A Rapid Micromethod for Bioautographic Assay

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The detection and identification of specific molecules may often be accomplished most conveniently by autographic methods. These procedures, in which nutritionally deficient bacterial cells suspended in solid minimal media show zones of growth in the presence of the required nutrilite, possess several advantages over liquid assay methods. These include simplicity of technique and the reduction of interference from inhibitory materials which may be present in crude samples as natural constituents or as residues from extraction procedures (Usdin et al., 1954), as well as direct applicability to paper chromatograms for the identification of spots (Winsten and Eigen, 1948). Their principal disadvantage, that samples of relatively high concentration are required, has been compensated for by the incorporation of tetrazolium salts in the solid medium. Reduction of these compounds to colored derivatives during growth of the test bacteria gives a criterion of response whose sensitivity to absolute amounts of growth factors is better than with tube assay (Ford and Holdsworth, 1953; Usdin et al., 1954). The method presented here offers a further increase in sensitivity and in economy of time and materials which may extend the usefulness of autography.

Methods
The test organism—in this case one of a series of autotrophic mutants of Klebsiella pneumoniae, or Lactobacillus arabinosus 2K8—is grown in a nutrient liquid medium. Cells (preferably from the logarithmic growth stage) are centrifuged, washed, and suspended in double-strength minimal medium. The M-9 medium (Anderson, 1946) was used for K. pneumoniae and pantothenate assay medium (Difco) for L. arabinosus. The cell suspension is now mixed with an equal volume of molten 3 per cent agar solution at 45°C. The density of the suspension is so adjusted that the seeded agar will contain about 10^6 or 10^7 cells per ml.

A sample of the growth factor to be assayed, in the form of a small section cut from a paper chromatogram or a measured quantity (up to 0.1 ml) of solution, is placed in the center of a sterile microscope slide. The slides may be kept conveniently in individual Petri dishes or placed in rows in large covered dishes. Solutions, especially those of relatively large volume, may be allowed to evaporate before the inoculum—one drop of seeded agar—is added. A warmed, sterile coverslip is immediately placed over the agar, which flows to the edges of the coverslip and solidifies almost at once. Prewarming of the slides by incubation or some other means will insure the agar’s spreading out in a thin, uniform layer. The edges of the coverslip are sealed to the slide with paraffin, and the slide is incubated at 37°C. Although growth response may be ascertained within a few hours, slides sealed in this manner may be preserved without noticeable drying of the agar for several days or even weeks after preparation.

Results
Detection of Growth Response
Response to relatively large quantities of sample may be detected upon gross examination of slides after incubation by a heavy clouding of the agar. Lesser amounts are detectable upon microscopic examination with a low power objective. Well-defined microcolonies are formed, especially by K. pneumoniae, in the presence of quite small quantities of the required nutrilite. The actual end point at which no response occurs can be detected only by careful comparison with control slides of seeded, unsupplemented minimal agar. With K. pneumoniae, results may be observed after 4 hours; overnight incubation was necessary for L. arabinosus.

No benefit was derived from the addition of tetrazolium salts to the agar, as microcolonies are formed well before any appreciable reduction of the dye occurs.

Sensitivity of the Method
Table 1 shows a comparison between the absolute quantities of four amino acids detectable by the slide method and by tetrazolium-containing plates prepared by the method of Usdin et al. (1954). The slide method appears to offer slightly greater sensitivity. Furthermore, the data shown represent the quantities which result in formation of easily observed microcolonies. Smaller quantities may be detected by careful observation of the slides, but this procedure is perhaps not suitable for routine work.

In the assay of pantothenic acid with L. arabinosus, about 10^{-5} μg could be detected by the tetrazolium...
TABLE 1. Minimum amounts detectable by two methods in the autographic assay of amino acids with mutant strains of Klebsiella pneumoniae

<table>
<thead>
<tr>
<th>Strain and Amino Acid Requirement</th>
<th>Minimum Absolute Amount Detectable (μg)</th>
<th>Plate (tetrazolium) autography</th>
<th>Slide autography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kp 82, tryptophane</td>
<td>10^-1</td>
<td>10^-2</td>
<td></td>
</tr>
<tr>
<td>Kp 17, histidine</td>
<td>10^-1</td>
<td>5 x 10^-2</td>
<td></td>
</tr>
<tr>
<td>Kp 27116, methionine</td>
<td>1.0</td>
<td>5 x 10^-2</td>
<td></td>
</tr>
<tr>
<td>Kp 88, arginine</td>
<td>1.0</td>
<td>10^-1</td>
<td></td>
</tr>
</tbody>
</table>

Technique, while 10^-4 μg and less was detectable by the slide method. The response in this case was less satisfactory than with K. pneumoniae, however. The latter species forms more compact and more easily observed microcolonies.

**DISCUSSION**

Careful determination of the optimum conditions for tetrazolium autography, as recommended by Usdin et al. (1954), might have permitted detection of more minute amounts of the compounds than were reported here. It appears, nevertheless, that the slide method will prove more sensitive in most cases, since diffusion is limited to the confines of a single drop of agar and growth response is observable on a micro level. In addition to the usual advantages of autographic procedures, the slide method offers a quick, sensitive test which can be performed with a minimum of equipment and supplies and requires only minute amounts of sample.

Slide methods should prove especially useful in identifying unknown spots on paper chromatograms. Since the minimum amount of most amino acids detectable as spots lies in the range of 0.1 to 5.0 μg (Berry et al., 1951), it would be possible in many cases to cut a spot area into pieces and screen it against several different test organisms. The high specificity of the nutrient requirements of mutant bacteria provides a precise means of identification which may often be applied in circumstances where limitations in time or availability of sample prohibit identification of spots by more conventional procedures.

By use of appropriate mutant bacterial strains, the slide method might be applied to identification of many types of compounds. The only apparent limitation is that the test bacteria must be anaerobic or facultative in their oxygen requirements.

**SUMMARY**

A rapid slide micromethod is presented for the bioautographic location and identification of specific molecules. The sensitivity of this procedure compares favorably with that of previously described methods. It is suggested that the slide method will prove especially useful in identification of paper chromatogram spots.

**REFERENCES**


