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BIOAUTOGRAPHY IN PAPER AND THIN-LAYER CHROMATOGRAPHY AND ITS SCOPE IN THE ANTIBIOTIC FIELD

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SUMMARY

A critical review of the application of bioautography as a special detection method in paper and thin-layer chromatographic studies on antibiotics is presented. Examples are given to show various aspects of the bioautographic detection of antibacterial, antifungal, antiprotozoal, antiphage, phage-inducing, antiviral and cytotoxic substances. Documentation possibilities and the use of bioautography in quantitative analysis are also discussed.

BIOAUTOGRAPHY AS A SPECIAL DETECTION METHOD

Bioautography is based on the biological effects of substances to be detected. In general, these effects can be growth inhibiting or growth promoting. Zones of inhibition in the former case and growth zones in the latter help in making the positions of the detected substances visible on chromatograms.

Of the substances commonly detected by bioautography, antibiotics are the most important. Several hundred antibiotics have been discovered during the last three decades and about 50 of them are now used in medicine. Both paper chromatography (PC) and thin-layer chromatography (TLC) have been used successfully for analysis of various antibiotics. Owing to the wide variations in the chemical properties of antibiotics, bioautography is the only general method for their detection on paper and thin-layer chromatograms.

Historically, PC and the bioautographic detection of antibiotics were used for the first time in 1946 (GOODALL AND LEVI[1]). Fifteen years later, FISCHER AND LAUTNER[2] and NICOLAUS *et al.*[3] carried out TLC in this field.

PC and TLC have become indispensable tools in the study of antibiotics mainly for the following purposes: (*i*) classification and identification in the search for new antibiotics; (*ii*) separation of mixtures of antibiotics; (*iii*) qualitative and quantitative control of biogenesis and biosynthesis; (*iv*) development of isolation procedures for unknown antibiotics; (*v*) preparative chromatography; (*vi*) purity control; (*vii*) systematic analysis; (*viii*) structural studies; and (*ix*) studies of degradation or transformation by microbial and animal enzymes.

As stressed above, bioautography is a general method for the detection of antibiotics on both paper and thin-layer chromatograms. In the first steps in the investigation of an unknown antibiotic, when it is not yet available as a pure sub-

stance, only this method of detection can be used. Applications of this type have been described elsewhere[4,5].

In addition to their antimicrobial properties, some antibiotics possess cytotoxic, antineoplastic and other biological effects. The antimicrobial activity of different antibiotics also varies; they may have antibacterial, antifungal, antiprotozoal, antialgal or antiviral properties. Bioautography is based on these biological effects, which is the reason why various biological systems, such as bacteria, yeasts, filamentous fungi, protozoa, algae, animal cells and virus-infected animal or bacterial cells, are used in bioautographic detection.

Synthetic antimicrobial compounds can also be detected bioautographically when other methods of detection are less convenient. In addition, a number of biologically active compounds, *e.g.*, antitumour and antifertility agents, tranquillizers and cholesterol depressants, were also found to have antimicrobial properties. Based on these properties, correlative microbiological assays, including bioautography, were developed for compounds for which simple and rapid assays were not otherwise available[6].

BIOAUTOGRAPHY IN PAPER CHROMATOGRAPHY

In a typical procedure in the bioautography of paper chromatograms, chromatographic strips or sheets are placed on the surface of large nutrient agar plates inoculated with microorganisms that are sensitive to the antibiotics being analysed. After 15–30 min, the sheets are removed, while the narrow strips can be left on the seeded surface. In both instances antibiotics diffuse from the paper into the agar layer and inhibit the growth of the test organisms. The plates are then incubated at an appropriate temperature until a thin film of the growing organisms is visible on the surface. Zones of inhibition are then clearly visible on the plates. With bacteria and some fungi, the incubation time is about 16–24 h. Common cultivation media for test bacteria and fungi are described elsewhere[7]. Commercially available media for bioautography are also used, and two layers of agarised media are recommended. The base layer contains about 2 % of agar and the top layer is obtained by pouring another medium containing 1 % of agar (cooled to 45–48° and seeded with a test microorganism) on to the solidified base layer[7].

Zones of inhibition can be made more conspicuous and visible earlier when indicators for dehydrogenases are used. The incubation time of test bacteria can be shortened from 16 h to about 5 h by using 2,6-dichlorophenolindophenol or 2,3,5-triphenyltetrazolium chloride (TTC) as follows[8]. After incubation for 4 h, the agar plates with chromatographic strips are sprayed with a solution of one of the above indicators. After an additional incubation for about 1/2 h, inhibition zones begin to become visible (Fig. 1). Similar colouring techniques have been described by other workers (for references, see BETINA[7,9]).

Various modifications of bioautography can be found in the literature, and only some of them will be mentioned here.

A bioautographic procedure for the detection of antibiotics inducing the λ bacteriophage of *Escherichia coli* K-12 (λ) was described by HEINEMANN *et al.*[10]. The top agar layer is inoculated with a suspension of the latter lysogenic bacterium along with a non-lysogenic culture of *E. coli* C600. After incubation, a halo of lysis around the

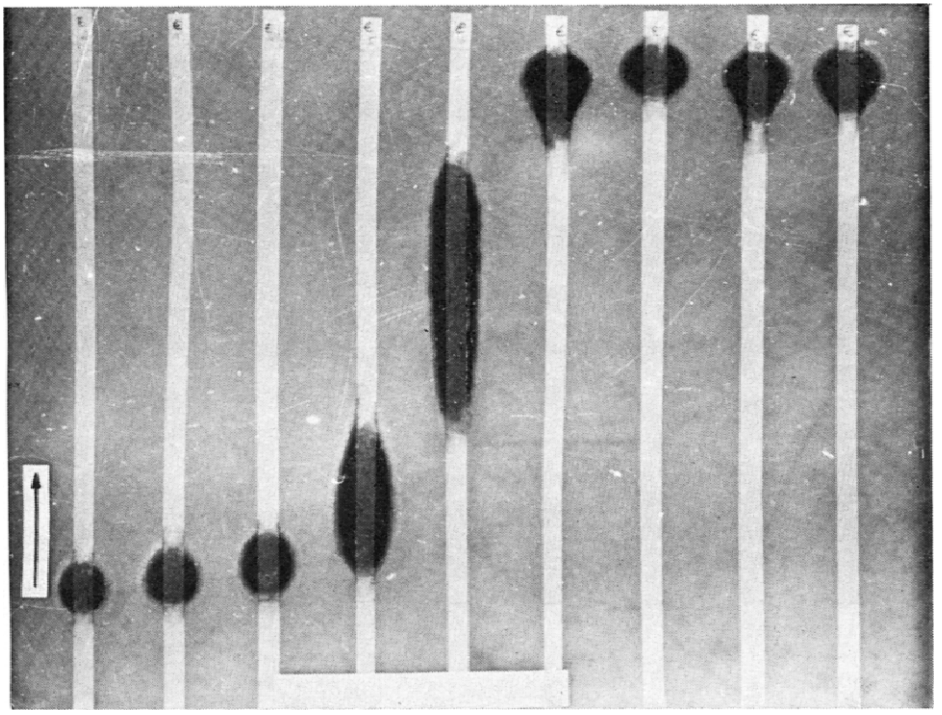

Fig. 1. Bioautographic detection of erythromycin with *Bacillus subtilis*. The paper strips were buffered before development to pH 2–10 (from left to right) to obtain a "pH-chromatogram" (for details and references, see BETINA[4]). Zones of inhibition were made visible with 2,6-dichlorophenolindophenol[8].

colonies of *E. coli* C600 indicates the position of the inducers studied on paper chromatograms.

Another special case of bioautography is that of 6-aminopenicillanic acid (6-APA), the "nucleus of the penicillin molecule". 6-APA has very weak antibacterial activity and must be converted, by phenylacetylation, into benzylpenicillin before bioautography. The developed chromatograms are sprayed with a solution of phenylacetyl chloride under mild alkaline conditions, dried and detected by means of *Staphylococcus aureus* or *Bacillus subtilis*[11].

The direct bioautography of antimicrobial substances on paper chromatograms has been described. According to one method, the developed chromatographic strips are dried in air, carefully immersed in a soluble agar medium inoculated with a sensitive microorganism and then incubated in a moist atmosphere at a suitable temperature. The growing microbial culture on the strips is then coloured by using an appropriate method. This technique is more sensitive than detection on agar plates[12]. Another procedure, recommended for synthetic fungicides, is as follows[13,14]. Developed chromatograms on Schleicher and Schüll 2043b or Whatman No. 3MM paper are dried in air and sprayed with a conidial suspension of filamentous fungi in an appropriate cultivation medium. The chromatograms are then incubated on glass plates in a moist atmosphere until zones of inhibition become visible.

With cytotoxic antibiotics, two methods of detection can be applied. SIMINOFF

AND HURSKY[15] used a monolayer culture of human HeLa cells covered with an agarized medium. After incubation with paper chromatograms on the plates, the cells were fixed and stained. Modifications of this procedure were described. On the other hand, ODA AND YAMAMOTO[16] simply brought a suspension of tumour cells into contact with the chromatograms and applied the colour reaction with dehydrogenases using 2,6-dichlorophenolindophenol in order to locate the zones of inhibition.

The correlative assays mentioned above[6] include the bioautography of antiviral substances, based on suppression of cytopathic effects of vaccinia virus by antiviral compounds under conditions similar to those used by SIMINOFF AND HURSKY[15]. A typical example is as follows. A certain filtrate from a microbial fermentation inhibited vaccinia virus *in vitro*. Correlative testing indicated that this beer contained an activity that was inhibitory to a yeast, *Saccharomyces pastorianus*. It was demonstrated that the same component in the fermentation was responsible for the inhibition both of the virus in the primary assay system and of the yeast in the correlative assay. This was accomplished by the use of PC and bioautographic detection. Duplicate paper strips were developed in parallel in five suitable solvent systems and placed on both seeded trays of *S. pastorianus* and of vaccinia-infected chick-kidney cells. It can be seen in Fig. 2 that the R_F values of the principal component were comparable in both test systems, thus establishing the correlation[6].

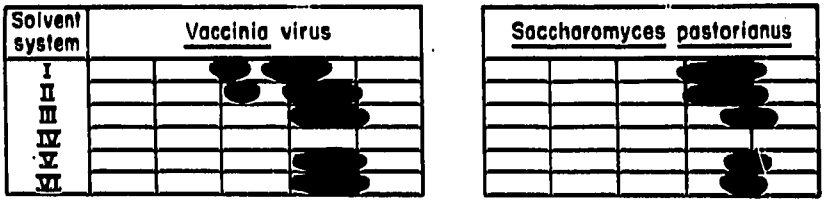

Fig. 2. Bioautography of an antiviral and antifungal substance in correlative assays according to HANKA AND SMITH[6] (schematic).

In special cases of bioautography, chromatographic strips are cut into short pieces, which are then placed into a series of test-tubes containing a liquid cultivation medium inoculated with sensitive microorganisms. The absence of growth in some test-tubes, after an incubation period, indicates the positions of antibiotics on the chromatograms. This technique was used for antileptospiral[17] and antiprotozoal[18] antibiotics in our screening programmes.

BIOAUTOGRAPHY IN THIN-LAYER CHROMATOGRAPHY

Several adaptations of bioautography are used for TLC. NICOLAUS *et al.*[3] poured an agar medium, containing TTC as a dehydrogenase indicator and seeded with test organisms, over the developed chromatographic plate, while KLINE AND GOLAB[19] sprayed a sufficiently cool agar medium on to the dried thin-layer plate so as to allow immediate solidification. Another agar medium cooled to 48° and seeded with a test organism was poured directly over the surface of the prepared plate. Zones of inhibition were identified after incubation by viewing the opaque plates directly. BICKEL *et al.*[20] pressed the chromatographic plate on to seeded agar plates.

MEYERS AND SMITH[21] have described a technique for TLC in which no binder is present in the sorbents. A wetted sheet of Whatman No. 1 paper is centred over a glass plate of the same size as the chromatographic plate. The filter-paper tabs, which extend beyond each end, are folded back over the glass plate and the assembly is placed on top of the chromatographic plate, thus resulting in a sandwich, with the filter-paper and sorbent layer between the glass plates. The filter-paper tabs are folded back over the ends of the chromatographic plate and the sandwich is inverted so that the chromatographic plate is on the top and the glass support plate on the bottom. The latter is removed, leaving the filter-paper adhering to the glass chromatographic plate by means of the filter-paper tabs. This assembly is carefully placed on the surface of the seeded agar. With *Streptococcus lactis*, which grows irrespective of the presence of oxygen, it proved possible to obtain results after overnight incubation at 37° with the chromatographic plate and filter-paper lying on the agar surface. With suitable test organisms, antibiotics that are active against Gram-negative organisms and antibiotics that are active against fungi were also detected. Some antibiotics that are active against Gram-positive organisms are non-inhibitory for *S. lactis* and the latter can be replaced by *Staphylococcus aureus* when the seed agar is supplemented with TTC and with KNO_3. The KNO_3 replaces oxygen in terminal respiration and *S. aureus* is able to grow on agar plates covered during incubation[22].

HAMILTON AND COOK[23] recommend the phytopathogenic bacterium *Xanthomonas pruni* for bioautography in TLC. This bacterium does not reduce tetrazolium dyes and is an obligate aerobe, but it hydrolyses gelatin and starch and produces acid from several sugars. These characteristics can be used as indirect indicators of growth when combined with colour reactions on the agar plates.

A combination of TLC on Sephadex and bioautography was applied by ZUIDWEG *et al.*[24]. After development, the Sephadex chromatoplate was removed from the chamber and pressed on to a seeded agar plate covered with a sheet of lens tissue-paper. After "printing" for 30 min, the chromatoplate was taken off, the lens tissue-paper was removed and the agar plate was incubated at the optimal growth temperature of the test organism.

WAGMAN AND BAILEY[25] used silicic acid–glass-fibre sheets (ChromAR) when much lower levels of antibiotics were required to be spotted, compared with TLC plates, for bioautography. ASZALOS *et al.*[5] used Eastman Chromagram sheets. In our laboratory, Silufol sheets (Kavalier, Czechoslovakia) proved to be useful for bioautography.

In preparative TLC, LEFEMINE AND HAUSMANN[26] located colourless neutramycin on a chromatogram by making a filter-paper print of the moist layer immediately after chromatography. A piece of glass was used to press the paper strip evenly against the support. After contact for 10 min, the print was removed, air-dried and bioautographed. The active component was then removed from the appropriate region of the original chromatogram. Independently, BETINA AND BARÁTH[27] described the same principle, which was used for the preparative TLC of gliotoxin and cyanein (detected with *Bacillus subtilis* and *Candida pseudotropicalis*, respectively). An example of such a detection is presented in Fig. 3.

PERLMAN *et al.*[28] described a procedure which can also be used for preparing bioautographs of paper chromatograms and thin-layer chromatograms of anti-

tumour antibiotics, and which is based on the cytocidal effect of the test compounds on tumour cells in an agar medium.

NICOLAUS *et al.*[3] used phenylacetylation to convert 6-APA into benzylpenicillin on thin-layer chromatograms and thus enabled it to be detected by bioautography.

HOMANS AND FUCHS[29] devised a direct bioautography on thin-layer chromatograms as a method for detecting fungistatic substances. The developed chromatograms are dried in air and sprayed with a conidial suspension of a sensitive fungus in a suitable medium. The thin-layer plates are then incubated in a moist atmosphere for 2–3 days at 25° and the positions of the inhibition zones are measured.

Details of some of the above procedures can be found in a chapter on antibiotics in a recent book on pharmaceutical applications of TLC and PC[7].

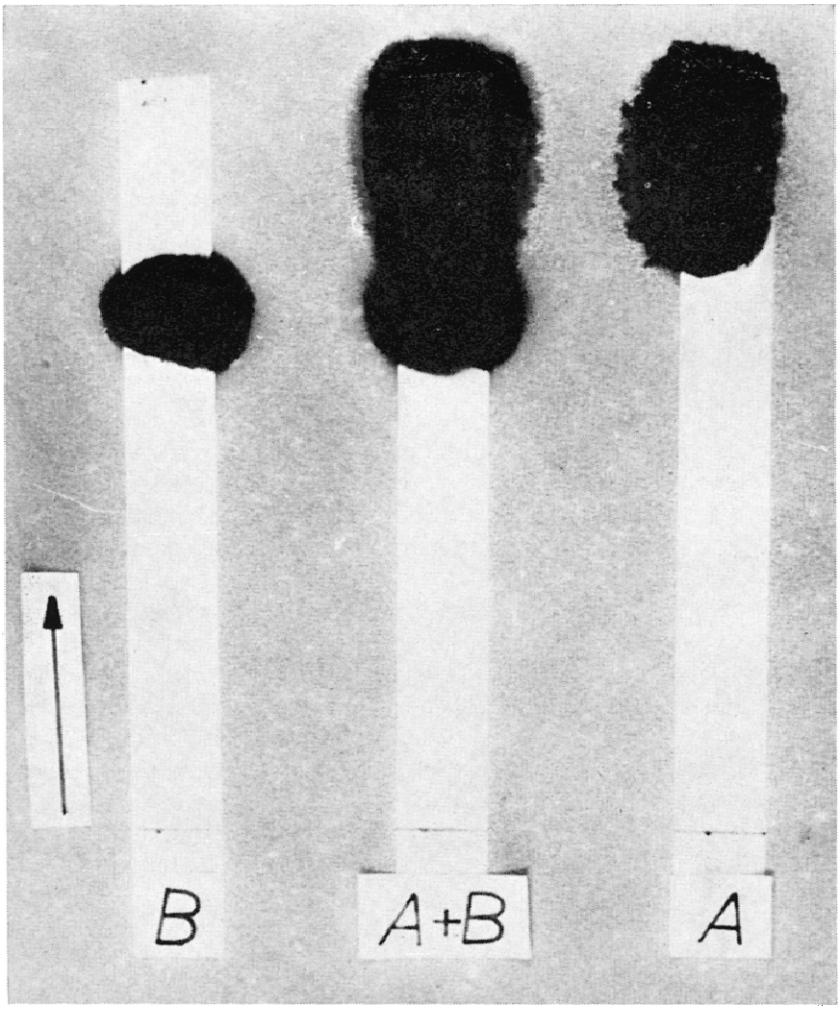

Fig. 3. Bioautography of "prints" from thin-layer chromatograms of gliotoxin (A) and an unknown antibiotic (B) before (A + B) and after (A, B) separation by means of preparative TLC. Test organism: *Bacillus subtilis*. Colouring as in Fig. 1.

DOCUMENTATION

Chromatograms detected bioautographically cannot be stored directly. For documentation purposes, agar test plates with inhibition zones can be photographed. DRAKE[30] photographed test plates under polarized light, which contrasted the inhibition zones more. Agar plates can also be photographed by direct contact with photographic papers[31]. Photographs of thin-layer chromatograms detected bioautographically can be found in the literature[19–21,25]. Contrast zones of inhibition are obtained when agar plates are coloured with 2,6-dichlorophenolindophenol[8]. Figs. 1 and 3 were prepared by photography after such procedures.

BIOAUTOGRAPHY IN QUANTITATIVE ANALYSIS

There are various possibilities for the use of bioautographic detection in quantitative PC and TLC.

The classical procedure of KARNOVSKY AND JOHNSON[34] for the determination of natural and biosynthetic penicillins is perhaps the best known method. After development, the positions and concentrations of the individual components are determined either by measurements of the zones of inhibition produced when the entire filter-paper strip is laid on a large agar plate seeded with *Bacillus subtilis*, or by cutting the strip into small uniform squares and measuring the circular inhibition zones produced by the individual squares. A convenient measure of the amount of each penicillin is given by the area under the relevant portion of the curve. The best known natural penicillins migrate with increasing mobilities in the sequence G, F, FH_2 and K.

ERICKSON AND BENNETT[35] described another method for determining 6-APA. Penicillins are separated from 6-APA by development of chromatograms, which are then air-dried and dipped in a solution of phenylacetyl chloride to convert 6-APA into benzylpenicillin. The chromatograms are dried and then bioautographed with *Sarcina lutea*. Levels of 6-APA in the samples are determined by plotting zone areas against a standard curve established by treating known amounts of 6-APA in the same manner as the samples. The evidence for the presence of 6-APA in a reaction mixture is based on comparisons with authentic 6-APA with respect to both chromatographic mobility and antibacterial properties, both before and after phenylacetylation and inactivation by penicillinase.

Cephalosporin C and related compounds can be determined according to the method of MILLER[36]. The chromatographic strips are detected with *B. subtilis* and the maximum widths of the zones of inhibition are measured. The average maximum diameters for each of three standard samples are plotted against the amount of antibiotic on semilogarithmic paper. As the relative specific activities for the various compounds are known, it is possible to determine the amount of parent compound (cephalosporin C), deacetylated compound and lactone simultaneously in an unknown sample from the single standard curve for the parent compound. Reaction mixtures containing cephalosporin C and 7-aminocephalosporanic acid (7-ACA) are analysed for 7-ACA in the same manner as described above for 6-APA after phenylacetylation[35].

Methods have been described for bioautography in the quantitative PC analysis

of gentamycins, lincomycin and related antibiotics (for references, see BETINA[7]). A PC procedure was developed by LARSON AND PETERSON[37] for the quantitative separation and determination of antifungal oligomycins A, B and C in fermented media. In this method, a filamentous fungus is used for bioautography.

Relatively few descriptions of bioautography in the quantitative TLC of antimicrobial substances can be found in the literature, because various factors have an adverse effect on the accuracy of such determinations.

BRODASKY *et al.*[38] described a quantitative method of analysis for 7-deoxy-7(S)-chlorolincomycin and its metabolite, developed for biological fluids, particularly serum. The analysis is based on the separation of the two activities on silica gel and subsequent quantitation by regression analysis of the zone sizes obtained by bioautography. In spite of obtaining relatively encouraging results, they observed that the response of both antibiotics varied markedly as a function of the age of the thin-layer plates used. This observation makes it imperative that all quantitations should be performed on plates that have been prepared in the same batch.

More recently, a simplified bioautographic procedure for antibiotics was described by BILLOW AND SPEAKER[39] that is adaptable for densitometric quantitation. Pre-coated commercial TLC media are used. The coated surface of the chromatographic plate is scored to produce parallel channels and the plate is developed as a whole and then cut so as to form separate chromatographic strips. For bioautography with bacteria, two layers of nutrient agar are used. As the level surface of the base agar layer approaches congelation, the chromatographic strips are gently placed in parallel on the agar surface with the coated sides uppermost. The agar is allowed to congeal fully and the strips and agar are overlaid with the top seeded agar. After the seeded agar has congealed, the chromatographic strips are isolated from one another by excision of a channel of agar between adjacent strips (Figs. 4a and

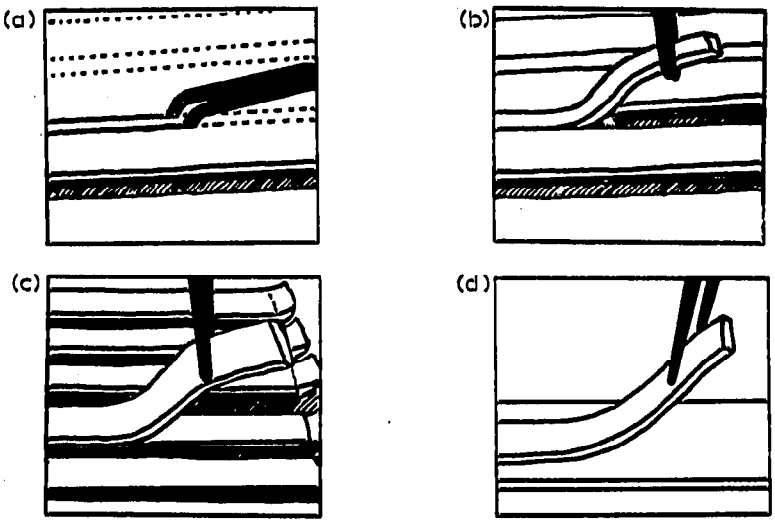

Fig. 4. Bioautography of antibiotics according to BILLOW AND SPEAKER[39]. (a) Cutting of barrier agar channel between chromatographic strips; (b) removal of agar channel; (c) lifting of seeded agar from the bioautogram; (d) supporting of seeded agar film on a glass plate for densitometric scan.

4b) in order to minimize the lateral diffusion of water-soluble antibiotics. After an incubation for 24 h, clear zones of inhibition are made visible by translumination of the intact bioautogram or of the seeded top layer, which is easily lifted (Fig. 4c) from the chromatogram with the aid of a blunt pair of forceps. The stripped agar film may be supported on a glass plate (Fig. 4d) and subjected to densitometric analysis. Although no analytical data were given by the authors, their procedure seems to be sufficiently simple and precise for quantitative analysis also.

CONCLUSIONS

With its advantages and limitations, bioautography is a specific and sometimes indispensable detection method in both PC and TLC. I have tried to show various aspects of its applications, hoping that such a review will be useful to those who do not use bioautographic detection in their chromatographic work.

The possibilities of the bioautographic detection of antibiotics with various biological activities are summarized in Table I. Test systems and biological effects indicating the positions of antibiotics on the chromatograms are given in general, as the details have been discussed above.

TABLE I

POSSIBILITIES OF THE BIOAUTOGRAPHIC DETECTION OF ANTIBIOTICS IN PC AND TLC

Antibiotics	*Bioautography*	
	Test systems	*Effects*
Antibacterial	Bacteria	Growth inhibition
Antifungal	Fungi	
Antialgal	Algae	
Antiprotozoal	Protozoa	
Antiphage	Bacteriophages plus host bacteria	Absence of plaques
Phage-inducing	Mixtures of lysogenic plus indicator strains	Halo of lysis on indicator strains
Antiviral	Virus-infected animal cells	Absence of cytopathic action
Cytotoxic	Monolayer animal cell cultures	Dead cells

In quantitative analysis, bioautography may be helpful, but the relatively high variability of biological quantitations must be borne in mind. As an illustration, for the two lincomycin antibiotics mentioned above[38], the mean standard errors were 7.3 and 7.7 %, respectively. In spite of such limitations, there are situations when bioautography is the only detection method possible in quantitative PC or TLC, *e.g.*, with samples of fermentation media or body fluids containing antibiotics that cannot be detected by specific chemical or physical methods.

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DISCUSSION

GOODALL (quoted from his opening remarks when chairing the session of the Symposium devoted to biological and enzymatic methods): Before the discovery of paper chromatography, fermentation mixtures of biologically active substances were assayed very approximately by differential response to various organisms. CONSDEN, GORDON AND MARTIN's paper in 1944 stimulated us at Manchester to extend the technique to penicillin analysis by buffering the paper and making the chromatograms visible by placing them on a sheet of nutrient agar seeded with *B. subtilis*. I can recall my excitement when we first saw the picture formed by the diffusion of separated penicillins into the agar and how growth was inhibited to a different extent according to the type of penicillin. Dr. LEVI (now deceased) applied his mathematical ability to the statistical interpretation of the results. Since those days, bioautographic methods have been developed for a wide variety of bioactive substances. Although those methods are generally highly sensitive, the limitations for

quantitation presumably still are in the diffusion stages and in the definition of the bacterial growth.

(To Dr. BETINA): Have you ever attempted to relate the sizes of the areas of inhibition with the distribution of the bioactive substances on the chromatogram? An example is the small area of fluorescence in an oxytetracycline chromatogram compared with the large and poorly differentiated areas of inhibition on *B. subtilis*.

BETINA: The bioautographic technique is composed of a number of different steps involving transfer from the chromatogram to the agar, diffusion in the agar, sensitivity of the organism, etc. All these factors influence the appearance of the zones of inhibition. If a sufficiently sensitive physical or chemical method for the determination of an antibiotic is available, it is given preference over bioautography. Bioautography is, of course, the most important detection method for new or unidentified antimicrobial compounds.

GOODALL: In my experience, the use of a radioactive isotope dilution technique is the most accurate independent way of checking the approximate bioautographic results, *e.g.*, for the major penicillin in a fermentation broth.