different mechanisms of action for cortisone and phenytoin must be involved in the inhibition of palate closure in this strain of mice. Additional studies involving high and low dietary intake of cobalt to confirm this hypothesis are being contemplated.

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Differences in Antibacterial Activity of Benzalkonium Chloride

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Abstract □ Benzalkonium solutions obtained from different manufacturers were shown to have different activities. This difference in activity was related to the composition of the benzalkonium chloride. The potential seriousness of this situation is emphasized, and a recommendation is made that the official monographs on benzalkonium chloride be amended appropriately, noting the apparently superior antibacterial activity of the tetradecyl (C_{14}) homolog.

Keyphrases □ Benzalkonium chloride—various commercial solutions, antibacterial activity evaluated and related to composition □ Antibacterial activity—evaluated in various commercial solutions of benzalkonium chloride, related to composition

The International Pharmacopoeia (IP 1967) (1) allows the alkyl chain of benzalkonium chloride to be a mixture of the alkyls from C_8H_{17} to $C_{18}H_{37}$. USP XIX (2) states that the alkyl chain may represent a mixture including all or some of the group beginning with C_8H_{17} and extending through higher homologs, with the $C_{12}H_{25}$ homolog representing not less than 40%, on the anhydrous basis, and the $C_{14}H_{29}$ homolog representing not less than 20% of the total alkylbenzyldimethylammonium chloride content; the two homologs together must comprise not less than 70% of the total alkylbenzyldimethylammonium chloride content.

BP 1973 (3) allows a mixture of alkylbenzyldimethylammonium chlorides, without specifying any particular homolog or percentage composition.

This permitted variation in the alkyl chain length may contribute to the variation in benzalkonium chloride activity (compare Refs. 4 and 5 and Refs. 6 and 7). Therefore, the comparative antibacterial activity of four commercial benzalkonium solutions was investigated to determine whether differences occur in the antibacterial activity due to variations in the number of carbon atoms in the alkyl radical and the relative percentage of particular chain lengths in a given benzalkonium chloride. Such investigations should also indicate whether a given formulation preserved with benzalkonium chloride might have different preservative capacities, depending on the commercial source of benzalkonium chloride.

Two benzalkonium solutions had approximately 50% tetradecyl (C_{14}) derivative, and the other two products had approximately 65% dodecyl (C_{12}). Therefore, with respect to the proportion of the C_{12} and C_{14} homologs, the four products were divided into two pairs. Other differences in composition, however, also existed among the four solutions.

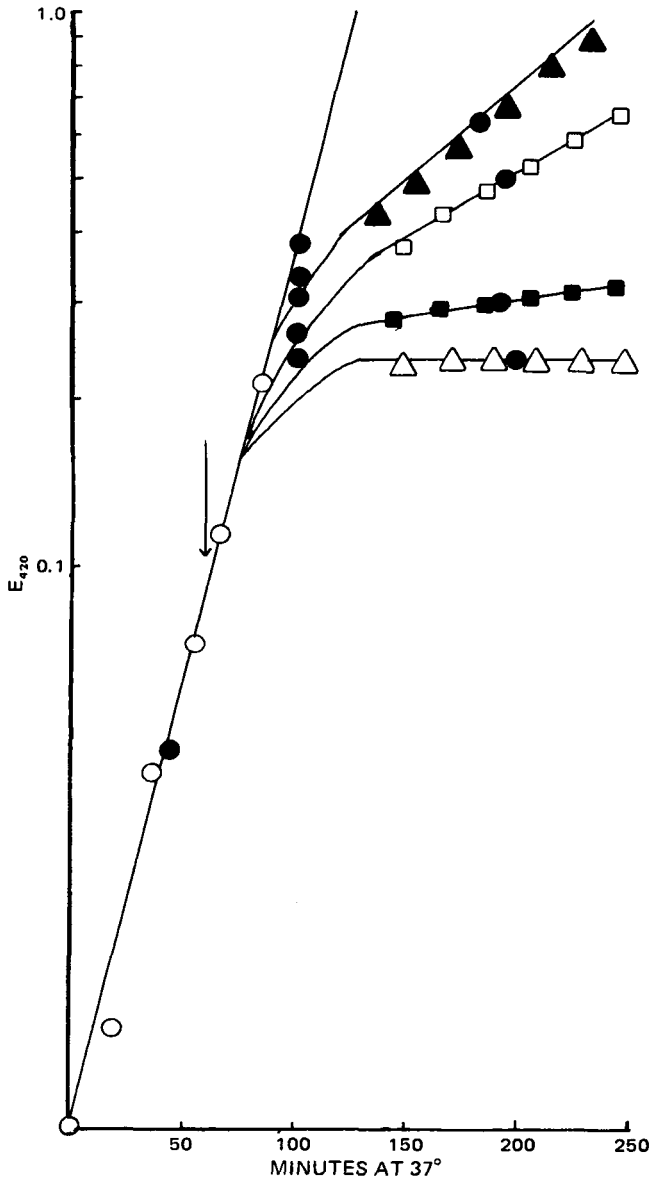

Figure 1—*Effect of a final concentration of 3 μg/ml of Solutions A, B, C, and D, separately, on the growth rate of* E. coli. *Key:* ○, *control culture (no benzalkonium added);* ■, *Solution A;* □, *Solution B;* △, *Solution C;* ▲, *Solution D; and* ●, E_{420} *values at given times calculated from the experimentally determined* E_{420} *values at known times for each culture. Addition of benzalkonium was made at the time indicated by the arrow.*

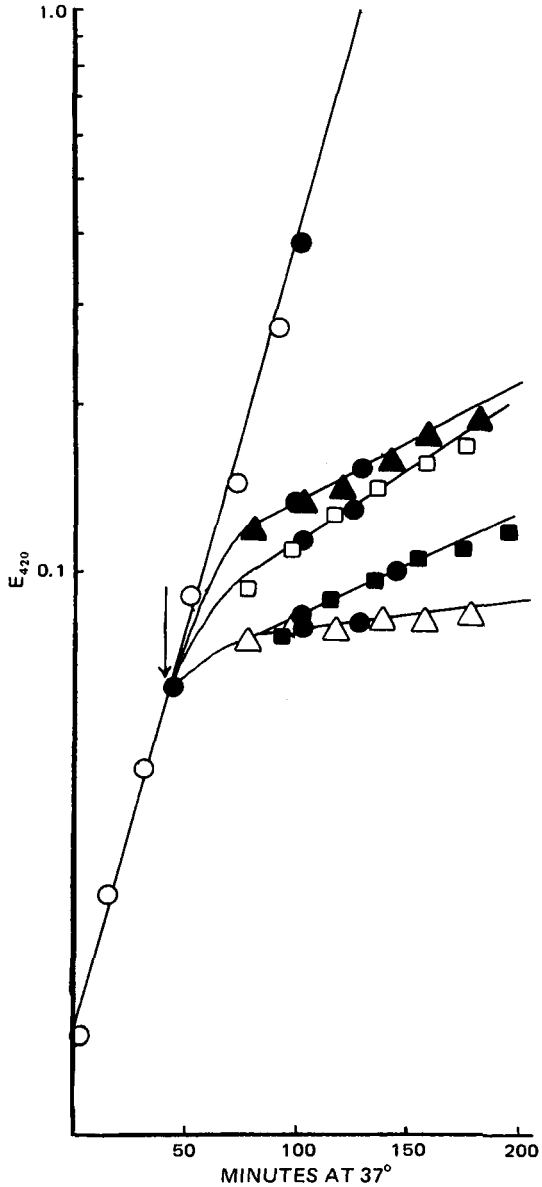

Figure 2—*Effect of a final concentration of 0.8 μg/ml of Solutions A, B, C, and D, separately, on the growth rate of* S. aureus. *Key:* ○, *control culture (no benzalkonium added);* ■, *Solution A;* □, *Solution B;* △, *Solution C;* ▲, *Solution D; and* ●, E_{420} *values at given times calculated from the experimentally determined* E_{420} *values at known times for each culture. Additions of benzalkonium was made at the time indicated by the arrow.*

EXPERIMENTAL

Chemicals, Organisms, and Media—The properties of the four solutions of benzalkonium chloride (A, B, C, and D) are given in Table I. Solutions B, C, and D comply with USP specifications, and all four comply with the BP and IP. *Escherichia coli*[1], *Staphylococcus aureus*[2], and *Pseudomonas aeruginosa*[3] were the test organisms. Nutrient broth No. 2[4] was the growth medium for liquid cultures, and nutrient agar[4] was the growth medium for solid cultures.

Methods—The evaluations used either log phase cultures for growth rate studies or 16-hr cultures for determinations of killing times. Cell numbers were determined by colony counts, and the cultures were maintained as already described (8).

Effect on Growth Rate—Log phase cultures were prepared, and the effects of 3 μg/ml of Solutions A, B, C, and D were determined on the growth rate of *E. coli* by the method described for determining the effect of three aromatic alcohols on log phase *P. aeruginosa* (9). The results are illustrated in Fig. 1.

The procedure was repeated using 0.8 μg/ml of Solutions A, B, C, and D against log phase *S. aureus* (Fig. 2).

The effects of equimolar concentrations of each solution against log phase *E. coli* and *S. aureus* cultures were determined, and the results were again expressed graphically (Figs. 3 and 4).

Effect on Cell Death—Tubes containing 9.9 ml of the aqueous solutions under test were equilibrated in a water bath at 22–23°. Then 0.1 ml of a 16-hr culture of either *P. aeruginosa, S. aureus,* or *E. coli* was added to give a final inoculum of approximately 2×10^6 cells/ml. At intervals of 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, and 360 min after inoculum addition, 0.5-ml samples were aseptically transferred to 4.5 ml of inactivating recovery medium [of the composition already described

[1] NCTC 8196, National Collection of Type Cultures, Colindale, London, England.
[2] NCTC 6571, National Collection of Type Cultures, Colindale, London, England.
[3] NCTC 6750, National Collection of Type Cultures, Colindale, London, England.
[4] Oxoid, Oxo Ltd., London, England.

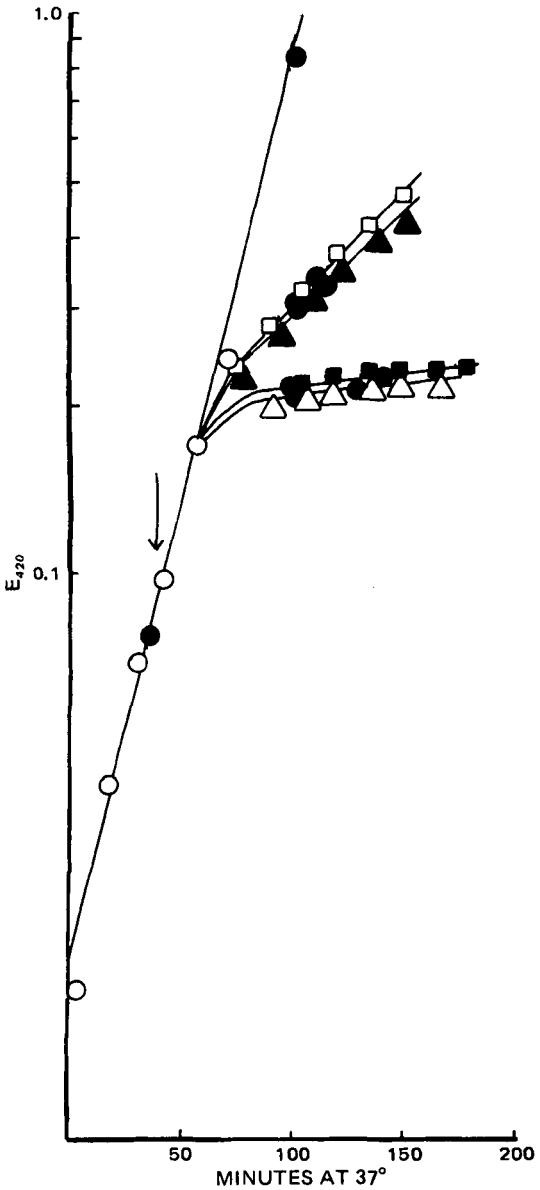

Figure 3—*Comparison of effect of equimolar concentrations of Solutions A, B, C, and D, separately, on the growth rate of* E. coli. *Key:* ○, *control culture (no benzalkonium added);* ■, *Solution A (3.12-μg/ml final concentration of benzalkonium);* □, *Solution B (2.91 μg/ml);* △, *Solution C (3.00 μg/ml);* ▲, *Solution D (2.96 μg/ml); and* ●, E_{420} *values of given times calculated from the experimentally determined* E_{420} *values at known times for each culture. Addition of benzalkonium was made at the time indicated by the arrow.*

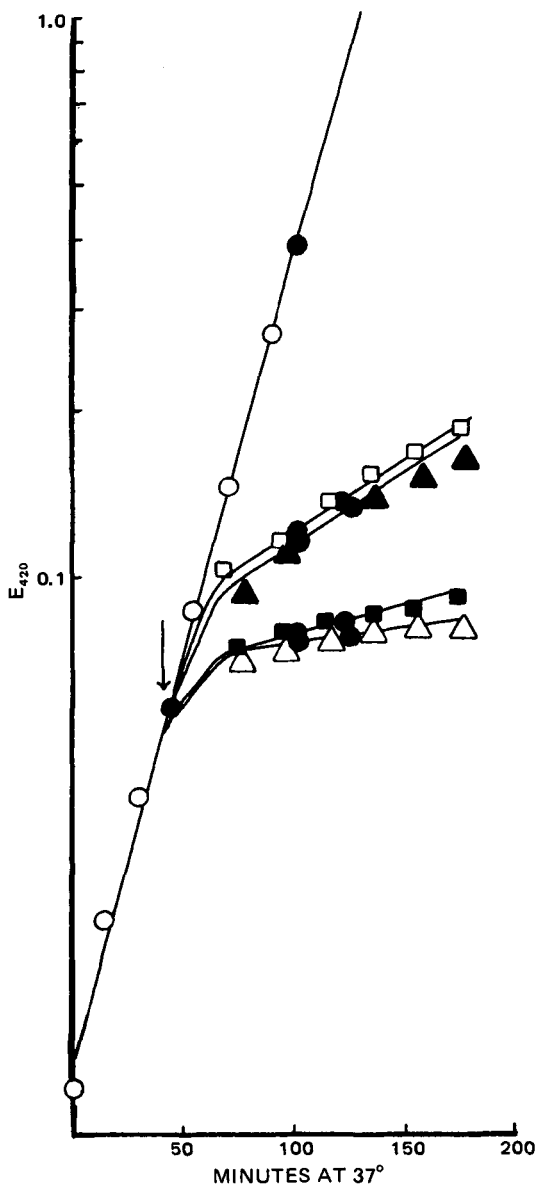

Figure 4—*Comparison of effect of equimolar concentrations of Solutions A, B, C, and D, separately, on the growth rate of* S. aureus. *Key:* ○, *control culture (no benzalkonium added);* ■, *Solution A (0.83-μg/ml final concentration of benzalkonium);* □, *Solution B (0.78 μg/ml);* △, *Solution C (0.80 μg/ml);* ▲, *Solution D (0.79 μg/ml); and* ●, E_{420} *values at given times calculated from the experimentally determined* E_{420} *values at known times for each culture. Addition of benzalkonium was made at the time indicated by the arrow.*

(5) but without the added agar] and incubated at 37° for 48 hr. Controls were carried out as described (9). The results are given in Table II.

RESULTS AND DISCUSSION

The addition of the same concentration of Solutions A, B, C, and D in subinhibitory amounts to log phase cultures of *E. coli* or *S. aureus* resulted in varying depressions of the growth rate (Figs. 1 and 2). When equimolar concentrations of A, B, C, and D were used, A and C produced a similar response and B and D had similar activity to each other (Figs. 3 and 4).

The results show that altering the composition of benzalkonium does result in different antibacterial activity and that solutions containing predominantly the tetradecyl (C_{14}) homolog apparently have greater activity than solutions consisting mainly of the dodecyl (C_{12}) homolog.

Further growth rate experiments with *S. aureus* cultures showed that Solutions B and D had to be added at twice the concentration as that of Solutions A and C to produce the same degree of inhibition on the growth rate. Thus, Solutions A and C were approximately twice as active as B and D against log phase *S. aureus* cultures.

Killing times for the four solutions against an inoculum of approximately 2×10^6 cells/ml of 16-hr cultures of *E. coli* or *S. aureus* showed a similar pattern (Table II). The concentration of A, B, C, and D used in these determinations was chosen to demonstrate the differences in activity. At high concentrations, all solutions would have a rapid killing time. However, low concentrations may have to be used in pharmaceutical preservation because of the constraints of the formulation, as in certain contact lens solutions, or the original concentration of the antibacterial may be depleted because of interactions with the container or other formulation components. The different levels of activity obtained with A, B, C, and D could have practical importance; at a concentration where two solutions exhibited fair antibacterial activity, the other two solutions were not able to kill the test inoculum of *S. aureus* during the 6-hr evaluation.

Minimum inhibitory concentration determinations and minimum bactericidal concentration determinations of the benzalkonium solutions

Table I—Properties of Four Benzalkonium Chloride Solutions as Supplied by Manufacturers

Property	Solution A	Solution B	Solution C	Solution D
Composition of alkyl chain	—	C_{10}, 3.5%	—	—
	C_{12}, 25–30%[a]	C_{12}, 67.5%	C_{12}, 40%	C_{12}, 60–65%
	C_{14}, 50–55%	C_{14}, 24.0%	C_{14}, 50%	C_{14}, 25–30%
	C_{16}, 15–20%	C_{16}, 5.0%	C_{16}, 10%	C_{16}, 10–15%
Molecular weight	375–382	348.04	359.6	355–362
Active matter, %	50.0–50.5	50 ± 1.5 (w/v)	50–52 (v/v)	50.0–50.5
Inert ingredients	3% maximum solubilizing agents/cloud-point modifiers	—	10% (w/v) ethyl alcohol 40% (w/v) water	2% maximum solubilizing agents/cloud-point modifiers
pH	7.0–7.5	8.0 ± 1.0 (5% v/v)	10.3–10.7	7.0–7.5
Free amine, %	0.5 maximum	0.3 maximum	Not given	0.5 maximum

[a] All percentages in this table are w/w unless otherwise specified.

in nutrient broth inoculated with *S. aureus* showed the same pattern of results. Solutions A and C were active at lower concentrations than Solutions B and D.

The problem was even greater with *P. aeruginosa* (Table II). At a concentration of benzalkonium chloride widely recommended for use in preserving multidose ophthalmic solutions, Solution C inactivated approximately 2×10^6 *P. aeruginosa* cells/ml in less than 15 min but Solution B required more than 4 hr to achieve the same result. Each solution had a different activity against this test organism, but the solutions containing mainly the C_{14} homolog were more active.

The pH of all reaction mixtures tested was similar (7.30–7.75), so differences in benzalkonium activity due to differences in pH were discounted.

Prior to 1956 (5), many misleading results were obtained in evaluating the antibacterial activity of benzalkonium chloride because of inadequate techniques, particularly in failing to inactivate the benzalkonium carried over with the bacteria sample to be tested for viability or death. Moreover, the previous history of the test organism, the inoculum size, the spacing of the contact times, the homogeneous mixing of organisms in the antibacterial solution, the pH and temperature of the bacteria–antibacterial reaction mixtures, the presence of other chemicals, the size of the test sample withdrawn in proportion to the volume of the reaction mixture sampled, the time period and temperature of incubation of the test sample, and the nutritional capacity of the culture medium used for culturing the damaged bacteria all influence the assessments of antibacterial activity.

When all of these factors have been understood and adequately provided for in the experimental technique, however, it appears that an investigator may be unwittingly deceived in any overall assessment of the activity of benzalkonium chloride based on the results obtained with one particular product. The inconsistencies existing in the literature due to this factor may be quite impossible to unravel. However, in the future the official monographs describing benzalkonium chloride should specify a product that has a much more precise composition and also note the apparently superior antibacterial activity of the C_{14} homolog.

Table II—Killing Times at 22–23° for Solutions A, B, C, and D, 0.0025% against 16-hr *S. aureus* and *E. coli* Cultures and 0.01% against 16-hr *P. aeruginosa*

Organism[a]	Antibacterial	Killing Time, min
S. aureus	A	90–120
	B	>360
	C	60–90
	D	>360
E. coli	A	150–180
	B	300–360
	C	120–150
	D	>360
P. aeruginosa	A	45–60
	B	240–300
	C	<15
	D	90–120

[a] Initial bacterial concentration in antibacterial solution was approximately 2×10^6/ml. The pH of all solutions was in the 7.30–7.75 range.

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