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STABILIZATION OF PERTUSSIS VACCINE IN THE PRESENCE OF BENZETHONIUM CHLORIDE

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OLSON, B. H. (Division of Laboratories, Michigan Department of Health, Lansing), GRACE ELDERING, AND BERNICE GRAHAM. Stabilization of pertussis vaccine in the presence of benzethonium chloride. J. Bacteriol. **87**:543-546. 1964.—Data are presented showing that pertussis vaccine preserved with benzethonium chloride (BC; Phemerol) was inactivated during storage. BC-preserved vaccine stored at 37 C showed no measurable mouse-protective potency at 16 weeks. That stored at 0 to 4 C lost approximately 80% of its potency within 1 year. Treatment of pertussis vaccines with aluminum, calcium, magnesium, choline, or DL-lysine before the addition of the BC prevented its uptake by the cells. Pertussis vaccines pretreated with 0.004 M Ca^{++} or 0.0004 M Al^{+++} retained 70% of the initial potency after 42 weeks of storage at 37 C. Similar vaccines showed no loss of protective antigens when stored for 1 year at 0 to 4 C.

It is a recognized finding that the potency of the pertussis antigen deteriorates in quadruple vaccine (diphtheria and tetanus toxoids, pertussis and polio vaccines) in which benzethonium chloride (BC; Phemerol) is the preservative. In 1960, the Massachusetts Department of Public Health (1960) issued a warning that its laboratories had found five of six lots of quadruple vaccine to have unsatisfactory pertussis components. The comprehensive report of Pittman (1962) indicated that approximately 6% of the potency of the pertussis antigen was lost each month. However, the loss was more rapid in samples exposed to the normal variation of market storage conditions than in those held constantly under refrigeration. Edsall et al. (1962) also presented data to show the significant loss of potency by the pertussis component in certain lots of quadruple vaccine. Although the cause of the loss of potency is not known, it seems reasonable to attribute it to the presence of the new preservative, BC, which has replaced the Merthiolate formerly used. The latter was replaced by BC when it was shown that Merthiolate inactivates the polio virus component of the quadruple vaccine.

The present work was predicated on two assumptions: (i) that the BC inactivates the antigen, and (ii) that the inactivation can be prevented. It is known from the work of Kivela, Mallmann, and Churchill (1948) that quarternary ammonium compounds function by attachment to the negatively charged sites on the bacterial cell surface, and, in this adsorption, a denaturation of the cell wall may occur. Mueller and Seeley (1951) reported that the germicidal action of a quarternary ammonium compound such as BC is much decreased in the presence of metallic ions. The degree of interference with the germicidal action is proportional to the valence of the metallic ion. The interfering activity of mono-, di-, and trivalent ions is approximately in the ratio of 1 per monovalent to 100 per divalent, and to 10,000 per trivalent ion. Mueller and Seeley (1951) stated that the results of their studies support the theory that any metallic cation can interfere with the adsorption of quarternary ammonium compounds by competing for the negative sites on the bacterial cell surface. The same authors stated that the higher the valence of the ion, the more strongly it is attached and held to the surface of the organism. The negative charge on the surface of the cell is reduced, thus lowering the attracting power of the cell for the quarternary ammonium compound.

These results suggested to the present authors that it might be possible to saturate the negative sites on the pertussis cell prior to the addition of the preservative, BC, thus preventing its uptake by the cell and thereby stabilizing the antigenic potency of the vaccine. It was with this theory in mind that the present experiments were designed.

MATERIALS AND METHODS

Chemical procedures. BC was detected and identified by its characteristic ultraviolet-absorption spectrum. A Cary model 14 spectrophotom-

eter was used for all ultraviolet-absorption determinations. Absorption maxima were found at 2,745, 2,683, and 2,630 A. Shoulders on the curve appeared at 2,820 and 2,560 A. To measure the BC concentrations quantitatively, a family of curves was obtained at concentrations between 25 and 200 ppm. The eosin yellowish quarternary ammonium dye complex method of Furlong and Elliker (1953) and Miller and Elliker (1959) was used to determine the concentration of unadsorbed BC. This method was modified from a titrimetric into a colorimetric method. When appreciable amounts of cation were present in the solution, the extract of the dye complex was washed with water to remove excess cations and thus allow for color development. Color standards of BC-dye complex containing 5, 10, 15, 20, and 25 μg of BC were prepared and used to determine concentration. The ultraviolet-absorption curves for pertussis vaccine supernatant were determined at pertussis cell concentrations from 15 billion to 480 billion cells per ml. When a small amount of ultraviolet-absorbing material was present, such as in the more dilute vaccines, BC was identified by the ultraviolet-absorption curve and measured by the increase in absorbance at 2,683 A. In vaccines which had appreciable ultraviolet absorption, as in the higher cell concentrations, the BC was measured by the dye complex method.

To determine unadsorbed BC, the pertussis vaccine was filtered through a Millipore filter, and the determination was carried out on the filtrate. A membrane filter of this type was used because ultrafine fritted-glass filters or Seitz filters adsorbed all of the BC from the solution.

Biological procedures. The potency of the pertussis vaccine was determined by the standard National Institutes of Health mouse-protection test developed by Kendrick et al. (1947) and Pittman (1954). White Swiss mice from the Michigan Department of Health colony were used in these tests.

TABLE 1. *Effect of cations on the adsorption of BC (25 PPM) by pertussis cells (15 billion cells per ml)*

Supplement added	Concn of supplement	Unadsorbed BC
	M	*ppm*
None	0	0
Calcium chloride	0.001	12
Calcium chloride	0.002	25
DL-Lysine	0.007	8
Aluminum potassium sulfate	0.0002	25

The pertussis vaccine concentrate employed in these experiments was MDH lot P1509A. The concentrate contained 480 billion cells per ml of *Bordetella pertussis* strain MDH 10536 grown on Bordet-Gengou medium and heat-killed, with agitation at 56 C for 30 min. This particular strain of *B. pertussis* was used because it has been studied intensively, both in our own and other laboratories. Its characteristics, particularly with respect to mouse-protective properties, are well established.

RESULTS

Table 1 indicates the ability of cations to interfere with the adsorption of BC on the pertussis cell. Each cation was added to a portion of vaccine (15 billion cells per ml) and thoroughly mixed prior to the addition of the BC. These experiments showed that the concentration of the cation is important. Calcium chloride present at 0.001 M allows the uptake of approximately 50% of the added BC but, when present at 0.002 M, almost all of the BC remained in the unadsorbed state. DL-Lysine competitively interfered with the adsorption when present at a molarity of 0.007. It is also apparent that aluminum present at a much lower concentration than calcium effectively prevented adsorption. Although not listed, magnesium and choline were also able to prevent adsorption of the preservative.

Based on the results reported in Table 1, a series of vaccines were prepared which contained cations added prior to the addition of the preservative. These vaccines were stored at 0 to 4 C, and their potencies were determined at intervals throughout a 12-month storage period. Table 2 summarizes the changes in mouse-protective units that occurred. The potency of the BC-preserved vaccine without added cations deteriorated from an initial level of 4.86 protective units per ml to approximately 1 protective unit per ml during 52 weeks of storage. The initial level of 4.86 units per ml was obtained by averaging the individual potencies of five vaccines tested at zero time. The values used in obtaining the average are given in Tables 1 and 2. When cations were added to the vaccine prior to BC addition, the potency did not decline significantly during the 52-week storage period.

TABLE 2. *Effect of cations or other additives on the stability of pertussis vaccine, preserved with 25 ppm of BC and stored at 0 to 4 C*

Supplement to BC-treated vaccine	Concn of supplement	Mouse-protective units per ml in vaccine with 10 billion cells per ml*						
		0 weeks†	5 weeks	10 weeks	15 weeks	30 weeks	39 weeks	52 weeks
	M							
Merthiolate‡		4.6	3.9	5.2	1.7	2.7	2.6	1.6
None		4.7	3.7	1.8	1.4	1.9	2.0	0.9
Calcium chloride	0.004	3.6	2.4	—	3.2	2.3	5.6	5.2
Aluminum potassium sulfate	0.0004	4.3	—	—	1.4	6.9	7.5	5.4
Magnesium sulfate·$7H_2O$	0.0008	7.1	—	—	5.1	6.9	3.8	5.0
Choline	0.0008	—	6.6	—	1.2	4.7	5.8	2.7
Calcium chloride	0.002	—	—	—	—	2.1	3.9	4.8
DL-Lysine	0.007							
LD_{50} of challenge (no. of organisms)		160	60	160	ca. 35	250	890	230

* National Institutes of Health Standard Vaccine #6 (potency of 8 units/ml) was used as control vaccine for each determination.

† The average of the five test vaccines was 4.86.

‡ No BC present.

TABLE 3. *Effect of cations or other additives on the stability of pertussis vaccine preserved with 25 ppm of BC and stored at 37 C*

Supplement to BC-treated vaccine	Concn of supplement	Mouse-protective units per ml in vaccine with 10 billion cells per ml*				
		0 weeks†	5 weeks	10 weeks	16 weeks	42 weeks
	M					
Merthiolate‡		4.6	2.1	2.1	—	No protectoin
None		4.7	2.8	.8	No protection	No protection
Calcium chloride	0.004	3.6	3.6	3.0	3.6	3.3
Aluminum potassium sulfate	0.0004	4.3	8.5	3.8	—	3.4
Magnesium sulfate·$7H_2O$	0.0008	7.1	2.8	2.8	1.9	0.9
Choline	0.0008	—	4.4	2.3	—	2.2
LD_{50} of challenge (no. of organisms)		160	60	160	1,050	230 (estimated)

* National Institutes of Health Standard Vaccine #6 (potency of 8 units per ml) was used as control vaccine for each determination.

† The average of five test vaccines was 4.86.

‡ No BC present.

One set of vaccines was stored at 37 C to accelerate the stability test and to subject the vaccines to conditions more severe than any expected market storage conditions. The BC-preserved vaccine used as control was without any measurable protective activity in 16 weeks, whereas the aluminum-protected vaccine retained more than 70% of its activity for 10 months (Table 3). The stability of the calcium- and aluminum-protected vaccines was approximately equivalent; however, a higher concentration of calcium was used. At this temperature, the magnesium-protected vaccine did not withstand the storage as well as did the aluminum and calcium vaccines, but the molar concentration of the magnesium was only one-fifth that of the calcium. It should also be pointed

out that under these conditions the Merthiolate-preserved pertussis vaccine was less stable than any of the protected vaccines.

DISCUSSION

It is realized that the number of vaccines used in this experiment was limited; however, supported by the chemical data, it is felt that these results permit the conclusion that the cations tested are capable of preventing the adsorption of BC onto the pertussis cells, thus stabilizing the protective antigen as measured by the mouse-protection test.

It is hoped that these data can be applied to the stabilization of pertussis vaccines for use in quadruple vaccine. The diphtheria and tetanus toxoid components of quadruple vaccines are normally alum-precipitated; therefore, the aluminum would be a normal component of the vaccine. If aluminum in the form of an aluminum phosphate precipitate added before BC protects the pertussis component to the extent reported for the cations in Table 2, the stability problem of the pertussis component in quadruple vaccine might be solved without the addition of any new substance to the vaccine.

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LITERATURE CITED

EDSALL, G., J. A. MCCOMB, L. H. WETTERLOW, AND J. IPSEN. 1962. Significance of the loss of potency in the pertussis component of certain lots of "quadruple antigen." New Engl. J. Med. **267**:687–689.

FURLONG, T. E., AND P. R. ELLIKER. 1953. An improved method of determining concentrations of quarternary ammonium compounds in water solutions and in milk. J. Dairy Sci. **36**:225–234.

KENDRICK, P. L., G. ELDERING, M. K. DIXON, AND J. MISNER. 1947. Mouse protection tests in study of pertussis vaccine: comparative series using intracerebral route for challenge. Am. J. Public Health **37**:803–810.

KIVELA, E. W., W. L. MALLMANN, AND E. S. CHURCHILL. 1948. Physical action of surface-active cations upon bacteria. J. Bacteriol. **55**: 565–572.

MASSACHUSETTS DEPARTMENT OF PUBLIC HEALTH. 1960. Pertussis immunization. New Engl. J. Med. **263**:410.

MILLER, D. D., AND P. R. ELLIKER. 1959. A test for quarternary ammonium compounds in milk and in detergent sanitizers, p. 188–190. *In* N. A. Malone [ed.], Sanitary practice laboratory manual and notes, part II. Department of Environmental Health, School of Public Health, The University of Michigan, Ann Arbor.

MUELLER, W. S., AND D. B. SEELEY. 1951. Effect of metallic ions on germicidal activity of quarternary ammonium germicides. Soap Sanit. Chemicals **27**:131–143.

PITTMAN, M. 1954. Variability of potency of pertussis vaccine in relation to number of bacteria. J. Pediat. **46**:57–69.

PITTMAN, M. 1962. Instability of pertussis vaccine component in quadruple antigen vaccine. J. Am. Med. Assoc. **181**:25–30.