tion by paper partition and thin-layer chromatography are described.

4. Screening of an aqueous extract as well as the total alkaloids of *V. major* in rats for possible leukopenic activity has confirmed a previous report of the absence of such activity.

REFERENCES

(1) Bisset, N. G., *Ann. Bogorienses*, **3**, 105(1958).
(2) Watt, J. M., and Breyer-Brandwijk, M. G., "The Medicinal and Poisonous Plants of Southern Africa," E. and S. Livingstone, Edinburgh, 1932, p. 144.
(3) Pannal, L. H., "Poisonous Plants of the World," Torch Press, Cedar Rapids, Iowa, 1911, p. 807.
(4) Fernald, M. L., "Gray's Manual of Botany," 8th ed., American Book Co., New York, N. Y., 1950, p. 1168.
(5) Noble, R. L., Beer, C. T., and Cutts, J. H., *Ann. N. Y. Acad. Sci.*, **1958**, 893.
(6) Janot, M. M., and LeMen, J., *Compt. rend.*, **238**, 2550(1954).
(7) Janot, M. M., and LeMen, J., *ibid.*, **240**, 909(1955).
(8) Janot, M. M., and LeMen, J., *Ann. pharm. franç.* **13**, 325(1955).
(9) Janot, M. M., and LeMen, J., *Compt. rend.*, **241**, 767(1955).
(10) Farnsworth, N. R., Draus, F. J., Sager, R. W., and Bianculli, J. A., THIS JOURNAL, **49**, 589(1960).
(11) Orechoff, A., Gurewitch, H., Norkina, S., and Prein, N., *Arch. Pharm.*, **272**, 70(1934).
(12) Djerassi, C., Gorman, M., and Woodward, R. B., *J. Am. Chem. Soc.*, **78**, 1259(1956).
(13) LeMen, J., and Pourrat, H., *Ann. pharm. franç.*, **11**, 449(1953).
(14) Plouvier, V., *Compt. rend.*, **251**, 131(1960).
(15) Rabate, J., *Bull. soc. chim. biol.*, **15**, 130(1933).
(16) Ibraham, R. K., and Towers, G. H. N., *Nature*, **184** 1803(1959).
(17) Quevauviller, A., LeMen, J., and Janot, M. M., *Ann. pharm. franç.*, **13**, 328(1955).
(18) Bein, H. J., *Pharmacol. Revs.*, **8**, 448(1956).
(19) Wall, M. E., Krider, M. M., Krewson, C. F., Eddy, C. R., Willaman, J. J., Corell, D. S., and Gentry, H. S., THIS JOURNAL, **43**, 1(1954).
(20) Krider, M. M., Monroe, H. A., Jr., Wall, M. E., and Willaman, J. J., *ibid.*, **46**, 304(1957).
(21) Wall, M. E., Krider, M. M., Krewson, C. F., Eddy, C. R., Willaman, J. J., Corell, D. S., and Gentry, H. S., U.S.D.A., Eastern Regional Research Laboratory, Philadelphia, Pa., Form 1AC-363, February 1954.
(22) Wall, M. E., Fenske, C. S., Garvin, J. W., Willaman, J. J., Jones, Q., Schubert, B. G., and Gentry, H. S., THIS JOURNAL, **48**, 695(1959).
(23) "United States Pharmacopeia," 15th rev., Mack Printing Co., Easton, Pa., 1955, p. 835.
(24) *Ibid.*, 13th rev., 1947, p. 712.
(25) *Ibid.*, p. 714.
(26) *Ibid.*, p. 715.
(27) Lepper, H. A., "Official Methods of Analysis of the Association of Official Agricultural Chemists," 5th ed., Washington, D. C., 1945, pp. 410, 571, 830.
(28) *Ibid.*, pp. 412, 850.
(29) *Ibid.*, pp. 571, 572.
(30) "United States Pharmacopeia," 15th rev., Mack Printing Co., Easton, Pa., 1955, p. 909.
(31) Korzun, B. P., St. Andre, A. F., and Ulshafer, P. R., THIS JOURNAL, **46**, 720(1957).
(32) Munier, R., and Macheboeuf, M., *Bull. soc. chim. biol.*, **31**, 1144(1949).

Stability of Antibacterial Preservatives in Parenteral Solutions I

Factors Influencing the Loss of Antimicrobial Agents from Solutions in Rubber-Stoppered Containers

By L. LACHMAN, S. WEINSTEIN, G. HOPKINS†, S. SLACK, P. EISMAN, and J. COOPER

Losses of preservative due to degradation and absorption in rubber-closed multiple-dose vials were investigated. The preservatives evaluated were benzyl alcohol, phenylethyl alcohol, *p*-chloro-β-phenylethyl alcohol, chlorobutanol, and methylparaben. The elastomer closures used in this study were intended to be representative of those most commonly employed for pharmaceuticals and were natural, neoprene, and butyl rubbers. The temperature dependency of the degradation and diffusion processes were studied. An attempt was made to correlate preservative loss and microbiological activity.

RUBBER CLOSURES are used extensively by pharmaceutical manufacturers to seal vials containing injectable solutions. It is well known that rubber may react with, absorb, or even dissolve substances in contact with it. Thus, incompatibilities between elastomer closures and injectable solutions occur frequently, thereby presenting many problems. One such very important problem is the interaction between rubber closures and bacteriostatic agents present in the solutions. These agents are added to multiple-dose parenteral preparations to insure bacteriostasis for the life of the product. Loss of preservative from solution by interaction with rubber closures could result in bacterial contamination of the injectable preparation if bacteria were accidently introduced.

The 1953 edition of the British Pharmacopoeia (1) recognizes the tendency of rubber to absorb preservatives from injectable solutions. It therefore directs that closures for parenteral solutions should be boiled in several changes of distilled

Received April 28, 1961, from the Research Department, Ciba Pharmaceutical Products, Summit, N. J.
Accepted for publication June 1, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
† Research Chemist, The West Co., Phoenixville, Pa.

water and then either boiled under a reflux condenser for 30 minutes, or stored for not less than 48 hours in a solution containing the same bacteriostat in the same concentration as in the injectable solution, or preferably in twice this concentration. However, the British Pharmacopoeia adds the further precaution that in prolonged storage, rubber so treated is still likely to absorb preservative from the injectable solution.

As early as 1923, Masucci and Moffat (2) reported on the substantial loss of cresol or phenol from rubber-capped vials of solutions stored at room temperature. The loss of preservative was explained as due to the vaporization of cresol or phenol through the rubber caps. In 1937 McGuire and Falk (3), studying certain biological products, found that 0.5% phenol was reduced to 0.3% after 237 days at 37°, while controls with glass-stoppered containers underwent no diminution of preservative content.

Within recent years, the influence of vial stoppers on the preservative content of parenteral products has received considerable attention. Wiener (4) studied the effect of various composition rubber closures on the bacteriostatic action of thiomersolate and observed great variability as to their interference with antibacterial properties. Berry (5) has shown that rubber caps could reduce the strength of a 0.1% chlorocresol solution by as much as 75% under normal conditions of storage. Wing (6–8) and Nielsen (9) studied the absorption of phenol and chlorocresol by elastomer caps. Wing (8) also investigated the effects of varying the chemical composition of the rubber mix on the absorption of these preservatives. Royce and Sykes (10) determined the loss of phenol, cresol, benzyl alcohol, chlorocresol, chlorobutanol, and phenylmercuric nitrate from rubber-closed multiple-dose containers. Of these preservatives, phenol and benzyl alcohol were the least affected and phenylmercuric nitrate the most affected. These investigators also evaluated the methods recommended in the British Pharmacopoeia for the pretreatment of rubber closures and found that they were not adequate to equilibrate the rubber with the preservative in solution. Wiener (4) also found this to be true in the case of thiomersolate.

It is evident from these studies that a considerable amount of work has been done relating the effects of rubber closures on preservative agents. In most instances, however, the investigators did not define the compositions of the rubber closures used. It must therefore be realized, when considering these data, that another composition closure might not produce the same effects. Siddell (11) reviewed the problems as they relate to chemical uniformity of vial stoppers. He attributed the existence of these problems to inadequate quality standards and poor control of methods of manufacture. Since 1954, attempts have been made in the United States and England to develop standards which would control the composition and properties of rubber closures. The lack of success in this endeavor is evident in the U.S.P. XVI, whose only specification for rubber closures is that "containers and closures shall not physically or chemically interfere with the preparations in any manner to alter the strength, quality, or purity beyond the official requirements."

In most of the previous work, little consideration was given to the possible degradation of preservative in solution. Generally, only the absorption or diffusion characteristics were evaluated. This study evaluates several preservatives with three commonly used rubber closures for (*a*) preservative degradation in solution, (*b*) loss of preservative due to absorption or diffusion into and through the closure, (*c*) influence of chemical structure on diffusion characteristics, (*d*) temperature dependency of the degradative and diffusion processes, (*e*) relationship of preservative loss to microbiological activity, and (*f*) changes in the physical properties of the rubber in contact with the preservative solutions.

EXPERIMENTAL

Materials.—0.275 *M* Citric acid-sodium phosphate buffer of pH 4.0. *p*-Chloro-*β*-phenylethyl alcohol, Ciba, b.p. 80–83° at 1.07 mm. Phenylethyl alcohol, Eastman Organic Chemicals. Benzyl alcohol, reagent grade, Fisher Scientific Co. Methylparaben U.S.P. Chlorobutanol, anhydrous, U.S.P. Elastomer closures: V-32-natural crepe, neoprene polymer, and butyl polymer, West Co. U.S.P. type I, 10-ml. amber ampuls and vials, Kimble Glass Co. Three piece aluminum caps for vials, West Co., No. 13-30.

Equipment.—Beckman spectrophotometer model DU. Beckman spectrophotometer model DK-2. Shore type A hardness tester. Beckman pH meter model G.

Preparation of Closures and Vials. The stoppers used in this study were immersed in a 1.0% solution of tetrasodium pyrophosphate and heated at 90° for 15 minutes under constant agitation. They were removed from the solution and washed 10 times with distilled water and then five times with filtered water for injection. The stoppers were dried at 40° for 48 hours before use.

The ampuls and vials were washed, placed into special pans, and covered. These pans of ampuls and vials were sterilized at 180° for 2 hours.

Preparation of Ampuls and Vials of Preservative Solution.—Solutions containing (*a*) 0.3% *p*-chloro-*β*-phenylethyl alcohol, (*b*) 0.5% phenylethyl alcohol.

(*c*) 1.0% benzyl alcohol, (*d*) 0.2% methylparaben, and (*e*) 0.5% anhydrous chlorobutanol, on a weight to volume basis, were prepared with water for injection buffered to pH 4.0. The preservative solutions were then passed through medium porosity sintered-glass filters. Each preservative solution was filled into 10-ml. amber ampuls and vials. The ampuls were closed by customary pull sealing techniques under an oxygen-gas flame. The vials of each preservative were stoppered with three different composition closures. The stoppered vials were then sealed with three-piece aluminum caps at a constant sealing head pressure of 60 p.s.i. with a Westcapper. Samples of buffer solution in ampuls and vials were filled and sealed similarly. The buffer and preservative solutions in ampuls and vials were placed into constant temperature ovens regulated at 25, 40, 50, and 60, ±1.5°. Half of the vials were stored upright and half inverted. The constant temperature equipment employed has been described in a previous publication (12). At designated time intervals, samples were withdrawn and tested for residual preservative content, microbiological activity, pH, and physical changes. In addition, physical tests were performed on the closures to determine whether or not changes had taken place as to hardness, shape, and color. As a control, buffer solutions in ampuls and vials were run concurrently.

Loss of Preservative Upon Sterilization.—Samples of each of the five preservative solutions in vials stoppered with natural, neoprene, or butyl rubber and in ampuls were autoclaved at 115°, 10 p.s.i., for 30 minutes. Assay for residual preservative content was then performed.

Apparent Distribution of Preservative Between Buffer Solution and Rubber.—Ten-millimeter aliquots consisting of (*a*) *p*-chloro-β-phenylethyl alcohol, (*b*) phenylethyl alcohol, and (*c*) chlorobutanol were filled into 20-ml. amber ampuls. The ampuls containing each preservative were separated into three groups. In one group, 5 Gm. of natural rubber stoppers (cut in half) were placed into each ampul; into each ampul of the second group cut neoprene polymer stoppers were added. No stoppers were added to the ampuls of the third group. The ampuls were pull sealed and placed into constant temperature ovens regulated at 25 and 40 ±1.5°. At the end of 4 weeks' storage the ampuls were removed and the solution assayed for residual preservative. Apparent distribution coefficients were calculated as follows

$$K_T = \frac{10(C_A - C_B)}{C_B V} = \frac{C_R}{C_B}$$

where K_T = apparent distribution coefficient at temperature T, C_A = concentration of preservative in ampul solution after storage without closures (mg./ml.), C_B = concentration of preservative in ampul solution after storage with 5 Gm. of closures (mg./ml.), V = volume of 5 Gm. of rubber, $10(C_A - C_B)$ = concentration of preservative in rubber (mg.), and $[10(C_A - C_B)]/V = C_R$ = concentration of preservative in rubber calculated as mg./ml.

To avoid discrepancies in the distribution values due to degradation of preservative in solution, the concentration of preservative in the ampul solution without closures, after 4 weeks' storage at the above temperature conditions (C_A), was taken as the total preservative concentration for the calculation of K_T.

Analytical Methods. — *Chlorobutanol.* — The method employed is essentially that of Rehm and Mader (13) with minor changes. The undegraded chlorobutanol in solution was determined by pipetting a 3-ml. aliquot of solution into a suitable microsteam distillation apparatus. One drop of concentrated sulfuric acid was added and the sample steam distilled for 3 minutes. The condenser was washed down with warm distilled water and the rinsings added to the distillate to make a total volume of 50 ml. The remainder of the procedure was according to the method of Rehm and Mader in the absence of interfering substances: mg. chlorobutanol/ml. = A sample/A standard × 0.5 × dilution factor measured at the absorption maximum of 500 mμ.

p-Chloro-β-phenylethyl Alcohol.—Residual preservative was determined by pipetting 5 ml. of solution into a microsteam distillation apparatus. The sample was steam distilled and 40 ml. was collected. Then 50 ml. of methanol was added to the distillate and brought up to volume with distilled water in a 100-ml. volumetric flask. Absorbance was measured at 267.5 mμ where $A(1\%, 1\text{ cm.}) = 22$.

Phenylethyl Alcohol.—The concentration of preservative was determined by pipetting 3 ml. of solution into a microsteam distillation apparatus and the sample steam distilled to 30 ml. The distillate was brought up to 50 ml. in a volumetric flask with distilled water. Absorbance was measured at 257 mμ where the $A(1\%, 1\text{ cm.}) = 18$.

Benzyl Alcohol.—The method employed was similar to that used for phenylethyl alcohol, except that 1 ml. of solution was pipetted into a microsteam distillation apparatus and steam distilled to 25 ml. and absorbance was measured at 257 mμ where $A(1\%, 1\text{ cm.}) = 18$.

Methylparaben.—A 5-ml. aliquot was pipetted into a 100-ml. volumetric flask and brought up to volume with methanol. Five milliliters of this solution was transferred to another 100-ml. volumetric flask and brought up to volume with methanol. Absorbance was measured at 256 mμ where $A(1\%, 1\text{cm.}) = 1{,}115$.

Zinc Mercaptobenzothiazole.—This substance was leached from the rubber and detected by the presence of a sharp peak at 318 mμ in the ultraviolet spectra of the preservative solutions. The concentration of this material was measured at 318 mμ where the $A(1\%, 1\text{ cm.}) = 16.7$.

Chromatography of Degraded Samples.—Thin-layer chromatography, according to the method of Stahl (14), was performed on each of the preservatives after storage in vial solutions at a pH of 4.0 and at 60° for 12 weeks. The concentrations of preservative placed on the layered plates were (*a*) 60 γ *p*-chloro-β-phenylethyl alcohol, (*b*) 100 γ phenylethyl alcohol, (*c*) 200 γ benzyl alcohol, (*d*) 40 γ methylparaben, and (*e*) 100 γ chlorobutanol. Silica gel G was used as the layer and the solvent system employed was benzene-acetone (1:1). The detecting reagent used for spraying the plates was composed of equal volumes of 1% aqueous solutions of ferric chloride and potassium ferrocyanide.

Measurement of Rubber Hardness.—A Shore type A-2 hardness tester was used. Measurements were made on the flange of the stoppers and readings were taken in triplicate and averaged.

Microbiological Tests.—*Bactericidal Activity of Preservatives.*—The phenol coefficient-type test employed involved the contamination of various concentrations (in pH 4 buffer) of the five preservatives under study with *S. aureus* and *E. coli*. The concentration of preservative necessary to destroy all the test organisms in less than 15 minutes, but not in 10 minutes, was determined by subculturing into fresh broth after contact periods of 5, 10, and 15 minutes.

Self-Sterilizing Properties of the Preservatives.—Solutions of the five preservatives, buffered to pH 4 and at the concentration noted earlier in this section, were evaluated as to the time required to destroy 50 and 95% of the following test organisms: *E. coli*, *S. aureus*, and the spores of *B. cereus* var. *mycoides*. Plate counts for the number of surviving organisms were performed after 1, 3, 5, 24, 48, 72, and 96 hours, and again after 7 days. In addition, at these time intervals, aliquots were tested for sterility by transferring to thioglycollate fluid medium.

Correlation of Chemical Assay to Microbiological Activity.—The microbiological assay method was investigated with only *p*-chloro-β-phenylethyl alcohol. It involved the development of a standard curve by using preservative concentrations in pH 4 buffer solution from 0.3 to 0.06% and diluting 1:7 with buffer solution before contamination. The solutions were contaminated by adding 1-ml. amounts of an 18-hour diluted culture (91% light transmission in a Bausch and Lomb Spectronic 20 colorimeter fitted with a No. 580 light filter) of *E. coli* to 14-ml. quantities of solution. At various time intervals, 1-ml. portions were removed and plated in trypticase soy agar for survivor count. Curves were plotted of per cent survivors *vs.* time for each concentration and the time required to produce 50% killing was obtained from the plots. A standard curve was then drawn of the time required to kill 50% of the organisms *vs.* concentration of preservative. Using the 50% killing time value of a degraded sample of preservative and placing it on the standard curve, an estimate of the residual concentration of preservative was obtained.

RESULTS AND DISCUSSION

The decrease in concentration of several bacteriological preservatives in aqueous buffered solutions stored in rubber-stoppered vials was investigated. These solutions were buffered to a pH of 4 in order to maintain a constant hydrogen ion concentration as well as to permit the study of chlorobutanol at a pH in which degradation in solution would be minimal.

The stoppers chosen for evaluation are representative of three rubber compositions commonly employed for multiple-dose vial closures. The composition and per cent rubber content of these stoppers are presented in Table I. Several of the physical properties of these closures are given in Table II.

Chemical and Physical Results.—An ascending thin-layer chromatographic technique was employed to distinguish decreases in preservative content in rubber-stoppered vial solutions which had been stored at 60° for 12 weeks. From the chromatogram in Fig. 1, it was estimated that in the case of chlorobutanol approximately a 90% reduction in concentration had occurred while for the other preservatives about a 50% reduction in concentration resulted. Consequently, additional studies were performed to ascertain the portion of preservative lost due to degradation in solution as well as the amount lost by reaction with the rubber stopper.

TABLE I.—CLOSURE COMPOSITION

Natural Rubber	Neoprene Rubber	Butyl Rubber
Natural crepe[a]	Neoprene polymer[b]	Butyl polymer[c]
Titanium dioxide	Sulfonated oil	Barium sulfate
Barium sulfate	Calcined clay	Calcined clay
Zinc oxide	Barium sulfate	Carbon black
Iron oxide	Zinc oxide	Titanium dioxide
Diphenylamine-acetone condensation product	Magnesium oxide	Stearic acid
	Iron oxide	Paraffin wax
Thiuram combined with aniline reaction product	Stearic acid	Sulfur
	Mineral oil	Thiazole-dithiocarbamate accelerator
	Imidazoline type accelerator	

[a] Rubber content, 50.4% of total composition. [b] Rubber content, 42.4% of total composition. [c] Rubber content, 36.9% of total composition.

TABLE II.—PHYSICAL CHARACTERISTICS OF THE RUBBER STOPPERS

Properties	Stopper Composition		
	Natural Rubber	Neoprene Rubber	Butyl Polymer
Specific gravity	1.600	1.490	1.609
Thickness[a]	0.125 inch	0.125 inch	0.124 inch
Weight[b]	618 mg.	548 mg.	595 mg.

[a] Measured microscopically and value is average of five measurements of different stoppers. [b] Value is average of 10 stoppers.

Chlorobutanol.—Chlorobutanol has been used widely as a bacteriostatic agent in parenteral solutions. The results of a number of studies on the stability of chlorobutanol in solution have been reported (15–20). The majority of these reports (15, 17–20) are concerned with the instability of chlorobutanol solutions during autoclaving. Several investigators (18, 19) have found that decomposition is minimal at pH 5 or below. Recently, a comprehensive quantitative study of the degradation of chlorobutanol was performed by Nair and Lach (21) who evaluated the kinetics of the degradation over a pH range from 2 to 7.5. From pH 2 to 4 the decomposition was found to be pH independent and having a half-life of about 90 years at 25°. However, at pH 7.5, the half-life dropped sharply to slightly less than 3 months. Therefore, for pharmaceuticals containing chlorobutanol as the preservative, it appears desirable to buffer the solution to pH 4 or below in order to minimize the degradation reaction.

In this investigation, the influence of natural, neoprene, and butyl rubber stoppers on the concentration of chlorobutanol in vial solutions buffered at pH 4 was studied. The residual concentrations of chlorobutanol in ampul solutions found at different time intervals and at several elevated temperatures are shown in Fig. 2. It is evident from the plot that the degradation reaction is first order with respect to chlorobutanol concentration. Using the integrated Arrhenius equation, the heat of activation was calculated employing the rate constants at

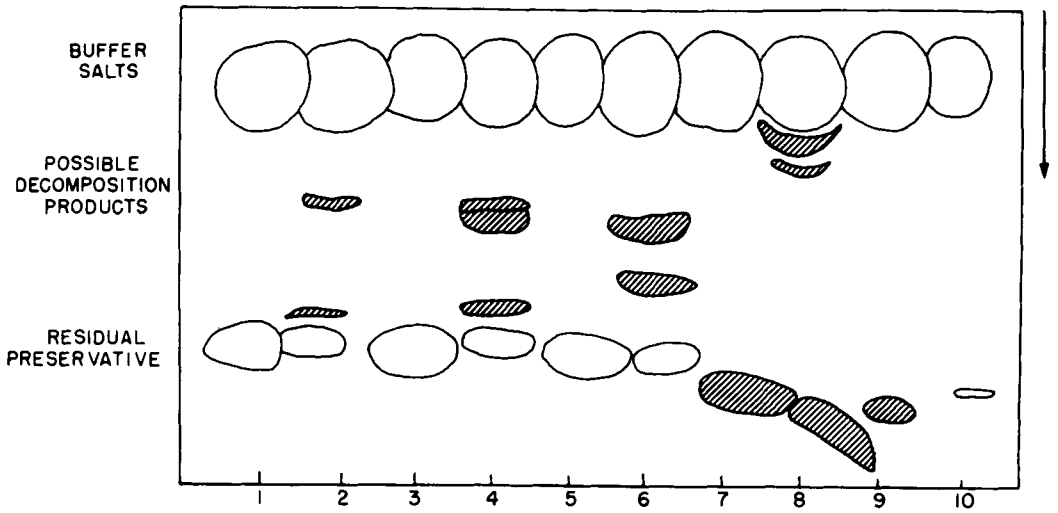

Fig. 1.—Thin-layer chromatograms of aged (60° for 12 weeks) and freshly prepared solutions of the five preservatives. 1, *p*-Chloro-β-phenylethyl alcohol standard solution; 2, *p*-chloro-β-phenylethyl alcohol after storage; 3, phenylethyl alcohol standard solution; 4, phenylethyl alcohol after storage; 5, benzyl alcohol standard solution; 6, benzyl alcohol after storage; 7, methylparaben standard solution; 8, methylparaben after storage; 9, chlorobutanol standard solution; 10, chlorobutanol after storage. ▨, Blue stain; □, white stain.

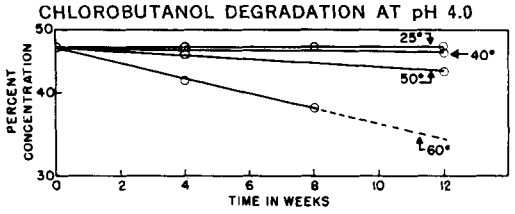

Fig. 2.—Plot of the logarithm of the per cent residual concentration of chlorobutanol against time at pH 4.0.

50 and 60°. A value of 27.6 Kcal. was obtained as the heat of activation for the degradation of chlorobutanol at pH 4. This is a reasonable value for a hydrolytic reaction of this nature and is in fair agreement with the value of 30.7 Kcal. obtained by Nair and Lach (21) for this reaction.

A comparison of chlorobutanol loss in vials stoppered with these different composition closures with that found for solutions in ampuls is presented in Table III. It is evident from these data that the closures have a marked deleterious effect on preservative concentration at all temperatures. This greater loss of preservative in the vial solutions can be attributed to (*a*) adsorption and absorption of preservative by the rubber, (*b*) possible diffusion of the preservative through the rubber and subsequent volatilization into the atmosphere, and (*c*) interaction of preservative with material extracted from the closure. Of these three factors, adsorption and absorption of the preservative by the closure is probably responsible for the greatest loss.

In Table IV are presented data indicating the per cent of chlorobutanol lost from solution due to the influence of the rubber stopper. These values were obtained by subtracting the per cent residual preservative found in the vial solutions from that found in the ampul solutions under the same storage conditions. From the data in this table and that in Table III, it is demonstrated that the major quantity of preservative lost due to rubber stopper effect takes place during the first 2 weeks of storage. Thereafter, very little if any, further loss occurs. This may be ascribed to the development of an apparent equilibrium state between the concentration in the solution and in the stopper.

It is interesting to note that the storage of vial solutions in an inverted position seems, for the most part, to cause a greater loss of preservative from solution than occurs in those vials stored in an upright position. Since it is not uncommon for vials to be on their side during storage in pharmacies, hospitals, or physicians' offices and thereby permitting the contents to come in contact with the rubber stopper, the losses of preservative in vials stored in the inverted position warrant serious consideration.

According to the 60° storage data in Table IV, it is evident that the preservative loss due to the rubber stopper decreases with the increase of preservative degradation as indicated by the ampul assays in Table III. This effect can be explained by the significant degradation of preservative in solution which, in turn, results in a reduction of the amount of available chlorobutanol to equilibrate between the solution and closure.

The data in Tables III and IV indicate that the neoprene rubber stopper is responsible for the greatest loss of chlorobutanol from solution. At 60°, after 12 weeks' storage, the vials in the inverted position show only an 8.5% residual concentration, whereas the ampuls under the same storage conditions contain 72.4%. These results are in agreement with the estimate of 10% residual preservative obtained from the thin-layer chromatogram. The other two stoppers, natural and butyl rubber, exert approximately the same effect on preservative loss with possibly the natural rubber stopper exerting a slightly lesser deleterious effect. This substantially

TABLE III.—INFLUENCE OF DIFFERENT COMPOSITION CLOSURES ON THE PER CENT RESIDUAL CONCENTRATION OF CHLOROBUTANOL IN VIAL SOLUTIONS AFTER STORAGE

Temp., °C.	Storage Time, wk.	Ampul Control	Stopper Composition: Natural Rubber Upright	Natural Rubber Inverted	Neoprene Polymer Upright	Neoprene Polymer Inverted	Butyl Polymer Upright	Butyl Polymer Inverted
25	2							
	8	100	87.3	83.0			74.5	63.8
	12	100	81.0	78.7	87.3	81.0	68.1	63.8
40	2	100	76.6	63.8	61.7	59.6	68.1	61.7
	8	100	76.6	63.8	57.5	51.0	68.1	61.7
	12	97.9	74.5	57.5	53.2	46.8	66.0	66.0
50	2	97.9	59.6	57.5	57.5	46.8	66.0	61.7
	8	93.6	57.5	....	59.6	46.8	66.0	59.6
	12	91.5	57.5	42.5	46.8	38.3	59.6	57.5
60	2	95.8	57.5	53.2	51.0	46.8	51.0	48.9
	8	81.0	59.6	51.0	53.2	42.5	51.0	48.9
	12	72.4	57.5	42.5	29.8	8.5	46.8	46.8

TABLE IV.—PER CENT CHLOROBUTANOL LOSS DUE TO STOPPER

Temp., °C.	Storage Time, wk.	Natural Rubber Upright	Natural Rubber Inverted	Neoprene Rubber Upright	Neoprene Rubber Inverted	Butyl Rubber Upright	Butyl Rubber Inverted
25	2						
	8	12.8	17.0			25.6	36.2
	12	19.1	21.3	12.8	19.1	31.9	36.2
40	2	23.4	36.2	38.4	40.0	31.9	38.4
	8	23.4	36.2	42.6	49.0	31.9	38.4
	12	23.4	40.0	44.7	51.1	31.9	31.9
50	2	38.4	40.0	40.0	51.1	31.9	36.2
	8	36.2	....	34.0	46.8	27.7	34.0
	12	34.0	49.0	44.7	53.2	31.9	34.0
60	2	38.4	42.6	44.7	49.0	44.7	46.8
	8	21.3	29.8	27.7	38.4	29.8	31.9
	12	14.9	29.8	42.6	63.8	25.6	25.6

greater effect caused by neoprene stoppers can essentially be explained by the physical properties of this rubber as compared with the other two. It is apparent from the data in Table II that neoprene rubber is of a more porous composition than either natural or butyl rubber. As a result of this greater porosity, chlorobutanol could diffuse into the closure with less impediment. The physical properties listed in Table II for the natural and butyl rubber stoppers indicate that the porosity of these two rubbers are approximately the same. Accordingly, if the chlorobutanol loss from solution is due essentially to absorption by the rubber, then the quantity absorbed should be approximately the same for both elastomers which, according to the data in Tables III and IV, proved to be the case.

Of particular interest are the results pertaining to the loss in preservative content from vial solutions at room temperature. Although the ampuls stored for 12 weeks at 25° show no loss of chlorobutanol content, the vial solutions stored in an inverted position undergo a loss ranging from 19 to 36%, depending upon the stopper used. These results are not surprising in the light of the report by Royce and Sykes (22) which indicated that a 0.5% aqueous solution of chlorobutanol was partitioned between rubber and water in the ratio of 85 to 15% after one month storage at room temperature. In their partition studies, these investigators used a ratio of 1 Gm. rubber to 3 ml. of solution.

During the study with chlorobutanol solution, it was found that zinc mercaptobenzothiazole, an accelerator used as part of the curing system in the butyl rubber stopper, was extracted by the solution, forming a fine colloidal precipitate in the vial solution. In addition, the portion of the closure in contact with the preservative solution became discolored and the solution developed a yellow color. Preliminary tests with other preservatives in solution showed similar results. As a result of the physical incompatibility of this rubber stopper with the various preservative solutions, it was eliminated from comprehensive evaluation with the other preservatives.

p-Chloro-β-phenylethyl Alcohol.—Hess and Speiser (23, 24) in their studies on the comparative efficiency of bactericidal compounds in buffer solutions, showed that *p*-chloro-β-phenylethyl alcohol exhibited promising potentialities as a new bactericidal agent. Studies performed in our laboratories have confirmed their findings, and additional data are presented in a later section of this paper.

In order to evaluate the stability of *p*-chloro-β-phenylethyl alcohol in vials stoppered with natural and neoprene rubber stoppers, a 0.3% solution buffered at pH 4 was employed. As controls, ampul solutions were used at each storage condition. According to the data given in Table V, the closures have a significant deleterious effect on preservative concentration at the several storage conditions. As in the case of the chlorobutanol solution, the neoprene closure causes a significantly greater loss of preservative content than does the natural rubber stopper.

The per cent *p*-chloro-β-phenylethyl alcohol lost due to the closure is summarized in Table VI. These values are representative of the per cent residual preservative in the vial subtracted from the

TABLE V.—INFLUENCE OF DIFFERENT COMPOSITION CLOSURES ON THE PER CENT RESIDUAL CONCENTRATION OF *p*-CHLORO-β-PHENYLETHYL ALCOHOL IN VIAL SOLUTIONS AFTER STORAGE

Temp., °C.	Storage Time, wk.	Stopper Composition: Natural Rubber — Ampul Control	Natural Rubber — Upright	Natural Rubber — Inverted	Neoprene Rubber — Ampul Control	Neoprene Rubber — Upright	Neoprene Rubber — Inverted
25	2						
	8	100	98.6	82.8	100	82.8	69.0
	12	100	82.8	76.0	100	76.0	65.5
40	2	100	98.6	86.2	100	86.2	72.5
	8	100	79.3	76.0	100	72.5	62.0
	12	89.6	76.0	72.5	89.6	62.0	51.7
50	2	100	86.2	82.8	100	79.3	65.5
	8	96.6	76.0	72.5	96.6	62.0	55.2
	12	89.6	72.5	69.0	89.6	55.2	44.8
60	2	96.6	82.8	79.3	96.6	72.5	62.0
	8	86.2	69.0	65.5	86.2	62.0	48.3
	12	86.2	65.5	58.6	86.2	48.3	41.1

TABLE VI.—PER CENT *p*-CHLORO-β-PHENYLETHYL ALCOHOL LOSS DUE TO STOPPER

Temp., °C.	Storage Time, wk.	Natural Rubber — Upright	Natural Rubber — Inverted	Neoprene Rubber — Upright	Neoprene Rubber — Inverted
25	2				
	8	10.4	17.2	17.2	31.0
	12	17.2	24.0	24.0	34.5
40	2	10.4	13.8	13.8	27.5
	8	20.7	24.0	27.5	38.0
	12	13.6	17.1	27.6	37.9
50	2	13.2	17.2	20.7	34.5
	8	20.6	24.1	34.6	41.4
	12	17.1	20.6	34.4	44.8
60	2	13.8	17.3	24.1	34.6
	8	17.2	20.7	24.2	37.9
	12	20.7	27.6	37.9	44.8

per cent remaining in the ampuls at the particular condition and time of storage. Again, as in the chlorobutanol solution study, the largest loss of preservative content takes place during the first 2 weeks of storage, with subsequent loss being relatively small. However, the loss of preservative due to closure effect is substantially less in the case of this preservative as compared to that for chlorobutanol. This may be explained by the apparent distribution coefficient of the preservatives between solution and rubber, where chlorobutanol favors the rubber to a greater extent than does *p*-chloro-β-phenylethyl alcohol. This is discussed in greater detail in a subsequent section of this report.

It is interesting to note (Table VI) that for the vials stoppered with the neoprene rubber and stored in an inverted position, there is a substantially greater loss in preservative content than occurs in the vials stored upright. However, for vials stoppered with natural closures, the loss of preservative from solution in the inverted vials does not differ significantly from that lost in the upright vials.

Of major importance is the loss of preservative content which takes place when the vial solutions are stored at room temperature for 12 weeks. It can be seen from Table VI that vials stored in an inverted position lose between 24 to 34% preservative from solution, the larger quantity being from the vials stoppered with neoprene closures. Because of this substantial loss in preservative content, it would seem that this preservative as well as chlorobutanol should not be used in vial solutions unless prior closure-preservative studies are performed. The ampul solutions used as controls showed no loss in preservative content during the storage period.

Phenylethyl Alcohol, Benzyl Alcohol, and Methylparaben.—Preliminary studies of the type just described for chlorobutanol and *p*-chloro-β-phenylethyl alcohol have been performed for phenylethyl alcohol, benzyl alcohol, and methylparaben. The results obtained indicate that these preservatives are also absorbed by the closures. Comprehensive studies of these preservatives are under way and the results will be reported at a later date.

Influence of Autoclaving Conditions on Preservative Loss.—Vial solutions of the several preservatives buffered at pH 4 and stoppered with neoprene, natural, and butyl rubber closures were autoclaved at 115°, 10 p.s.i. for 30 minutes. The residual preservative content in these solutions was determined and the data are summarized in Table VII. It is apparent from these results that the vial solutions containing *p*-chloro-β-phenylethyl alcohol are most deleteriously affected with respect to residual preservative concentration. Of the closures tested, natural rubber is the least reactive. It should be noted that the only ampul solution undergoing loss in preservative content after autoclaving was chlorobutanol. A 5% loss in preservative content took place in the ampul solutions of chlorobutanol. Nair and Lach (21), in their kinetic studies of chlorobutanol degradation in solution, found that at pH 5 there occurred a 13% loss of chlorobutanol content upon autoclaving at 115°, 10 p.s.i. for 30 minutes.

TABLE VII.—EFFECT OF AUTOCLAVING AT 115°, 10 p.s.i. FOR 30 MINUTES ON THE PRESERVATIVE CONTENT IN VIAL SOLUTIONS STOPPERED WITH DIFFERENT COMPOSITION RUBBER CLOSURES

Preservative[a]	% Residual Preservative — Ampul Control	Natural Rubber	Neoprene Rubber	Butyl Rubber
p-Chloro-β-phenylethyl alcohol	100	91	88	88
Phenylethyl alcohol	100	100	96	98
Chlorobutanol	95	92	92	92
Benzyl alcohol	100	100	90	100
Methylparaben	100	100	100	100

[a] Preservative solutions were buffered to pH 4.0.

Since the half-life of the degradation reaction at pH 4 is about 2.2 times that at pH 5, the value of 5% degradation obtained in this study seems to be entirely in order.

Apparent Distribution of Preservative Between Rubber and Solution.—It has been reported by Wing (7) and Royce and Sykes (22) that the proportion of chlorocresol absorbed by rubber is much higher than that of phenol. This results in partition coefficients which are almost 20 times greater than for phenol with the same rubber. It would appear that the chlorine substituent on the cresol molecule greatly influences the diffusion of the preservative into the rubber.

In order to determine whether a similar situation existed for *p*-chloro-β-phenylethyl alcohol and phenylethyl alcohol, distribution studies were performed. The results obtained are summarized in Table VIII. These data indicate that the chlorinated compound distributes more strongly into the rubber than does the nonchlorinated analog. In addition, the neoprene rubber absorbs to a greater degree than does the natural rubber. The data show that the distribution values decrease with a rise in temperature, with the exception of neoprene rubber in contact with *p*-chloro-β-phenylethyl alcohol solution. In this case the 40° distribution value is greater than for the 25° value.

TABLE VIII.—APPARENT DISTRIBUTION OF PRESERVATIVES BETWEEN RUBBER AND BUFFER SOLUTION[a] AFTER 4 WEEKS STORAGE

$K_T = C_R/C_B$

Temperature, °C.	Phenylethyl Alcohol[b]		*p*-Chloro-β-phenylethyl alcohol[c]	
	Natural	Neoprene	Natural	Neoprene
25	1.72	4.23	6.05	16.4
40	1.39	4.13	5.70	21.8

[a] Solutions buffered to a pH of 4.0. [b] Ampul concentration at end of 4 weeks: 25° = 0.46%, 40° = 0.43%. [c] Ampul concentration at end of 4 weeks: 25° = 0.26%, 40° = 0.25%.

Of the closures investigated, the butyl polymer was the only one to exhibit significant physical incompatibilities. With each of the preservative solutions, zinc mercaptobenzothiazole was leached from the closure, forming a colloidal precipitate in the vial solutions. That portion of the closure in contact with the solution became discolored and the solutions developed a yellow color. Hardness testing of the three closures in contact with the preservative solutions at different temperature conditions showed no significant changes when compared to original values.

Results of Microbiological Tests.—In order to evaluate the comparative bactericidal activities of the five preservative solutions under study, a phenol coefficient type test was performed with *S. aureus* and *E. Coli.* The data obtained from this study are summarized in Table IX. It is evident from these results that *p*-chloro-β-phenylethyl alcohol is the most active of these preservatives. In comparison to its nonhalogenated analog, phenylethyl alcohol, it is approximately five times more active when tested with *S. aureus* and approximately three times more active when the test organism is *E. coli.* This superior activity of the halogenated molecule may be explained according to the theory of Hess and Speiser (24) who have postulated the existence of a lipid barrier at the cell surface of the organisms. Since the halogenated molecules distribute more readily into nonpolar solvents, it would be expected to penetrate the lipid barrier more rapidly than the nonhalogenated molecule and, hence, require a smaller concentration to achieve the same degree of activity. It is probably this same property which causes chlorinated preservatives to be absorbed by rubber closures to a greater extent than the nonhalogenated analogs.

TABLE IX.—RELATIVE BACTERICIDAL ACTIVITY OF PRESERVATIVES

Preservative[a]	Lowest Concentration, %, Required to Destroy All Test Organisms in 15 Minutes or Less, But Not in 10 Minutes	
	S. aureus	*E. coli*
p-Chloro-β-phenylethyl alcohol	0.10	0.15
Chlorobutanol	0.28	0.30
Phenylethyl alcohol	0.50	0.50
Benzyl alcohol	0.90	0.80
Methylparaben	0.25	0.25

[a] All preservative solutions were buffered to pH 4.0.

Studies of the self-sterilizing properties of the five preservatives were carried out at concentrations most commonly used for parenteral solutions. The results of this study are given in Table X. These data show that all the preservatives tested are very effective against *S aureus* and *E. coli.* However, against spores of *B. cereus*, benzyl alcohol seems to be the most effective preservative, with *p*-chloro-β-phenylethyl alcohol next in effectiveness. Of the five preservatives, phenylethyl alcohol is the least effective as a sporicidal agent.

Assays based on the chemical analysis of preservative in pharmaceutical solutions may, in certain instances, yield data of questionable significance. It is conceivable that a chemical breakdown of an effective preservative, as for example the loss of a chlorine substituent, may yield a product which *per se* possesses intrinsic antibacterial properties. Thus, an assay which is indicative of chemical breakdown or alteration of a preservative can be misleading, since the degraded product may exert demonstrable antimicrobial activity.

In view of these theoretical considerations, it was of interest to attempt the correlation of a chemical breakdown of *p*-chloro-β-phenylethyl alcohol in solution with a loss, if such should prove to be the case, of antibacterial activity as determined by microbiological methods. The method used for determining the latter activity involved the establishment of a standard curve obtained by plotting the ET_{50} (time required for freshly prepared standard solutions to destroy 50% of the test organisms) *vs.* the logarithm of preservative concentration. The best fitting straight line was computed by the method of least squares. Because of difficulties experienced thus far in reproducing the slope of the standard curve, the microbiological data with respect to the assay of chemically degraded solutions are not available at this time for interpretation. The results of this study, however, will be presented at a later date.

TABLE X.—SELF-STERILIZING PROPERTIES OF SELECTED PRESERVATIVES

Preservative	Time Required to Destroy the Following Percentages of Test Organisms,[a] hr. 50%			95%		
	E. coli.	*S. aureus*	Spores of *B. cereus*	*E. coli*	*S. aureus*	Spores of *B. cereus*
pH 4 Buffer solution	1–3	1–3	168	5–24	3–5	>168
p-Chloro-β-phenylethyl alcohol (0.3%)	<1	<1	5–24	<1	<1	48–72
Phenylethyl alcohol (0.5%)	<1	<1	48	<1	<1	<168
Benzyl alcohol (1.0%)	<1	<1	5	<1	<1	48–72
Methylparaben (0.2%)	<1	<1	24–48	<1	<1	<168
Chlorobutanol (0.5%)	<1	<1	24–48	<1	<1	>168

[a] Inoculum: *E. coli*, 700,000; *S. aureus*, 500,000; *B. cereus* spores, 50,000.

It is evident from this investigation that the choice of a rubber stopper for a particular vial solution requires considerable study to select one which exhibits optimal stability. Such a study should include the testing of the vial solutions stored in an upright as well as in an inverted position. In order to accomplish this most effectively, close cooperation must exist between the pharmaceutical and rubber stopper manufacturers.

Additional studies are under way to determine the effect of coated stoppers on preservative loss from solution and the influence of nonaqueous parenteral vehicles on closure properties.

SUMMARY

In this investigation an attempt was made to determine the contributing factors responsible for the loss of preservative from multiple-dose vial solutions. The preservatives evaluated were (*a*) chlorobutanol, (*b*) *p*-chloro-β-phenylethyl alcohol, (*c*) phenylethyl alcohol, (*d*) methylparaben, and (*e*) benzyl alcohol. Solutions of these preservatives in multiple-dose vials were stoppered with three commonly used rubber closures representing natural rubber, neoprene polymer, and butyl polymer. The results obtained are summarized as follows:

1. Preservative solutions in vials stoppered with rubber closures exhibited a substantially greater loss in preservative content than ampul solutions of the same preservative.

2. Vials stored in an inverted position demonstrated a greater loss in preservative content than those stored upright.

3. The natural rubber stoppers exerted the least deleterious effect on preservative content in solution.

4. The butyl rubber closure was found to be physically incompatible with the preservative solutions tested due to a leaching of an accelerator residue.

5. Vial solutions of chlorobutanol and *p*-chloro-β-phenylethyl alcohol stored in an inverted position at room temperature exhibited as much as 30% reduction in preservative content in less than 8 weeks. Since it is not unusual for vials to be stored in a manner whereby their contents are in direct contact with the rubber stopper, these results warrant serious consideration when formulating multiple-dose vial preparations.

6. Distribution studies on *p*-chloro-β-phenylethyl alcohol and phenylethyl alcohol have shown that the chlorinated compound is absorbed into the rubber to a substantially greater degree than the nonchlorinated analog.

7. Results obtained from studies on the comparative bactericidal activity of the preservative solutions have shown that *p*-chloro-β-phenylethyl alcohol was the most effective agent aginst *E. coli* and *S. aureus*.

8. An attempt was made to relate loss in preservative content with corresponding changes in antimicrobial activity.

REFERENCES

(1) "British Pharmacopoeia," 1953 ed., The Pharmaceutical Press, 17 Bloomsbury Square, London W.C. 1, 1953, p. 850.
(2). Masucci, P., and Moffat, M. I., THIS JOURNAL, **12**, 117(1923).
(3) McGuire, G., and Falk, K. G., *J. Lab. and Clin. Med.*, **22**, 641(1937).
(4) Wiener, S., *J. Pharm. and Pharmacol.*, **7**, 118(1955).
(5) Berry, H., *ibid.*, **5**, 969(1953).
(6) Wing, W. T., *ibid.*, **7**, 648(1955).
(7) Wing, W. T., *ibid.*, **8**, 734(1956).
(8) Wing, W. T., *ibid.*, **8**, 738(1956).
(9) Nielsen, A. B., *Dansk Tidsskr. Farm.*, **32**, 109(1958).
(10) Royce, A., and Sykes, G., *J. Pharm. and Pharmacol.*, **9**, 814(1957).
(11) Siddell, D. F., *Bull. Parenteral Drug Assoc.*, **8**, 17 (Nov.-Dec. 1954).
(12) Lachman, L., and Cooper, J., THIS JOURNAL, **48**, 227 (1959).
(13) Rehm, C. R., and Mader, W. J., *ibid.*, **46**, 621(1957).
(14) Stahl, E., *Pharmazie*, **11**, 633(1956).
(15) Ray, N., and Basu, U. P., *Indian J. Pharm.*, **12**, 6 (1950).
(16) van Esveld, L. W., *Pharm. Weekblad.*, **71**, 1318 (1934).
(17) Gershenfeld, L., *Am. J. Pharm.*, **124**, 363(1952).
(18) Taub, A., and Luckey, W. H., THIS JOURNAL, **32**, 28 (1943).
(19) Murphy, J. T., Allen, H. F., and Mangiaricine, A. B., *Arch. Ophthalmol.*, **53**, 63(Jan.-June 1955).
(20) Gladhart, W. R., Jr., Wood, R. M., and Purdum, W. A., *Bull. Am. Soc. Hosp. Pharmacists*, **12**, 534(1955).
(21) Nair, A. D., and Lach, J. L., THIS JOURNAL, **48**, 390 (1959).
(22) Royce, A., and Sykes, G., *J. Pharm. and Pharmacol.*, **9**, 814(1957).
(23) Hess, H., and Speiser, P., *ibid.*, **11**, 650(1959).
(24) Hess, H., and Speiser, P., *ibid.*, **11**, 694(1959).