NOTES

Analysis of Quaternary Ammonium Compounds and Hexachlorophene by Thin-layer Chromatography and Agar Diffusion Bioautography

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As part of a program to evaluate the antimicrobial effectiveness of various oral hygiene formulations, a rapid thin-layer chromatographic method was developed for the separation and identification of quaternary ammonium compounds and hexachlorophene (G-11). The chromatographic locations of antibacterial components of commercial mouthwashes and dentifrices was facilitated by coupling a bioautographic technique with relatively nonspecific staining reactions.

A dye marker consisting of 0.04% (w/v) bromthymol blue and 0.2% (w/v) methyl red in ethyl alcohol was chromatographed routinely as an aid in locating and identifying the compounds under investigation. This was particularly important when bioautographs were to be obtained from unsprayed plates.

Three solvent systems were used on two types of migration media. **System 1:** Prepare samples in ethyl alcohol (95%, v/v)-chloroform (1:1, v/v); develop on Eastman Chromagram Sheet type K 301 R2 (Silica Gel) in ethyl alcohol (95%)-chloroform-water (105:75:10, v/v/v); time, 30 min for 10 cm (Fig. 1) when chromatographed in the Eastman developing apparatus. **System 2:** Prepare samples in ethyl alcohol (95%)-chloroform (3:5, v/v); develop on Silica Gel G, 250 μ chromatoplates with ethyl alcohol (95%)-chloroform (3:5); time, 25 min for 10 cm; activation of the plate at 110°C for 2 hr increases the RF values (Fig. 2). **System 3:** Prepare most samples in ethyl alcohol; quaternary ammonium standards separate better when dissolved and applied in ethyl alcohol-water (15:85, v/v); develop in ethyl alcohol (95%)-chloroform-water (36:60:1) on Silica Gel G plates deactivated by storing in a chamber over a saturated solution of NaBr (58% relative humidity).

Quaternary ammonium compounds were detected by spraying with a potassium iodoplatinate solution (K. Randerath, *Thin-Layer Chromatography*, p. 157, Academic Press, Inc., New York, 1963) prepared by mixing 45 ml of 10% potassium iodide with 5 ml of 5% platinic chloride and diluting to 100 ml with water. Characteristic
blue, green, or purple spots appeared. Since these faded rapidly, a photographic record was made immediately.

Hexachlorophene was detected by spraying the plate with ammoniacal silver nitrate (S. M.

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**FIG. 2.** Thin-layer chromatogram of benzalkonium chloride (2), benzethonium chloride (3), cetyl pyridinium chloride (4), and a mixture of the three (1) on Silica Gel G. The bromthymol blue spot (5) was scored before spraying the plate with potassium iodoplatinate reagent. (See system 2 in text.)

**FIG. 3.** Thin-layer chromatography of quaternary ammonium compounds. Cetyl pyridinium chloride (2), benzethonium chloride (3), and benzalkonium chloride (4) react with potassium iodoplatinate (top) and produce zones of inhibition with Staphylococcus aureus (bottom). The mixture (5) gives a pattern similar to that in an experimental mouthwash (7) containing the three quaternary compounds. Only one of the quaternaries is readily detectable in an extract of buccal tissues (6) obtained several hr after gargling with the mouthwash. Hexachlorophene (8) is shown for comparison. The methyl red and bromthymol blue spots (1) were scored before pouring the agar (see System 3 in text).

**FIG. 4.** Thin-layer chromatography of hexachlorophene (G-11) and sodium lauryl sulfate (SLS). Exposure to iodine vapors produces a brown spot (upper photo) with 5 μg of G-11 (3) but not with 0.05 (4) or 0.025 (5) μg. Only the lowest concentration of G-11 was applied on the bioautogram (lower). Numbers indicate: (1) Methyl red, (2) bromthymol blue, (6) extract of buccal tissue obtained several hr after brushing with a dentifrice containing G-11 and SLS, and (7) 5 μg of SLS. Detergent and bisphenol produce zones of inhibition but are too dilute to react with iodine, a nonspecific stain which reacts with other unidentified tissue components.
Partridge, Biochem. J. 42:238, 1948) and heating, or by exposure to iodine vapors. Shortwave, ultraviolet light may be used when hexachlorophene is chromatographed on plates with fluorescein incorporated into the silica gel.

No satisfactory spray reagent was found for the detection of sodium lauryl sulfate.

For bioautography, Silica Gel G plates (100 × 100 mm) were used. Untreated chromatograms were placed on a hardened base layer of agar in round phage typing glass or plastic dishes (150 × 25 mm). Approximately 40 ml of Trypticase Soy Agar (BBL), liquified at 48 C and seeded with an 18- to 24-hr culture of Staphylococcus aureus ATCC 6538 was poured carefully over the chromatogram and allowed to harden. After 18 to 24 hr of incubation at 37 C, the dish was flooded with 2 ml of 0.1% (w/v) aqueous solution of Neotetrazolium chloride (Nutritional Biochemicals Corp., Cleveland, Ohio) to facilitate location of the zones of inhibition.

The methods described above were used successfully to study the persistence of antibacterial substances in oral tissues and plaque subsequent to use of a mouthwash or dentifrice. At intervals up to 15 hr after brushing or gargling, buccal scrapings were obtained by using a curette with a capacity of approximately 7 μliters. The tissue was extracted with ethyl alcohol and the eluate was concentrated and chromatographed quantitatively (Fig. 3 and 4).

The bioautographic method developed by the authors is similar to that originally described for antibiotics by J. R. Nicholaus, C. Coronelli, and A. Binaughi [Farmaco (Pavia), Ed. Prat. 8:349, 1961] and refined by R. M. Kline and T. Golab (J. Chromatog. 18:409, 1965). The use of smaller chromatoplates placed directly into large petri dishes and the application of vital stain after incubation are important simplifications of the technique.

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