Vaccine Additives and Manufacturing Residuals in Vaccines Licensed in the United States

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In addition to one or more immunogens,[\*](#_bookmark0) a vaccine may contain any of several added substances such as an adjuvant or a preservative. Residual components from the manufactur- ing process, in varying amounts, are also present in the vaccine. This chapter addresses the types and amounts of additives that are present in vaccines, the rationale for their inclusion, and the applicable federal regulations. Additionally, residual mate- rials from the manufacturing process that are present in the final formulation of the vaccine, as well as relevant federal regulations regarding these residuals, are discussed. Finally, albeit to a limited extent, several issues and concerns that cur- rently pertain to the use of, or presence of, some of these additives and residuals are examined.

This chapter focuses on vaccines licensed in the United States; vaccines licensed outside the United States may contain the same types of additives and residuals, although the amounts that are present in any given vaccine may differ. For the pur- poses of this chapter, the term “additives” refers to materials that are added to the immunogen by the manufacturer for a specific purpose. Additives include adjuvants, preservatives (i.e., antimicrobial agents), and stabilizers, as well as materials that are added to affect pH and isotonicity. In addition to addi- tives, vaccines contain residuals that remain from the licensed manufacturing process. The final formulation—immunogen plus additives and residuals—defines the specific vaccine; although not all manufacturing residuals can be identified and quantified, their presence and quantity is assumed to be con- stant because of the constancy of the manufacturing process. Some information regarding additives and residuals is consid- ered to be a trade secret and thus confidential, and cannot be discussed in this chapter.

Vaccine manufacturing includes in-process and release

tests, along with their respective specifications, for the allow- able quantity of additives and certain residuals that may be present in the vaccine. These tests and their accompanying specifications are detailed in the product’s Biologic License Application (BLA); some of the specifications may be provided in the vaccine’s package insert. A manufacturer must report each change in the manufacturing process, including removal or adjustment in the quantity of an additive, to the U.S. Food and Drug Administration (FDA). FDA biologics regulations, found in 21 CFR §610.61, address whether the use of, and quantity of, additives and residuals must be disclosed in vaccine labeling. These regulations state:

*The following shall appear on the label affixed to each package containing a product …*

*(e) The preservative used and its concentration …*

1. *Known sensitizing substances, or reference to an enclosed circular containing appropriate information;*

\*An immunogen is a preparation consisting of all or a portion of a disease-containing organism, or the nucleic acid that encodes one or more of the proteins from that organism, or all or a portion of a human tissue, and it is administered to an individual to induce an immune response to the immunogen for the treatment or prevention of a disease or condition.

1. *The type and calculated amount of antibiotics added during manufacture;*
2. *The inactive ingredients when a safety factor, or reference to an enclosed circular containing appropriate information;*
3. *The adjuvant, if present;*
4. *The source of the product when a factor in safe administration;*
5. *The identity of each microorganism used in manufacture, and, where applicable, the production medium and method of inactivation ….*

# VACCINE ADDITIVES

## Preservatives

Preservatives are added to vaccine formulations to prevent the growth of bacteria or fungi that may inadvertently be intro- duced into the vaccine during use. In some cases, preservatives are used during the manufacturing process (e.g., in buffers and column washes) to prevent microbial growth. Improvements in manufacturing technology, however, have decreased this need for the addition of preservatives to control bioburden during the manufacturing process. The Code of Federal Regu- lations (CFR) requires that, with certain defined exceptions, or with the approval of the Center Director (discussed later), preservatives must be added to multidose vials of vaccines. In the past, tragic consequences followed the use of multidose vials that did not contain a preservative, and served, in part, as the impetus for this requirement (see Wilson1 for a discus- sion of incidents related to the lack of preservatives in vaccines). Specifically, 21 CFR §610.15(a) states; “Products in multiple-dose containers shall contain a preservative, except that a preservative need not be added to Yellow Fever Vaccine; Polio-virus Vaccine Live Oral; viral vaccines labeled for use with the jet injector; dried vaccines when the accompanying diluent contains a preservative; or to an Allergenic Product in 50 percent or more volume in volume (v/v) glycerin.”

Although the regulation does not specify a quantity, it does

require that the preservative used “shall be sufficiently non- toxic so that the amount present in the recommended dose of the product will not be toxic to the recipient, and in the com- bination used it shall not denature the specific substances in the product to result in a decrease below the minimum accept- able potency within the dating period when stored at the recommended temperature.”

The CFR does not, however, provide a definition of a pre- servative. The definition (i.e., antimicrobial effectiveness) that has been used by the FDA for vaccines and other biologicals is found in the U.S. Pharmacopoeia (USP).2 This is a func- tional definition, wherein the final formulation of the vaccine, including the preservative, is challenged with specified quanti- ties of the following organisms: *Candida albicans, Aspergillus brasiliensis, Escherichia coli, Staphylococcus aureus,* and *Pseudo- monas aeruginosa*. The test sample (preservative-containing vaccine plus the microorganism) is incubated at 20° to 25°C,

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and the number of viable microorganisms is determined on days 7, 14, and 28. A preservative is deemed acceptable if the following are achieved:

* Bacteria: a reduction of not less than 1.0 log10 from the initial count at 7 days, and not less than a 3.0 log10 reduction from the initial count after 14 days, and no increase in the 14-day count at 28 days.
* Yeasts and molds: remain at or below the level of the initial inoculum on days 7, 14, and 28.

Note that the antimicrobial agent is not tested by itself; rather, it is the final vaccine formulation that is tested.

Preservatives cannot completely eliminate the risk of bacte- rial or fungal contamination of vaccines; moreover, they do not address any potential viral contamination. Although it occurs rarely, and not in the recent past, the scientific litera- ture does contain reports3,4 (see also Wilson1) of bacterial contamination of vaccines despite the presence of a preserva- tive, emphasizing the need for meticulous attention to tech- nique when withdrawing vaccines from multiuse vials. At present, the following preservatives are used in U.S.-licensed vaccines: phenol, benzethonium chloride plus formaldehyde, 2-phenoxyethanol, and thimerosal (termed *thiomersal* in some other countries). Recently, the FDA amended the biologics regulations to permit exceptions or alternatives to the regula- tion for constituent materials (21 CFR §610.15), which includes preservatives and adjuvants. The following section was added to the regulation: “(d) The Director of the Center for Biologics Evaluation and Research (CBER) or the Director of the Center for Drug Evaluation and Research (CDER) may approve an exception or alternative to any requirement in this Section. Requests for such exceptions must be in writing.”

The amended regulation could, as an example, allow the

use of particular vial adaptors to prevent contamination of products in multidose vials without the use of preservatives. As noted in the final rule,5 the Director of CBER or the Director of CDER “would not approve an exception or alternative when the data or conditions of use, including the indication and patient population, do not provide a sufficient scientific and regulatory basis for such an approval.” The amended regula- tion took effect in May 2011.

As noted, a preservative “shall not denature the specific substances in the product to result in a decrease below the minimum acceptable potency within the dating period when stored at the recommended temperature.” Certain preserva- tives are not compatible with certain antigens; compatibility must be established. For example, it has been known for a number of years that thimerosal has a deleterious effect on the potency of inactivated poliovirus vaccine (IPV).6,7 An alterna- tive preservative is necessary for IPV. A preservative that is used in other products, 2-phenoxyethanol,8 has been found to be compatible with IPV vaccine formulations; it is used as a pre- servative in both of the currently U.S.-licensed IPV vaccines (IPOL [Sanofi Pasteur SA] and Poliovax [Sanofi Pasteur Ltd.; not currently marketed in the United States]).

Phenol is currently used in three U.S.-licensed vaccines: the polysaccharide vaccines Pneumovax 23 (a 23-valent pneumo- coccal polysaccharide vaccine manufactured by Merck Sharp & Dohme Corp.) and Typhim Vi (*Salmonella typhi* capsular polysaccharide vaccine manufactured by Sanofi Pasteur SA), and ACAM2000 (the smallpox vaccine from Sanofi Pasteur Biologics Co.); each of these vaccines contains 0.25% phenol as a preservative (phenol is contained in the diluent for

products.[\*](#_bookmark1) This requirement is also reflected in other regula- tions or requirements, such as those of the World Health Organization (WHO).11 It has been reported that phenol affects diphtheria toxoid, “so that its immunizing power falls rapidly.”12 Benzethonium chloride with formaldehyde is cur- rently used in only one U.S.-licensed vaccine, anthrax vaccine adsorbed (BioThrax; the preservative is 25 µg/mL benzetho- nium chloride and 100 µg/mL formaldehyde), manufactured by Emergent BioDefense Operations Lansing LLC.

In recent years, considerable controversy has surrounded the use of thimerosal, an organomercurial, in vaccines. Although allergic responses to thimerosal have been described,13 a controversy, arising in the late 1990s, centered on the hypothesis that exposure to thimerosal, a derivative of ethyl mercury, may be causally linked to autism and other neurodevelopmental disorders in children. Although there were no clear or definitive data to support a link between thimerosal and neurodevelopmental disorders, the U.S. Public Health Service (PHS), first in July 199914 and again in June 2000,15 in an effort to reduce mercury exposure in children from all sources, recommended that thimerosal be removed from pediatric vaccines as expeditiously as possible. The July 1999 PHS statement was issued jointly with the American Academy of Pediatrics; the June 2000 PHS statement was issued jointly with the American Academy of Pediatricians, the American Academy of Family Physicians, and the Advisory Committee on Immunization Practices (ACIP). Letters from CBER of the FDA, in 199916 and again in 2000,17 to the various vaccine manufacturers noted that the removal of thimerosal from vaccines was merited and requested manufacturers’ time- lines for thimerosal removal or submission of an explanation as to why thimerosal removal was not currently feasible.

In 2004, the Institute of Medicine (IOM)’s Immunization

Safety Review Committee of the National Academy of Sci- ence’s IOM issued its final report, examining the hypothesis that, inter alia, thimerosal-containing vaccines are causally associated with autism. The committee concluded that the body of evidence favors rejection of a causal relationship between thimerosal-containing vaccines and autism, and that the hypotheses that were generated concerning a biological mechanism for such causality were theoretical only.18 The European Medicines Agency also noted, as a precautionary measure, “that, although there is no evidence of harm caused by the level of exposure from vaccines, it would be prudent to promote the general use of vaccines without thiomersal and other mercury-containing preservatives.”19 Of note, the WHO continues to recommend the use of vaccines containing thimerosal because the need for multidose preservative- containing vaccines and, thus, the benefit of using such vac- cines outweighs the theoretical risk of toxicity.20 Additionally, the Global Advisory Committee on Vaccine Safety has stated that it remains of the view that there is no evidence supporting a causal association between neurobehavioral disorders and thimerosal-containing vaccines.21 A more comprehensive update on the thimerosal-autism hypothesis for vaccines may be found in Chapter 82.

At present, with the exception of the inactivated influenza

vaccine, all of the U.S.-licensed, routinely recommended pedi- atric vaccines (hepatitis B, diphtheria–tetanus toxoid–acellular pertussis [DTaP], *Haemophilus influenzae* type b, IPV, pneu- mococcal conjugate, human papillomavirus [HPV], hepatitis A, rotavirus, measles-mumps-rubella [MMR] and varicella) are thimerosal free or contain only trace amounts (<1 µg

ACAM2000). According to the Minimum Requirements of the

National Institutes of Health (NIH),9,10 phenolic compounds (such as phenol or the various creosols) are not permitted as preservatives in diphtheria- and tetanus toxoid–containing

\*At the time of the writing of these requirements for diphtheria and tetanus toxoids, U.S. vaccine regulation was the responsibility of the NIH.

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| **TABLE 7.1** Preservatives, Adjuvants, and Inactivation Residues Noted in Labels of Selected U.S.-Licensed Vaccines | | | | |
| **Vaccine** | **Trade Name** | **Preservative (Amount/ Dose) and µg Hg/Dosea (if Noted)** | **Adjuvant and Aluminum Content Per Dose** | **Inactivation Residues (Amount Per Dose if Noted)** |
| **Bacterial Vaccines** |  |  |  |  |
| Td (adult) | DECAVAC | None  ≤0.3 µg Hg | Alum [KAl(SO4)2]  Al: ≤0.28 mg | Formaldehyde (≤0.1 mg) |
|  | None/MassBiologics | None  ≤0.3 µg Hg | AlPO4  Al: ≤0.53 mg | Formaldehyde (<0.1 mg) |
| DTaP | DAPTACEL | None | AlPO4  Al: 0.33 mg | Formaldehyde (≤0.5 µg) Glutaraldehyde (<50 ng) |
|  | Infanrix | None | Al(OH)3  Al: ≤0.625 mg | Formaldehyde (≤0.1 mg) Glutaraldehyde (%NN) |
| Tdap | Adacel | None | AlPO4  Al: 0.33 mg | Formaldehyde (≤5 µg) Glutaraldehyde (<50 ng) |
| **Viral Vaccines** |  |  |  |  |
| Hepatitis A | Havrix  0.5-mL pediatric dose | None | Al(OH)3  Al: 0.25 mg | Formalin (≤0.05 mg) |
|  | VAQTA  0.5-mL pediatric dose | None | Aluminum (mixed salt) Al: 0.225 mg/dose | Formaldehyde (<0.4 µg) |
| Human papillomavirus | Cervarix | None | AS04 (MPL + Al(OH)3):  0. 5mg  Al: 0.17 mg | N/A |
|  | Gardasil 9 | None | Aluminum (mixed salt) Al: 0.5 mg/dose | N/A |
| Influenza | Afluria (2014-2015) (0.5 mL, single dose) | None | None | β-Propiolactone (≤2 ng) |
|  | Afluria (2014-2015) (multidose vial) | Thimerosal  24.5 µg Hg | None | β-Propiolactone (≤2 ng ng) |
|  | Agriflu (2013-2014) (0.5 mL, single dose) | None | None | Formaldehyde (≤10 µg) |
|  | Fluarix (2014-2015) (0.5 mL, single dose) | None | None | Formaldehyde (≤5 µg) |
|  | Fluvirin (2014-2015) (0.5 mL, single dose) | None  ≤1 µg Hg | None | β-Propiolactone (≤0.5 µg) |
|  | Fluvirin (2014-2015) (multidose vial) | Thimerosal 25 µg Hg | None | β-Propiolactone (≤0.5 µg) |
| Japanese encephalitis virus | Ixiaro | None | Al (OH)3 (250 ug) Al: NN | Formaldehyde (≤200 ppm,  ≤0.1 mg/0.5 mL dose) |
| Polio | IPOL | 2-Phenoxyethanol (2.5 mg) +  formaldehyde (≤0.1 mg) | None | Formaldehyde ≤0.1 mg |
| DTaP, diphtheria–tetanus toxoid–acellular pertussis; MPL, 3-*O*-desacyl-4′-monophosphoryl lipid A; NN, not noted on label; %NN, amount in final container not noted on label; Td, tetanus-diphtheria vaccine for adolescents and adults; Tdap, tetanus, diphtheria, and pertussis for adolescents and adults.  aDose is 0.5 mL. | | | | |

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of mercury per dose) as a residual from the manufacturing process ([Table 7.1](#_bookmark2)). Of the U.S.-licensed trivalent injectable influenza vaccines, six are approved for pediatric use: Fluzone (Sanofi Pasteur Inc.), for use in infants 6 months of age and older; Fluvirin (Seqirus Vaccines, Ltd.), for use in children 4 years of age and older; Afluria (Seqirus Pty Ltd.), for use in children 5 years of age and older; Fluarix (GlaxoSmithKline Biologicals), for use in children 3 years of age and older; Flu- Laval (ID Biomedical Corporation of Quebec), for use in chil- dren 3 years of age and older; and Flucelvax (Seqirus, Inc.), for use in children 4 years of age and older. These six inactivated influenza vaccines are available in either thimerosal-free pre- sentations (Fluzone, Fluarix, Afluria, FluLaval, and Flucelvax) or with trace thimerosal (≤1 µg of mercury per 0.5 mL dose) (Fluvirin). Additionally, there are four U.S.-licensed quadri- valent inactivated influenza vaccines (FluLaval Quadrivalent, Fluzone Quadrivalent, Fluarix Quadrivalent, and Flucelvax Quadrivalent) available for children; the age indications are

as above for the trivalent presentations and single-dose pre- sentations of these vaccines contain no thimerosal. FluMist Quadrivalent (MedImmune, LLC) is a live, attenuated quad- rivalent influenza vaccine and does not contain thimerosal.

Two Tdap (tetanus, diphtheria, and pertussis for adoles- cents and adults) vaccines (tetanus, diphtheria, and acellular pertussis vaccines, with the lowercase letters indicating a reduced antigen content for the diphtheria toxoid and one or more of the pertussis antigens) for use in adolescents and adults, Adacel (Sanofi Pasteur Ltd.) for use in persons 10 through 64 years of age, and Boostrix (GlaxoSmithKline Bio- logicals) for use in persons 10 years of age and older, are licensed in the United States. Neither product contains thimerosal. The quadrivalent (Groups A, C, Y, and W135) meningococcal conjugate vaccines, Menactra (Sanofi Pasteur Inc.) and Menveo (Novartis Vaccines and Diagnostics S.r.l.) do not contain any preservative. MenHibrix (GlaxoSmithKline Biologicals) is a bivalent meningococcal conjugate vaccine

(against serogroups C and Y), additionally containing an *H. influenzae* type b capsular polysaccharide (polyribosyl-ribitol- phosphate) conjugate, and is likewise thimerosal free. Simi- larly, the two meningococcal serogroup B vaccines BEXSERO (Novartis Vaccines and Diagnostics S.r.l.) and TRUMENBA (Wyeth Pharmaceuticals Inc.) and the HPV vaccines Gardasil 9 (Merck Sharp & Dohme Corp.) and Cervarix (GlaxoSmith- Kline Biologicals) do not contain any preservative.

A diphtheria and tetanus toxoid vaccine (DT) for use in children younger than 7 years of age is available from Sanofi Pasteur Inc. It contains only a trace amount of thimerosal as a manufacturing residual.

## Adjuvants

Adjuvants are materials that enhance and direct the immune response (see Chapter 6). Vaccine adjuvants are not licensed separately; rather, the adjuvant is a constituent of the licensed vaccine, and it is the vaccine formulation, in toto, that is tested in clinical trials and is licensed. An adjuvant cannot be added or removed, or its amount in a licensed vaccine changed, without submitting a supplement to the vaccine license and obtaining approval from the FDA. The various aluminum salts (aluminum hydroxide, aluminum phosphate, alum [potassium aluminum sulfate], or mixed aluminum salts) are the most commonly used adjuvants in U.S.-licensed vaccines. One vaccine, Cervarix (GlaxoSmithKline Biologi- cals), which contains AS04, an adjuvant system composed of an aluminum salt and monophosphoryl lipid A, a detoxified lipopolysaccharide (LPS), has been licensed. In 2013, as part of the U.S. government’s national pandemic preparedness ini- tiative, an H5N1 monovalent influenza vaccine, approved for use in persons 18 years of age and older, containing the unique adjuvant, AS03, manufactured by ID Biomedical Cor- poration of Quebec was licensed. The AS03 adjuvant is an emulsion composed of squalene, DL-α-tocopherol, and poly- sorbate 80. In 2015, an inactivated influenza vaccine, Fluad (Seqirus Vaccines Ltd.), which contains the squalene-based adjuvant MF59C.1, was licensed.

Despite worldwide use of aluminum salts for more than

50 years, surprisingly little has been known about their mecha- nism of action as adjuvants (see, e.g., Chapter 6 and Hogen- Esch22). For many years, the prevailing thought was that the aluminum salts functioned as depots for the vaccine immu- nogens. More recently, it was shown that the aluminum salts also activate inflammasomes, clusters of proteins found inside certain cells. Inflammasomes respond to stresses such as infec- tion or injury by releasing cytokines, which, in turn, stimulate an immune response.23

[Table 7.1](#_bookmark2) presents the specific aluminum salt (hydroxide, phosphate, sulfate, or mixed) and the quantity of aluminum that is contained in a number of commonly used vaccines. (The aluminum content that is listed for some vaccines noted in [Table 7.1](#_bookmark2) represents the upper limit of the specification; the vaccine may routinely contain less aluminum.) By regulation (21 CFR §610.15[a]), the aluminum content of a vaccine cannot exceed 0.85 mg of aluminum per dose if the amount is assayed, or 1.14 mg/dose if determined by calculation based on the amount of the aluminum compound that is added. To harmonize with WHO recommendations, this regulation was amended in 1981 to permit up to 1.25 mg of aluminum per dose. However, the higher amount was permitted only “pro- vided that data demonstrating that the amount of aluminum used is safe and necessary to produce the intended effect are submitted to and approved by the Director, Center for Biolog- ics Evaluation and Research” (21 CFR §610.15[a]). The Euro- pean Pharmacopoeia likewise restricts the aluminum content to 1.25 mg per dose. It should be noted that the above

regulation for aluminum content refers to an individual dose of a biological product; thus, for example, for a hypothetical combination vaccine derived from licensed components, the aluminum content for the combination vaccine may still not exceed 0.85 mg.

Recently, the FDA amended the CFR requirements5 for con- stituent materials including adjuvants to permit, when justi- fied, exceptions or alternatives such as the use of an increased aluminum content in a vaccine; this change may have a greater impact on certain therapeutic vaccines than on the preventive vaccines.

Concerns have been raised in recent years about the use of aluminum in vaccines and potential adverse outcomes that may be associated with its use at the levels that exist in indi- vidual vaccines and through the additive effects of multiple vaccinations. These concerns about the use of aluminum in vaccines prompted a workshop that was sponsored by the National Vaccine Program Office in May 2000. The general use of aluminum salts in vaccines24 and aluminum toxicokinet- ics25 were reviewed during the workshop. In their overall summary of the workshop, Eickhoff and Meyers26 noted that “based on 70 years of experience, the use of salts of aluminum as adjuvants in vaccines has proven safe and effective.” A more recent study by FDA scientists, using updated parameters for toxicokinetic assessments, including current recommended vaccines and aluminum excretion data, concluded that the risk from aluminum exposure from vaccines and the environment to infants was extremely low.27

## Stabilizers

Various stabilizers are added to vaccines to help protect them from adverse conditions such as the freeze-drying process (for those vaccines that are freeze-dried) or heat. For freeze-dried (lyophilized) preparations of vaccines, it is also necessary to add materials that provide a bulk matrix for the vaccine. The amount of an immunogen that is contained in a vaccine can be extremely small, on the order of tens of micrograms or less. If sufficient amounts of various materials were not added to the vaccine before lyophilization, the vaccine would not be readily observable and would most likely adhere to the vial wall. By way of illustration of vaccine immunogen masses, ActHIB (Sanofi Pasteur SA), a polysaccharide conjugate vaccine that is marketed in a freeze-dried presentation, con- tains approximately 10 µg of purified polysaccharide conju- gated to 24 µg of tetanus toxoid. The immunogen mass for live viral vaccines is even less, on the order of nanograms (approximately 103 to 105 viral particles per dose). Thus, there is a need to provide a matrix to contain these vaccines during freeze-drying.

The types of material that are added to vaccines as stabiliz-

ers include sugars (such as sucrose or lactose), amino acids (such as glycine or the monosodium salt of glutamic acid), and proteins (such as gelatin). [Table 7.2](#_bookmark4) lists the stabilizers that are used for a number of common vaccines.

Added proteins may be of concern for two principal reasons. The first concern arises from the potential for animal- and human-derived protein to contain one or more adventi- tious agents. The second concern arises from the potential for these proteins to elicit an allergic reaction. The two animal- or human-derived proteins that are currently used as stabilizers in U.S.-licensed vaccines are human serum albumin (HSA) and gelatin. The FDA has, as of this writing, required that, if blood-derived HSA is used in vaccine manufacture, only U.S.- licensed HSA may be used. Additionally, an FDA guidance recommends that the following statement appear in the Warn- ings section of the package insert for blood-derived HSA- containing products28: “This product contains albumin, a

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| **TABLE 7.2** Vaccine Stabilizers, Manufacturing Residuals, and Cell Lines Noted in the Labels of Selected U.S.-Licensed Vaccines | | | | |
| **Vaccine** | **Trade Name** | **Stabilizers Per Single Dosea** | **Manufacturing Residuals (Except Inactivating Agents) Per Dosea** | **Cell Line** |
| **Live Bacterial Vaccines** | | | | |
| Typhoid | Vivotif (1 capsule) | 3.3–34.2 mg Sucrose; 0.2–2.4 mg ascorbic acid; 0.3–3.0 mg amino acid  mixture | NN | NA |
| **Live Viral Vaccines** |  |  |  |  |
| Mumps, measles, rubella | MMR-II | 1.9 mg Sucrose  14.5 mg hydrolyzed gelatin;  14.5 mg sorbitol;  0.38 mg monosodium  L-glutamate; sodium phosphate;  ≤0.3 mg recombinant human albumin | 25 µg Neomycin;  <1 ppm fetal bovine serum albumin;  other buffer and media componentsb | Chick embryo cell culture (measles and mumps)  WI-38 cells (rubella) |
| Yellow fever | YF-Vax | Sorbitol and gelatin (%NN) | NN | Chicken embryos |
| **Inactivated Viral Vaccines** | | | | |
| Polio | IPOL | NN | <5 ng Neomycin;  <200 ng streptomycin;  <25 ng polymyxin B;  <1 ppm calf serum protein | Vero cells |
| Japanese encephalitis virus | Ixiaro | None added | ≤50 ng Bovine serum albumin;  ≤100 pg host cell DNA;  ≤200 ppm sodium metabisulfite; host cell proteins (≤300 ng/6 µg  of protein);  ≤0.5 µg protamine sulfate | Vero cells |
| Hepatitis A | Havrix (1mL dose) | NN | ≤5 µg/mL MRC-5 cellular proteins; ≤40 ng/mL neomycin sulfate | MRC-5 cells |
|  | VAQTA (1 mL  dose) | 70 µg sodium borate/mL | <0.1 µg Nonviral protein/mL;  <4 × 10−6 µg DNA/mL;  <10−4 µg bovine albumin/mL | MRC-5 cells |
| Rabies | RabAvert | <12 mg polygeline;  1 mg potassium glutamate | <0.3 mg Human serum albumin  <3 ng ovalbumin;  <1 µg neomycin;  <20 ng chlortetracycline  <2 ng amphotericin B | Chicken fibroblasts |
|  | Imovax | NN | <100 mg Human serum albumin;  <150 µg neomycin sulfate;  <20 µg phenol red | MRC-5 cells |
| **Recombinant Protein Viral Vaccines** | | | | |
| Human papillomavirus | Gardasil 9 | NN | <7 µg Yeast protein | *Saccharomyces cerevisiae* |
|  | Cervarix | NN | <40 ng Insect cell and viral proteins;  <150 ng bacterial proteins | *Trichoplusia ni* |
| Hepatitis B | Engerix-B (1 mL dose) | NN | ≤5% Yeast protein | *S. cerevisiae* |
|  | Recombivax HB (1 mL dose) | NN | <1% Yeast protein | *S. cerevisiae* |
| Influenza | Flublok | NN | ≤28.5 µg Baculovirus and insect cell proteins;  ≤10 ng baculovirus and insect cell DNA;  ≤100 µg Triton X-100 | *Spodoptera frugiperda* |

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| **TABLE 7.2** Vaccine Stabilizers, Manufacturing Residuals, and Cell Lines Noted in the Labels of Selected U.S.-Licensed Vaccines *(Continued)* | | | | |
| **Vaccine** | **Trade Name** | **Stabilizers Per Single Dosea** | **Manufacturing Residuals (Except Inactivating Agents) Per Dosea** | **Cell Line** |
| **Viral Vaccines** |  |  |  |  |
| Influenza (trivalent) | Afluria | NN | ≤10 ppm Na taurodeoxycholate;  <1 µg ovalbumin;  ≤3 ng neomycin sulfate;  ≤0.5 ng polymyxin B | Embryonated chicken eggs |
|  | Flucelvax | NN | ≤8.4 µg MDCK cell protein;  ≤120 µg non-HA protein;  ≤10 ng MDCK cell DNA;  ≤1125 µg polysorbate 80;  ≤13.5 µg cetyltrimethylammonium bromide | Madin-Darby Canine Kidney (MDCK) cells |
|  | Fluarix | NN | ≤0.0016 µg Hydrocortisone;  ≤0.15 µg gentamicin sulfate;  ≤0.05 µg ovalbumin;  ≤50 µg sodium deoxycholate | Embryonated chicken eggs |
|  | Fluzone | NN | ≤150 µg octylphenol ethoxylate | Embryonated chicken eggs |
| NA, not applicable; NN, none noted; %NN, percentage not noted in labeling. aEach vaccine dose listed in the Table is 0.5 mL unless otherwise noted. bAmounts of all ingredients not specified in labeling. | | | | |

derivative of human blood. Based on effective donor screening and product manufacturing processes, it carries an extremely remote risk for transmission of viral diseases. Although there is a theoretical risk for transmission of Creutzfeldt-Jakob disease (CJD), no cases of transmission of CJD or viral disease have ever been identified that were associated with the use of albumin.”

For HSA produced as a recombinant DNA protein, it would not be necessary for the albumin to be separately licensed by the FDA nor would the above-quoted warning be included in the package insert (as it is not derived from blood). In August 2005, the FDA approved a license supple- ment for use of recombinant HSA in MMR-II (Merck Sharp & Dohme Corp.).

Gelatin or processed gelatin is also used as a stabilizer. Gelatin may be bovine or porcine derived. Despite the use of a harsh manufacturing procedure (extremes of heat and pH) in the production of gelatin, there is concern about the pres- ence of the bovine spongiform encephalopathy (BSE) agent in bovine-derived material. Thus, any bovine-derived gelatin that is added to vaccines, or used in the vaccine manufacturing process, must not be sourced from countries reporting BSE *or* from countries that do *not* meet the latest BSE-related stan- dards of the Office International des Epizooties (OIE) (see “Transmissible Spongiform Encephalopathy Agents” later).29

A second concern for gelatin relates to allergic responses. Allergic responses to gelatin, although rare, have been described in the medical literature.30–33 It has been hypothe- sized that in Japan, use of partially hydrolyzed gelatin, which contained a small amount of high-molecular-weight gelatin, contributed to an increase in the incidence of allergic reac- tions.33,34 Nakayama and Aizawa noted that a change to hydro- lyzed modified porcine gelatin, together with discontinuation of the use of gelatin-containing DTaP vaccines, may have con- tributed to a decrease in the incidence of allergic reactions after administration of monovalent measles and mumps vaccine in Japan.34 A severe allergic reaction to gelatin is a contraindication to receiving gelatin-containing vaccines.

Various buffers (e.g., phosphate buffer) are also used in vaccines to maintain a particular pH range, and salts (e.g., NaCl) may be added to achieve isotonicity.

# MANUFACTURING RESIDUALS

In principle, any or all of the materials that are used in the manufacturing process may be present in the final vaccine formulation. For the purposes of this chapter, materials that are present in the final vaccine formulation that derive from the manufacturing process are termed *residuals*. Various steps in the manufacturing process may remove or reduce the amounts of many of these residuals. However, for various vac- cines, an acceptable technology to remove these manufactur- ing residuals may not exist, or there may be no perceived need (e.g., with regard to safety or a potential for an adverse effect on efficacy) for their removal. Additionally, and as a general principle, it is not possible to remove a particular substance completely, nor is it possible to demonstrate that a particular substance has been removed completely. For many substances, the residual amount may be below the limits of detection by current analytic technologies, and may, for practical reasons, be considered absent.

Bacterial and viral inactivation substances must be noted

in the package label (21 CFR §610.61[q]). Residual bacterial or cellular culture components, such as antibiotics that are used during manufacture, as well as sensitizing substances (generally proteins), and other inactive ingredients when con- sidered a safety factor, also must be noted in the label (21 CFR

§610.61 [l][m][n]). There may be some overlap between these categories; however, they are grouped in this manner for con- venience and to aid a discussion of these materials as they are affected by current regulations. Residual bacterial or cell culture components may be included in these categories, but other residuals, such as DNA and endotoxin, are not generally noted in labeling.

## Inactivation Residuals

Various agents may be used to inactivate bacteria and viruses or to detoxify bacterial toxins. The goal of these chemical treat- ments is to inactivate the bacterium or virus or to remove toxic activity while still preserving the antigenicity of the product against the homologous organism or toxin. After inactivation, a virus or bacterium may be processed further to furnish par- ticular antigens. For example, after inactivation, influenza

viruses may be split by various chemical treatments (e.g., detergent) so that more purified inactivated influenza vaccines can be produced.

Formaldehyde has a long and extensive history of use in the preparation of bacterial and viral vaccines. It was used by Ramon in 1923 to detoxify diphtheria, yielding a diphtheria toxoid vaccine termed an *anatoxine*.35 Requirements for the use of formaldehyde and the permitted residual amount of form- aldehyde that is allowed for diphtheria toxoid are provided in the NIH Minimum Requirements.9 Similar NIH Minimum Requirements exist for tetanus toxoid.10 These documents note that residual, free formaldehyde in the finished product should not be in excess of 0.02% (i.e., 0.1 mg for a 0.5-mL vaccine dose). Formaldehyde is also used to inactivate viruses (e.g., polio and influenza viruses) when preparing vaccines. [Table](#_bookmark2)

[7.1](#_bookmark2) lists the amount of residual formaldehyde that is present or allowed in these and various other U.S.-licensed vaccines; in the diphtheria and tetanus toxoids, the amount does not exceed 0.02%.

Similar requirements for residual formaldehyde exist else- where, for example, in Europe, as noted in the European Phar- macopoeia for various vaccines. Concern about the presence of residual formaldehyde in vaccines stems from the known toxic effects of formaldehyde, and from its carcinogenicity potential. The U.S. Environmental Protection Agency (EPA) has established a reference dose (RfD) for formaldehyde through the oral route.36 The RfD is defined by the EPA as “an estimate (with uncertainty spanning perhaps an order of mag- nitude) of a continuous inhalation exposure or a daily expo- sure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious noncancer effects during a lifetime.”37 The RfD for formalde- hyde (oral administration) is 0.2 mg/kg of bodyweight per day.36 The amount of residual formaldehyde in vaccines (which are administered infrequently, not daily) is below this level.

Formaldehyde has been further classified by the EPA as a

“probable human carcinogen” (the EPA’s B1 classification).36 The bulk of the carcinogenicity studies on formaldehyde have focused on chronic respiratory exposure because this is the primary route of industrial and routine household expo- sure. There are fewer data regarding ingested or parenteral exposure to formaldehyde, and the EPA has not developed a risk estimate for either oral or parenteral exposure.36 Data regarding carcinogenicity studies may be found in documents from the EPA36 and the International Agency for Research on Cancer.38

One point regarding formaldehyde should be made. Form- aldehyde is naturally present in the human body as the result of various biochemical processes38,39 and the steady-state level of formaldehyde in the bloodstream of humans is approxi- mately 2.6 mg/L.40 The amount of formaldehyde that is natu- rally, continuously present in the blood of humans, or turned over in a particular day, is markedly in excess of the amount that is present as a vaccine residual. A 2013 FDA-authored study41 of the risk posed by the residual formaldehyde in vac- cines concluded, “In the absence of any known adverse health effects from endogenously produced formaldehyde, which exists in blood and extravascular water at steady-state concen- trations that are more than 100-fold higher [than are residual in vaccines], we consider vaccine-related, exogenous formal- dehyde to be a miniscule part of the daily formaldehyde turn- over by the body, and, therefore, do not find it plausible that vaccine-related formaldehyde represents an unsafe compo- nent of infant vaccines.”

Inactivating agents other than formaldehyde are used in

various U.S.-licensed vaccines and include glutaraldehyde, which is used to inactivate pertussis toxin (PT) in seven

acellular pertussis-containing vaccines (Adacel [Sanofi Pasteur Ltd.], Boostrix [GlaxoSmithKline Biologicals], DAPTACEL [Sanofi Pasteur Ltd.], Infanrix, KINRIX, Pediarix [each manu- factured by GlaxoSmithKline Biologicals], and Pentacel [Sanofi Pasteur Ltd.]), and β-propiolactone, which is used in the inac- tivation of three seasonal influenza virus vaccines (Afluria [Seqirus Pty Ltd.] and Fluvirin and Flucelvax [manufactured by Seqirus Vaccines Ltd. and Seqirus, Inc., respectively]), and two rabies vaccines (RabAvert [Novartis Vaccines and Diagnos- tics GmbH] and Imovax [Sanofi Pasteur SA]).

## Residual Cell Culture Materials

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Antibiotics

The CFR permits the addition of antibiotics (with the excep- tion of penicillin) in viral vaccine manufacture (21 CFR

§610.15[c]). Antibiotics that have been used include strepto- mycin, polymyxin B, neomycin, and gentamicin. Although a manufacturer need not specifically test for these antibiotics in the final container, the calculated amount of residual antibiot- ics (based on dilutions of the amount that was added) must be noted on the package label (21 CFR §610.61[m]). [Table 7.2](#_bookmark4) lists the amount of residual antibiotics in several U.S.-licensed vaccines. Although the amended regulation for Constituent Materials5 (21 CFR §610.15) applies equally to antibiotics, no examples of potential changes for the antibiotic regulation, unlike for preservatives and adjuvants, were presented in the *Federal Register* notice.

# SENSITIZING SUBSTANCES

The CFR’s biologics labeling regulations (21 CFR §610.61[l]) provide that “known sensitizing substances” should be listed on the product label. Furthermore, 21 CFR §610.15(b) states that “extraneous protein known to be capable of producing allergenic effects in human subjects shall not be added to a final virus medium of cell culture produced vaccines intended for injection.” These regulations address the possibility that animal-derived proteins present in the final formulation of a vaccine can cause allergic reactions in some vaccine recipients. (Other sensitizing substances, such as preservatives, are addressed in other sections of these regulations.) Animal- derived materials are used extensively in vaccine manufactur- ing, particularly in viral cultures. When viral vaccines are grown in embryonated chicken eggs (influenza and yellow fever vaccines) or chick embryo cell culture (measles or mumps virus vaccines), the label will state that residual chicken pro- teins may be present in the final formulation (see [Table 7.2](#_bookmark4)). Although hypersensitivity to any component of the vaccine is a contraindication, the MMR-II and yellow fever vaccine (YF- Vax) package inserts address the vaccination of persons with hypersensitivity to eggs or egg protein.

The two U.S.-licensed hepatitis B vaccines, Engerix-B (Glaxo-

SmithKline Biologicals) and Recombivax HB (Merck Sharpe & Dohme Corp.), are recombinant DNA–derived proteins and are produced in yeast; their package inserts note that residual yeast protein may be present (Engerix-B contains not more than 5% and Recombivax HB not more than 1% yeast protein). Hepatitis B vaccine is contraindicated in persons with a history of hypersensitivity to yeast; however, the ACIP has noted that “no evidence exists that documents adverse reactions after vaccination of persons with a history of yeast allergy.”42 The HPV vaccines, Gardasil (Merck Sharp & Dohme Corp.) and Gardasil 9 (Merck Sharp & Dohme Corp.), are also manufac- tured in yeast cells; according to the package inserts, both vaccines contain less than 7 µg of yeast proteins per dose; hypersensitivity to yeast is given as a contraindication to

receiving the vaccine. The other HPV vaccine, Cervarix (Glaxo- SmithKline Biologicals), is manufactured in insect cells; levels of insect cell proteins are reduced to less than 40 ng/dose.

It has been reported43 that anaphylactic reactions to DTaP and Tdap vaccines may be caused by the presence of residual amounts of casein, a milk protein that is used in the culture media for these vaccines; the reactions occurred in children who already had high levels of milk allergy. The authors43 note, however, that these anaphylactic reactions are exceed- ingly rare, and that many highly sensitive children with milk allergy tolerate the vaccines. Further studies will be necessary to define whether there is a subpopulation of children with milk allergies who might be at risk for an anaphylactic reac- tion. Slater and colleagues44 have, however, noted several potential confounding factors in this analysis.

Vaccine vial stoppers or syringe components (plunger or tip caps) may contain latex, a component of natural rubber; certain persons are sensitive to latex. Vaccine presentations that contain or may contain latex have statements in the Warning and Precautions section of the package insert to the effect that the vaccine may cause allergic reactions in latex- sensitive individuals.

The Food, Drug and Cosmetic Act (Section 502[e][1][A] [iii]) states that all inactive ingredients should be noted in labeling; it also states that this requirement is not necessary if trade secret information would be disclosed. The CFR addi- tionally notes that an inactive ingredient should be listed in the labeling if the ingredient’s presence is considered a safety factor (21 CFR §610.61[n]). In some cases, even in the absence of any evidence that a particular material might pose a safety factor, manufacturers have elected to disclose the presence of residual materials such as detergents, solvents, and chelating agents (see [Table 7.2](#_bookmark4) for examples of manufacturing residu- als). In addition, many manufacturers provide a brief summary of manufacturing methods, including the reagents used in various steps, such as precipitation (ammonium sulfate) or bacterial culture (e.g., an antifoam agent such as polydimeth- ylsiloxane). Many of these substances are removed or mark- edly reduced in subsequent manufacturing steps.

## Bacterial and Cellular Residuals

For bacterial vaccines, manufacturing residuals may include various bacterial cell constituents. Naturally, whole-cell vaccines—such as the previously used whole-cell pertussis vaccine—contain high levels of these components. At present, no parenteral whole-cell bacterial vaccine is in use in the United States; an oral, live attenuated bacterial vaccine, Typhoid Vaccine Live Oral Ty21a (Vivotif, Crucell Switzerland Ltd.), is approved for use.

Vaccines derived from gram-negative bacteria may contain LPS, commonly termed *endotoxin*, a component of the bacte- rium’s outer membrane. Stimulation of the innate immune system by LPS can produce an inflammatory response that can result in fever, shock, and death.45 Different organisms have different LPS structures, so their respective response potentials differ. Two tests are currently used to detect LPS in vaccines: the rabbit pyrogen test (which is a test for all pyrogens) and the limulus amebocyte lysate (LAL) test. The lysate from the amebocytes of the horseshoe crab, *Limulus polyphemus*, clots in the presence of LPS and forms the basis for this test.46,47 In cases where pyrogenicity would be attributable to the presence of LPS, the LAL test is more commonly used. The limulus lysate that is used to test for bacterial endotoxin in U.S.- licensed vaccines (and other FDA-regulated biological prod- ucts) is itself a U.S.-licensed product. The amount of endotoxin remaining in a final vaccine formulation depends on a number of factors, including the purification steps that are used in

vaccine production. Although endotoxin testing of antigens derived from gram-negative bacteria is performed during the manufacturing process—and there may be a release specifica- tion for this test—the labeling may not contain this informa- tion. One of the DTaP vaccine labels (Tripedia [Sanofi Pasteur Inc.], which is no longer available) includes the amount of endotoxin contributed by the inactivated pertussis compo- nents (<50 endotoxin units/mL).

Residual bacterial protein may be present in the final vaccine formulation of bacterial vaccines. The consequences of this residual protein can vary, and its presence may be neutral or harmful. For example, it has been recognized for many years that the presence of residual protein may contrib- ute to increased reactogenicity of diphtheria toxoid.48,49 However, it has also been held that any such protein contrib- uted to the immunogenicity of the vaccine.50

Polysaccharide, conjugated polysaccharide, and purified protein vaccines undergo a number of purification steps that reduce the amount of residual bacterial protein. However, these purification steps may not totally eliminate cellular or media protein components. During development of the product, a number of assessments of purity are performed, such as silver staining or polyacrylamide gel electrophoresis (PAGE) gel immunoblotting. After licensure, purity and quality of the vaccine antigen is often assessed by sodium dodecylsulfate (SDS)–PAGE as a release test. However, there is a limit to the sensitivity of these methods, as is illustrated in publications of the National Institute of Allergy and Infec- tious Disease–sponsored multicenter acellular pertussis trial,51,52 which show that some children vaccinated with a fourth and fifth dose of Tripedia (DTaP [Sanofi Pasteur Inc.]; a two-component pertussis vaccine containing PT and fila- mentous hemagglutinin [FHA]), after previous doses of Tripe- dia, or a fourth dose of Tripedia after a primary series with whole-cell pertussis vaccine, had a booster response to pertac- tin and fimbriae, suggesting that there was sufficient antigen in Tripedia to stimulate an immune response.

Cell substrates used in viral vaccine manufacture include

two diploid cell strains of human origin (MRC-5 and WI-38); a simian-derived, continuous cell line (Vero cells); a dog kidney derived cell line (MDCK [Madin-Darby canine kidney] cells); and chick embryo cells. Several recombinant DNA- derived viral vaccines are manufactured in insect cell lines. Residual protein from these differing cell lines is present to varying degrees in the vaccines produced from them. There is a concern, as noted previously, for residual egg proteins in sensitive individuals.

Residual cellular DNA from normal primary diploid cells or diploid cell strains, as well as from bacterial cells, may be present in the final vaccine formulation, and this DNA is not considered to pose a risk. Residual DNA from continuous cell lines, such as Vero cells, has been considered by a WHO study group.53 The WHO Expert Committee on Biological Standard- ization assessed the risk of a transformational event as negli- gible and concluded that levels of up to 10 ng/dose of injected product are acceptable.52 This limit was a revision of an earlier, more conservative limit (≤100 pg per parenteral dose) pro- posed by the 1986 WHO Expert Committee.54 In revising this limit, the 1997 Expert Committee considered data from human and animal experience. This included data from non- human primates showing that milligram amounts of DNA containing an activated oncogene from human tumor cells had not caused a tumor during 10 years of evaluation; consid- eration that human blood contains substantial amounts of DNA in plasma; and consideration that contaminating DNA in a biological product would probably be in small fragments that are unlikely to encode a functional gene.53 The committee concluded that continuous-cell-line DNA could be considered

a contaminant rather than a significant risk factor requiring removal to extremely low levels; hence the revised limit of 10 ng/dose.

Manufacturers do not necessarily need to demonstrate that each lot meets this specification through specific testing on each lot; they may be able to validate that the purification process can remove DNA to this level or below. This limit of 10 ng of residual DNA per dose does not apply to products derived from microorganisms, diploid cell strains, or primary animal cells, or to oral vaccines made with continuous cell lines.53 The U.S.-licensed Japanese encephalitis virus vaccine (Ixiaro, Valneva Scotland Ltd.), IPV vaccine (IPOL, Sanofi Pasteur SA), and the IPV component of the DTaP-HepB-IPV vaccine, Pediarix (GlaxoSmithKline Biologicals) are produced in the continuous Vero cell line. IPOL contains less than 10 pg of DNA per dose.55 One influenza virus vaccine, Flucelvax (Seqirus Inc.), is produced in MDCK cells, a canine continu- ous cell line; the vaccine contains less than 10 ng of MDCK DNA per dose.

## Adventitious Agents

Use of animal-derived materials, such as gelatin, fetal bovine serum (commonly referred to as fetal calf serum), or primary animal-derived cells, in vaccine manufacture raises concerns about the potential presence of adventitious contaminants. Regulations (21 CFR §610.18) require that cultures used in the manufacture of products be free from extraneous organisms, and that cell lines should be tested for the presence of detect- able microbial agents. A 1993 FDA guidance document56 stated that master cell banks should be tested for adventitious agents and that animal-derived materials should be free from contaminants and adventitious agents, including viruses and the agent of BSE. A final guidance was published in February 2010.57

Manufacturers are required to perform such testing as nec- essary, and to ensure that the certification provided with any raw material is adequate (e.g., documentation that bovine- derived ingredients have been tested for extraneous viruses). Adventitious agent testing, performed to ensure that cell sub- strates used in vaccine manufacture are free from bacteria, fungi, mycoplasma, and mycobacteria, is described in detail in U.S.56,57 and international58,59 guidance documents. The use of polymerase chain reaction–based reverse transcriptase assays to test for adventitious retroviruses is addressed in a

CBER letter to manufacturers.60 The testing that has been carried out for adventitious agents is not described in the product labeling. However, the manufacturing method, including the cell lines and culture media used, are described.

## Transmissible Spongiform Encephalopathy Agents

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The lack of sensitive, specific, or readily accessible premortem diagnostic tests for transmissible spongiform encephalopa- thies (TSEs), such as BSE or CJD, limits surveillance and the ability to test for contamination of products for these agents. Diagnosis of a TSE disease is definitively confirmed by post- mortem examination of brain tissue. However, even this exam- ination is limited in sensitivity because, at earlier stages of disease, the TSE agent may be present in undetectably low concentrations. Because of these limits on testing, potential contamination of animal-derived materials with TSE agents is controlled through restricted sourcing.29

In 2013, the U.S. Department of Agriculture (USDA) pub- lished a final rule to base the conditions for importation of products derived from bovines with regard to BSE on the inherent risk of the particular product, as well as the BSE risk status of the region the product came from (9 CFR §92). This rule removed the USDA list of countries where BSE exists or that present an undue risk of BSE, and describes regions of risk generally similar to those classified by the OIE. A pro- posed rule, published in January 2007, harmonizes require- ments for bovine materials for pharmaceutical products with those of USDA-regulated meat and FDA-regulated foods and animal feeds.61

# SUMMARY

A final vaccine contains materials in addition to the active immunogen. Some of these materials are added by the manu- facturer to effect a specific purpose—for example, stabilizers and adjuvants. Others are residual materials from the manu- facturing process. Although not all of the results of all final release and in-process testing are contained in the labeling that accompanies a product, the CFR does specify which infor- mation should be included. Package inserts also contain infor- mation on manufacturing methods and growth conditions.

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