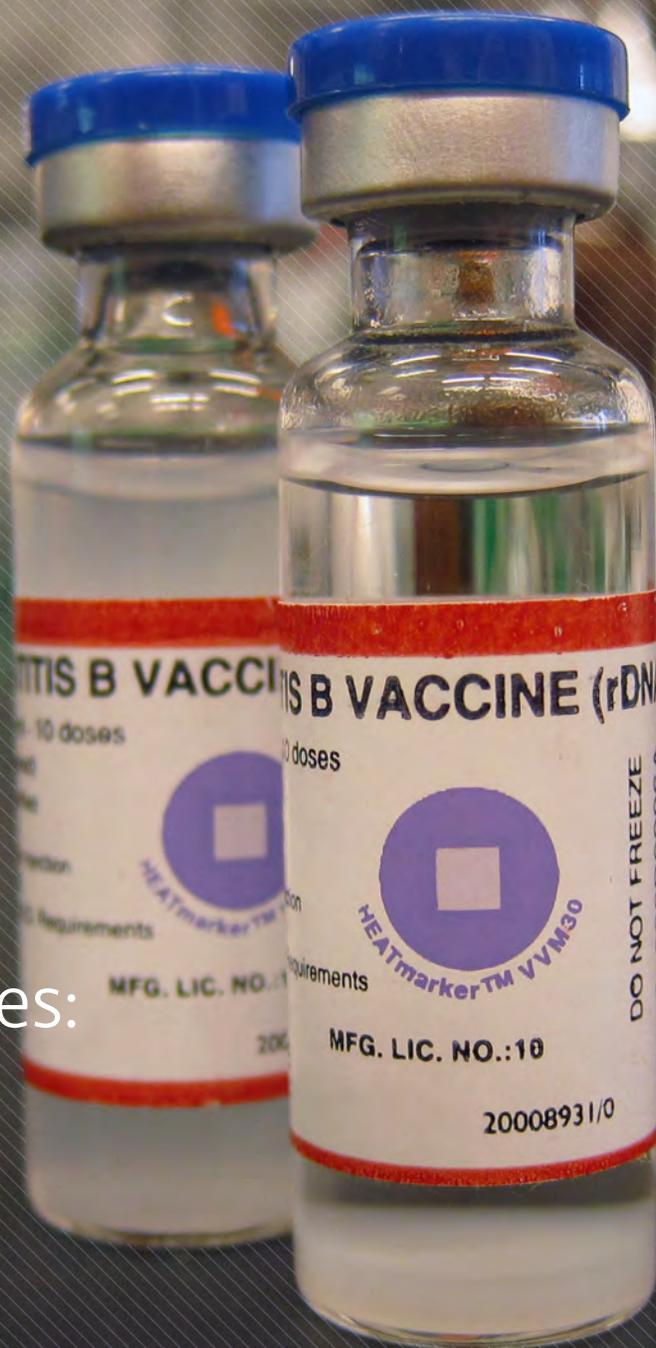


# Freeze-protection of aluminum-adjuvanted vaccines: PATH formulation technology

TECHNICAL DOSSIER



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# Summary

PATH has developed a low-cost and straightforward approach for protecting aluminum-adjuvanted vaccines from irreversible damage that results from freezing. Many current vaccines of importance to global health contain an aluminum-salt adjuvant (such as aluminum hydroxide or aluminum phosphate) and could benefit from this freeze-protection technology. There is no intellectual property barrier for manufacturers wishing to adopt the technology; it has been placed in the public domain. PATH is ready to assist vaccine producers and product-development partnerships in applying this technology to improve the stability and effectiveness of these important vaccines.

This technical dossier summarizes the rationale for freeze protection, application of the technology to specific vaccines, the data obtained to date, and comments on the likely regulatory pathway for vaccines incorporating the technology.

# Framework for advancing vaccine freeze-protection technologies at PATH

## INTRODUCTION

Vaccines typically are kept at 2°C to 8°C to preserve their potency. The cold chain that provides this continuous temperature control during transport, storage, and distribution is, however, subject to breaks and disruptions, especially in low-resource countries. Exposure to temperatures above this range, common in many low-resource country settings, can result in a reduction in a vaccine's immunogenicity. However, some measures that are used to protect vaccines from excessive heat, such as using frozen ice packs in transport containers, and placing vaccine vials adjacent to these, can inadvertently expose them to sub-zero temperatures. Vaccines can also be accidentally frozen when placed too close to the walls of ice-lined refrigerators, the evaporator in certain refrigerators, or during transportation in regions with naturally cold ambient temperatures. Freezing, like heating, can reduce the effectiveness of some vaccines, which then either need to be discarded or, if used, may have suboptimal potency.<sup>1,2,3</sup>

With funding from the Bill & Melinda Gates Foundation, formulation scientists at PATH are exploring and advancing a variety of approaches to improve vaccine stability. One major project has focused on freeze protection or cryoprotection of liquid vaccines,<sup>4</sup> particularly those containing aluminum-salt adjuvants, many of which are at the core of the routine childhood vaccination schedule of all countries.

With the research of its freeze-protection technology now at an advanced stage, PATH has the following goals in providing this dossier:

- Summarize the results of research conducted to date.
- Invite inquiries from vaccine producers, regulatory agencies, and vaccine purchasers in order to move forward with the objective of protecting vaccines of major importance to global health.
- Lay the groundwork for exploring partnerships with vaccine manufacturers to develop and test freeze-protected vaccines for use worldwide.

## FEATURES OF THE FREEZE-PROTECTION TECHNOLOGY

Some vaccines need to be formulated with an adjuvant in order to induce a protective immune response. The most commonly used adjuvants in licensed vaccines are based on aluminum salts, such as aluminum hydroxide or aluminum phosphate.

The freeze-protection technology developed at PATH involves the addition of one of three polyol excipients—propylene glycol (PG), glycerin (also known as glycerol), or polyethylene glycol 300 (PEG 300)—to aluminum-salt adjuvanted liquid vaccines during the formulation stage of vaccine production.

...formulation scientists at PATH are exploring and advancing a variety of approaches to improve vaccine stability.

## Molecular mechanisms of freeze protection

The damage caused by freezing of vaccines that contain aluminum-salt adjuvants has been attributed, at least in part, to agglomeration of the adjuvant that occurs when the vaccine is frozen and thawed. The agglomeration and loss of colloidal structure of the antigen-adjuvant complex results in a reduction in the immunogenicity of the vaccine.

The three cryoprotective excipients that are the basis of PATH's freeze-protection technology are believed to work through one or two mechanisms:

- At “high” concentrations (e.g., 40 to 50 percent) the cryoprotectants lower the freezing point of the vaccine to below -20°C, thereby preventing agglomeration of the adjuvant and also preventing damage to the tertiary structure of the protein antigen, thereby preserving its antigenicity.<sup>5,6</sup>
- At lower concentrations (e.g., 10 percent or less) the excipients do not necessarily prevent freezing, but still prevent damage caused by freezing by inhibiting agglomeration of particles.<sup>5,6</sup>

## Compatibility with technologies for protecting vaccines against damage by heat

The freeze-protection technology is not expected to improve the stability of vaccine antigens exposed to high temperatures (i.e., improve heat stability). However, preliminary work has shown that one of the freeze-protection excipients (PG) is compatible with at least one formulation developed by PATH's collaborators, to provide heat stability to hepatitis B vaccine.<sup>6</sup> This is discussed in more detail below.

## Applicability of the technology

The PATH freeze-protection technology is likely to be appropriate for use with human as well as animal vaccine products that are formulated as liquids and contain an aluminum-salt adjuvant or its equivalent, such as aluminum hydroxide, aluminum phosphate, or calcium phosphate. The technology might also be appropriate for use with some non-adjuvanted but freeze-sensitive liquid vaccines.

Commonly used human vaccines that might benefit from PATH's freeze-protection technology include:

- Diphtheria, tetanus, pertussis-based combination vaccines.
- Diphtheria toxoid.
- *Haemophilus influenzae* type b (Hib), (liquid formulations).
- Hepatitis A.
- Hepatitis B.
- Human papillomavirus.
- Inactivated influenza (pandemic).
- Inactivated poliovirus.
- Meningococcal polysaccharide-protein conjugate.
- Pertussis (acellular [aP] or whole-cell [wP]).
- Pneumococcal polysaccharide-protein conjugate.
- Tetanus toxoid.

Data demonstrating freeze protection with at least one of the three cryoprotective excipients have been obtained with diphtheria, tetanus, pertussis (aP and wP), hepatitis B and Hib antigens. The following sections of this document summarize studies conducted by PATH and its collaborators using the excipients, primarily with hepatitis B vaccine. Results from studies with other vaccines are also included.

The PATH freeze-protection technology is likely to be appropriate for use with human as well as animal vaccine products...

# Development of PATH's freeze-protection technology

The following steps were carried out by PATH and its collaborators to develop the freeze-protection technology:

- Hepatitis B vaccine was used as a model to understand the processes that occur during freezing and thawing that cause damage to aluminum adjuvanted vaccines. For this work, *in vitro* and *in vivo* assays were developed for detecting the damage.
- A list of candidate cryoprotective excipients was drawn up based on literature reviews and discussions with experts.
- From this list, three excipients were chosen for testing with the hepatitis B vaccine.
- Following detailed studies with hepatitis B as a model, testing of the excipients was extended to other vaccines, namely diphtheria, tetanus and whole-cell pertussis (DTwP) and diphtheria, tetanus, and acellular pertussis (DTaP) vaccines.
- PATH and its collaborators also assessed the freeze-protection technology with a hepatitis B vaccine that had been formulated with buffers that protect against heat damage to investigate the feasibility of producing vaccines resistant to damage caused by heat and freeze-thawing.
- Initial studies to evaluate the safety and toxicity of the excipients were conducted.

Findings from these studies are summarized below and many are available in peer-reviewed publications.

## SELECTION OF ASSAYS TO DETECT FREEZE DAMAGE

A number of *in vitro* assays have been used by PATH and collaborators to analyze and characterize the structural changes brought about by freezing and thawing, namely:

- **Sedimentation assays**, for detecting the agglomeration of antigen and/or adjuvant particles.
- **Fluorescence spectroscopy**, for detecting conformational changes of the tertiary structure of antigens.
- **Laser diffraction and particle counting**, for assaying the size distribution of particles.
- **Differential scanning calorimetry**, for determining the equilibrium freezing-point of solutions.
- **Zeta potential analysis**, for detection of changes in average surface charge of particles.
- ***In vitro* potency by conformation-dependent enzyme-linked immunosorbent assay (ELISA)**, for detection of changes to the antibody-binding potential of vaccine antigen(s).

The most relevant test of whether or not a vaccine has been damaged by freeze-thawing is whether it induces the appropriate immune response and/or provides protection against the pathogen of interest. Therefore *in vivo* assays were also used:

- ***In vivo* immunogenicity of the vaccine in mice, measured by ELISA** of specific antibodies. This is a good correlate of efficacy for some vaccines.
- ***In vivo* potency of the vaccine**, determined by testing its ability to protect animals against a challenge with the pathogen of interest.

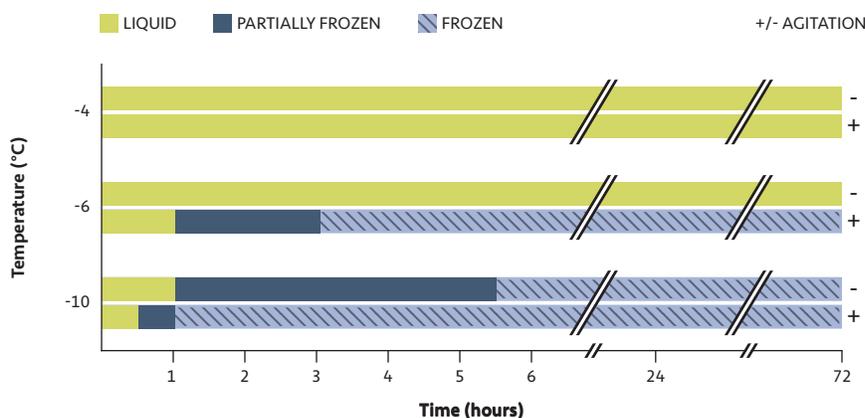
Examples of the use of these assays in the study of freeze protection can be found in Braun et al. 2009a, Braun et al. 2009b, and Chen et al. 2009.<sup>5,6,7</sup>

## DEFINING FREEZE DAMAGE: HEPATITIS B VACCINE AS A MODEL

Before beginning to test candidate cryoprotective excipients, it was important to understand the processes involved when an aluminum-salt adjuvanted vaccine freezes and thaws and the stages at which damage to the vaccine occurs. Therefore, a commercially available hepatitis B vaccine (Shanvac-B, Shantha Biotechnics Ltd., India) was subjected to a range of freeze-thaw treatments in the laboratory, and *in vitro* and *in vivo* assays were used to analyze the physical or structural changes in the vaccine and changes in its immunogenicity. The data described in this section have been published.<sup>7</sup>

Experiments showed that the type of freeze exposure was important; mild freezing ( $-4^{\circ}\text{C}$  or warmer) followed by thawing did not damage the vaccine, and the freezing point of the liquid vaccine was lower (below  $-6^{\circ}\text{C}$ ) if the vaccine was not agitated during freezing (Figure 1). Agitation, which was performed to simulate movement during the transport of vaccine, resulted in complete freezing to a solid at  $-6^{\circ}\text{C}$  (Figure 1). Damage increased with exposure to lower temperatures, for longer periods, with repeated freeze-thaw cycles, and with agitation.

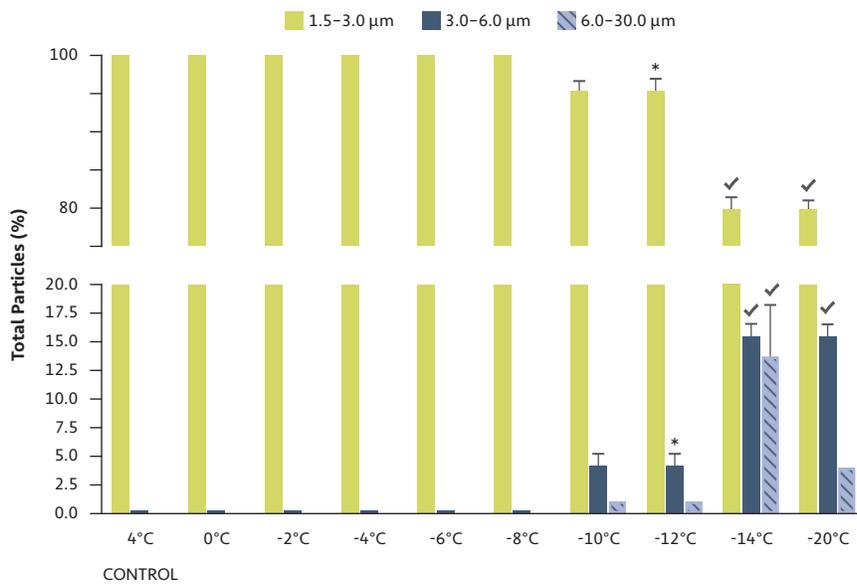
Because agglomeration of adjuvant-antigen complexes into larger particles is known to occur during freezing, the number of particles in different size categories, from 1.5 to 30  $\mu\text{m}$ , was determined in hepatitis B vaccine exposed to a single freezing and thawing event.



**FIGURE 1. Freezing kinetics of hepatitis B vaccine.** Freezing status (flowability and formation of solid vaccine) was ascertained visually after exposure to  $-4^{\circ}\text{C}$ ,  $-6^{\circ}\text{C}$ , or  $-10^{\circ}\text{C}$  for the times indicated, with or without agitation (one to two revolutions per second).<sup>7</sup>

In undamaged hepatitis B vaccine, greater than 99 percent of the antigen was associated with aluminum-adjuvant particles having a total diameter of less than 3  $\mu\text{m}$ . There was a trend for particle size to increase with lower freezing temperatures (Figure 2) or with longer exposure to freezing temperatures.<sup>7</sup>

In this experiment, vaccine was physically frozen only at temperatures of  $-10^{\circ}\text{C}$  or below; therefore agglomeration of particles appeared to correlate with whether or not

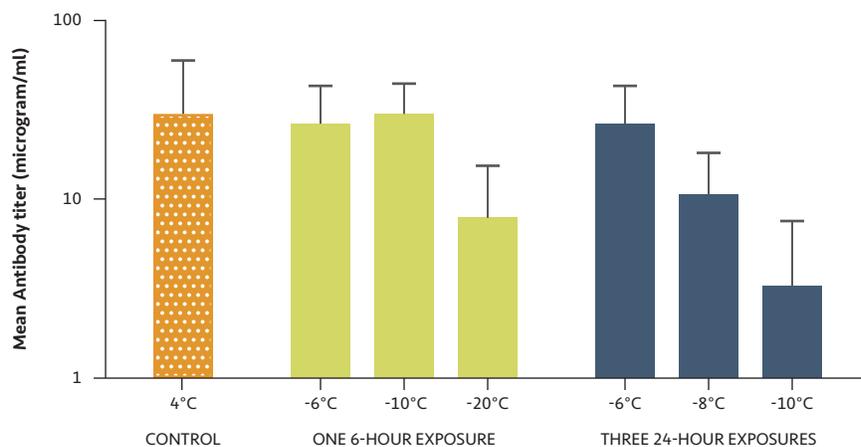


**FIGURE 2. Effect of different freezing temperatures on particle size distribution in hepatitis B vaccine.** Vaccine was exposed for 24 hours to the temperatures shown and then thawed. Bars represent the percentage of particles in various size fractions. A particle size greater than 3 μm indicates some degree of aggregation and therefore assumes physical “freeze damage” compared with the control (unfrozen and maintained at 4°C). \* $p < 0.05$ ,  $\sqrt{p} < 0.01$  (one-way analysis of variance [ANOVA]). Error bars represent standard errors of the mean.<sup>7</sup>

physical freezing had occurred. The proportion of larger particles also increased with longer duration of freezing and increasing numbers of freeze-thaw cycles.<sup>7</sup>

The hepatitis B antigen appeared to be less sensitive to damage by freezing than the adjuvant. The amount of antigen measured using the *in vitro* potency assay was only reduced following repeated freeze-thaw events (data not shown, see Chen et al. 2009).<sup>7</sup>

An *in vivo* immunogenicity assay was established in mice to test the effect of a number of different freeze-thaw cycles on the stimulation of antibody responses (Figure 3). A single freezing event at -20°C or three freeze-thaw cycles at -10°C or below appeared to reduce the immunogenicity of the vaccine. Milder treatments, in which the vaccine was exposed to a single or repeated freezing events at -6°C, did not appear to adversely affect the immunogenicity of the vaccine in mice.



**FIGURE 3. Effect of different freeze-thaw treatments on the immunogenicity of hepatitis B vaccine.** Bars represent the geometric mean antibody titers of eight mice (measured at day 43), immunized on days 1 and 29 with 1 μg of hepatitis B vaccine that had been subjected to specific temperatures and cycles of freeze-thaw treatment. The first bar shows antibody titers induced by the control vaccine, maintained at 4°C. Those with six-hour exposure had one cycle of freeze-thaw and those at 24 hours had three cycles, each of 24 hours. Error bars represent standard deviations.<sup>7</sup>

### Summary of studies characterizing the freezing and thawing of hepatitis B vaccine

- Damage caused by freezing and thawing of vaccines such as hepatitis B occurs by a complex process that is affected by the duration and temperature of freezing, the number of freeze-thaw cycles and whether the vaccine is agitated while it is being frozen.

- Under the conditions tested, physical changes to the adjuvant-antigen complex can occur before the conformation of the antigen or immunogenicity of the vaccine is affected.
- Agglomeration correlated with whether or not physical freezing, visible to the naked eye, had occurred.
- Immunogenicity of the vaccine in mice was reduced as the freezing temperature decreased and/or the number of freeze-thaw cycles increased.

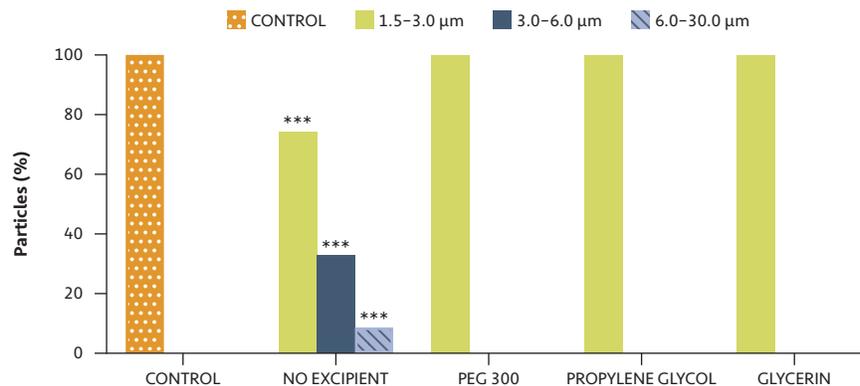
### SELECTING CRYOPROTECTIVE EXCIPIENTS

Three excipients that have been or are used in products approved by the US Food and Drug Administration (FDA) that are injected intramuscularly (IM) or subcutaneously (SC) were identified for our studies: PG, glycerin, and PEG 300. Key characteristics of these agents (reviewed in Braun et al. 2009a)<sup>5</sup> include:

- Miscibility with water in any ratio.
- Very low freezing points.
- Very low cost (estimated to be less than US\$0.001 per vaccine dose).
- Availability at clinical grade.
- Generally recognized as safe (GRAS).
- Broad use in other parenteral, IM, and SC drugs, including for pediatric use (reviewed in Braun et al. 2009a).<sup>5</sup>

### EVALUATING CRYOPROTECTIVE EXCIPIENTS WITH HEPATITIS B VACCINE

Initial experiments were conducted with the three candidate excipients—PG, PEG 300, and glycerin—at a high concentration to see whether they could protect aluminum hydroxide-adjuvanted hepatitis B vaccine (Shanvac-B, Shantha Biotechnics Ltd., India) against freeze damage. The data in this section are presented in full in Braun et al. 2009a.<sup>5</sup>



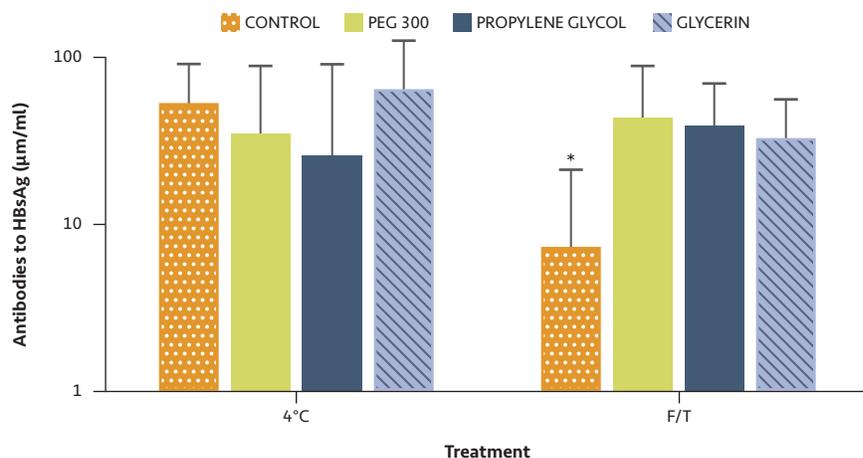
**FIGURE 4. Effect of cryoprotective excipients on particle size distribution in hepatitis B vaccine.** Vaccine preparations contained either no cryoprotectant (no excipient), or 50 percent (v/v) PEG 300, propylene glycol, or glycerin and were subjected to three cycles of freeze-thawing ( $-20^{\circ}\text{C}$  for at least 12 hours to room temperature for at least two hours). The particle size distribution was measured using a Coulter Counter. ‘Control’ shows the particle distribution the standard hepatitis B vaccine kept at  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$ . \*\*\* $p < 0.001$ . One-way ANOVA analyses with Dunnett’s posttest was used to compare the mean values to the untreated control.<sup>4</sup>

When any of the excipients was added at 50 percent final concentration (v/v), they prevented visually apparent freezing and prevented agglomeration of the vaccines, as assessed by particle sizing (Figure 4).

In untreated hepatitis B vaccine, 99 percent of the particles were between 1.5 and 3.0  $\mu\text{m}$  in size. In the absence of cryoprotective excipients, three freeze-thaw cycles increased the proportion of larger-sized particles: 20 percent of particles were 3.0 to 6.0  $\mu\text{m}$  and some particles were as large as 25 to 30  $\mu\text{m}$ . In contrast, hepatitis B vaccine that had been frozen and thawed in the presence of any of the cryoprotective excipients had particle-size distributions similar to the unfrozen control (Figure 4).

In addition to preventing agglomeration of the adjuvant, the excipients (used at 50 percent v/v) also preserved the tertiary structure of the antigen, as assessed by the *in vitro* potency assay, when the vaccine was treated with three freeze-thaw cycles at temperatures as low as -20°C (data not shown, see Braun et al. 2009a).<sup>5</sup>

Finally, using the excipients at 50 percent v/v also prevented loss of *in vivo* immunogenicity of the hepatitis B vaccine caused by freeze damage (Figure 5). Following freeze-thaw treatment, vaccine without excipients induced significantly lower antibody titers in mice, compared with non-frozen vaccine ( $p < 0.05$ ); this loss of immunogenicity was not seen following freeze-thaw treatment in the presence of the cryoprotective excipients (Figure 5).

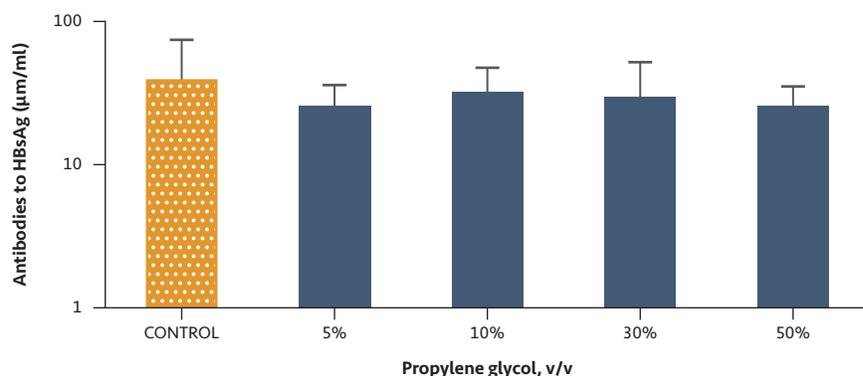


**FIGURE 5. Effect of three cryoprotective excipients on hepatitis B vaccine immunogenicity.** Hepatitis B vaccine was experimentally freeze-thaw treated by three cycles of exposure to -20°C (as described for Figure 4), or stored at 4°C as a control. Mice were immunized and assays performed as described for Figure 3. \* $p < 0.05$ , compared to 4°C control with no excipient (one-way ANOVA with Dunnett's posttest).<sup>4</sup>

### Studies with PG

The ability of PG to protect against freeze damage when used at lower concentrations was also investigated. This was of interest because adding less PG should simplify the formulation process by reducing PG's impact on pH and osmolality, as well as minimizing the additional cost of incorporating the excipient. The key findings were:

- PG at 10 and 30 percent v/v protected against particle agglomeration caused by three and six freeze-thaw cycles to temperatures as low as -80°C (data not shown, see Braun et al. 2009a).<sup>5</sup>
- PG at concentrations as low as 5 percent prevented agglomeration and loss of *in vitro* potency of hepatitis B vaccine exposed to three freeze-thaw cycles (-10°C to 4°C) (data not shown, see Braun et al. 2009a).<sup>5</sup>
- Furthermore, vaccine that had been exposed to three freeze-thaw cycles (-10°C to 4°C) in the presence of 5 percent v/v PG (or more) induced a similar antibody response in mice as control vaccine that had been stored at 4°C (Figure 6).



**FIGURE 6. Effect of 5 to 50 percent PG on hepatitis B vaccine immunogenicity after freezing and thawing.** Aluminum-salt adjuvanted hepatitis B vaccine was experimentally freeze damaged by three cycles of exposure to -10°C to 4°C (at least 18 hours at each temperature), or stored at 4°C as a control. Mice were immunized and assays performed as described for Figure 3. One-way ANOVA analyses to compare the mean values to the untreated control revealed no statistically significant differences.<sup>5</sup>

## Studies with glycerin

The cryoprotective properties of glycerin have also been analyzed in more detail (PATH, unpublished data). These studies used concentrations of 4 to 30 percent v/v glycerin and hepatitis B vaccine adjuvanted with aluminum hydroxide. The ability of glycerin to protect the commercially available adjuvants, Alhydrogel (aluminum hydroxide) and Adju-phos, (aluminum phosphate), without adsorbed antigen was also investigated.

Concentrations of glycerin as low as 6 percent v/v could protect adjuvanted hepatitis B vaccine against physical damage caused by three freeze-thaw cycles to  $-20^{\circ}\text{C}$  assessed by sedimentation and assays of particle size distribution (PATH, unpublished data). The *in vitro* potency of the vaccine was protected against freeze damage by concentrations of glycerin of 10 percent (v/v) or greater; 5 percent v/v glycerin was also tested, but the data from this concentration were inconclusive. *In vivo* immunogenicity assays in mice were not performed.

Sedimentation of the commercial adjuvants Alhydrogel and Adju-phos, caused by freeze damage, was prevented by 8 or 10 percent glycerin, but not 4 percent (6 percent glycerin was not tested) (PATH, unpublished data).

## Summary

- The three candidate excipients (PG, glycerin, and PEG 300) protected aluminum hydroxide-adjuvanted hepatitis B vaccine from freeze damage when used at high concentrations, 40 to 50 percent v/v.
- At concentrations of  $\geq 5$  percent v/v, PG protected against freeze damage following exposure to temperatures as low as  $-10^{\circ}\text{C}$ ; PG at 10 percent v/v protected against damage caused by exposure to  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ .
- The concentrations of glycerin required for freeze protection were similar to PG;  $\geq 8$  percent v/v glycerin prevented damage to the two adjuvants and the adjuvanted hepatitis B vaccine that were tested, following freezing at  $-20^{\circ}\text{C}$ .

## USING THE SELECTED EXCIPIENTS WITH OTHER VACCINES

The cryoprotective properties of the three excipients (PG, glycerin, and PEG 300) have also been demonstrated with other vaccines.

### DTaP and DTwP vaccines

Initial experiments tested high concentrations of each of the three excipients for their ability to provide protection from freeze damage for two different DTaP vaccines, containing either aluminum phosphate (Daptacel, Sanofi Pasteur) or aluminum hydroxide (Infanrix, GSK) adjuvants. Agglomeration in both vaccines was prevented by 30 percent v/v of either PG, PEG 300, or glycerin (data not shown, see Braun et al. 2009a).<sup>4</sup>

More recently, PG (7.5 percent v/v) was evaluated with DTaP and DTwP vaccines by a major vaccine producer in China in collaboration with PATH (PATH, manuscript in preparation). The key findings were as follows:

- PG prevented agglomeration in both vaccines following three cycles of freezing and thawing ( $-20^{\circ}\text{C}$  for 20 hours and  $22^{\circ}\text{C}$  to  $25^{\circ}\text{C}$  for four hours), based on particle-size analysis.
- Freeze-thaw treatment of the DTwP and DTaP formulations without PG resulted in an immediate drop in potency of wP and aP antigens to below the 8 IU/ml minimum requirement.
- In the presence of PG, the potency of wP remained above the 8 IU/ml threshold after three freeze-thaw treatments and throughout three-month stability tests at  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$  and  $22^{\circ}\text{C}$  to  $25^{\circ}\text{C}$ .

- In the presence of PG, aP also retained its potency immediately after freezing-thawing and during the three-month stability study, with the possible exception of freeze-thawed samples stored at 22°C to 25°C for three months, which had a slightly reduced potency.
- PG also prevented loss of potencies of diphtheria and tetanus toxoids, which remained equivalent to the untreated controls and above the minimum required potency.

### Pentavalent vaccine

Studies with PG have been carried out by a WHO prequalified vaccine manufacturer in collaboration with PATH to evaluate whether it is feasible to develop a freeze-stable formulation for a licensed liquid pentavalent (DTwP- hepatitis B-Hib) vaccine, adjuvanted with aluminum phosphate.

The results (PATH, unpublished data) indicated the following:

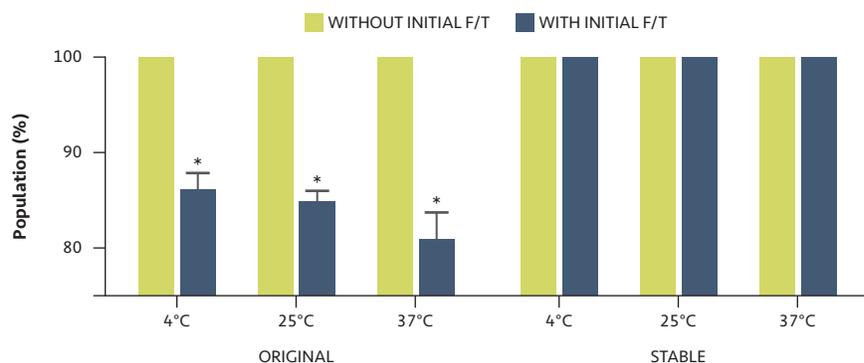
- Addition of PG (2.5 percent v/v to 10 percent v/v) slightly raised the pH of the vaccine, but it remained within specification.
- The osmolality of the vaccine formulation increased with increasing concentration of PG.
- PG at 10 or 7.5 percent, but not 5 or 2.5 percent v/v, protected against agglomeration of the vaccine caused by six freeze-thaw cycles, each consisting of storage at -20°C for 22 hours, followed by thawing at ambient temperature for four hours.
- PG preserved the *in vivo* potency of the diphtheria, tetanus, and pertussis (wP) antigens (assessed using standard challenge models for each component) at concentrations  $\geq$  2.5 percent v/v PG (for tetanus and pertussis) or  $\geq$  5 percent v/v PG (for diphtheria).
- *In vivo* Hib potency was maintained after freeze-thaw even in the absence of PG, and so the cryoprotective effect of PG on Hib could not be determined.
- The data obtained with the hepatitis B component in the pentavalent vaccine used in these experiments were inconclusive due to low responses in the positive control group and requires further testing before any firm statements can be made.
- Although 5 and 7.5 percent v/v PG preserved the potency of wP in a mouse intracerebral challenge assay, higher concentrations of PG appeared to reduce the stability of the wP component:
  - From these initial results, it was not clear, however, whether PG was detrimental to the stability of wP or whether the results were close to the normal level of variability seen.
  - PG did not have an adverse effect on the stability of wP in subsequent experiments using DTwP from a different manufacturer.

### Summary

The cryoprotective excipients, especially PG, were shown to protect DTaP and DTwP vaccines and even pentavalent vaccines formulated with aluminum hydroxide or aluminum phosphate adjuvants from freeze damage, providing preliminary evidence that the technology could be widely applicable to vaccines with aluminum-salt adjuvants.

### ACHIEVING FREEZE PROTECTION AND HEAT STABILITY IN THE SAME FORMULATION

PATH and collaborators also have developed a heat-stable, liquid formulation of hepatitis B vaccine. The vaccine retained *in vitro* potency after storage at 37°C for 12 months, whereas the standard formulation's potency was significantly reduced after one month's storage at 37°C.<sup>6</sup> Experiments were performed to test whether a novel formulation

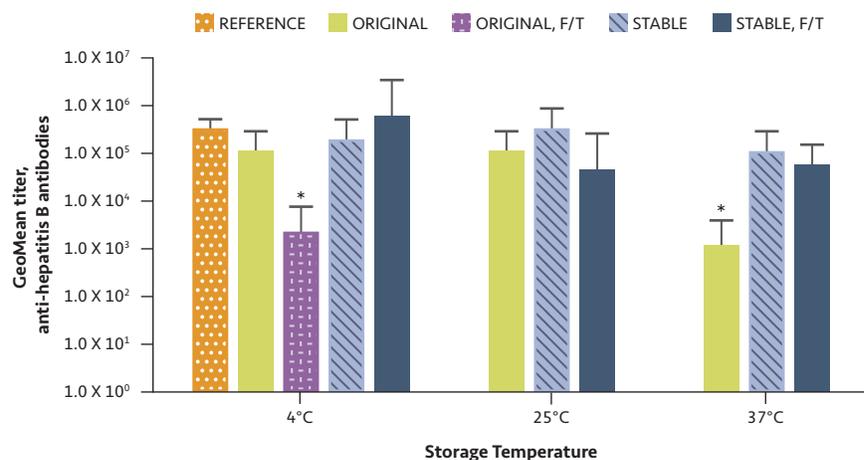


**FIGURE 7. Effect of freeze-thaw cycles and long-term storage on the particle size of antigen-adjutant complexes.** Freeze-thaw (F/T) treatments consisted of three cycles of freezing ( $-20^{\circ}\text{C}$ , 20 hours) and thawing ( $22^{\circ}\text{C}$ , four hours). The heat-stable PG formulation contained hepatitis B antigen and aluminum hydroxide adjuvant reformulated with 20 percent PG, 40 mM phosphate, and 40 mM histidine buffers at pH 5.2. Freeze-thaw treatment was performed prior to initiation of the stability study. The bars represent the percentage of 1.5 to  $3.0\ \mu\text{m}$  particles following 12-month storage at specific temperatures ( $*p < 0.001$  compared with the original formulation stored at  $4^{\circ}\text{C}$ ).<sup>6</sup>

combining both technologies would provide both freeze protection and heat stability. The data presented in this section are described in more detail in Braun et al. 2009b.<sup>6</sup>

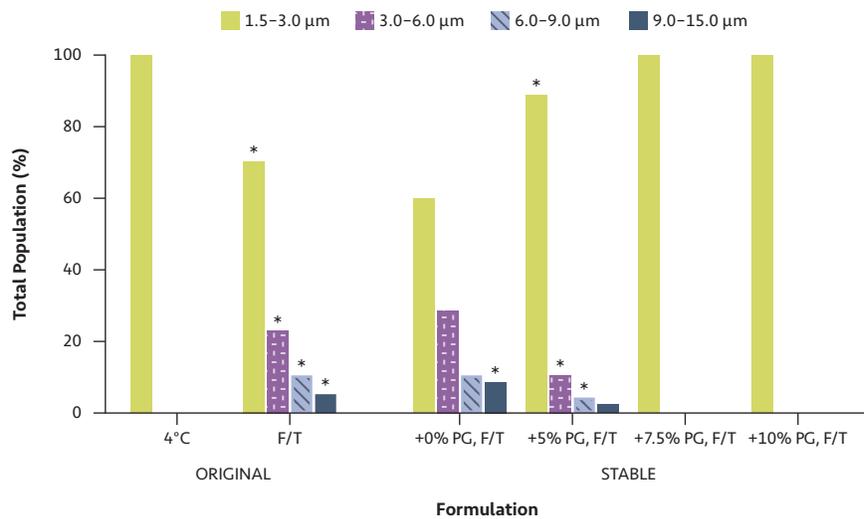
The cryoprotective excipient PG and the heat-stable formulation were found to be chemically compatible. As in earlier experiments, PG (20 percent v/v) prevented particle agglomeration following freeze-thaw treatment (three cycles of freezing at  $-20^{\circ}\text{C}$  for 20 hours and thawing at  $22^{\circ}\text{C}$  for four hours), whereas particle size increased in formulations that did not contain PG (Figure 7). Agglomeration appeared to worsen in non-PG-containing formulations during subsequent storage for 12 months at  $4^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$ , and  $37^{\circ}\text{C}$ , but this was not statistically significant (data not shown, see Braun et al. 2009b).<sup>6</sup>

The hepatitis B vaccine formulations were tested for immunogenicity in an *in vivo* mouse model (Figure 8). The immunogenicity of the standard vaccine formulation was reduced more than 100-fold after storage at  $37^{\circ}\text{C}$  for 12 months. The original vaccine, subjected to freeze-thaw treatment, also had an approximately 100-fold drop in immunogenicity, immediately after the freeze-thaw treatment and after storage at  $4^{\circ}\text{C}$ . In contrast, all the heat-stable formulations containing PG, including those that received the freeze-thaw treatment, were fully immunogenic after 12 months of storage at all three temperatures ( $4^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$ , and  $37^{\circ}\text{C}$ ).



**FIGURE 8. Effect of freeze-thaw treatment and long-term storage on the immunogenicity of hepatitis B formulations.** Hepatitis B vaccine was reformulated with 20 percent v/v PG plus heat-stabilizing excipients. The vaccine was freeze-thawed for three cycles ( $-20^{\circ}\text{C}$  for 20 hours and up to  $22^{\circ}\text{C}$  for four hours), and then stored for 12 months at  $4^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$ , or  $37^{\circ}\text{C}$ . Immunizations and assays were carried out as described for Figure 3.  $*p < 0.001$  compared with the original “reference” vaccine, without the freeze-thaw treatment. Error bars represent standard deviations.<sup>6</sup>

The minimum PG concentration required for physical freeze protection in the combined formulation was evaluated *in vitro* by particle size distribution (Figure 9). PG at 5 percent v/v reduced agglomeration, but concentrations of 7.5 and 10 percent completely prevented particle agglomeration following six cycles of freeze-thawing (20 hours at  $-20^{\circ}\text{C}$  followed by ambient temperature storage at temperatures up to  $22^{\circ}\text{C}$  for four hours).



**FIGURE 9. Effect of PG concentration on particle-size distribution in heat-stable hepatitis B vaccine formulations.** Hepatitis B vaccine was reformulated with 0, 5, 7.5, and 10 percent v/v of PG, plus fixed concentrations of heat-stabilizing excipients. After six cycles of freeze-thawing ( $-20^{\circ}\text{C}$  for 20 hours and up to  $22^{\circ}\text{C}$  for four hours), particle size was measured using a Coulter Counter.  $*p < 0.001$  (two-way ANOVA with Bonferroni posttests) compared with original formulation stored at  $4^{\circ}\text{C}$ .<sup>6</sup>

### Summary

- The minimum concentration of PG required for freeze protection of a heat-stable, aluminum hydroxide-adsorbed hepatitis B vaccine was 7.5 percent v/v. This is consistent with data obtained with DTP-containing vaccines (PATH unpublished data, described above).
- Incorporation of PG into a heat-stable formulation of aluminum hydroxide-adsorbed hepatitis B vaccine resulted in a vaccine that was resistant to physical damage caused by freezing and that could be stored for 12 months at  $37^{\circ}\text{C}$  without loss of *in vivo* immunogenicity, even following an initial freeze-thaw treatment.

### TOXICITY AND SAFETY STUDIES

The data from the studies performed to date with hepatitis B, DTaP, DTwP, and pentavalent (DTwP-hepatitis B-Hib) vaccines suggest that PG concentrations of 7.5 to 10 percent v/v should be sufficient to provide protection against freeze damage caused by exposure to temperatures as low as  $-20^{\circ}\text{C}$  followed by thawing.

Each of the three excipients tested for their cryoprotective properties have GRAS designation, and there are precedents for their inclusion as inactive ingredients in other injected (SC or IM) formulations:

- Glycerin is used at concentrations of 15.36 percent and 32.5 percent in products injected IM and SC, respectively (FDA Center for Drug Evaluation and Research database).
- PEG 300 is present at 50 percent in IM injected product(s) (FDA Center for Drug Evaluation and Research database).
- PG is present at concentrations up to 40 percent in some drug formulations given by the IM route, although not all of these have been used or tested in infant or pediatric populations (reviewed in Braun et al. 2009a).<sup>5</sup>

PATH and collaborators have performed a number of studies to assess possible local and systemic toxicity of PG combined with vaccines:

- Single-dose toxicity studies in rabbits with hepatitis B vaccine containing PG at 7.5 and 15 percent v/v showed the vaccine to be well tolerated, with no adverse findings or indications of acute local or systemic toxicity.<sup>6</sup>
- In a repeat-dose toxicity study in rabbits, five fortnightly IM injections with a pentavalent (DTwP-hepatitis B-Hib) vaccine containing PG at 7.5 percent v/v resulted in inflammatory injection-site reactions, which partially or completely resolved

during the recovery period in the study. There were no systemic reactions (PATH unpublished data).

- Acute- and repeat-dose toxicity studies with hepatitis B vaccine in a freeze-resistant, heat-stable formulation have been conducted in mice and in guinea pigs by one of PATH's collaborators. No abnormal signs or mortality were observed in either species following SC injection, and there was no observable local or systemic toxicity, other than injection-site reactions, which were comparable to those seen with the standard formulation of the vaccine.

PATH has commissioned preliminary literature reviews on the toxicology and safety of PG and glycerin as well as their use in other vaccines or pharmaceuticals. PATH has also consulted with key opinion leaders in toxicology and allergy. The conclusion of the experts consulted so far is that the excipients should be safe for use in vaccines.

#### Supply and cost of cryoprotective excipients

All three cryoprotective excipients (PG, PEG, and glycerin) are commonly used to formulate pharmaceutical drugs. For this reason, there are a number of qualified sources that supply pharmacopeia-grade and non-animal derived materials (e.g., DOW Chemical Company). The cost of cryoexcipient in a dose of vaccine is negligible. One dollar's worth of excipients may be sufficient to formulate several thousand doses of vaccine.

# Future development of the freeze-protection technology

## REGULATORY PATHWAY

In general, the regulatory pathway for vaccines incorporating PG or the other cryoprotective excipients is expected to be straightforward because: the freeze-protection technology uses excipients that have established safety records, would only require a small change in production equipment and/or processes, and does not require a change in packaging or administration method.<sup>10</sup>

PATH has sought guidance from independent consultants on the regulatory requirements for vaccine formulations incorporating the freeze-protection technology.<sup>11</sup> The advice received to date is summarized below.

For “novel” vaccines that are in development:

- A vaccine producer will need to demonstrate that the cryoprotective excipient is compatible with other vaccine components. The vaccine producer must generate laboratory data to support the freeze-stability claim. The additional laboratory work is unlikely to affect the product-development course and product launch date.
- Integration of the freeze-protection technology with a new product is expected to have negligible impact on the development or product costs.
- No additional preclinical or clinical work solely related to the freeze-protection technology is expected to be required.

For an already-licensed vaccine:

- Preclinical studies will be needed to demonstrate the immunogenicity of the freeze-protected vaccine and lack of interference with other concurrently administered vaccines.
- A bridging clinical study is thought to be necessary to demonstrate the non-inferiority of the freeze-stable vaccine to the current vaccine in use. The endpoints of the clinical study and the number of subjects needed will be dependent on the specific vaccine and the requirement of the regulatory authority involved.

The WHO is very aware of the damage caused by the freezing of freeze-sensitive vaccines. The Quality, Safety, and Standards (QSS) group at WHO has included the statement that “Vaccines that are not damaged by freezing temperatures (<0°C) are preferred” in their guidelines titled “Assessing the Programmatic Suitability of Vaccine Candidates for WHO Prequalification.”<sup>18</sup> PATH and collaborators have held discussions with QSS on the need to develop guidelines for the preclinical and clinical testing of freeze-protected formulations of vaccines that will lead to WHO prequalification. WHO/QSS is willing to have early discussions with vaccine suppliers interested in pursuing such products.

PATH wants to advance the application of this technology, particularly to vaccines of importance to low- and middle-income country, public-sector markets.

## **INTELLECTUAL PROPERTY**

There is no intellectual property barrier for manufacturers wishing to adopt the PATH freeze-protection technology. The formulation has been placed in the public domain.<sup>10</sup>

## **NEXT STEPS**

PATH wants to advance the application of this technology, particularly to vaccines of importance to low- and middle-income country, public-sector markets. Therefore, PATH is seeking vaccine manufacturers interested in collaborative research and development leading to implementation of the freeze-protection technology with the combination vaccines (such as pentavalent and hexavalent formulations) and/or with other freeze-sensitive vaccines in development.

PATH's role potentially could include assistance with technology transfer, market research, regulatory analyses, preclinical studies, clinical trials, or advocacy.

## **FURTHER INFORMATION**

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