Preservatives for Poliomyelitis (Salk) Vaccine II

Formaldehyde and Esters of *p*-Hydroxybenzoic Acid

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During the manufacture of poliomyelitis vaccine, 92 p.p.m. of formaldehyde was added to inactivate the virus. After 2 years at 4°, 70–80 p.p.m. was still present. If not neutralized by bisulfite, this concentration of formaldehyde was a potent antibacterial agent, but had little antimycotic activity. The addition of toxoids and pertussis vaccine to poliomyelitis vaccine to form DPT polio caused no immediate loss of formaldehyde but after about 10 months at 4° and 25° there were losses of about 50 and 80 per cent, respectively. The loss of 50 per cent of the formaldehyde decreased the preservative activity. The methyl and propyl parabens in a ratio of 10:1 and a final concentration of 0.165 per cent were only moderately antibacterial, but were effective antifungal agents. The addition of parabens to vaccine containing nonneutralized formaldehyde gave a mixture of preservatives which was inhibitory against high concentrations of both bacterial and fungal contaminants. Parabens also prevented loss of formaldehyde from DPT polio vaccine.

A PREVIOUS STUDY (1) showed that benzethonium chloride possessed limited usefulness as a preservative for inactivated poliomyelitis vaccine (Salk). However, the antibiotics and formaldehyde added to the vaccine during the manufacturing process possessed considerable antibacterial activity. The antibiotics are added to the tissue culture used for proliferation of the poliomyelitis virus to prevent the growth of bacterial contaminants; in the finished vaccine 135 p.p.m. of streptomycin and 10 p.p.m. of neomycin were found. Formalin is added to a concentration of 1:4000 (92 p.p.m. of formaldehyde) to inactivate the virus. The residual formaldehyde (70–80 p.p.m.) may be neutralized with bisulfite or it may be left without neutralization. If not neutralized, the formaldehyde serves as an antibacterial agent.

Neither the antibiotics nor the formaldehyde, at the concentration used in poliomyelitis vaccine, possess marked antimycotic activity. However, the esters of *p*-hydroxybenzoic acid (parabens) have excellent antimycotic activity (2, 3) and do not destroy poliomyelitis antigen (4). Because of the possibility of complementary activity of antibiotics, formaldehyde, and parabens against both bacteria and fungi, we studied mixtures of these materials in poliomyelitis vaccine. This report presents the results of the study.

EXPERIMENTAL

Poliomyelitis vaccine with and without neutralization of formaldehyde by bisulfite, the combination of pertussis vaccine, toxoids, and poliomyelitis vaccine (DPT polio vaccine), HB597 medium, and the microbial cultures have been described previously (1).

Antibiotics, added to the tissue cultures used for growing the poliomyelitis virus, were present in the finished vaccine (1). To obtain comparable concentrations of antibiotics in HB597 medium, we added 140 p.p.m. of streptomycin and 2 p.p.m. of neomycin.

Cultures used for challenge were four strains of *Pseudomonas aeruginosa*, four unidentified *Pseudomonas* species, a *Proteus* species, *Shigella flexneri*, *Salmonella paratyphi C*, two strains of *Staphylococcus aureus*, *Saccharomyces ellipsoideus*, *Debaromyces kloeckeri*, *S. rouxii*, *Rhodotorula glutinis*, *Rhizopus oryzae*, *Fusarium lini*, and *Circinella spinosa*. Preparation of cultures, numbers of cells used in the challenge, and criteria for growth in the challenged preparations have been described (1).

Formaldehyde was measured by the method of Nash (5) and a modification of this method (6). The former measures total formaldehyde, *i.e.*, free formaldehyde plus that which is combined with bisulfite, while the latter method measures only free formaldehyde.

Esters of *p*-hydroxybenzoic acid (parabens) were added as a 29% w/v ethanolic solution to obtain a mixture containing 1.5 mg. of the methyl ester and 0.15 mg. of the propyl ester per milliliter. Higher concentrations crystallized when the vaccine was stored at 4°. Parabens were assayed by the method of Jones, *et al.* (7).

RESULTS

The antimicrobial activity of formaldehyde and antibiotics was tested in HB597 medium. This medium is a component of the tissue culture used for growing poliomyelitis virus and comprises most of the volume of the finished poliomyelitis vaccine.

Table I shows that HB597 medium supported growth of all bacteria, even when the challenge dose was diluted to 10^{-6} (approximately 100 viable cells per challenge), but the medium was less suitable for growth of small inocula of yeasts and molds. The addition of 46 p.p.m. of formaldehyde to HB597 medium inhibited the growth of bacteria, but did not affect yeasts and molds. Increasing the form-

Received September 6, 1963, from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, Canada.
Accepted for publication September 20, 1963.
The authors acknowledge the technical assistance of Mr. A. von Seefried.

TABLE I.—PRESERVATIVE EFFECT OF FORMALDEHYDE AND ANTIBIOTICS IN HB597 MEDIUM

Formaldehyde Added, p.p.m.	Dilution[a] of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged — Antibiotics Absent: Bacteria	Antibiotics Absent: Yeasts and Molds	Antibiotics Present: Bacteria	Antibiotics Present: Yeasts and Molds
0	None	13/13	7/7	11/13	7/7
	10^{-3}	13/13	6/7	6/13	6/7
	10^{-6}	13/13	2/7	5/13	4/7
46	None	7/13	7/7	3/13	7/7
	10^{-3}	1/13	6/7	1/13	6/7
	10^{-6}	0/13	4/7	0/13	2/7
92	None	2/13	6/7	1/13	6/7
	10^{-3}	1/13	3/7	0/13	2/7
	10^{-6}	0/13	2/7	0/13	1/7

[a] Undiluted challenge cultures furnished challenge doses of approximately 1×10^8 bacterial cells or 1×10^7 yeast or mold cells.

aldehyde to 92 p.p.m. gave an increased antibacterial activity and possibly some antimycotic activity. The addition of antibiotics to HB597 medium furnished moderate antibacterial activity but no antimycotic activity. Mixtures of antibiotics and formaldehyde possessed greater antibacterial activity than either preservative by itself, but the antimycotic activity of the mixture appears to be due only to the formaldehyde.

Table II shows that the residual formaldehyde in poliomyelitis vaccine contributed considerable antibacterial activity and that neutralization of formaldehyde by bisulfite destroyed this antibacterial activity. The loss of antibacterial activity resulting from neutralization of the formaldehyde confirmed the work of Taylor and Moloney (6), who showed that the Nash method of analysis for formaldehyde neutralized by bisulfite was not a true analysis of the free formaldehyde, whereas their modification of the assay measured free formaldehyde.

Table III shows that formaldehyde in poliomyelitis vaccine was stable for 2 years and that the addition of pertussis vaccine and toxoids to form DPT polio vaccine caused no immediate loss of formaldehyde. As will be shown later, however, prolonged storage of DPT polio vaccine may result in loss of formaldehyde.

The failure of formaldehyde to inhibit yeasts and molds showed that some antimycotic compound should be added. Table IV shows that parabens possessed a marked antifungal activity in HB597 medium but only moderate antibacterial activity. The mixture of parabens with formaldehyde, how-

TABLE II.—EFFECT OF NEUTRALIZING FORMALDEHYDE IN POLIOMYELITIS VACCINE[a] BY ADDITION OF BISULFITE

Vaccine Tested	Formaldehyde, p.p.m. Nash	Formaldehyde, p.p.m. Modified Nash	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
Poliomyelitis vaccine (Lot 155)	82	73	None	2/13	6/7
			10^{-3}	0/13	2/7
			10^{-6}	0/13	1/7
Poliomyelitis vaccine (Lot 155) with bisulfite	63	8	None	11/13	7/7
			10^{-3}	3/13	5/7
			10^{-6}	0.13	6/7

[a] Antibiotics present.

TABLE III.—STABILITY OF THE ANTIBACTERIAL ACTIVITY OF FORMALDEHYDE IN POLIOMYELITIS VACCINE

Prepn. Tested	Storage Period at 4° C.	Formaldehyde, p.p.m. Nash	Formaldehyde, p.p.m. Modified Nash	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
HB597[a]	0	4	4	None	13/13	7/7
				10^{-3}	13/13	6/7
				10^{-6}	13/13	5/7
Poliomyelitis[b] vaccine	0	82	73	None	2/13	6/7
				10^{-3}	0/13	2/7
				10^{-6}	0/13	1/7
Poliomyelitis[b] vaccine	1 yr.	69	62	None	2/13	6/7
				10^{-3}	0/13	4/7
				10^{-6}	0/13	1/7
Poliomyelitis[b] vaccine	2 yr.	76	70	None	1/13	6/7
				10^{-3}	0/13	4/7
				10^{-6}	0/13	0/7
DPT[b] polio vaccine	7 days	76	76	None	3/13	6/7
				10^{-3}	0/13	5/7
				10^{-6}	0/13	3/7

[a] Formaldehyde and antibiotics absent. [b] Formaldehyde not neutralized; antibiotics present.

TABLE IV.—ANTIMICROBIAL EFFECT OF PARABENS MIXED WITH FORMALDEHYDE IN HB597 MEDIUM

Parabens, %	Formaldehyde, p.p.m. Nash	Formaldehyde, p.p.m. Modified Nash	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
0	4	8	None	13/13	7/7
			10^{-3}	13/13	5/7
			10^{-6}	13/13	5/7
0	52	53	None	10/13	7/7
			10^{-3}	4/13	4/7
			10^{-6}	0/13	3/7
0.165	4	8	None	7/13	3/7
			10^{-3}	5/13[a]	0/7
			10^{-6}	3/13[b]	0/7
0.165	50	54	None	0/13	1/7
			10^{-3}	0/13	0/7
			10^{-6}	0/13	0/7

[a] 4/5 cultures which grew were *Ps. aeruginosa*; 1/5 was *S. aureus*. [b] 2/3 cultures which grew were *Ps. aeruginosa*; 1/3 was *S. aureus*.

ever, inhibited growth of all bacteria and six of seven yeasts and molds when the undiluted inoculum was used for challenge.

Table V confirms that formaldehyde with the antibiotics is an excellent antibacterial mixture, even in DPT polio, but that the addition of parabens is necessary for antifungal activity.

The stability of preservatives in DPT polio vaccine was studied. Table VI shows that there was no immediate loss of parabens when poliomyelitis vaccine was mixed with other antigens to form DPT polio but within 7 months there was a decrease from 0.165 to 0.14%. After 11 months' storage at 4°, there was a further decrease to 0.12% (Table VII). This loss of parabens did not result in a significant decrease in antimicrobial activity but did cause a drop in pH, shown by the phenol red indicator present in the vaccine.

Table VII shows that the stability of formaldehyde in DPT polio vaccine during storage for 9 to 11 months depended on both temperature and the presence of parabens. In the absence of parabens, about 50 and 85% of the formaldehyde was lost when temperatures of storage were 4° and 25°, respectively. This resulted in a marked loss of antibacterial activity. In the presence of parabens, formaldehyde was stabilized and retained its antibacterial activity.

Studies similar to those reported in Table VII were done by adding 25 p.p.m. of benzethonium chloride (BEC) to DPT polio vaccine. The BEC was reduced to 3–4 p.p.m. by the addition of pertussis vaccine (1), and this residuum had no effect on the stability of the formaldehyde.

TABLE V.—ANTIMICROBIAL ACTIVITY OF PARABENS IN DPT POLIO VACCINE

Prepn. Tested	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
HB597[a]	None	13/13	7/7
	10^{-3}	13/13	6/7
	10^{-6}	13/13	4/7
DPT[b] polio vaccine	None	1/13	6/7
	10^{-3}	0/13	6/7
	10^{-6}	0/13	3/7
DPT polio vaccine with 0.165% parabens	None	1/13	3/7
	10^{-3}	0/13	0/7
	10^{-6}	0/13	0/7

[a] Formaldehyde and antibiotics absent. [b] Formaldehyde not neutralized; antibiotics present.

DISCUSSION

The most important requirements for preservatives in vaccines are activity against diverse microorganisms, compatibility with the vaccine, stability, and lack of toxicity.

Antimicrobial activity is necessary to prevent the growth of microorganisms which may be introduced when samples are withdrawn from multiple-dose containers (1, 8–10). The antimicrobial agent should prevent the growth of large numbers of a wide variety of bacteria, yeasts, and molds. Preferably, it should destroy them quickly, although failure to act quickly should not invalidate an otherwise useful compound.

Compatibility of the preservative with the vaccine may be considered from several standpoints. The preservative must not lower the potency of the vaccine below permissible levels; it should not form a precipitate; and it should not be inactivated by the vaccine. The compatibility must be maintained for periods of time and under conditions of storage and transportation which would not adversely affect the antigen if the preservative were not added.

Adequate stability of the preservative during the shelf life of the vaccine is essential. However, if breakdown occurs, it should not result in a marked decrease of the antimicrobial activity, visible changes, or destruction of the antigen. In addition, the preservative should not precipitate in the cold or be absorbed by the rubber stopper.

These numerous and exacting requirements make it unlikely that a universal preservative will be found which will be suitable for all vaccines. In-

TABLE VI.—PARABEN CONTENT OF POLIOMYELITIS AND DPT POLIO VACCINES

Poliomyelitis Vaccine: Lot No.	Paraben content, %	DPT Polio Vaccine: Lot No.[a]	Paraben Content, %
1	0.15	1A	0.16
2	0.16	2A	0.16
3	0.17	3A	0.16
4	0.17	4A	0.17
5	0.17	5A	0.17
6	0.17	6A	0.14

[a] Lots 1A–5A were analyzed within 2 weeks, and Lot 6A was analyzed 7 months after preparation.

TABLE VII.—RECOVERY OF PRESERVATIVES IN DPT POLIO VACCINE AFTER PROLONGED STORAGE

Vaccine No.	Preservatives Added	Preservatives Recovered—Formaldehyde, p.p.m.	Preservatives Recovered—Parabens, %	Tubes with Growth / Tubes Challenged B[c]	Tubes with Growth / Tubes Challenged Y and M[d]
		11 Mo. at 4° C.			
486	Form[a]	25		9/13	4/6
467	Form	45			
468	Form	40			
484	Form + *para*[b]	71	0.13	0/13	1/6
488	Form + *para*	71	0.12	1/13	0/6
		9[f] Mo. at 25° C.			
486	Form	11		11/13	6/6
484	Form + *para*	67	0.10	1/13	1/6
488	Form + *para*	64	0.10	1/13	0/6
HB597	None[e]			13/13	6/6

[a] 92 p.p.m. formaldehyde. [b] 92 p.p.m. formaldehyde and 0.165% parabens. [c] Bacteria—10^{-3} dilution of inoculum used for challenge. [d] Yeasts and molds—10^{-1} dilution of inoculum used for challenge. [e] Control on ability of inoculum to initiate growth in HB597 medium. [f] Stored 1–2 months at 4° C. and then 9 ± 1 month at 25° C.

deed, it is difficult to find a single preservative which fulfills all requirements for a single vaccine.

Falk and Applington (11) recognized the deficiencies of a single preservative for vaccines and showed that mixtures of thimerosal with phenol were superior to either compound alone. We also have shown that mixtures of preservatives are useful in poliomyelitis vaccine. The mixtures we have used include the two antibiotics, formaldehyde and methyl and propyl esters of *p*-hydroxybenzoic acid.

The antibiotics added to the tissue culture to prevent bacterial contamination during manufacture have considerable stability (1, 12). The mixture of streptomycin and neomycin has the advantages of a wide spectrum of antibacterial activity, low toxicity, and apparent compatibility with the antigen. The antibiotics fail, however, to control heavy contamination with most bacteria or even light contamination with fungi and some strains of *Ps. aeruginosa*.

Formaldehyde, which may also be present in poliomyelitis vaccine (13–16) has excellent antibacterial activity against most bacteria and is especially important because of its activity toward *Ps. aeruginosa* (17). This species is one of the most difficult organisms to inhibit (18–20), and even a few cells of some strains can initiate growth in the presence of both the antibiotics and *p*-hydroxybenzoate esters at the concentrations used in this investigation. The excellent stability of formaldehyde in poliomyelitis vaccine, its compatibility with other antigens, its ready solubility, and its lack of toxicity at levels which are adequate for antibacterial activity make it a useful preservative. Formaldehyde has been used for the preservation of solutions of penicillin and streptomycin (21) and suspensions of procaine penicillin (22). Schmidt-Lorenz has recently reviewed the use of formaldehyde as a preservative (23).

The concentration of formaldehyde in poliomyelitis vaccine may vary considerably. Although formalin is added to a concentration of 1:4000 (92 p.p.m.) to inactivate the virus, the residual formaldehyde may be neutralized by bisulfite (16) or it may be left without neutralization. If left without neutralization, we have found 60–80 p.p.m. Other workers have found more (13, 14). It is permissible to inactivate the virus for vaccine without using formaldehyde (16), and some manufacturers use β-propiolactone for inactivation.

The antimicrobial activity of the parabens has been described in numerous publications; a comprehensive review is available (3). The main advantages of the parabens are their demonstrated ability to inhibit fungi, their lack of harm to antigens and, in DPT polio vaccine, their stabilizing effect on formaldehyde. Their disadvantages are their low solubility at temperatures at which vaccines are usually stored (4–10°), their moderate antibacterial activity, especially against *Ps. aeruginosa* (18, 19), and a lowering of pH when vaccines containing parabens are stored.

The stability of formaldehyde in poliomyelitis vaccine and its instability in DPT polio vaccine during prolonged storage is unexplained. Also unexplained is the ability of parabens to stabilize the formaldehyde in DPT polio vaccine. Any hypothesis concerning these phenomena should take into account the following observations: (*a*) formaldehyde is stable in DPT polio vaccine for at least 7 days after the poliomyelitis vaccine is mixed with the toxoids and pertussis; (*b*) pertussis vaccine alone or mixed with toxoids is far more stable than pertussis vaccine mixed with polio vaccine (24); and (*c*) the addition of Versene to DPT poliomyelitis vaccine decreases degradation of pertussis (25). It is possible that the instability of formaldehyde and pertussis vaccine in DPT polio vaccine are related.

One hypothesis concerning the instability of formaldehyde and pertussis vaccine is that poliomyelitis vaccine contains proteolytic enzymes which slowly break down pertussis cells. This breakdown produces sites which react with formaldehyde. Unfortunately, this and several other hypotheses do not accommodate all of the known observations and additional work must be done.

The value of a preservative in a vaccine can only be established when adequate tests have been conducted concerning not only its antimicrobial activity but also its compatibility with the antigens which it preserves. Such tests have been conducted by various departments in these laboratories. To date, there has been no contraindication concerning the use of the antibiotics, formaldehyde, and parabens in the concentrations mentioned.

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Quantitative X-Ray Diffraction Analysis of Intact Tablets

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An investigation into the possible use of X-ray diffraction techniques to analyze intact tablets was made. The analysis of various pharmaceutical compounds in tablet form by X-ray diffraction was considered. Of those tested, only in glutethimide tablets, where the percentage of active component weight in total formulation weight is high, was the analysis of the intact tablet feasible. Methods of improving the reproducibility of this intact tablet analysis were considered. A procedure which makes it possible to assay 10 individual glutethimide tablets in 25 minutes was developed.

THE DEVELOPMENT of rapid analysis of unit dose formulations is a problem which has only recently come into the forefront. In 1961, Head (1) investigated the possible use of solid state fluorescence for the analysis of individual intact tablets and demonstrated that fluorescence was not totally acceptable for this purpose. The author did, however, suggest that X-ray diffraction techniques might possibly be more useful.

Taking advantage of this suggestion, an investigation into quantitative X-ray diffraction was initiated in this laboratory with the view that it might be possible to develop this technique to assay individual intact tablets both rapidly and automatically. It was felt that this investigation could uncover the answers to the following questions: What adaptations and precautions are necessary to obtain acceptable reproducibility in the analysis of an intact tablet by X-ray diffraction techniques? What concentration of a drug within the normal tablet matrix can be detected by these techniques? What precision can be obtained and how rapid can an analysis be run on individual intact tablets?

The theory of quantitative X-ray diffraction analysis is authoritatively considered by Klug and Alexander (2), while a brief but lucid account has recently been given by Shell (3). Consequently, a treatment of the underlying theory will not be considered here. However, it must be noted that the attainment of high precision in intensity measurements of diffracted X-rays demands careful attention to a number of factors, even when one is dealing with a carefully packed powdered sample. These factors—the particle size of the crystallite, preferred orientation, etc.—were considered in this work but could not be dwelled upon because of the nature of the sample. It is understood that an attempt at doing quantitative X-ray diffraction work without careful precautionary sample preparation is unusual and may be viewed with distress by a purist.

The work that is reported here is essentially a two-part study. The first part consists of an X-ray examination of various representative tablet formulations to determine what tablets might be assayed by this technique. The second part is a report on the analysis of glutethi-

Received May 16, 1963, from the Research Department, Ciba Pharmaceutical Co., Summit, N. J.
Accepted for publication, September 6, 1963.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.