It appears that two double bonds are present in the sterol, although unequivocal evidence must await further data. One double bond is at position 5(6)-; rotational differences between the new sterol and its acetate and benzoate coincide with established differences for Δ^5-stenols (Table III). This is substantiated by the similarity of the infrared spectrum to β-sitosterol and the rate of the Liebermann-Burchard reaction. The exact position of the second double bond cannot be ascertained with the available data, which were limited by the supply of sterol on hand. However, nuclear positions 7(8)-, 8(9)-, 8(14)-, and 9(11)- can be ruled out because of the rotational values in Table III and because of the negative Tortelli-Jaffe reaction (Reference 5, p. 101). Positions 7(8)- and 3(4)- can be eliminated since no conjugation was indicated in the ultraviolet spectrum, and position 11 can be eliminated because of the lack of identity with the $\Delta^{5,(11^7)}$-stigmastadienol of Idler *et al.* (6). Hydrogenation in neutral medium converted the sterol to a compound (unidentified) that was not Δ^5-stigmastenol (β-sitosterol). Therefore, the second (presumed) double bond must have been more resistant to hydrogenation than Δ^5. This eliminates the side chain positions 22(23)-, 24(25)-, or 24(28)- (6). It also eliminates position 14(15)- (7). With these eliminations ring A, positions 1(2)- or 2(3)- become possibilities, since some "vicinal" effect (Reference 5, p. 209) is indicated by the M_D for both the free sterol and acetate (Table III). The significance of the peak at 14.3 μ in the infrared shows promise for later identification; at the moment its significance is unknown.

The other substance new to *M. vulgare*, a strongly polar sesquiterpene which probably contains two nonconjugated double bonds, was not isolated from the crude extracts but was readily isolated from nonsaponifiable fractions of extracts of the plant. Neither the sterol nor the sesquiterpene has yet been equated with known substances; we are describing them as "unidentified," pending further experimentation.

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Preservatives for Poliomyelitis (Salk) Vaccine III

2-Phenoxyethanol

By HILLIARD PIVNICK, J. M. TRACY, A. L. TOSONI, and D. G. GLASS

Poliomyelitis vaccine contains antibiotics, but the antibiotics are inadequate for preventing the growth of heavy contamination with bacteria or light contamination with fungi. The addition of 0.375 per cent v/v of 2-phenoxyethanol to poliomyelitis vaccine furnished a stable mixture of preservatives (streptomycin, neomycin, and 2-phenoxyethanol) which was inhibitory to both bacteria and fungi. This mixture was also effective when poliomyelitis vaccine was mixed with diphtheria and tetanus toxoids and pertussis vaccine.

VACCINE packaged in multiple-dose vials must contain a preservative to prevent the growth of microbial contaminants which may be introduced when samples are withdrawn. In studies of preservation of poliomyelitis vaccine and DPT polio vaccine (1, 2) we showed that antibiotics, benzethonium chloride, formaldehyde, and esters of *p*-hydroxybenzoic acid (parabens) alone, or in various combinations, had deficiencies as preservatives.

The present report concerns 2-phenoxyethanol[1] (ethylene glycol monophenyl ether) (2-POE) as a preservative for poliomyelitis vaccine. This compound was chosen for study because of its activity against *Pseudomonas aeruginosa* (3–6) a potential pathogen which may grow in vaccines and other medicinals (7–9).

MATERIALS AND METHODS

2-Phenoxyethanol was sterilized by Seitz filtration, added to vaccines or media and the mixture was stirred slowly for a few hours to effect solution.

A method of assay for 2-POE in poliomyelitis

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[1] Marketed as Phenoxetol by Nipa Laboratories Ltd., Treforest Industrial Estate, near Cardiff, Wales, U.K.

TABLE I.—ANTIBACTERIAL ACTIVITY OF 2-PHENOXYETHANOL IN HB597 MEDIUM[a]

Challenge Culture	No. of Culture	2-Phenoxyethanol, % 0	0.5	0.75	1.0
Pseudomonas species	C-1	+[b]	—	—	—
Pseudomonas species	C-2	+	—	—	—
Pseudomonas species	C-3	+	—	—	—
Pseudomonas species	C-5	+	—	—	—
Pseudomonas aeruginosa	C-4	+	—	—	—
Pseudomonas aeruginosa	C-6	+	+	—	—
Pseudomonas aeruginosa	C-13	+	+	—	—
Pseudomonas aeruginosa	C-16	+	+	+	—
Shigella flexneri	C-8	+	+	—	—
Proteus species	C-9	+	+	—	—
Salmonella paratyphi C	C-12	+	+	—	—
Staphylococcus aureus	C-10	+	+	—	—
Staphylococcus aureus	C-11	+	+	+	—

[a] No antibiotics present. [b] + = growth within 60 days at room temperature; — = no growth.

vaccine was developed. A standard curve of 2-POE, distilled in a fractionating column, was prepared. The relation between the absorbance at 271 mμ in reagent grade ethylene chloride compared to a solvent blank was found to be linear in the range from 0 to 0.005% v/v. To assay poliomyelitis vaccine, a suitable volume of sample was extracted with an equal volume of ethylene chloride by thoroughly shaking in a separator for 1 minute. The ethylene chloride extract was diluted 1:100 with ethylene chloride and the absorbance read on a Beckman DK-2 spectrophotometer at 271 mμ. Centrifugation was usually necessary to obtain clear extracts when working with DPT polio vaccine. Poliomyelitis vaccine which did not contain 2-POE gave no absorbance and it was found that one extraction gave essentially quantitative recovery.

All other materials and methods have been previously described (1, 2).

RESULTS

All inoculated media and vaccines were incubated at room temperature, unless otherwise stated, and examined after 4, 11, and 60 days of incubation. When HB597 medium without preservatives was inoculated, growth was visible within 4 days. When medium or vaccine containing preservatives was inoculated, only about two-thirds of all tubes which eventually contained visible growth did so by the fourth day. Most of the remainder were positive by the eleventh day. Subcultures were usually done on the eleventh day after inoculation and about 5–10% of those tubes which did not show macroscopic evidence of growth contained viable cells. Rarely did these viable cells proliferate sufficiently to give macroscopic evidence of growth within 60 days after inoculation.

Table I shows that all 13 bacterial challenge cultures grew when approximately 25×10^6 cells were added to each milliliter of HB597 medium. This medium with added antibiotics is used in monkey kidney tissue culture for growing poliomyelitis virus and is a major part of the finished vaccine. This table also shows that 0.75% v/v of 2-POE did not inhibit growth of all the cultures but 1% did.

Table II shows the effect of challenging, with three concentrations of 12 different bacterial cultures, poliomyelitis vaccine containing 2-POE. Poliomyelitis vaccine is mainly HB597 medium with killed virus and antibiotics. This vaccine contained 135 p.p.m. of streptomycin and 2 p.p.m. of neomycin (1). The vaccine itself had considerable antibacterial activity and this was greatly increased by 0.375% but not by 0.25% of 2-POE. A concentration of 0.5% of 2-POE gave the same results as 0.375% but 0.75% prevented growth of all cultures. For further work we used 0.375% v/v of 2-POE.

The preservative activity of the mixture of antibiotics and 2-POE was stable. Vaccine containing 0.375% of 2-POE was challenged with the 13 bacterial cultures using the three concentrations of cells previously employed. After 60 days at room temperature those tubes in which growth was not evident were rechallenged with freshly prepared inoculum. As a result of the first challenge, growth occurred in 3 of the 39 tubes. When the tubes without growth were rechallenged 60 days later, growth occurred in only one.

2-Phenoxyethanol was effective as a preservative when challenged poliomyelitis vaccine was

TABLE II.—ANTIBACTERIAL ACTIVITY OF 2-PHENOXYETHANOL IN POLIOMYELITIS VACCINE[a]

2-Phenoxyethanol, %	Approximate Number of Cells per ml. of Vaccine	No. of Tubes with Growth / No. of Tubes Challenged	
0	25×10^5	12/12	
	25×10^3	4/12	(C1, C2, C3,
	25	0/12	C13)[b]
0.25	25×10^5	9/12	
	25×10^3	5/12	(C2, C3, C4, C6,
	25	0/12	C13)
0.375	25×10^5	3/12	(C4, C6, C13)
	25×10^3	0/12	
	25	0/12	

[a] Formaldehyde neutralized. [b] Numbers in brackets refer to cultures listed in Table I and identify the cultures which grew.

TABLE III.—PRESERVATIVE EFFECT OF 2-*para*-CHLOROPHENOXYETHANOL[a] AND 2-PHENOXY-1-METHYLETHANOL[b] IN POLIOMYELITIS VACCINE

Vaccine	Approximate Number of Cells per ml. of Vaccine	No. of Tubes with Growth / No. of Tubes Challenged
Poliomyelitis	25×10^5	12/13
	25×10^3	8/13
	25	8/13
Poliomyelitis + 0.2% of 2-*para*-[a]	25×10^5	0/13
	25×10^3	0/13
	25	0/13
Poliomyelitis + 0.3% of 2-phenoxy-[b]	25×10^5	10/13
	25×10^3	8/13
	25	6/13

TABLE IV.—PRESERVATIVE ACTIVITY OF 2-PHENOXYETHANOL IN VACCINE CONTAINING NONNEUTRALIZED FORMALDEHYDE

Preparation Tested	Bacteria: Approximate Number of Cells per ml. of Vaccine	Bacteria: Growth[b] Challenged	Yeasts and Molds[a]: Approximate Number of Cells per ml.	Yeasts and Molds[a]: Growth Challenged
HB597	25 × 10^6	13/13	2.5 × 10^6	7/7
	25 × 10^3	13/13	2.5 × 10^3	6/7
	25	13/13	2.5	4/7
DPT polio[c] vaccine	25 × 10^6	1/13	2.5 × 10^6	6/7
	25 × 10^3	0/13	2.5 × 10^3	6/7
	25	0/13	2.5	3/7 (C28, C33, C35[d])
DPT polio vaccine + 0.375% of 2-phenoxyethanol	25 × 10^6	0/13	2.5 × 10^6	3/7 (C28, C33, C35)
	25 × 10^3	0/13	2.5 × 10^3	0/7
	25	0/13	2.5	0/7

[a] C27—*Saccharomyces ellipsoideus;* C28—*Debaromyces kloeckeri;* C29—*Saccharomyces rouxii;* C30—*Rhodotorula glutinis,* C32—*Rhizopus oryzae;* C33—*Fusarium lini;* C35—*Circinella spinosa.* [b] No. of cultures with growth/No. of cultures used for challenge. [c] DPT polio vaccine was made by combining 100 volumes of poliomyelitis vaccine with 4 volumes of a mixture of diphtheria and tetanus toxoids and pertussis vaccine. [d] Bracketed figures identify cultures which grew.

stored at 4°. When the vaccine without 2-POE was challenged with 12 different bacterial cultures, 8 of the 12 grew within 10 weeks at 4° but when the vaccine contained 0.375% of 2-POE, none grew within 15 weeks.

Two other compounds similar to 2-POE were also tested briefly as preservatives in poliomyelitis vaccine in which the formaldehyde had been neutralized. These were *2-para*-chlorophenoxyethanol (Para-phenoxetol) and 2-phenoxy-1-methylethanol (Propylene phenoxetol). Table III shows that the former, at a concentration of 0.2% v/v had excellent antibacterial activity while the latter was useless at 0.3% v/v. The high antibacterial activity of 2-*para*-chlorophenoxyethanol indicates that further study of this material may be rewarding.

Formaldehyde may be added to poliomyelitis virus to inactivate the virus in order that it may be used for Salk vaccine. The residual formaldehyde (70–80 p.p.m.) may be left or it may be neutralized by bisulfite (2). If not neutralized the formaldehyde supplements the antibacterial activity of the antibiotics in the vaccine but has little antimycotic activity (2). Table IV shows that 2-POE provided antifungal activity for DPT polio vaccine which contained nonneutralized formaldehyde and already posessed adequate antibacterial activity. This table also gives evidence that the 2-POE was not inactivated by the toxoids and pertussis vaccine in DPT polio vaccine or by the formaldehyde.

Studies were done to determine the stability of 2-POE in DPT polio vaccine stored for 11 months at 4°. During this time the 2-POE decreased from 0.375 to 0.33% but the antimicrobial activity of the vaccine was not diminished.

DISCUSSION

2-Phenoxyethanol has not been used widely as a preservative for biologicals and would not be useful by itself unless about 1.0% were added. Its solubility at 20° in water is only 2.4%. As part of a mixture of preservatives in poliomyelitis and DPT polio vaccine it does, however, appear to be useful. It supplements the antibacterial activity of the antibiotics, provides adequate antifungal activity, and has excellent stability. Moreover, it is not inactivated by components of DPT polio vaccine nor, unlike parabens, does it cause a change of pH in the vaccine during prolonged storage. Besides its preservative activity, 2-POE has other necessary attributes of a preservative for vaccines—it is nontoxic and it does not destroy antigenicity of any of the components of DPT polio vaccine when used at a concentration of 0.375% and stored at 4° (10). Thus, 2-POE appears to be a satisfactory preservative for this vaccine.

2-Phenoxyethanol may have further application as a preservative for biologicals which contain high concentrations of proteins. Berry (3) has stated that the bacteriostatic concentration is not greatly increased by 20% of serum. It must be emphasized, however, that the bacteriostatic concentrations are about 0.8% for *S. aureus.* We believe that the best method of using 2-POE is in conjunction with stable antibiotics such as streptomycin and neomycin, in which mixtures it may be satisfactory for a variety of products.

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