CHROM. 5752

The use of tetrazolium salts in bioautographic procedures

WALLHÄUSSER[1] has pointed out that TTC (2,3,5-triphenyl-2H-tetrazolium chloride) as well as other redox indicators such as 2,6-dichlorophenol–indophenol can be successfully employed either as visualizing sprays or as additions to the assay media. Although the use of tetrazolium salts in bioautography is not novel, our experience leads us to believe that their use as visualizing systems in bioautographic procedures could be profitably increased.

Recently, we developed a bioautographic procedure which, due to the microorganism used for detection, gave indistinct zones of inhibition. This difficulty, plus the much greater variety of tetrazolium salts now commercially available, precipitated a study on our part to ascertain if use of one or more of these reagents would resolve our problem.

Materials

TNBT (Tetranitro Blue Tetrazolium) (2,2′,5,5′-tetra-*p*-nitrophenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene]-ditetrazolium chloride), INT (*p*-iodonitrotetrazolium violet) (2-[*p*-iodophenyl]-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride), NBT (Nitro Blue Tetrazolium) (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene]-ditetrazolium chloride), TTC (Tetrazolium Red) (2,3,5-triphenyl tetrazolium chloride), TB (Tetrazolium Blue) (2,2′,5,5′-tetraphenyl-3,3′-[3,3′-dimethoxy-4,4′-biphenylene]-ditetrazolium chloride), and MTT (Methyl Thiazolyl Tetrazolium) (MTT Tetrazolium) (3-[4,5-Dimethyl Thiazolyl-2]-2,5-diphenyl tetrazolium bromide) were obtained from Sigma Chemical Company.

Bioautographs were prepared essentially according to the procedure of KLINE AND GOLAB[2]. Monensin was tested using *Bacillus subtilis* (ATCC 6633), tylosin using *Sarcina lutea* (ATCC 9341), and hygromycin B using *Pseudomonas syringae* (ATCC 12885). The following media were used: *B. subtilis*: $K_2HPO_4 \cdot 3H_2O$, 0.69 g; KH_2PO_4, 0.45 g; yeast extract, 2.5 g; glucose, 10.0 g; Ionagar No. 2, 15.0 g; distilled water, 1,000 ml; adjust pH to 6.0 before use. *S. lutea*: beef extract, 1.5 g; yeast extract, 3.0 g; trypticase, 4.0 g; peptone, 6.0 g; dextrose, 1.0 g; agar, 17.5 g; distilled water, 1,000 ml; adjust pH to 7.8 before use. *Ps. syringae*: peptone, 6.0 g; NZ case (Sheffield), 4.0 g; yeast extract, 6.0 g; beef extract, 1.5 g; agar, 17.5 g; distilled water, 1,000 ml; adjust pH to 7.0 before use. Developers used were: monensin, carbon tetrachloride–benzene–ethylene glycol monomethyl ether (80:10:10); tylosin, ethyl acetate–diethylamine (95:5); hygromycin B, methanol–chloroform–conc. ammonium hydroxide (50:40:40).

The respective bioautographs were sprayed with aqueous tetrazolium solutions of the following concentration: TNBT, 2 mg/ml; INT, 2 mg/ml; NBT, 1 mg/ml; TTC, 20 mg/ml; TB, 1 mg/ml; MTT, 1 mg/ml. Bioautographs were then allowed to react for approximately 30 min (except TTC and TB which were 2 h) in the case of *B. subtilis* and *S. lutea* and 2 h in the case of *Ps. syringae*.

Apparatus

Silica gel thin-layer chromatographic plates, prepared from Merck Silica Gel (Brinkmann Instruments, Inc.), were used for monensin and tylosin. Commercially

prepared cellulose thin-layer chromatographic plates (Brinkmann Instruments, Inc.) were used for hygromycin B.

Results and discussion

As can be seen from Figs. 1A, 1B, 2A, and 2B, the growth of organisms such as *B. subtilis* and *S. lutea* usually provides sufficient contrast to photograph satisfactorily in bioautographs. On the other hand, an organism such as *Ps. syringae* fre-

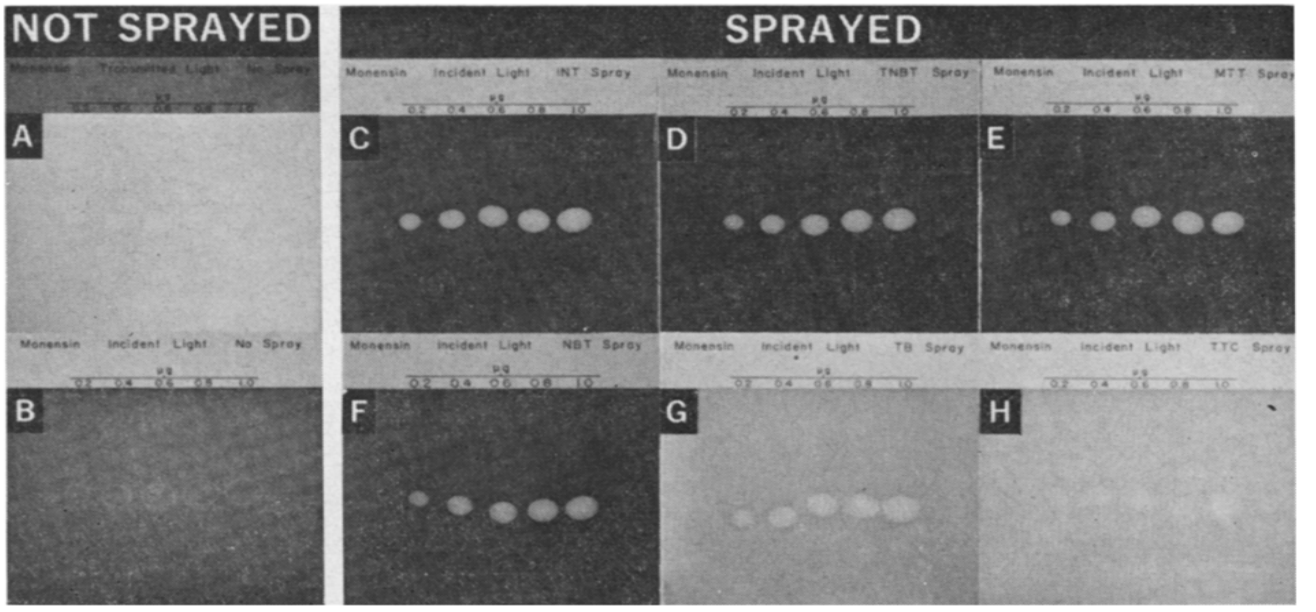

Fig. 1. *B. subtilis* bioautograph of monensin. Lanes 1–5: 0.2, 0.4, 0.6, 0.8, and 1.0 μg, respectively. A and B are not sprayed and photographed under transmitted and incident light, respectively. C–H are all photographed under incident light and sprayed with INT, TNBT, MTT, NBT, TB, and TTC, respectively.

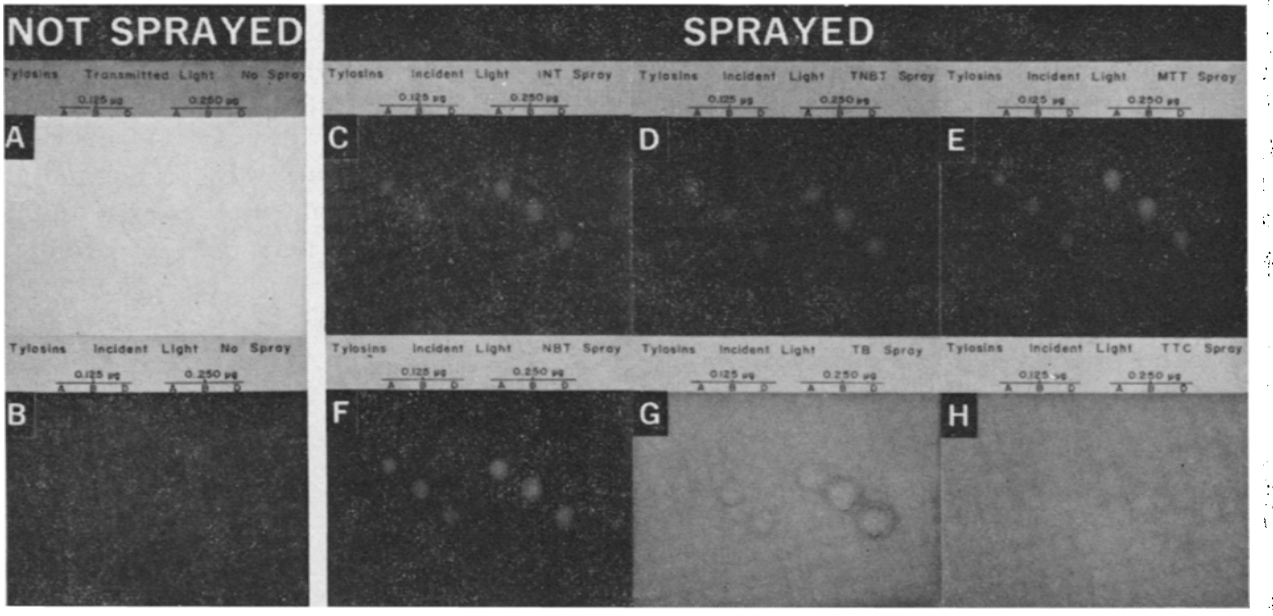

Fig. 2. *S. lutea* bioautograph of tylosins. Lanes 1–3: tylosins A, B, and D (0.125 μg). Lanes 4–6: tylosins A, B, and D (0.250 μg). A and B are not sprayed and photographed under transmitted and incident light, respectively. C–H are all photographed under incident light and sprayed with INT, TNBT, MTT, NBT, TB, and TTC, respectively.

quently provides insufficient contrast in photographs as shown in Figs. 3A and 3B.

We attempted to improve the contrast by spraying the bioautographs with various tetrazolium salts on completion of the incubation period. Although contrast is for the most part satisfactory in the cases of *B. subtilis* and *S. lutea* even without spraying, it can be seen by comparing Figs. 1C–1F with 1A and 1B and Figs. 2C–2F with 2A and 2B that the contrast can be improved greatly by the use of several

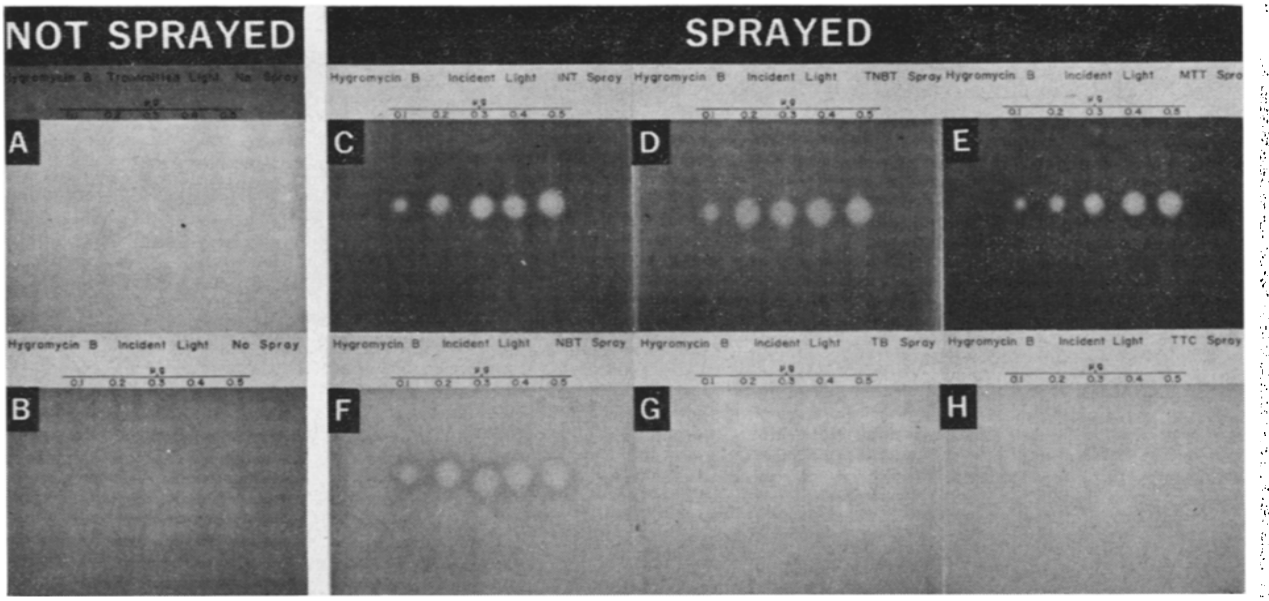

Fig. 3. *Ps. syringae* bioautograph of hygromycin B. Lanes 1–5: 0.1, 0.2, 0.3, 0.4, and 0.5 μg, respectively. A and B are not sprayed and photographed under transmitted and incident light, respectively. C–H are all photographed under incident light and sprayed with INT, TNBT, MTT, NBT, TB, and TTC, respectively.

different tetrazolium salts. More importantly, in the case of *Ps. syringae* in which there was no contrast previously (Figs. 3A and 3B) and hence no means of noting areas where inhibition had occurred, zones of inhibition after spraying with certain tetrazolium salts were vividly depicted (Figs. 3C–3F). It is equally important to note that not all tetrazolium salts will produce the desired results either due to no reaction occurring or insufficient reaction (Figs. 1G, 1H, 2G, 2H, 3G, and 3H). Hence, each investigator may need to find the best agent, experimentally, for his own system. Of those tetrazolium salts that we have tried, INT appears to be the most serviceable.

We wish to acknowledge the constructive suggestion of Dr. G. W. PROBST and the technical assistance of JOHN MORAN.

Eli Lilly and Company, Agricultural Analytical Chemistry Department, Greenfield Laboratories, P.O. Box 708, Greenfield, Ind. 46140 (U.S.A.)

WILLIAM J. BEGUE
RICHARD M. KLINE

1 K. H. WALLHÄUSSER, in E. STAHL (Editor), *Thin-Layer Chromatography*, 2nd Ed., Springer-Verlag, New York, 1969, p. 569.
2 R. M. KLINE AND T. GOLAB, *J. Chromatogr.*, 18 (1965) 409.

Received October 5th, 1971