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Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation

(protein stability/controlled release/single-dose vaccine/formaldehyde/Mannich reaction)

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ABSTRACT **The progress toward single-dose vaccines has been limited by the poor solid-state stability of vaccine antigens within controlled-release polymers, such as poly(lactide-*co*-glycolide). For example, herein we report that lyophilized tetanus toxoid aggregates during incubation at 37°C and elevated humidity—i.e., conditions relevant to its release from such systems. The mechanism and extent of this aggregation are dependent on the moisture level in the solid protein, with maximum aggregation observed at intermediate moisture contents. The main aggregation pathway is consistent with formaldehyde-mediated cross-linking, where reactive electrophiles created and stored in the vaccine upon formalinization (exposure to formaldehyde during vaccine preparation) react with nucleophiles of a second vaccine molecule to form intermolecular cross-links. This process is inhibited by the following: (*i*) succinylating the vaccine to block reactive amino groups; (*ii*) treating the vaccine with sodium cyanoborohydride, which presumably reduces Schiff bases and some other electrophiles created upon formalinization; and (*iii*) addition of low-molecular-weight excipients, particularly sorbitol. The moisture-induced aggregation of another formalinized vaccine, diphtheria toxoid, is also retarded by succinylation, suggesting the generality of this mechanism for formalinized vaccines. Hence, mechanistic stability studies of the type described herein may be important for the development of effective single-dose vaccines.**

Vaccines against tetanus and diphtheria are among the most successful developed and have eliminated these diseases in those countries where they are widely used. In addition to being potent antigens, tetanus and diphtheria toxoids (TT and DT, respectively) are also used as carrier proteins for polysaccharides and haptens to make these immunogenic (1, 2). Despite the success of these vaccines, neonatal tetanus is still a major killer in developing countries (3), and diphtheria has emerged in epidemic form in the countries of the former Soviet Union during the last few years (4, 5). A major factor for the occurrence of these diseases in developing countries is the logistical difficulty of delivering the two or three doses of these vaccines required to achieve protection (3). Therefore, recent efforts have focused on developing controlled-release formulations that can deliver the necessary vaccine in a single dose (6–9).

Previous investigations in our laboratory have demonstrated high neutralizing antibody levels in mice and guinea pigs following a single dose of TT encapsulated in poly(lactide-*co*-glycolide) microspheres (7). However, in that study, incomplete release profiles of the TT protein from this biodegradable polymer *in vitro* indicated aggregation of the antigen within the microspheres. Thus, the immunogenic potential of controlled-release preparations incorporating TT may be improved by enhancing stability under conditions which mimic its release environment.

Protein aggregation can occur at various stages from encapsulation to release from polymer microspheres (10). We have focused on the rehydration step and subsequent exposure to moisture within the polymeric carrier during release since (*i*) these conditions take place with any lyophilized microsphere preparation and (*ii*) moisture induces the aggregation of many solid proteins (11–14). We report that lyophilized TT becomes insoluble upon exposure to elevated humidity and physiological temperature. The principal pathway responsible for this deleterious process has been found to be distinct from any reported previously for moisture-induced aggregation. Finally, we have devised rational approaches for stabilization against this mechanism, which may be applicable to other formalinized vaccines, such as DT.

MATERIALS AND METHODS

Chemicals. A purified form of TT (≈150 kDa; ≈85% monomer) was obtained from the Massachusetts Public Health Biologic Laboratory (MPHBL, Boston) [lot PS-21; 1663 limit flocculation units (Lf) per ml; 3.9 μg/Lf]. This purified TT was employed for all experiments, except for water-sorption measurements, where a partially purified TT (≈50% monomer; from MPHBL; lot LP-943P; 420 Lf/ml; 4.0 μg/Lf) was used. DT (≈58.3 kDa) was also from MPHBL (lot DCP-54; 540 Lf/ml; 3.1 μg/Lf). All other biochemicals and chemicals were of analytical grade or purer and obtained from commercial suppliers.

Modification of Vaccines. Reduction of disulfide bonds of TT was performed in excess dithiothreitol (DTT; 140 mM) at 37°C for 2 h in 0.29 M Tris buffer, pH 8.6. To protect free amino groups, TT or DT was treated with a 2.4 weight excess of succinic anhydride, while manually maintaining a pH of 7 (15). Reduction of TT (1 mg/ml) with sodium cyanoborohydride (CBH; 1 mg/ml) was performed under gentle stirring at pH 7 (0.04 M sodium phosphate) and 37°C for 1 h as described (16), except that formaldehyde was absent in the reaction mixture. Modifying agents were removed by desalting on a Sephadex G-50 column equilibrated with phosphate-buffered saline or by dialysis (see below).

Lyophilization of Vaccines. Vaccines were dialyzed (12,000–14,000 molecular weight cut-off membrane) against 1 mM phosphate buffer at pH 7.3 (4°C) and diluted to roughly 1 mg/ml (Bio-Rad protein assay). Excipients, if any, were added,

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Abbreviations: TT, tetanus toxoid; DT, diphtheria toxoid; DTT, dithiothreitol; CBH, sodium cyanoborohydride.

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and samples were flash frozen in liquid N_2 in 0.25-ml aliquots and lyophilized. No aggregation was observed upon lyophilization as determined by size-exclusion chromatography (HP Series II 1090 liquid chromatograph with a TSK-G3000SW column). Water content in the lyophilized TT was 5–7 g of water per 100 g of dry protein (see below).

Assays. The exposed free thiols of the vaccines were quantitated by Ellman's reagent, 5,5′-dithio-bis(2-nitrobenzoic acid), under nondenaturing conditions (17). The exposed amino groups were measured with 2,4,6-trinitrobenzenesulfonic acid (18) by using N^{α}-acetyl-L-lysine methyl ester as a standard. In both spectrophotometric determinations, background corrections were necessary to account for vaccine and reagent absorption at 412 and 335 nm. Protein water content was measured by Fischer titration (19). Protein concentrations were measured by either the Bio-Rad or bicinchoninic acid method; when denaturing and/or thiol agents were present, the Bio-Rad assay was used exclusively (20).

Solid-State Aggregation at Elevated Humidity. Lyophilized TT or DT (0.25 mg) was placed in a controlled-humidity environment maintained by saturated salt solutions in water (19). The standard conditions were 37°C and 86% relative humidity (R.H.), although other humidities were also used, as described below. Incubated samples were reconstituted in 0.5 ml of 0.1 M phosphate buffer, pH 7.3, by stirring at 37°C for 2 h and filtered (0.22-μm pore size, low protein-binding filter). Aggregation was quantified from the recoverable soluble fraction of incubated protein (11, 14, 19). In those instances where the reconstitution media contained denaturant (6 M urea) and/or reductant (10 mM DTT, 1 mM EDTA) stirring was carried out for only 30 min to avoid precipitation upon extended exposure to the denaturing agent.

Amino Acid Composition of the Aggregates. Prior to their acid hydrolysis, TT aggregates (obtained after a 9-day incubation at 86% R.H. and 37°C) were centrifuged, washed with phosphate buffer, and lyophilized. Acid hydrolysis of the aggregates or unincubated vaccine was accomplished in 6 N HCl in sealed and evacuated ampoules for 20 h at 110°C. The hydrolysates were neutralized, lyophilized, reconstituted in 1:1 acetonitrile/water, and injected into an amino acid analyzer equipped with a phenylisothiocyanate precolumn derivatizer and a 22-cm C_{18} column with a 5-μm pore size. The amino acid composition of each sample was determined by using the amount of recovered leucine (stable to acid hydrolysis) as an internal standard.

Water Sorption Isotherms. Lyophilized samples (1–4 mg) were stored at various relative humidities, and sorbed water was titrated after reaching the equilibrium water uptake (10 days) at 37°C. Saturated aqueous salt solutions used to attain the following constant relative humidities were as follows (21, 22): LiCl, 11%; NaBr, 54%; $SrCl_2$, 67%; $NaNO_3$, 72%; NaCl, 75%; $(NH_4)_2SO_4$, 80%; K_2CrO_4, 86%; KNO_3, 90%; and K_2SO_4, 97%. The water sorption isotherm for TT was fit by nonlinear regression according to the Brunauer–Emmett–Teller gas adsorption equation (23). Theoretical monolayer coverage of TT was 4.8 g of water per 100 g of dry protein, and the constant C (19) was 86.

RESULTS AND DISCUSSION

Moisture-Induced Aggregation of Tetanus Toxoid. To investigate the role of moisture in TT solid-state aggregation, we followed the loss of soluble protein and water uptake at 37°C for 10 days at various humidities. As seen in Fig. 1 *A* and *B*, at low water activity (11% R.H.), which corresponded to low water uptake in the solid protein (4 ± 2 g of water per 100 g of dry protein), no aggregation of TT was observed. However, when the humidity was raised to intermediate levels (66–90% R.H.), the extent of aggregation dramatically rose, as did the water content in the protein powder. The parallel trends that were observed suggest that the aggregation of lyophilized TT may be caused by moisture or, more specifically, by the water sorbed in the lyophilized powder. As the humidity was further raised from an intermediate to a high level—e.g., 97% R.H.—the aggregation declined sharply, while water content increased markedly. Similar bell-shaped dependence to that seen in Fig. 1*A* has been reported for a number of proteins (13) and rationalized mechanistically (12).

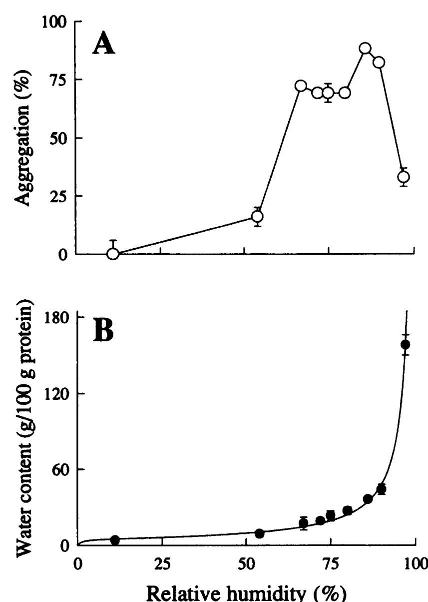


FIG. 1. Effect of relative humidity on the extent of solid-state aggregation of TT (*A*) and on the water content in the TT powder (*B*) after a 10-day incubation at 37°C. The Brunauer–Emmett–Teller gas adsorption equation (23) was fit to the sorption data by nonlinear regression. TT was lyophilized from 1 mM sodium phosphate buffer, pH 7.3. Each point is the mean of two or three measurements and error bars are SEM.

Effect of Excipients on the Moisture-Induced Aggregation of Tetanus Toxoid. A profound effect of the water content in the protein powder on aggregation (Fig. 1) suggests that one could affect the aggregation by controlling the water level in the incubated protein. For example, co-lyophilization with excipients that possess stronger water-sorbing capacity than the protein should increase the water uptake. If the latter were increased to high levels—e.g., >100 g of water per 100 g of dry protein—then stability should rise relative to that at intermediate water contents (15–50 g of water per 100 g of dry protein), as predicted by the descending portion of the curve shown in Fig. 1*A*. To test this hypothesis, we co-lyophilized TT with three excipients (0.2 g of excipient per g of protein) and examined the time course of its aggregation during incubation at 86% R.H. The excipients selected represent a range of water-sorbing powers, as evidenced by the following experimentally obtained (at 86% R.H. at equilibrium) data in parentheses: high, NaCl (349 g of water per 100 g); medium, D-sorbitol (47 g of water per 100 g); and low, polyethylene glycol 20000 (PEG; 5.3 g of water per 100 g). As predicted, the addition of co-lyophilized NaCl inhibited TT solid-state aggregation and the addition of PEG was ineffective, both relative to TT lyophilized without excipient (Fig. 2*A*). However, the presence of sorbitol, which should not alter appreciably the water content in, and thus the stability of, the vaccine powder (Fig. 1), had a remarkable stabilizing effect (Fig. 2*A*). This indicates that the water-sorbing capability is not the only important characteristic of the excipient which influences solid-state aggregation. Sorbitol and other sugars have been reported to preserve the conformation of proteins upon lyo-

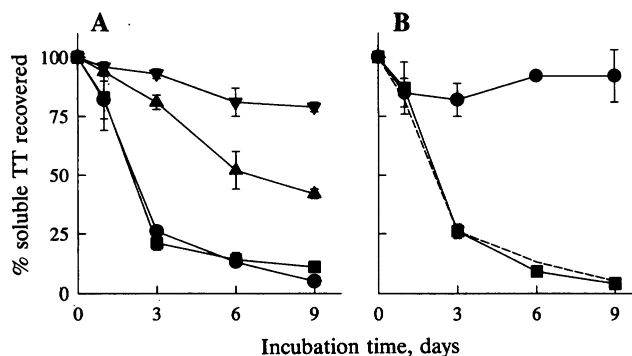


FIG. 2. Effect of excipients co-lyophilized with TT and modification agents on the moisture-induced aggregation of the vaccine in the solid state. (*A*) Time courses of the loss of soluble TT when co-lyophilized with sorbitol (▼), NaCl (▲), or polyethylene glycol (■) are compared with that for TT lyophilized without excipient (●) after exposure to 86% R.H. and 37°C. Excipient to protein ratio was 1:5 (wt/wt), and equilibrium water uptakes (after 14 days) of individual excipients under the same conditions were as follows: 5.3 ± 0.8 g of water per 100 g of PEG; 47 ± 2 g of water per 100 g of sorbitol; and 349 ± 6 g of water per 100 g of NaCl. (*B*) Time courses of moisture-induced aggregation of TT that was either reduced (with DTT) (■) or reduced and succinylated (●) are compared with the unmodified vaccine (- - -) replotted from *A*. For other conditions, see the legend to Fig. 1.

philization (24–27) which could improve solid state stability if aggregation requires prior unfolding (13, 14).

Analysis of Aggregates. Previously reported mechanisms for moisture-induced aggregation of proteins include disulfide-mediated and noncovalent routes (13). To examine whether these pathways were involved in TT aggregation, vaccine aggregates formed at various humidities (10-day incubation) were reconstituted in reducing and/or denaturing solvents. We selected humidities that corresponded to high and intermediate water contents in the lyophilized TT powder, namely, 97% and 80% R.H., resulting in 158 ± 8 and 27 ± 3 g of water per 100 g of dry powder, respectively (Fig. 1). (The low water-uptake case, $<$10 g of water per 100 g of dry protein, was not examined since no appreciable aggregation occurred, even after 10 days.)

Aggregates obtained after exposure to 97% R.H. were soluble in the various media (all in phosphate buffer, pH 7.3) to the following extents: 48% ± 8% in 6 M urea (denaturing), 20% ± 5% in 10 mM DTT/1 mM EDTA (reducing), and 55% ± 2% in 6 M urea/10 mM DTT/1 mM EDTA (both denaturing and reducing). Thus, the two pathways mentioned above, namely disulfide and noncovalent bond formation, are indeed involved in TT aggregation at high water contents. In contrast, the aggregates formed at the intermediate water content were not appreciably soluble under either reducing or denaturing conditions, and only 8% ± 3% soluble in the combined denaturing and reducing medium. Hence, in this instance a third, heretofore undescribed, mechanism of moisture-induced aggregation involving the formation of non-disulfide covalent bonds is dominant.

To shed light on this aggregation pathway, we determined the amino acid composition of the aggregate formed following a 9-day incubation at 86% R.H. We found a noticeable loss of lysine and histidine residues in the moisture-induced TT aggregates relative to the vaccine that had not undergone cross-linking (Fig. 3). In addition, ≈30% more tyrosine residues were recovered in the aggregate samples. Since formaldehyde, which is used to prepare the formalinized TT vaccine, is known to react strongly with these three amino acid residues (28), these data suggest that formaldehyde may be involved in the aggregation mechanism.

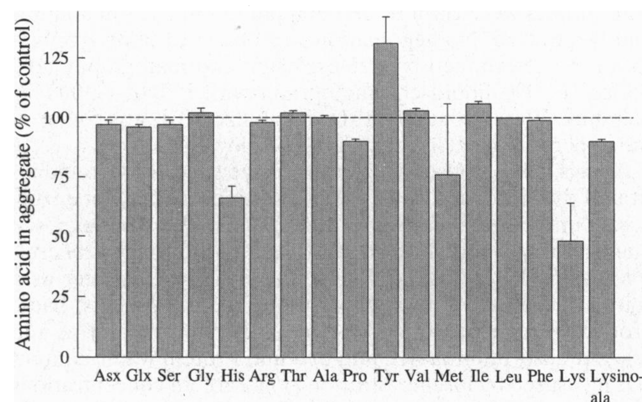


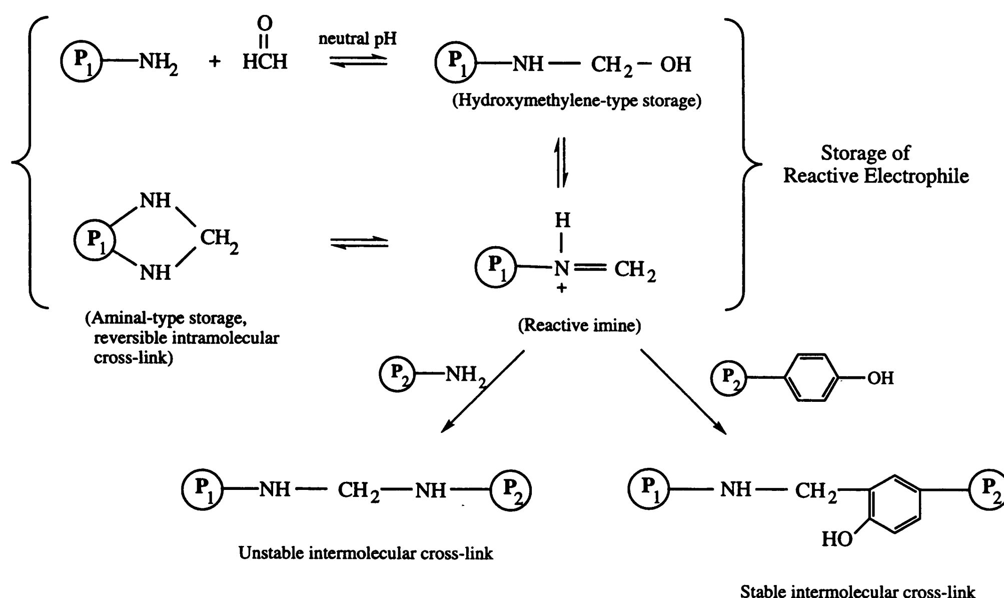
FIG. 3. Changes in the amino acid composition of TT due to moisture-induced aggregation. Aggregates were obtained following a 9-day incubation at 37°C and 86% R.H. The amino acid composition of the acid hydrolysate of the aggregated TT is given relative to that of the hydrolyzate of the vaccine that had not undergone the moisture treatment. All amino acid concentrations were normalized for the concentration of leucine. Each data point is the mean of two measurements and error bars are SEM.

Approximately 0.03 mol equivalent of lysinoalanine (eluting just after lysine) was observed in the control TT vaccine. This is evidence that the lysinoalanine cross-link may form during formalinization (the process of detoxifying tetanus toxin with formaldehyde). Importantly, the levels of lysinoalanine in the control and in the aggregate were very similar (Fig. 3), indicating that no additional cross-links of this kind formed as a result of aggregation.

Cross-Linking Reactions in Proteins. The number of lysine residues missing in the aggregate compared with the control was greater than for any other amino acid residue. Two well-known reactions involving lysine include transamidation and formation of lysinoalanine cross-links (13). Since the latter did not increase in the aggregate (Fig. 3), this pathway is unlikely to occur during TT moisture treatment. Likewise, the loss of lysine in the aggregate is not consistent with transamidation, since amide bonds are hydrolyzed under the conditions of acid hydrolysis used for the amino acid analysis (29) and, therefore, the free lysine residues would have been recovered. In search of an explanation for the missing lysine residues, we examined formaldehyde, a potent and nonspecific cross-linking agent used to prepare the toxoid from the native toxin, as a potential participant in the aggregation pathway.

The reactions that occur during the treatment of proteins with formaldehyde have been investigated in detail (28, 30, 31). Among all protein amino acid residues, the side chains of cysteine, lysine, histidine, and tyrosine possess the highest reactivity toward formaldehyde (28). They belong to two distinct types: (*i*) standard nucleophiles capable of forming new C—N or C—S bonds with formaldehyde, such as lysine, cysteine, and histidine residues, and (*ii*) aromatic side chains capable of forming new C—C bonds with formaldehyde (via direct addition to the aromatic ring), such as tyrosine, tryptophan, and histidine (28).

Under neutral conditions, formaldehyde may react with either type of side chain to form a hydroxymethylene derivative, as illustrated for lysine residues in scheme **I**. Following condensation, the electrophilic methyl imine (Schiff base) or equivalent is formed. If the electrophilic group is an imine, attack from a second amino group gives an aminal (Eschweiler-type chemistry) (32, 33), which is a labile, hydrolyzable cross-link (scheme **I**). However, if the imine is attacked by tyrosine, the resultant cross-link is stable (Mannich-type chemistry) (31, 34). Finally, if the electrophile is enolizable and is attacked by a second enolate, the result is also a stable



Scheme I

cross-link (Lederer–Manasse-type chemistry) (34). Model studies with free lysine and tyrosine demonstrate the Mannich cross-link to be the dominant of the two types of stable cross-links (31); the formation of the unstable, aminal-type cross-links was not evaluated.

Formaldehyde-Mediated Aggregation Hypothesis. Any hypothesis linking formaldehyde to aggregation of TT requires that a reactive species be either present or stored in a latent form within the protein—e.g., a hydroxymethylene or aminal derivative (35). The existence of formaldehyde stored in labile linkages of TT is supported by reports of formaldehyde liberated during acid hydrolysis of formalinized proteins (31) and well-known reversion of the formalinized vaccines back to the toxic form when the formaldehyde exposure is carried out over too short a time period (36). [In fact, it is common practice to add sodium borohydride when labeling proteins with [^{14}C]-formaldehyde to reduce the unstable electrophiles (37).] The likely candidate functional groups for the reversible storage of formaldehyde are the hydroxymethylamine and the aminal, the latter of which can form ring structures of alternating N—C bonds (33).

Consequently, we hypothesize the following molecular mechanism of moisture-induced aggregation of TT (scheme **I**): a stored electrophile in one TT molecule becomes exposed and is attacked by a nucleophilic or aromatic side chain on a second TT molecule to form either a N—C bond (labile to acid hydrolysis) or a C—C bond [stable to acidic or basic conditions (31)]. This process proceeds until water-insoluble aggregates ensue. This hypothesis is consistent with both the formation of intermolecular covalent, nondisulfide bonds (as reflected by the insolubility of the aggregates in the combined denaturing and reducing solvents) and the loss of lysine and histidine residues in the aggregate (Fig. 3). (Histidine can be substituted for lysine in the storage of the electrophile in scheme **I**.) Moreover, the lower level of tyrosine observed in the control compared with the aggregates (Fig. 3) may also be explained as follows. Upon acid hydrolysis, formaldehyde is liberated (31), probably from storage in reversible hydroxymethylene or aminal linkages (scheme **I**). Liberation from the (unincubated) control would be greater than from the aggregates, since in the latter case some formaldehyde had been lost due to its involvement in irreversible, non-acid-hydrolyzable cross-links. In turn, this larger pool of formaldehyde in the control would lead to a lower level of tyrosine since formaldehyde is known to react with phenols (34), such as tyrosine (31), in acidic media.

Devising Rational Stabilization Strategies. Several stabilization strategies may be proposed based on the formaldehyde-mediated aggregation pathway hypothesized above. One would be to block lysine residues, which form both reversible and irreversible cross-links (scheme **I**). Consequently, the exposed electrophiles would remain idle until they combine with water to yield the hydroxymethylene-inactivated form. This approach was verified (Fig. 2*B*) by demonstrating that the succinylation of DTT-reduced TT virtually abolishes its aggregation during incubation at 86% R.H. The reduction of cystines (with DTT, which increases the number of accessible thiols from nearly 0 to 1.2 ± 0.2 mol equivalents) alone did not affect TT solid-phase stability. This reduction may arrest potential disulfide-dependent pathways, such as β-elimination followed by thiol-catalyzed disulfide exchange and thiol-disulfide interchange [the native toxin molecule has six free thiols and two disulfides (38)].

To quantify the covalent modifications to the vaccine, the number of accessible amino groups was examined (Table 1). Succinylation of free amino groups was highly effective, resulting in an 85% drop in the titratable amino groups in TT (note that the reduction itself increases the number of accessible NH_2 groups almost by half). In addition to blocking surface nucleophiles by succinylation, another approach to inhibiting the aggregation via the proposed mechanism would be to convert the reactive electrophile, e.g., a Schiff base, to an inactive amine. To this end, we examined a reducing agent, CBH, for its ability to stabilize TT. The CBH-reduced vaccine (lyophilized from 10 mM phosphate buffer, pH 7.3) was tested for moisture-induced aggregation and found to aggregate only

Table 1. Determination of accessible amino groups of TT and DT

Vaccine	Free amino groups, mol-equivalents*
TT	12
Reduced with DTT	17
Reduced with DTT and succinylated	2.6
Reduced with CBH	14
DT	5.2
Succinylated	0.12

*Determined with 2,4,6-trinitrobenzenesulfonic acid and using N^{α}-acetyl-L-lysine methyl ester as a standard.

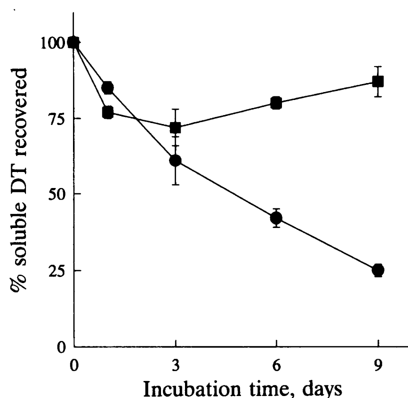


FIG. 4. Time course of moisture-induced aggregation of unmodified DT (●) and succinylated DT (■) after exposure to 86% R.H. and 37°C. Both vaccines were lyophilized from 10 mM phosphate buffer, pH 7.3. Each data point is the mean of two measurements and error bars are SEM.

27% ± 5% and 41% ± 1% after 6 and 9 days, respectively, whereas the unmodified TT lyophilized under the same conditions aggregated 68% ± 0% and 86% ± 6%, respectively, after the same time periods. These data again are consistent with the proposed mechanism.

Since formaldehyde is involved in the aggregation pathway, our stabilization approaches should also apply to other formalinized vaccines, provided no other aggregation mechanism—e.g., disulfide interchange—is predominant at the intermediate water content. To test this, we heavily succinylated DT in the same manner as TT (Table 1), and its aggregation tendency was then compared with that of the unmodified DT. After 9 days of exposure to 86% R.H., only 25% of unmodified DT but 87% of the succinylated DT was recovered as a soluble protein (Fig. 4). Hence, as was the case with TT (Fig. 2), the detoxification of diphtheria toxin with formaldehyde gives rise to an otherwise impossible mechanism of aggregation in the solid state.

In closing, as tetanus and diphtheria toxoids undergo the transformation from rigid unreactive species in the dry state to more conformationally flexible and reactive species in the wet powder, they begin to aggregate. The dominant covalent mechanism at the intermediate water content, where the aggregation is most rapid, appears to be formaldehyde-mediated cross-linking, where a formaldehyde-modified electrophile is exposed and attacked by surface nucleophiles that can form stable intermolecular crosslinks.

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