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The application of tetrazolium bioautography to the identification of folic acid derivatives

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SYNOPSIS Tetrazolium bioautography has been applied to the identification of folates in solutions of known concentration, and satisfactory resolution has been obtained between pteroyl-L-monoglutamic acid, calcium leucovorin, and N-5-methyltetrahydrofolic acid. The technique is suitable for the identification of folates in biological solutions, and is more specific than differential microbiological assay.

Bioautographic techniques combine paper chromatography with the response of microorganisms to the presence of growth factors. The procedure was first used to separate and identify members of the vitamin B_c group by Winsten and Eigen (1948), and later applied semi-quantitatively to the growth factors for *L. casei*, *S. faecalis*, and *Pediococcus cerevisiae* by Usdin, Shockman, and Toennies (1954). Tetrazolium compounds are reduced to deeply coloured formazan derivatives by growing bacteria as shown by Kuhn and Jerchel (1941), and this principle is used to identify the zones of enhanced growth at the site of concentration of growth factors on paper chromatograms. These techniques have been applied to the separation and identification of folic acid derivatives in various solvent systems.

Materials and Methods

TRIPHENYL-TETRAZOLIUM CHLORIDE MEDIUM (TTC)

Single strength folic acid casei medium (Difco) was made up to 500 ml per plate containing 50 ml 0·1 M phosphate buffer *p*H 6·1, 0·5 g ascorbic acid, 11 g agar (Oxoid Ionagar no. 2), 10 ml 2% aqueous tetrazolium salt (2:3:5-triphenyl-tetrazolium chloride), and 10 ml washed suspension of *L. casei* (N.C.1.B 8010) prepared to an optical density of 0·128 at a wavelength of 650μ. The last two ingredients were added after the basic medium had been prepared, sterilized, and cooled to 45°C.

PLAIN AGAR

For each plate, 3·5 g agar (Oxoid Ionagar no. 2) was dissolved in 300 ml distilled water.

STOCK SOLUTION OF FOLIC ACID DERIVATIVES

The following solutions were prepared at a concentration of 1 mg per ml in 1% aqueous ascorbic acid and stored at −20°C in 0·5 ml aliquots: pteroyl-L-monoglutamic acid (Sigma), calcium leucovorin (Lederle), and N-5-methyltetrahydrofolic acid (Department of Chemistry, University of Aston).

SOLVENT SYSTEMS TESTED

The following solvent systems were used: (1) 3% aqueous ammonium chloride; (2) 5% aqueous acetic acid; (3) 0·5% sodium bicarbonate; (4) 0·1M phosphate buffer *p*H 6·0; (5) 0·1M phosphate buffer *p*H 6·5; (6) 0·1M phosphate buffer *p*H 7·0; (7) saturated aqueous disodium hydrogen phosphate; (8) 0·2M acetate buffer *p*H 5·0; (9)

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2% aqueous sodium chloride; and (10) 1% aqueous ascorbic acid. Except for solvent system no. 10, all systems contained 1% ascorbic acid as an anti-oxidant.

Preparation of the Bioautographs

Ascending paper chromatograms were prepared on Whatman no. 1 chromatography paper and were developed for 60 minutes at room temperature in the dark. Each bioautograph plate was prepared by pouring 250 ml of the TTC medium described above into a culture dish measuring 30 cm × 30 cm × 2·5 cm and allowing to set. The developed and partially dried chromatogram was then laid on top of this layer and a further 250 ml of TTC medium was poured on. The whole preparation was then covered with 300 ml plain agar. Each plate was then incubated at 37°C overnight.

In assessing the suitability of the various solvent systems, the stock solutions of pteroyl-L-monoglutamic acid, calcium leucovorin, and N-5-methyltetrahydrofolic acid were diluted in 1% aqueous ascorbic acid to give the following working solutions: pteroyl-L-monoglutamic acid 0·1 μg per ml; calcium leucovorin 1·0 μg per ml; and N-5-methyltetrahydrofolic acid 1·0 μg per ml.

A mixture containing all three of the above solutions in similar final concentration was also prepared.

In testing the solvent systems, 10 μl of each folate was applied to the chromatograms and developed simultaneously in the manner described above.

To test the sensitivity of the technique, serial 10-fold dilutions of the solutions of folates were prepared and at each stage of dilution 10 μl volumes were added to strips of chromatography paper. These strips were not developed chromatographically, but were incorporated into the bioautograph plates in an identical manner to the developed chromatograms. Chromatograms were developed with 3% aqueous ammonium chloride and 0·1M phosphate buffer *p*H 7·0 as solvents in which 10 μl of solutions of pteroyl-L-monoglutamic acid, calcium leucovorin, and N-5-methyltetrahydrofolic acid had been applied at the origin. This series was repeated using increasing dilutions of each solution, such that the concentrations applied were 5 ng per 10 μl, 1 ng per 10 μl, 500 pg per 10 μl, 100 pg per 10 μl, and 50 pg per 10 μl.

Solvent System	*Calcium Leucovorin*	*5-Methyltetrahydrofolic Acid*	*Folic Acid*
1 3% Aqueous ammonium chloride	0·59 (0·14)[1]	0·69	0
2 5% Aqueous acetic acid	0·70 (0·22)[1]	0·75	0·10[2]
3 0·5% Aqueous sodium carbonate	0·78	0·75	0·47
4 0·1 M Phosphate buffer *p*H 6·0	0·59 (0·21)[1]	0·70	0
5 0·1 M Phosphate buffer *p*H 6·5	0·59 (0·29)[1]	0·66	0
6 0·1 M Phosphate buffer *p*H 7·0	0·72 (0·32)[1]	0·72	0·31
7 Saturated aqueous disodium hydrogen phosphate	0·62	0·60	0
8 0·2 M Acetate buffer *p*H 5·0	0·70 (0·32)[1]	0·75	0
9 2% Sodium chloride	0·52	0·60	0
10 1% Aqueous ascorbic acid	0·53	0·55	0

Table *R_f values for folic acid derivatives in 10 solvent systems*

[1]Minor spot.
[2]Elongated spot.

Results

TESTS OF SUITABILITY OF SOLVENT SYSTEMS

After incubation at 37°C overnight the position of each folate was revealed as a dark red spot. The R_f values obtained for each folic acid derivative in the 10 solvent systems used are shown in the Table. The best separation and definition of spots was obtained using 3% aqueous ammonium chloride and 0·1M phosphate buffer *p*H 7·0.

TESTS OF SENSITIVITY OF THE TECHNIQUE

Using strips of chromatography paper impregnated with 10 μl of pteroyl-L-monoglutamic acid, calcium leucovorin, and N-5-methyltetrahydrofolic acid in serial 10-fold dilutions using 1% aqueous ascorbic acid as diluent, it was found that a concentration of 100 pg per 10 μl could be detected unequivocally as a zone of red colorization after incubation.

In a series of progressive dilutions of known concentration as above, using 3% aqueous ammonium chloride and 0·1M phosphate buffer *p*H 7·0, it was found that after chromatographic development both calcium leucovorin and N-5-methyltetrahydrofolic acid cound be detected at a concentration of 500 pg per 10 μl and pteroyl monoglutamic acid at a concentration of 100 pg per 10 μl.

Discussion

The techniques whereby semi-quantitative measurements may be applied to bacterial growth zones using differential microbiological assay have previously been described by Usdin *et al* (1954). Tetrazolium bioautography has now been applied to the identification of folic acid derivatives using solutions of known content and concentration. A satisfactory bioautographic technique has been established with two solvent systems (nos. 1 and 4) which provides adequate resolution between the three folates studied. Identification of calcium leucovorin and N-5-

methyltetrahydrofolic acid is thus possible in the range 10-50 ng per ml, and identification of pteroyl-L-monoglutamic acid in the range of 5 to 10 ng per ml. Although these levels are still in excess of those in serum, suitable levels can be attained by concentration and the various folates present readily identified. Folates are present in urine in sufficient quantities to allow identification without concentration.

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