NOTE

THE USE OF PHENOLPHTHALEIN MONOPHOSPHATE TO ENHANCE BIOAUTOGRAPHY WITH *TRICHOMONAS VAGINALIS*

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(Received for publication May 21, 1979)

Bioautography to detect antibiotics on chromatograms or electrophorograms is of singular importance in antibiotic research, especially in the early stages when pure material is usually not available[1,2]. It is the only technique that can readily distinguish between the desired antimicrobial agents and the accompanying inert components of a fermentation broth or crude preparation.

In some bioautographic systems, however, the contrast between the zones of inhibition and the surrounding lawn of living cells is not always sharply defined. To enhance this contrast many investigators have used redox indicators such as tetrazolium dyes[3], 2,6-dichlorphenolindophenol[1,2] or resazurin[4]. Usually the dye is incorporated into the agar[1], at other times it is added to the already-grown organism after incubation[5]. Either technique results in an enhanced differentiation between areas of growth and inhibition, with clear zones of inhibition contrasting with reddish areas of growth in the case of tetrazoliums and blue zones of inhibition contrasting with a colorless area of growth in the case of 2,6-dichlorphenolindophenol. Although indicators of dehydrogenase activity are most frequently used, several other indicator systems have also been tried. Thus, both starch and gelatin hydrolysis have been used as indirect indicators of growth of *Xanthomonas pruni*,[6] aesculin hydrolysis has been helpful in delineating zones of inhibition in a lawn of *Erwinia*[7], and neutral red was favored in studies with agents cytotoxic to KB cells[8].

In our studies with the protozoan *Trichomonas vaginalis*, the contrast between the zones of inhibition and surrounding areas of growth on bioautographic plates was low, often times rendering interpretation of results difficult. As a consequence, we studied several methods in an attempt to accentuate the contrast between the zones of inhibition and growth. The redox dyes, *i.e.*, the tetrazolium, 2,6-dichlorphenolindophenol and resazurin, did not give the desired response. When 2,3,5-triphenyl tetrazolium chloride (TTC), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and resazurin were tested, autoreduction of the dyes occurred, presumably as a result of the highly reduced state of the medium. With tetrazolium blue [(3,3'-dimethoxy-4,4'-biphenylene)-bis(2,5-diphenyl 2H-tetrazolium chloride)], tetrazolium violet [2,5-diphenyl-3,2 naphthyl tetrazolium chloride] and methyl viologen, no color change was observed.

In this note, we describe a method for the enhancement of the contrast between the zones of inhibition and areas of growth on bioautographic plates of *T. vaginalis*. The rationale for this technique is the hydrolysis of phenolphthalein monophosphate by the organism growing in agar and the subsequent development of the deep-red color of the liberated phenolphthalein upon addition of alkali. No color development occurs in those areas in which the cells have been killed; *i.e.*, the zones of inhibition; these areas retain the color of the medium. Phenolphthalein phosphate had been used previously as a substrate for the phosphatase reaction and as an aid in the identification of microorganisms[9,10,11].

Although a number of antitrichomonal agents were involved in this study, the major portion of the work was done with metronidazole. A variety of chromatographic media were examined; paper, silica gel, silicic acid and alumina. The procedure consists of placing the developed and air-dried chromatograms on the surface of agar seeded with *T. vaginalis* in bioautographic trays. The chromatograms are removed after 30~60 minutes incubation at room temperature, and the agar trays are further incubated at 37°C for 48 hours in an anaerobic chamber. Alternatively, the entire assembly is placed in an anaerobic chamber and incubated for 48 hours at 37°C after which the chromatograms are removed. The surface of the agar is then flooded with a shallow layer of a 1% solution of phenolphthalein monophosphate. After incubation at 37°C

for 1.5 hours aerobically, the surface of the plate is flooded with a shallow layer of a 2 M solution of sodium carbonate, thereby producing color from any free phenolphthalein. In areas where growth has occurred, a deep-red color appears within one minute after the addition of the base, while the zone of inhibition retains the normal faint yellow color of the agar medium. The contrast is very good, the zones of inhibition becoming readily visible especially when viewed against a white background. The color and hence the differentiation is stable over the course of at least 3~4 hours, after which red color does appear within the area of inhibition. The length of the incubation period allowing hydrolysis of the phosphate ester (1.5 hours) and the concentration of the phenolphthalein monophosphate used (1%) were deemed optimal for our conditions. These may change depending upon a number of other factors, *e.g.*, medium, inoculum density, *etc.*

When other phenolphthalein derivatives were similarly evaluated, *i.e.*, the diphosphate, the disulfate and the glucuronate, little or no color change was observed. Cell-free preparations of *T. vaginalis*, made by sonication, were incapable of hydrolyzing the diphosphate or disulfate esters, indicating that permeability was not the problem. Attempts were made to induce synthesis of phosphatases capable of hydrolyzing the diesters by supplementing growing broth cultures of *T. vaginalis* with low levels (0.05%) of either substrate. However, neither whole cells nor cell-free preparations made from them gave rise to the deep-red color of phenolphthalein when each of the substrates was evaluated. Examination of the culture supernates after removal of the cells by centrifugation again failed to reveal any color development with the diesters, although as expected, a deep-red color was obtained with phenolphthalein monophosphate.

A limited survey was done to determine the applicability of the technique to other microbial systems. Included in the survey were *Trichomonas foetus, Staphylococcus aureus, Bacillus cereus, Micrococcus luteus, Clostridium perfringens, Escherichia coli, Bacteroides fragilis, Neisseria gonorrhoea* and *Candida albicans.*

No evidence was obtained with either of the 2 strains of *T. foetus* tested for hydrolysis of any of the phenolphthalein derivatives. On the other hand, both *S. aureus* and *C. perfringens* were able to hydrolyze both the mono and diphosphates, while *B. cereus* and *B. fragilis* were able to hydrolyze the monophosphate, producing a pinkish color resulting in a moderate degree of enhanced visibility of the zones of inhibition. No color change with either substrate was noted with *M. luteus, E. coli* or *N. gonorrhoea.*

The inclusion of phenolphthalein phosphate in agar is not novel[9,10], however, its use as an aid in the enhancement of zones of inhibition in bioautography has, to our knowledge, not been reported previously. Although not a universal indicator, as evidenced by its failure with *T. foetus* and a number of other microorganisms, phenolphthalein monophosphate can be hydrolyzed by a variety of microbial species including *T. vaginalis.* It is hoped that this procedure might find applicability in the study of other protozoans capable of being cultivated in acellular media.

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