltered protein structure and feasibility of upscaling for large scale production.

Gamma irradiation as a physical means of virus inactivation is not a novel method as it has been used extensively in the past for investigational vaccine purposes (Campbell 1985; Marennikova and Macevic 1975; Reitman and Tonik 1971; Reitman et al. 1970; Wiktor et al. 1972). It is argued that $\gamma$-irradiation is superior to the conventional chemical methods (formalin and BPL) due to the view that organisms can be rendered incapable of replication by generating strand breaks in genetic material without structural destruction of proteins (Furuya 2012). Furthermore, $\gamma$-irradiation is associated with high penetration capacity allowing viral inactivation of large volumes, stored in closed containers, and even in frozen state. Moreover, there is no need to remove a chemical compound after inactivation (Furuya 2012). Despite all these advantages no $\gamma$-irradiated vaccine exists today, this presumably due to the success of formalin and BPL which represent well-established and regulatory-accepted inactivation methods, which manufacturer’s may prefer for vaccine production. Furthermore, concerns surrounding the safety of $\gamma$-irradiation may also have inhibited its use in the vaccine industry, however, these concerns may diminish as more research on the application of $\gamma$-irradiation is increasing (Frenzen et al. 2001). Recently, a $\gamma$-irradiated Venezuelan equine encephalitis virus (VEE) strain has shown to convey protection against subcutaneous challenge at low doses in mice, however, despite the hypothesis that $\gamma$-irradiation should not affect epitopes, a decrease in antigenicity was observed after inactivation and protection against aerosol challenge was suboptimal (Martin et al. 2010a), nonetheless, this VEE vaccine candidate will be further pursued by the authors, as well as a formalin-inactivated counterpart (Martin et al. 2010b).
Gamma irradiation has also been proposed for whole inactivated influenza where improved heterotypic immunity was observed, primarily mediated by cross reactive T cells (Alshariﬁ and Mullbacher 2010; Furuya et al. 2010).

2.6.2 New Targets for Whole Inactivated Vaccine Development

2.6.2.1 Inactivation of Attenuated Viruses

As described earlier in the chapter, attenuated vaccine viruses may revert to a virulent form which would make them capable of causing the disease against which they should protect. There are multiple licensed attenuated vaccine viruses that are currently being considered for inactivation for vaccine purposes.

The oral polio vaccine (OPV) displays frequent reversion to virulence in vaccine recipients and there are estimates of approximately 400–800 vaccine-associated paralytic poliomyelitis (VAPP) cases per year globally (John 2002). Despite the immediate recognition of the fact that OPV strains can revert readily into a pathogenic phenotype (Henderson et al. 1964), OPV has been used since the 1960s and still is being used extensively. However, recently, it has been acknowledged that use of the oral live attenuated vaccine is at odds with global eradication of poliomyelitis. Indeed, the number of vaccine-associated poliomyelitis cases is in the range of wild-type PV induced poliomyelitis cases (WHO 2006a). Although IPV is a safe alternative, the costs of currently available IPV are too high to implement its use in low income countries (Heinsbroek and Ruitenberg 2010; Zehrung 2010) and several options to reduce costs of IPV are being considered (WHO 2009b). In the era after eradication, IPV use will have to be continued at least for a certain amount of time. At that time, production of IPV from wild-type PV strains will fall under strict biosafety measures (WHO 2009c). Even though it is currently not clear whether an IPV based on OPV strains may be produced at lower biosafety level after eradication as compared to a wild-type based IPV, there is much research going on to the inactivation of the OPV strains with formalin to eventually replace the inactivated PV vaccine based on the wild-type strains. Not only would the lowering of biosafety level decrease potential costs of goods, replacing the wild-type strains greatly reduces the risks of poliomyelitis upon accidental outbreaks from the manufacturing facility, after eradication. The manufacture of Sabin-IPV is essentially identical to the Salk-IPV process with slight modifications (Westdijk et al. 2011). The WHO encourages the development of this Sabin-IPV vaccine (Bakker et al. 2011) and multiple clinical trials have been or are being performed (Verdijk et al. 2011), moreover, in Japan a Sabin based IPV has recently been licensed in combination with diphtheria, tetanus, and acellular pertussis (DTaP-Sabin IPV) (Mahmood et al. 2013). In general, Sabin-IPV displays higher immunogenicity for serotype 1, lower for type 2, and similar for type 3 in comparison to Salk-IPV, licensure of more Sabin derived IPV’s is foreseen in the near future.
Monath et al. describe the results of a Phase I study of a BPL-inactivated Yellow Fever (YF) vaccine, based on the licensed attenuated 17D strain (Monath et al. 2011). The 17D vaccine was developed in 1936 by Max Theiler and today 20 million doses are issued per year. However, yellow fever vaccine-associated viscerotropic disease (YF-AVD) and yellow fever vaccine-associated neurological disease (YF-AND) occurring at a frequency of 0.4 and 1.8 per 100,000 doses, respectively (Lindsey et al. 2008), instigate a need for safer vaccines. Inactivated vaccines will reduce the adverse effects associated with the vaccine and is predicted to be less reactogenic as it has been cultivated on Vero cells instead of eggs (Hayes 2010). The alum-adjuvanted, BPL-inactivated vaccine induced neutralizing antibodies in a high percentage of subjects, albeit lower titers than the live vaccine, whether the lower titers will be compensated for by the higher safety profile is yet to be determined (Monath et al. 2011).

The oral rotavirus vaccines have also been shown to be disadvantageous with respect to safety and regional inefficacy to induce an adequate immune response, consequently, an inactivated counterpart has been proposed for development (Jiang et al. 2008a). As “conventional” chemical inactivation of rotavirus with formaldehyde or BPL treatment has shown to destroy integrity of the immunogenic epitopes (Offit and Dudzik 1989; Zissis et al. 1983), a novel heat inactivated rotavirus vaccine (IRV) has been proposed for development. Rotavirus inactivated at 60 °C for 2 h has shown to be immunogenic in mice (Jiang et al. 2008b) and gnotobiotic piglets when administered together with an aluminum adjuvant (Wang et al. 2010).

Another example of inactivation of attenuated virus strains for vaccine purposes is the inactivation of the attenuated Varicella-Zoster Virus (VZV) Oka/Merck strain. The live attenuated vaccine provides protection against varicella (chicken pox) and zoster (shingles), however, despite the high safety profile (Galea et al. 2008), there was stimulus to inactivate this attenuated strain for use in prevention of zoster in immunocompromised patients (Hata et al. 2002; Redman et al. 1997). Currently Phase III clinical trials are ongoing for this heat inactivated vaccine which has been reported to be (almost completely) inactivated by heating at 56 °C for 7 days which reduced the pfu/ml of the live vaccine preparation from 4,000 to 2 pfu/ml in the “inactivated” vaccine (Levine et al. 2000).

2.6.2.2 Inactivation of Wild-Type Viruses

The successes of the current inactivated viral vaccines have stimulated the research and development of other virus inactivated vaccines using formalin or BPL. A selection of interesting candidate vaccines is listed below.

Enterovirus 71 (EV71) is a picornavirus capable of inducing hand, foot, and mouth disease (HFMD) which can lead to serious neurological complications and even death (McMinn 2002). As with the other picornaviridea vaccines (PV and HAV) formalin was chosen as inactivating agent to develop an inactivated EV71 vaccine, propagated on Vero cells. In Taiwan, a pilot production procedure has been developed resulting in an optimized USP and DSP procedure. Inactivation studies
demonstrated that inactivation of the entire vaccine bulk would require 2.2, 12.6, and 31.6 days at 37, 25, and 4 °C, respectively (Chong et al. 2012). A Phase I clinical study has been scheduled using this inactivated bulk. In China, there are three Phase III clinical studies ongoing with a formalin-inactivated EV71 C4 strain grown on either Vero cells (Li et al. 2012; Zhu et al. 2013) or on human diploid KMB-17 cells (Dong et al. 2011), all adsorbed to aluminum hydroxide as an adjuvant.

The inactivated flavivirus vaccines against JEV and TBEV, have encouraged researchers to examine formalin inactivation of another member of the flavivirus family, namely Dengue viruses. A cell culture adapted Dengue virus type 2 (DENV2) that was propagated on Vero cells was inactivated with 0.05 % formalin for 10 days at 22 °C and adsorbed to alum hydroxide. This candidate elicited neutralizing antibodies as well as protection in macaques (Putnak et al. 2005). However, this monovalent candidate has not been further pursued since its development as it has been recognized that Dengue requires a potent vaccine that can elicit equally protective immune response against all 4 serotypes without risks of waning immunity (Heinz and Stiasny 2012).

Mosquito-transmitted alphaviruses such as Venezuelan, Western and Eastern equine encephalitis virus (VEE, WEE, and EEE virus) have been formalin-inactivated for use as vaccines for horses (Zacks and Paessler 2009). However, there are no commercially available alphavirus vaccines licensed for human use despite their development and use as investigational vaccines against bioterrorist threats by the US army. The reason for halted development most likely lies in the only moderate immunogenicity of this candidate (Edelman et al. 1979; Steele et al. 2007). Research focusing on the less known alphavirus Ross River virus (RRV), which is endemic in Australia and Papua New Guinea, causing epidemic polyarthritis (Harley et al. 2001), has lead to a phase 1/2 dose escalation study using an adjuvanted, formalin-inactivated RRV vaccine which showed high seroconversion rates in naïve healthy young adults (Aichinger et al. 2011). Another inactivated alphavirus vaccine in development is a chikungunya virus (CHIKV) inactivated vaccine, which has been studied in mice. Formalin, as well as BPL, inactivated CHIKV vaccines in combination with three different adjuvants were compared. The alum-adjuvanted, BPL-inactivated vaccine induced highest antibody titers, however, all inactivated CHIKV vaccine formulations conveyed some degree of reduction in viral replication after homologous challenge (Kumar et al. 2012).

Inactivation of respiratory syncytial virus (RSV) with formalin in the 1960s resulted in hospitalization and even deaths of infants as described earlier in this chapter. Therefore, inactivation was deemed an unsuitable and even dangerous method for RSV vaccine development. Nonetheless, a BPL-inactivated RSV vaccine supplemented with toll-like receptor (TLR) ligands and with total respiratory tract (TRT) administration has recently been proposed in mice as a nonreplicating RSV vaccine without the enhanced disease due to proper immune stimulation (Shafique et al. 2012). Inactivation was achieved by a 0.025 % BPL concentration for 16 h at 4 °C. Administration of this vaccine in combination with innate receptor ligands, induced a protective Th1 immune response. In addition, the protection was achieved without priming for enhanced disease.
Another example of a novel BPL-inactivated pathogen is the Severe Acute Respiratory Syndrome (SARS) Corona Virus propagated on Vero cells. When immunized in combination with an adjuvant mice and golden Syrian hamsters conveyed the induction of high titer neutralizing antibodies and protection from challenge (Roberts et al. 2010).

2.7 Concluding Remarks

Time will tell whether one or more of these vaccines in development will contribute to the struggle against infectious diseases in the form of a licensed inactivated vaccine. The extensive knowledge, and experience achieved with the existing viral inactivation procedures for vaccine manufacture will continue to serve as a foundation of vaccinology for novel inactivated vaccines. Today millions of people are, and will be, protected worldwide with inactivated viral vaccines. Furthermore, this number will presumably continue to grow as research in novel inactivated vaccines expands, as this method of vaccine preparation remains a relatively straightforward way to produce safe and effective vaccines. The concept of virus inactivation for vaccine production can be therefore seen as the low-hanging fruit within the tree of vaccine design, bearing in mind that complete understanding of viral inactivation and immunogenicity of the resulting particle are essential for success.

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