more sensitive, the spots tend to fade rapidly one to two hours after spraying. Charring with H_2SO_4 is preferable if the spots should remain visible for documentation.

Erythromycin A and B, erythromycin A "Hemiketal"[2], anhydroerythromycin[6], and several erythromycin acetates were separated using the methylene chloride–methanol–benzene–formamide solvent system (Figs. 1 and 2).

Thin-layer chromatography on silica gel G plates using the methylene chloride–methanol–benzene–formamide system has proven to be an efficient and sensitive method for the separation of erythromycins. This procedure is preferable to paper chromatography since the time of analysis is greatly decreased.

Acknowledgement

The author wishes to thank Dr. P. H. JONES for supplying the erythromycin samples and giving guidance and encouragement.

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Received August 5th, 1963

J. Chromatog., 14 (1964) 127–129

Bioautography of antibiotic spread-layer chromatograms

Thin-layer chromatography (TLC) of antibiotics is receiving increasing attention[1–6]. In comparison with paper chromatography, TLC has greater resolving power, is the more rapid of the two techniques and can be more easily scaled up to preparative scale. A necessary adjunct to any chromatographic technique is a means of detection; with TLC, U.V. absorption, U.V. fluorescence, color producing reagents, charring with mineral acids and heat, and bioautography are methods in use. For antibiotics, bioautography is preferable and necessary because it is usually more sensitive than chemical methods and detects only biologically active components.

Several reports in the literature describe bioautography of antibiotics separated by TLC. NICOLAUS, CORONELLI AND BINAGHI[1] poured agar seeded with *Bacillus subtilis* over the developed chromatographic plate, while BICKEL *et al.*[3] and BRODASKY[5] pressed the chromatographic plate onto seeded agar plates. These investigators used adsorbents containing binder. In our studies, we have used the spread-layer technique[7–9], in which no binder is present in the adsorbent. Some advantages of this method are: alumina plates can be prepared and used within a matter of minutes, alumina of the desired activity can be used and the need for costly spreading devices

is eliminated. The purpose of this communication is to describe a means for the bioautography of such chromatograms. The applicability of this method to antibiotics with different biological activities will be demonstrated.

Experimental

Alumina (Woelm) and silica gel H (Merck) were spread on 3 × 8 in. glass plates by means of a glass rod. A double thickness of adhesive tape was wrapped about each end of the glass rod so that the tape rested on the edges of the glass plate when the rod was held in a horizontal position. The thickness of the tape wrapping determines the clearance between the glass rod and the surface of the plate, and hence the thickness of the adsorbent layer. The rod is drawn back and forth over the plate several times, removing any surface imperfections and excess adsorbent. The adsorbent layers used in our studies were approximately 0.5 mm thick. Alumina, of the required activity, was spread as a dry powder and silica gel H as an aqueous slurry prepared by the addition of 2–2.5 parts water. The alumina plates were ready for immediate use whereas the silica gel H plates were air dried at room temperature overnight before use. The antibiotics and chromatographic systems used were: thiostrepton — neutral alumina adjusted to activity IV, developed with 5 % methanol in chloroform; erythromycin, nystatin and polymyxin B sulfate — silica gel H developed with methanol.

For bioautography with bacteria, 200 ml of Base Agar and 100 ml Seed Agar (Baltimore Biological Laboratories, Baltimore, Maryland), each with added 0.5 % sodium chloride, were poured into 8 × 13 in. sterile Pyrex baking dishes covered with aluminum covers. The Seed Agar contained 1 ml of an aqueous 2 % (w/v) solution of 2,3,5-triphenyl-2H-tetrazolium chloride and 2.5 ml of a 24 h broth culture of the indicator organism. *Staphylococcus aureus* 209 P and *Escherichia coli* ATCC 10536 were grown in Penassay Broth (Difco Laboratories, Detroit, Michigan) and *Streptococcus lactis* (Squibb Culture Collection No. 1783) in Micro Inoculum Broth (Difco). For the yeast, *Saccharomyces cerevisiae* (Squibb Culture Collection No. 1600), the agar medium was of the following composition: tryptone, 5 g; malt extract, 3 g; glucose, 10 g; yeast extract, 3 g; agar, 15 g; distilled water, 1 l. This medium, lacking agar, was used to prepare the inoculum, which was 2.5 ml of a 24 h culture incorporated into the seed agar. No dye was added to this medium.

For bioautography, a 3 × 11 in. sheet of Whatman No. 1 paper, wetted by dipping through distilled water, was centered over a clean glass plate of the same size as the chromatographic plate. The filter paper tabs, extending beyond each end of the glass plate, were folded back over the glass plate and the assembly carefully placed on top of the chromatographic plate, resulting in a sandwich, with the filter paper and adsorbent layer between the glass plates. The filter paper tabs were folded back over the ends of the chromatographic plate, and the sandwich inverted so that the chromatographic plate was on top and the glass support plate on bottom. The latter was set aside, leaving the filter paper adhering to the glass chromatographic plate by means of the filter paper tabs. This assembly was carefully placed on the surface of the seeded agar.

Initially, our technique consisted of incubating the developed chromatographic plate on the agar surface seeded with *S. aureus* 209 P for 16 h at 37°. This method was unsuccessful because the organism did not grow under the chromatographic plate. The technique was modified by removing the chromatographic plate and filter paper

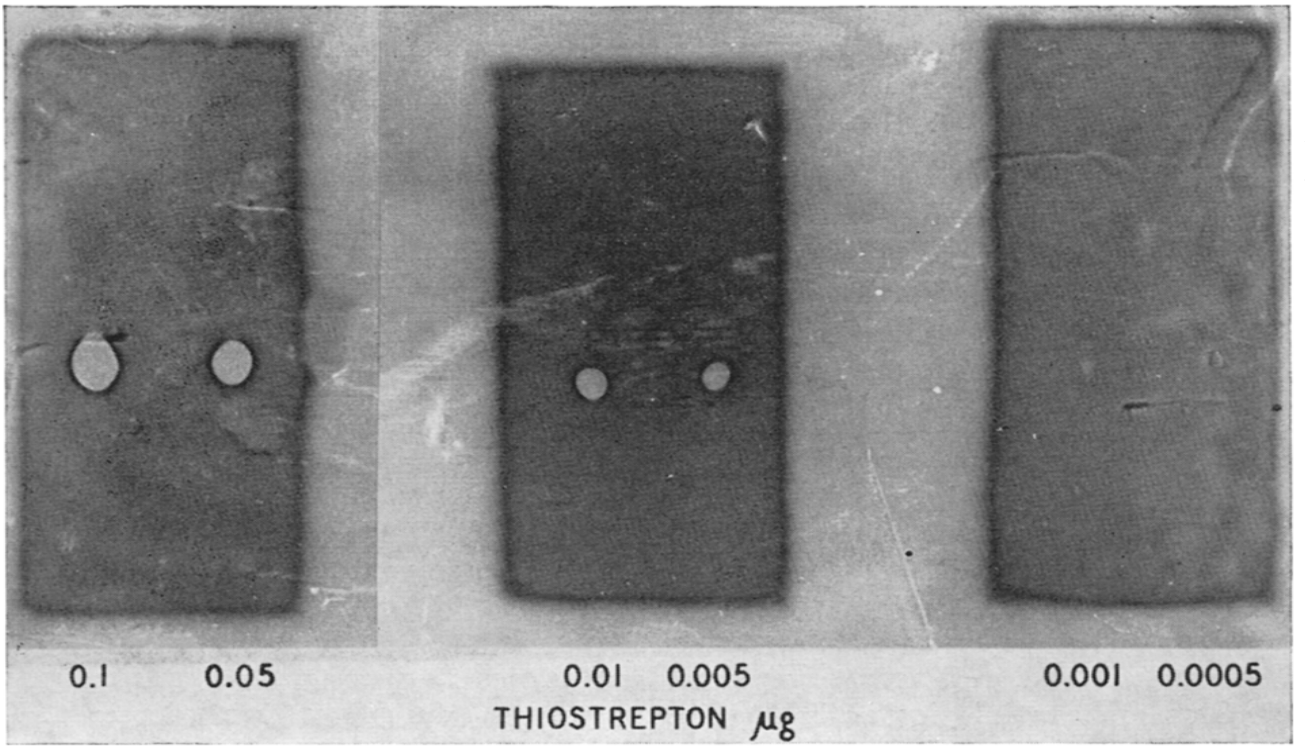

Fig. 1. Bioautogram of TLC plates of varying amounts of thiostrepton.

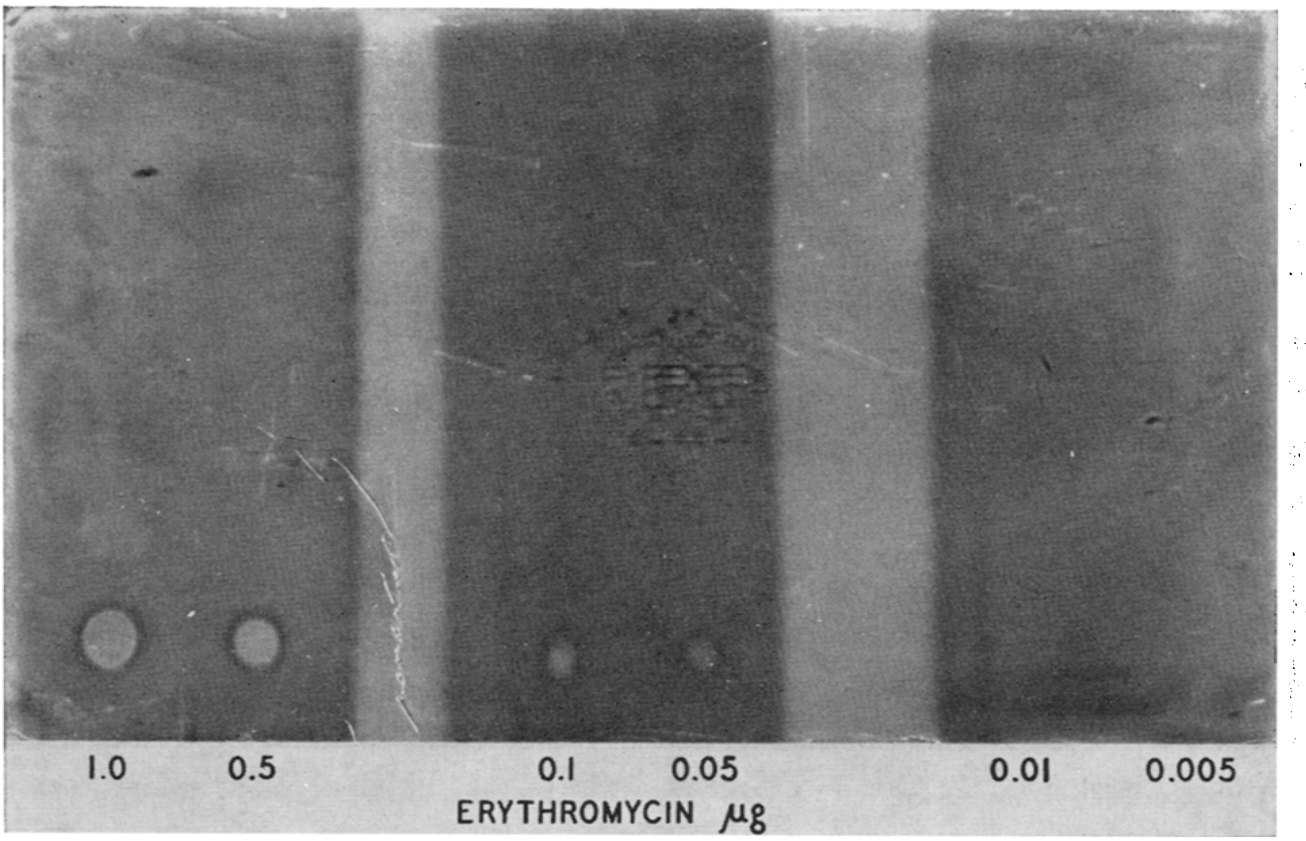

Fig. 2. Bioautogram of TLC plates of varying amounts of erythromycin.

after one hour diffusion time at room temperature, and subsequent incubation at 37° for 16 h. However, this method did not yield consistent results. The procedure as developed with *S. aureus* 209 P was to incubate the chromatographic plate on the seed agar surface for 16 h at 4° to allow diffusion of the antibiotic into the agar. The chromatographic plate and filter paper were removed and the seeded agar examined after an additional overnight incubation at 37°. Sharp, well defined antibiotic zones of inhibition were consistently obtained. Because two overnight incubation periods were inconvenient, a more rapid procedure was sought. *Streptococcus lactis*, facultative in respect to oxygen, was substituted for *Staphylococcus aureus*, and it proved possible to obtain results after overnight incubation at 37° with the chromatographic plate and filter paper lying on the agar surface. Growth of the organism occurred only under the area covered by the glass plate. Sharp, well defined antibiotic zones of inhibition were easily obtained.

The sensitivity of this technique is of the same order of magnitude as that obtained with paper chromatography. Figs. 1 and 2 show the size of the inhibition zones when graded amounts of thiostrepton and erythromycin respectively were chromatographed by TLC and bioautographed against *S. lactis*. As little as 0.001 μg thiostrepton and 0.05 μg erythromycin were detected.

With suitable indicator organisms, antibiotics active against gram negative organisms and antibiotics active against yeast and fungi could be detected. For example, *Escherichia coli* was used to detect polymyxin B sulfate and *Saccharomyces cerevisiae* served as a useful organism for the bioautography of nystatin.

Basic alumina, neutral alumina and silica gel posed no problems in bioautography. None of these adsorbents showed any adverse effect upon growth of the indicator organisms and concomitant reduction of the tetrazolium dye. Acid washed alumina, on the other hand, inhibited the growth of the organisms. With the agar seeded with bacteria, the inhibition was overcome by incorporation of either 0.1 *M* Tris buffer or 0.1 *M* K_2HPO_4 into both Base and Seed Agar, and adjusting the pH to 7.5 prior to sterilization. With agar seeded with the yeast, incorporation of 0.1 *M* KH_2PO_4 into the medium and adjustment of the pH to 6.0 prior to sterilization resulted in good growth under the chromatographic plate.

These bioautographic techniques for spread-layer chromatograms are proving very satisfactory. The convenience of spread-layer chromatography and the specificity and sensitivity of bioautography makes them valuable tools in our studies of antibiotics.

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Received August 2nd, 1963