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Some techniques for bioautography of antimicrobial substances on thin-layer chromatograms

Bioautography is extremely useful for the detection of antimicrobial substances on thin-layer chromatograms and the literature contains several reports on this method of detection and identification. NICHOLAUS, CORONELLI AND BINAUGHI[1] and HENRY, JACOBS AND ACHMETELI[2] poured agar seeded with a sensitive microorganism over the developed chromatographic plate while BRODASKY[3] pressed the chromatographic plate onto seeded agar. The former technique can result in the antimicrobial substance spreading over the plate to produce vast areas of inhibition. KLINE AND GOLAB[4] overcame this by spraying a thin base layer of agar over the chromatoplate before pouring the inoculated agar. Pressing of the chromatoplate on the agar requires the use of microorganisms that can grow anaerobically[5] or the removal of the chromatoplate after diffusion of the antimicrobial substance into the seeded agar. The removal of the plate usually results in the adherence of the adsorbent to the agar surface. The adhering material later can be washed and scraped from the surface[3] to allow inspection of the inhibitory zones. BICKEL *et al.*[6] and MEYERS AND SMITH[5] avoided this problem by inserting a sheet of moistened filter paper between the chromatoplate and the seeded agar. Another approach to improving the zone definition in bioautography has been the incorporation into the agar of a tetrazolium dye[1,5] which is reduced to a colored material by the growing microorganism. A variation has been to flood the agar surface with the vital stain after incubation[2].

During an investigation of the antibacterial substances in the hosts of the phytopathogenic *Xanthomonas pruni*, it became apparent that the above procedures were not applicable. *X. pruni* did not reduce tetrazolium dyes, it was an obligate aerobe, and its normally slow growth was even slower and scantier in the subsurface conditions of a pour plate. Nevertheless, four techniques based on standard microbiological procedures were found to give improved zone definition.

X. pruni hydrolyzes gelatin and starch and produces acid from several sugars[7] and these characteristics can be made indirect indicators of growth. Gelatin hydrolysis as an indicator of microbial growth was ascertained by incorporating 0.4 % gelatin into the nutrient agar. After the incubation period, the agar was flooded with a solution of 10 % mercuric sulfate in 2.5 *N* hydrochloric acid which causes the unhydrolyzed gelatin to form a white precipitate. Hence the zones of inhibition are white and the growth areas have the normal slight turbidity of nutrient agar. Starch hydrolysis as an indicator of microbial growth was determined by incorporating 0.2 % soluble starch into the nutrient agar. After incubation the agar was flooded with 1.0 % iodine in 2.0 % aqueous potassium iodide. The unhydrolyzed starch forms a blue complex with iodine, hence the zones of inhibition are blue and the areas of growth have the normal faint yellow color of nutrient agar. Acid production was detected by incorporating brom cresol purple (1 ml of a 1.6 % solution in ethanol per l of medium) into nutrient agar containing 1.0 % glucose. This microorganism produced only slight amounts of diffusible acids and the zones of inhibition were purple and the growth zones were yellow. A similar technique which was not tried but which should

work would be based on the breakdown and hydrolysis of fat emulsions since this microorganism is lipolytic[7].

The other new technique found to be useful does not utilize seeded agar but rather depends on the formation of a "lawn" on the agar surface similar to those used in phage work. The surface of the hardened but unseeded agar is inoculated with a heavy suspension of the microorganism. After incubation the confluent growth areas are raised on the surface of the agar and the zones of inhibition appear clear and depressed. If the colonial form is opaque or pigmented like *X. pruni*, the contrast between zones of growth and inhibition is very noticeable even against a background of silica gel.

The problem of spreading the antimicrobial substances over the chromatoplates as a result of pouring agar on the chromatoplates was solved by laying preformed sheets of nutrient agar on the surface of the adsorbent. If thin-layers of silica gel on microscope slides were used, the slides were placed in the bottom of a petri dish and a rectangle of nutrient agar cut from another plate was placed on their surfaces with a sterile spatula. Somewhat larger blocks of agar could be handled by using larger spatulas and by increasing the agar concentration to provide a more rigid substrate. If bioautography of a 20 cm × 20 cm plate was desired, a corresponding empty plate was covered with aluminum foil and molten agar of the desired thickness was poured on the foil. After the agar hardened, the developed chromatoplate was pressed onto the agar and the two plates with the agar and foil sandwiched between were inverted. The empty plate was removed and then the foil was peeled from the agar which remained in contact with the adsorbent.

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1 J. R. NICHOLAUS, C. CORONELLI AND BINAUGHI, *Farmaco Pavia, Ed. Prat.*, 8 (1961) 349.
2 S. M. HENRY, G. JACOBS AND H. ACHMETELI, *Appl. Microbiol.*, 15 (1967) 1489.
3 T. F. BRODASKY, *Anal. Chem.*, 35 (1963) 343.
4 R. M. KLINE AND T. GOLAB, *J. Chromatog.*, 18 (1965) 409.
5 E. MEYERS AND D. A. SMITH, *J. Chromatog.*, 14 (1964) 129.
6 H. BICKEL, E. GAUMANN, R. HUTTER, W. SACKMANN, E. VISCHER, W. VOSER, A. WETTSTEIN AND H. ZAHNER, *Helv. Chim. Acta*, 45 (1962) 1396.
7 A. S. BREED, E. G. D. MURRAY AND A. P. HITCHENS, *Bergey's Manual of Determinative Bacteriology*, 6th Ed., Williams and Wilkins, Baltimore, 1948.

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