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Volatile Preservatives for Culture Media.

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During investigations of microbial nutrition centering about growth factors and trace elements, contaminants were repeatedly detected growing in stock solutions of many cp inorganic and organic compounds even when the solutions had been treated with toluene and chloroform, and stored at 6°C. The need for more effective preservatives motivated the study herein reported.

The use of volatile antiseptics is of long standing, especially in work with enzymes. Yet few comparative studies in this field have been described. The common alkyl halide solvents were the compounds tested in some typical studies.[1,2] Propylene oxide and ethylene oxide were recently proposed for sterilizing culture media not to be subjected to heating.[3]

As the solutions to be protected were for use in compounding culture media sterilized by steam, completeness of volatilization of preservative was essential. For the past year, the addition of a mixture of *o*-fluorotoluene, *n*-butyl chloride and 1,2-dichloroethane, has enabled us to store putrescible solutions more or less indefinitely at refrigerator temperatures, and also at room temperature. A description of the development of this preservative may be helpful to others confronted with a similar problem, and may indicate some interesting possibilities in the field of volatile antiseptics.

Materials and Methods. a. *Compounds.* The following considerations guided selection of compounds to be tested:

1. Boiling point $< 121°C$.

2. Immiscibility with water to ensure removal by steam distillation on autoclaving.

3. Satisfactory chemical stability in contact with water at room and sterilization temperature.

4. Low reactivity towards most compounds used in culture media. Where some decomposition might occur, the products to be nutritionally inert.

5. Moderate cost and ready availability.

At the beginning of this investigation a specific gravity near 1.0, to allow a more stable dispersion in dilute aqueous media, was considered a desideratum, but later experiments indicated advantages in blends of light and heavy compounds, and hence less importance was attached to this property. Compounds boiling at or below room temperature were too easily lost from opened containers. Hydrocarbons were excluded because they were too easily metabolized by microorganisms.[4] Nevertheless because the use of toluene was traditional in enzyme studies, some preliminary experiments with it were conducted. It proved very inefficient: toluene-saturated broth allowed growth of *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Streptococcus faecalis,* and *Torula utilis;* however it did check more or less completely *Bacillus cereus* and *Mycobacterium lacticola.* It was decided to concentrate attention on alkyl and aryl halides because these appeared best to fulfill the above listed requirements and they already had shown some effectiveness. Few tests were conducted with compounds with allylic halogen atoms because of their instability. Some sulfur-containing compounds were investigated despite their instability and unpleasant odors. Nearly all compounds tested were redistilled in an all-glass apparatus. "Practical" grade chemicals were distilled over a 3° range; nearly all other chemicals boiled over a much narrower range, agreeing well with the constants in the litera-

1 Joachimoglu, G., *Biochem. Z.*, 1921, **124**, 130.

2 Lihnell, D., *Arch. Mikrobiol.*, 1935, **6**, 326.

3 Hansen, H. N., and Snyder, W. C., *Phytopath.*, 1947, **37**, 369.

4 Zobell, C. E., *Bact. Revs.*, 1946, **10**, 1.

TABLE I.
Compounds Tested.

Toluene	1-bromo-2-methyl-propane (*iso*-butyl bromide)
Diethyl ether	4-bromo-2-methyl butane (*iso*-amyl bromide)
Iso-propyl ether	1,2-dibromoethylene
Carbon tetrachloride	1,1-dibromoethane (ethylidene bromide)
Chloroform	1-bromo-1-propene
Dichloromethane (methylene chloride)	2-bromo-1-chloro-propane
1-chloropropane (*n*-propyl chloride)	chlorobromomethane
2-chloropropane (*iso*-propyl chloride)	1,2-chlorobromoethane (1,2-ethylene chlorobromide)
1-chlorobutane (*n*-butyl chloride)	Iodoethane (ethyl iodide)
Monochlorbutenes	1-iodo-propane (*n*-propyl iodide)
2-chloro-2-methyl-1-propane (*t*.-butyl-chloride)	2-iodo-butane (*sec*.-butyl iodide)
4-chloro-2-methyl butane (iso-amyl chloride)	*p*-fluorotoluene
1-chlorohexane (*n*-hexyl chloride)	*o*-fluorotoluene
1,1-dichloroethane (ethylidene chloride)	Fluorobenzene
1,2-dichloroethane (ethylene chloride)	Benzotrifluoride
1,1,1-trichloroethane (methyl chloroform)	1,1,2-trichloro-3-trifluoro-1-propene
1,2-dichloroethylene	Thiophene
1,1,2,2-tetrachlorethylene (carbon dichloride)	2-methyl thiophene
1-chloro-1-propene	3-methyl thiophene
Bromoethane (ethyl bromide)	Dimethyl disulfide
1-bromo-1-butane (*n*-butyl bromide)	Methyl ethyl sulfide
2-bromo-2-methyl propane (*t*.-butyl bromide)	Methyl orthoacetate

TABLE II.
Test Organisms

Routine test organisms	Source	
Escherichia coli	R. R. Roepke	ATCC* 9723
Protaminobacter alboflavum b	C. B. van Niel	
Pseudomonas riboflavina	J. W. Foster (available from ATCC)	
Corynebacterium pseudodiphthericum		ATCC 6981
Staphylococcus aureus	Merck and Co., Inc.	" 9144
Streptococcus faecalis		" 8043
Actinomyces scabies	W. H. Burkholder	" 3021
Bacillus subtilis		" 6633
Torula utilis		" 8206

* ATCC—American Type Culture Collection, Georgetown University School of Medicine, Washington, D.C.

ture and commercial catalogs. The compounds tested are listed in Table I. For the sake of brevity, source and physical constants are omitted. Most of the chemicals were obtained either from the Eastman Kodak Co., the Amend Drug and Chemical Co., or the Columbia Organic Chemical Co., Inc., Columbia, S.C. The methyl chloroform and thiophenes were gifts of the Dow Chemical Co. and the Socony-Vacuum Laboratories respectively. Where alternate names are in use, the one employed by *Chemical Abstracts* is given first.

b. *Test Organisms.* The organisms were selected to represent some of the genera commonly encountered as air contaminants in the ordinary course of laboratory work. Mycelial fungi were not included because they never appeared in preservative-treated solutions. It was supposed that acid-fast bacteria might be important contaminants, but they were not encountered, and hence a petroleum-utilizing strain of *Mycobacterium lacticola* and a rapidly-growing strain of *M. avium* were dropped from the roster of routine test organisms. They were killed by nearly all the halogenated compounds tried. The organisms selected for routine use are listed in Table II. Additional organisms were used in later tests.

c. *Test Procedure.* Ten ml amounts of broth, whose composition is given in Table III, were distributed in 125 x 20 screw-capped tubes and sterilized for 20 minutes at 117 to 121°C. The medium allowed heavy rapid growth at room temperature of all the routine test organisms. The tubes were inoculated with a drop of fresh slant growth suspended in broth. After 4 hours at room temperature,

TABLE III.
Composition of Test Medium.

	%
K_2HPO_4	0.05
$MgSO_4 \cdot 7H_2O$	0.01
Na_3 citrate $\cdot 2H_2O$	0.1
Trypticase*	0.6
Lactate	0.25
Glycerol	0.5
Solubilized Liver†	0.1
pH 6.9 to 7.1	

* Baltimore Biological Laboratory.
† Wilson's Liver "L."

the compounds to be tested were aseptically pipetted in, to a final concentration by volume of 1%. The tubes were shaken vigorously and then allowed to stand at room temperature (21 to 28°C) for 96 to 120 hours. Growth was then recorded. To test for completeness of removal of the preservative on autoclaving, and absence in the medium of toxic residual decomposition products, the materials under test were added to broth, which was then autoclaved according to the routine technic. With this method, after sterilization, the exhaust valve of the autoclave was kept shut until the partial vacuum which develops on cooling was subsequently dissipated by the gradual influx of air. If necessary the exhaust valve was opened very slightly. This procedure was equivalent to steam distillation under reduced pressure and no difficulty was experienced in complete removal of preservatives, even in amounts well over the 1% level. To illustrate the effectiveness of the method, trials with *n*-hexyl chloride (b.p. 132.6°C) showed it completely removed under the standard conditions. In orientation experiments it was convenient to test for non-volatilized preservative by adding a few drops of I-KI solution; free iodine dissolving in the preservative made tiny droplets conspicuous.

Mixtures were compounded on a volume basis. In practice, a few drops of preservative were added to the solution in a glass-stoppered bottle and the bottle was then shaken vigorously. Volumes of culture media up to 2 liters were thus successfully preserved for months in the refrigerator.

Results. After conducting killing tests in this manner on 16 compounds, the majority of them halogenated hydrocarbons, it ap-

TABLE IV.
Killing Tests with Mixtures of *o*-Fluorotoluene, *n*-Butyl Chloride, and 1,2-Dichloroethane.

	None	Routine No. 3*	*o*-Fluoro-toluene	*n*-Butyl chloride	1,2-dichloro-ethane	*o*-Fluorotoluene—*n*-Butyl chloride—1,2-dichloroethane 1:1:1	1:2:1	1:3:1	1:1:2
Escherichia coli	+	0	0	+	+	0	0	0	0
Protaminobacter alboflavum b	+	0	0	+	+	0	0	0	0
Pseudomonas riboflavina	+	0	0	+	+	0	0	0	0
Corynebacterium pseudodiphthericum	+	0	0	+	0	0	0	0	0
Staphylococcus aureus	+	0	+	+	+	0	0	0	0
Streptococcus faecalis	+	0	+	+	+	0	0	0	0
Actinomyces scabies	+	0	0	+	0	0	0	0	0
Bacillus subtilis	+	0	+	+	+	0	0	0	0
Torula utilis	+	0	+	+	+	0	0	0	0

* Routine No. 3
33% 1-Bromo-1-propene
67% *n*-Butyl chloride

0 = growth
\+ = growth

peared that a 3:1 mixture of *n*-butyl chloride and CCl_4 might be satisfactory. Need for a better preservative later became evident, since contaminations of stock solutions, while much less frequent, still appeared; and under test conditions there was some growth of *E. coli, Staphylococcus aureus, Streptococcus faecalis* and *Torula utilis*. The next mixture extensively used consisted of equal parts of CCl_4, *n*-butyl chloride, and 1,2-dichloroethane. This was much more effective but did not completely check the growth of *E. coli* and *Strep. faecalis*. Complete effectiveness was shown by a mixture of 1 part 1-bromo-1-propene and 2 parts *n*-butyl chloride. This not only completely inhibited the routine test organisms and many others, but no contaminants appeared in practice; 1-bromo-1-propene was not altogether stable, yellowing slowly at room temperature. This did not significantly alter its antiseptic properties, and volatility tests with yellowed samples remained satisfactory. However on continued use it became extremely irritating to the eyes and nose, and had to be discarded. Several of the somewhat less effective halides were tried singly, and many were tried in combination with *n*-butyl chloride and 1,2-dichlorethane. *O*- and *p*-fluorotoluene appeared unusually effective in repeated trials and were then tested in detail in combination with other halides. The *o*-isomer appeared somewhat more effective and is part of the preservative now in use. A typical experiment with the present preservative is shown in Table IV. About 50 experiments in all were performed on this scale.

The sulfur compounds were almost completely non-inhibitory. No consistent differences emerged between bromo and chloro homologues. The iodo derivatives were not effective enough and were too unstable to encourage further work in that direction.

The preservative finally adopted consisted of a mixture of 1 part *o*-fluorotoluene, 2 parts *n*-butyl chloride, and 1 part 1,2-dichlorethane.

Discussion. Rhodotorulae had previously been isolated as principal contaminants of solutions preserved with the less effective alkyl halide-containing mixtures. Their abundant growth in such seemingly unfavorable media as 3% boric acid has been described.[5] They have not appeared in any of the solutions preserved with the new mixture. Exposure of varied agar plate media indicated that rhodotorulae were quantitatively insignificant but constantly present in our laboratory air. As they have been reported to be pyrogenic,[6] they may perhaps constitute an important source of pyrogenic contamination of therapeutic preparations.

Certain limitations attend the use of alkyl and aryl halide preservatives. Compounds of this class react slowly with –SH groups.[7] This does not appear to affect the practical usefulness of these compounds in preservation of natural materials such as yeast extract and protein hydrolysates, probably because most of the potential –SH compounds are in the disulfide form. This reaction became evident when concentrated solutions of Na thiosulfate were treated with the preservative: the solutions became toxic and acquired peculiar odors. Slow interaction with –SH compounds may be of significance in accounting for the effectiveness of the preservative. Little more can be said at present as to their mode of action. In the recommended preservative mixtures, the most effective component is *o*-fluorotoluene. The *n*-butyl chloride, lighter than water, is a poor preservative but serves to bring the specific gravity of the mixture closer to that of water, besides being an inexpensive extender for the relatively expensive *o*-fluorotoluene. The 1,2-dichloroethane may function as a contact agent because of its relatively good solubility in water (*ca.* 0.9% at room temperature) bringing the less soluble *o*-fluorotoluene into more effective contact with the cell. The obvious fat-solvent properties of these compounds appear inadequate to account for their striking differences in effectiveness.

The tremendous recent expansion in the

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5 Skinner, C. E., *et al.*, 2nd ed. *Henrici's Molds, Yeasts, and Actinomycetes*, 1947, John Wiley and Sons, N.Y.

6 Co Tui, F. W., and Schrift, M. H., *J. Lab. Clin. Med.*, 1942, **27**, 569.

7 Hickinbottom, W. J., *Reactions of Organic Compounds*, p. 106, Longmans, Green and Co., 1936, New York.

laboratory and industrial chemistry of organic fluorine compounds[8] may, in the light of the results reported here, encourage further work in the rather neglected field of the water-immiscible volatile antiseptics.

[8] Symposium on Organo-fluorine Chemistry, 1947, *Ind. Eng. Chem.* (many authors), **39**, 235.

Summary. A mixture by volume of 1 part *o*-fluorotoluene, 2 parts *n*-butyl chloride, and 1 part 1,2-dichlorethane, is recommended as a chemically nearly inert but nevertheless effective preservative for biological solutions. This preservative, used at approximately the 1% level, is completely removed under the usual conditions of steam sterilization.

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Isolation of an Agent in Chicken Embryo Causing Infectious Sinusitis of Turkeys.

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The term infectious sinusitis of turkeys was suggested by Dickenson and Hinshaw[1] to differentiate it from sinusitis which may occur in connection with reduced vitamin A intake[2] or follow inoculation with *Hemophilus gallinarum*.[3] Infectious sinusitis occurs in all sections of the country and although mortality is low, morbidity varies from 10% to 90% of a flock.[1,4] Thus, losses to the grower resulting from inferior quality of the birds are often quite heavy. According to the literature Delaplane[5] has reported a *Pasteurella*-like organism to be associated with an infectious type of sinusitis. In transmission experiments Hart[6] and Hinshaw[7] were unable to filter the causal agent through collodion membranes, Chamberland or sintered glass filters.

Serial transmission of the disease in this laboratory from a case of sinusitis submitted to the laboratory for examination was carried out in the usual manner.[7] Sinus exudate was aspirated from infected turkeys and 0.5 ml amounts were inoculated into the right infraorbital sinus of normal turkeys. The inoculated sinus became swollen 5 to 10 days after inoculation and contained a viscous exudate which resembled egg albumen and was bacteriologically sterile. Later the sinuses became greatly distended and often contained caseated material from which a variety of bacteria were isolated. On the assumption that these organisms represented secondary bacterial invaders an attempt was made to isolate the causal agent early in the course of the disease from the third serial turkey passage. Accordingly, sinus exudate was aspirated on the second day after the appearance of clinical symptoms and was inoculated into embryonating eggs by the following routes: onto the chorioallantois, into the allantoic cavity and into the yolk sac. No embryo deaths or significant macroscopic lesions were observed in any of the eggs inoculated onto the chorioallantois or into the allantoic cavity. However, all of the 10 embryos inoculated into the yolk sac died 6 to 12 days after inoculation. Yolk sacs from these embryos were bacteriologically sterile and were subsequently passed in

[1] Dickenson, E. M., and Hinshaw, W. R., *J. Am. Vet. Med. Assn.*, 1938, **93**, 151.

[2] Hinshaw, W. R., and Lloyd, W. E., *Hilgardia*, 1934, **8**, 281.

[3] Beach, J. R., and Schalm, O. W., *Poult. Sc.*, 1936, **15**, 466.

[4] Lee, C. D., *No. Am. Vet.*, 1942, **23**, 715.

[5] Delaplane, J. P., *Poult. Sc.*, 1944, **23**, 247.

[6] Hart, L., *Austral. Vet. J.*, 1940, **16**, 163.

[7] Hinshaw, W. R., from *Diseases of Poultry*, edited by H. E. Biester and L. Devries, The Iowa State College Press, 1945.