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Thermal inactivation of diphtheria toxoid following drying by sublimation *in vacuo*

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The denaturation of proteins consists of a physical rearrangement of the peptide molecule; primarily an unfolding of peptide chains (Putnam, 1953; Haurowitz, 1963). If denaturation is heat induced, the peptide chains unfold only if water flows into the spaces between the chains (Haurowitz, 1963). Denaturation can be minimized if the water content of the protein is reduced by sublimation *in vacuo* prior to heating (Greaves, 1946; Greiff & Pinkerton, 1954; Greaves, 1960). In this case, stability of the protein at increased temperatures is a function of the amounts of water in the dried product (Greaves, 1960). Because residual moisture is not the only factor affecting the stability of the dried product, the stability of dried antisera, toxins, virus, etc., is based frequently on the measurement of a biologic parameter and not on a direct analysis of residual moisture (Jerne & Perry, 1956; Fisek, 1959; Neumann, 1960; Greiff & Rightsel, 1965). The present study reports the effects of elevated temperatures on the stability of dried diphtheria toxoid, the stability of dried diphtheria toxoid when in combination with tetanus toxoid, and the stability of diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine.

MATERIALS AND METHODS

A purified preparation of diphtheria toxoid was used in all investigations (Holt, 1950). Activities of diphtheria toxoid were measured by L_f values, that amount of toxoid which gives most rapid flocculation with one standard unit of antitoxin, and K_f values, the time required in a flocculation test (Ramon technique) for particles to become visible to the unaided eye (Boyd, 1956). In those cases where weakly combining toxoid was present, flocculation was aided by the addition of an active diphtheria toxin (Regamey, 1957). When diphtheria toxoid was a component of diphtheria–tetanus–pertussis vaccine, the flocculation test was carried out after centrifugation to remove bacteria.

The concentration of diphtheria toxoid component, alone or in combination with tetanus toxoid, was adjusted so that each 1 ml. sample dried by sublimation *in vacuo* contained 350 L_f units. For testing, each dried preparation was rehydrated with 10 ml. of a concentration of sodium chloride sufficient to result in an isotonic solution and an activity of 35 L_f/ml. In the case of diphtheria toxoid in combination with tetanus toxoid and pertussis vaccine, the concentration of the former was adjusted so that 2 ml. of the suspension contained 290 L_f. Two ml. volumes of the

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suspension above were dried by sublimation *in vacuo* and rehydrated with 10 ml. of a suitable concentration of sodium chloride to give an isotonic concentration of salts in the final suspension and an activity of 29 L_f/ml.

All preparations, contained in ampoules, were frozen in an alcohol bath at −45° C before drying. Following freezing the material was dried by sublimation *in vacuo* in a chamber freeze-dryer (Usifroid, Model SMU) to a residual moisture content of less than 1 % and sealed under dry nitrogen (Rey, 1960; Rieuford, 1960).

In those preparations of diphtheria toxoid plus tetanus toxoid alone or in combination with pertussis vaccine, dried by sublimation *in vacuo* and sealed under air, the concentration of the starting material was adjusted so that following rehydration each ml. of the former contained 21 L_f units and each ml. of the latter 35 L_f units.

The activity of dried toxoid was determined before exposure to elevated temperatures and after exposure to (1) 100° C. for 60, 80, 100, 120 and 140 min., (2) 110° C. or 120° C. for 60 min., (3) 130° C. for 60, 120 or 180 min.

RESULTS

The effects of exposure to elevated temperatures for 60 min. on the L_f or K_f values of dried diphtheria toxoid alone and in combination with tetanus toxoid or tetanus toxoid plus pertussis vaccine are shown in Table 1. The L_f values for all preparations sealed under nitrogen, exposed to elevated temperatures and tested immediately thereafter did not vary significantly from those of the control. Significant decreases in L_f values were observed in those samples sealed under air. The greatest change occurred in dried diphtheria toxoid in combination with tetanus toxoid when heated to 130° C. for 60 min. (57 %). The L_f values of the foregoing preparations did not change following storage at room temperature (20° C.) for 2 years. The decline in the diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine was 17 % when heated to 130° C. for 60 min.

K_f values increased with each increment of increased temperature in preparations of dried diphtheria toxoid only (nitrogen), dried diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine (nitrogen) or dried diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine (air). Results similar to the above were observed for dried diphtheria toxoid plus tetanus toxoid (nitrogen) heated to a final temperature of 120° C.; the K_f value of 130° C. was obtained following the addition of untreated diphtheria toxin and consequently the time required for flocculation was shortened.

In the samples stored at room temperature for 2 years and tested with the addition of untreated diphtheria toxin, the time for flocculation increased significantly following exposure at 100° C. or 110° C. and declined significantly following exposure at 120° C. or 130° C.

In experiments similar to the above, the L_f values for samples sealed under nitrogen did not change following heating to 100° C. for 80, 100, 120 or 140 min.; significant decreases were observed in samples sealed under air. The L_f values for samples of diphtheria toxoid only and diphtheria toxoid when in combination

with tetanus toxoid, both sealed under nitrogen, did not change following heating to 130° C. for 120 and 180 min.; flocculation was aided by the addition of untreated diphtheria toxin.

The K_f values of the preparation above increased with prolonged exposure to

Table 1. *The effects of exposure to elevated temperatures for* 60 *min. on the* L_f *and* K_f *values of diphtheria toxoid alone and in combination with tetanus toxoid or tetanus toxoid plus pertussis vaccine dried by sublimation* in vacuo *and sealed under dry nitrogen or air*

	L_f/ml.					K_f in min.				
Temperature (°C)	Control (dried and rehydrated only)	100°	110°	120°	130°	Control* (dried and rehydrated only)	100°	110°	120°	130°
Diphtheria toxoid (nitrogen)	35	35	35	35	35	80	90	200	255	720
Diphtheria toxoid +tetanus toxoid (nitrogen)	35	35	35	35	35	110	165	370	595	320‡
Diphtheria toxoid +tetanus toxoid†‡ (air)	21	21	15	13	9	165	250	380	210	210
Diphtheria toxoid +tetanus toxoid +pertussis vaccine (nitrogen)	29	29	28	27	27	125	145	205	300	490
Diphtheria toxoid +tetanus toxoid +pertussis vaccine (air)	35	34	31	30	29	100	120	175	275	480

* The K_f values of the original fluid preparations before freezing and freeze-drying were approximately 30 minutes.

† These samples were stored for two years at room temperature.

‡ Flocculation aided by the addition of active diphtheria toxin.

Table 2. *The effects of the delayed additions of serum (antitoxin) on the* L_f *and the* K_f *values of diphtheria toxoid dried by sublimation* in vacuo *and exposed to* 100° *C. for* 60 *min. before rehydration and testing*

	Flocculation at 20° C				Flocculation at 45° C			
	Antitoxin added immediately		Antitoxin delayed		Antitoxin added immediately		Antitoxin delayed	
Antigen tested	L_f/ml.	K_f in min.	L_f/ml.	K_f in min.	L_f/ml.	K_f in min.	L_f/ml.	K_f in min.
Dried diphtheria toxoid before heating (control)	30	342	30	365	30	91	29	141
Dried diphtheria toxoid following heating at 100° C. for 60 min,	30	357	29	345	30	168	30	177

100° C. The values for K_f of preparations heated to 130° C. for 120 min. declined following the addition of untreated diphtheria toxin; an increase in K_f was observed in preparations exposed for 180 min.

In other studies, constant amounts of antigen (diphtheria toxoid dried by sublimation *in vacuo*, exposed to 100° C. for 60 min. and rehydrated with 10 ml. of a concentration of NaCl sufficient to result in an isotonic solution) were placed in two sets of tubes and varying amounts of serum were added immediately to one set. Several tubes of both sets were kept at 20° C. (room temperature) or at 45° C. When flocculation appeared in the set of tubes containing the antigen-antibody complex, varying amounts of serum were added to the tubes containing antigen only.

The variations in the values of L_f for the above were not significant (Table 2). The variations in the values of K_f between sets of tubes containing diphtheria toxoid treated similarly and differing only in the time at which serum was added and the temperature at which the test was carried out, with one exception, were not significant.

DISCUSSION

The flocculation reaction is based primarily on the specific, mutual attraction of antigen and antibody. Although the reaction is usually completed in 1–2 min., in some cases several hours may elapse. In our studies the latter occurred when a weakly combining diphtheria toxoid antigen was used. To increase the sensitivity of the test system an active diphtheria toxin of known L_f value was added to the weakly reacting system. Through the use of 'helper' toxin we were able to demonstrate residual activity in preparation of diphtheria toxoid dried by sublimation *in vacuo* and stored under adverse conditions (sealed under air) or exposed to high temperatures. The need for 'helper' toxin may indicate that various degrees of denaturation of antigens exist. If increased K_f values are a sign of slight damage, the necessity for the addition of an active antigen, diphtheria toxin, to obtain flocculation is probably a sign of greater damage. The observation that original L_f values can be obtained in preparations stored under adverse conditions (dried diphtheria toxoid when in combination with tetanus toxoid, sealed under air and stored two years) supports the above.

In the preparations dried by sublimation *in vacuo* and sealed under nitrogen, heating did not alter L_f values; K_f values were increased. These data indicate that heating did not affect the total amount of diphtheria toxoid protein available for flocculation, but resulted in some denaturation of protein components responsible for their specific attraction to antibody. The finding that slight alterations in the structure of a hapten caused considerable changes in the affinity of hapten for antibody lend support to the above (Haurowitz, 1963). These results were similar to those obtained when dried antibody was exposed to elevated temperatures following freeze-drying (Damjanovic & Iovicic, 1964). It is to be noted also that freezing and drying by sublimation *in vacuo* of itself resulted in denaturation as shown by the increased K_f values for non-heated, dried preparations.

If we assume that the principal factor involved in the heating of dried diphtheria toxoid was reversible changes in protein structures of the antigen responsible, in

part, for the affinity of antigen for antibody, one would expect the K_f's of heated dried diphtheria toxoid to give normal values if rehydrated and stored before the addition of serum. The results reported by Holt (1950), decreased K_f on prolonged storage at room temperature, support this hypothesis. That the above did not occur in our system is shown by the results obtained when the addition of the serum was delayed (Table 2). The differences observed in the direction of K_f values may be a function of the temperatures and the periods of storage.

Accelerated deleterious changes in the stabilities of biologic materials have been reported for dried suspensions sealed under air in contrast to those sealed under vacuum or dried nitrogen (Sololov, Kulikova, Kholeva & Azadova, 1958). In our studies decreased L_f and increased K_f values were observed in dried toxoid sealed under air and exposed to elevated temperatures. The decline in activity of the former occurred as a first-order reaction. It is recommended, therefore, that dried diphtheria toxoid be stored under nitrogen rather than air for increased stability.

The combination of diphtheria toxoid with tetanus toxoid or with tetanus toxoid plus pertussis vaccine does not seem to have any significant influence on the stabilities of dried diphtheria toxoid. The difference in K_f values between diphtheria toxoid only and diphtheria toxoid when in combination with tetanus toxoid could be anticipated; the flocculation of the latter is usually slower (Regamey, 1957). The slight decrease in L_f values when diphtheria toxoid is in combination with tetanus toxoid plus pertussis vaccine (nitrogen) does not seem sufficient reason to assert that it was caused by the presence of pertussis vaccine.

SUMMARY

The L_f values of diphtheria toxoid alone and in combination with tetanus toxoid or tetanus toxoid plus pertussis vaccine, dried by sublimation *in vacuo*, sealed under nitrogen, exposed to elevated temperature and rehydrated thereafter were not altered. L_f values declined in samples sealed under air. The values for K_f in the above preparations increased in relation to increased temperatures of exposure for a given time or following exposure to a given temperature for increased time intervals. The sensitivity of the system of testing used was greater following the addition of 'helper', a fast flocculating solution of diphtheria toxin, and in the case of dried diphtheria toxoid stored under adverse conditions (sealed under air) for two years, the addition of 'helper' was necessary to obtain a flocculation reaction. In general, the results obtained indicated a very high stability for preparations sealed under nitrogen, and a significantly lower stability for preparations sealed under air.

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