A technique for the location of egg-white lysozyme on zone electrophoresis papers using plates containing *Micrococcus lysodeikticus*

A strain of *Bacillus subtilis* has been isolated which liberates a lysozyme-like enzyme into the growth medium during exponential growth[1]. In the course of the development of a method of purification of this enzyme, it was necessary to detect lysozyme on zone-electrophoresis papers.

SALTON[2] has described the use of agar plates containing cell wall preparations of various bacteria for the isolation of cell wall degrading micro-organisms. The present communication describes the use of similar plates containing a cell wall preparation of *Micrococcus lysodeikticus* (NCTC 2665) to determine the position of egg-white lysozyme on electrophoresis papers run in the apparatus described by MARKHAM AND SMITH[3]. Cell wall preparations are prepared by the method reported by SALTON AND HORNE[4].

Double-layer agar plates are prepared as follows:

1. Lower layer: 15 ml molten 2% (w/v) washed agar in 0.1*M* Na_2HPO_4/KH_2PO_4 buffer, pH 6.5, is poured in Petrie dishes of 14 cm diameter and allowed to set before adding the upper layer.

2. Upper layer: 5 ml of a distilled water suspension of a preparation of *M. lysodeikticus* cell wall (containing about 30 mg dry weight of cell wall/ml) is mixed with 10 ml 2.5% (w/v) washed agar in 0.1*M* phosphate buffer, pH 6.5 and poured as the upper layer. The cell wall suspension and the agar are heated separately and mixed thoroughly immediately before pouring, otherwise flocculation of the cell wall suspension may lead to uneven opacity of the plates.

To locate the lysozyme after electrophoresis, the strip of paper is blotted to remove excess liquid and placed in contact with the surface of the cell wall agar plate. The plate, with the paper *in situ*, is then incubated at 37° for 3 h and under these conditions the lysozyme diffuses out of the electrophoresis paper into the agar gel where it digests the cell wall to produce a clear area in the opaque plate. Fig. 1 is a photograph of a cell wall agar plate used to detect the position of egg-white lysozyme (EWL) on an electrophoresis paper developed in 0.1*M* veronal-acetate buffer, pH 8.3, containing 0.05*M* NaCl.

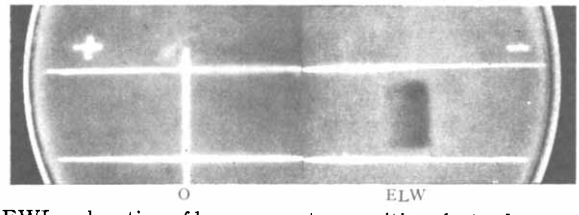

Fig. 1. O = Origin; EWL = location of lysozyme; + = positive electrode; — = negative electrode. The parallel white lines indicate the limits of the electrophoresis paper when applied to the plate.

The large scale preparations of *M. lysodeikticus* cell wall are time consuming to make and may be replaced in the upper layer by heat-killed cells. For this purpose, *M. lysodeikticus* is grown for 48 h at 37° on agar containing a tryptic digest of casein supplemented with 1.0% glucose. The cells are washed off the surface of the agar in distilled water, filtered through glass wool, washed three times with distilled water and suspended in distilled water to a density of about 30 mg dry weight of cells/ml. The suspension is heated in a boiling water bath for 10 min and 5 ml samples are added to the agar in the upper layer.

SALTON has shown that cell wall preparations of *M. lysodeikticus* are digested to soluble products by lysozyme[5] but that heat-killed preparations are only partially degraded[6]. However, lysozyme treatment renders heat-killed cells of *M. lysodeikticus* susceptible to digestion by trypsin[6] so that digestion with lysozyme followed by treatment with trypsin causes complete dissolution of the heat-killed cells to soluble products. Because the degradation is only partial, the zone of clearing produced on heat-killed cell agar plates by lysozyme is not very distinct but may be developed by removing the electrophoresis paper, spraying with trypsin (0.1 mg/ml) and re-incubating at 37°. Bacterial contamination may be suppressed if necessary, by spraying the plates before incubation with 1.0 ml of a mixture of penicillin (30 units/ml) + streptomycin (30 μg/ml) + chloramphenicol (30 μg/ml).

These methods may be used to detect the position of lysozyme bands on electrophoresis papers at 1/20 the concentration of enzyme that can be detected by the staining technique for proteins described by DURRUM[7] and modified by KUNKEL AND TISELIUS[8].

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O-Methylation, the principal pathway for the metabolism of epinephrine and norepinephrine in the rat

Previous studies have shown that epinephrine and norepinephrine are methylated on the 3-hydroxy position *in vitro* and *in vivo*[1]. Estimation of the extent to which O-methylation occurs in the intact animal was made possible by the development of a method for the determination of meta-O-methylepinephrine (metanephrine) and meta-O-methyl-norepinephrine (normetanephrine) in urine.

Metanephrine or normetanephrine was isolated from the urine at pH 10 by gently shaking for 20 min with 20 volumes ethylene dichloride* containing 2% isoamyl alcohol*. After the separation of the 2 phases by centrifugation, the aqueous layer was removed by aspiration and an aliquot of the organic phase was shaken with 1/10 volume 0.1 *N* HCl. The lower layer was removed by aspiration and the acid extract was washed twice with 10 volumes isoamyl alcohol to remove interfering substances. The acid extract was transferred to a quartz cuvette and metanephrine or normetanephrine was determined by measuring the fluorescence in an Aminco-Bowman fluorospectrophotometer at 335 mμ after activation at 283 mμ.

In order to correct for the partition of the methylated metabolites of the catechol amines in the two-phase system described above and the quenching of fluorescence that occurs occasionally, a known amount of synthetic metanephrine or normetanephrine was added to the urine to be assayed and carried through the procedure.

Glucuronides of metanephrine and normetanephrine were hydrolyzed by incubation of 1 ml of urine with 2,000 units of bacterial β-glucuronidase for 3 h at 37°.

After the administration of L-epinephrine or L-norepinephrine to rats, the methylated metabolites isolated from the urine by the procedure described above had the same R_F values, partition coefficients, and fluorescent spectra as authentic samples of metanephrine or normetanephrine[2].

TABLE I

O-METHYLATION OF EPINEPHRINE IN THE RAT

Each rat received 10 μg/g dibenamine hydrochloride intraperitoneally. After 30 min each rat was given a total of 5 μmoles of epinephrine or metanephrine intraperitoneally. The compounds were administered in 5 divided doses every hour for 5 h. Iproniazide (200 μg/g) was given intraperitoneally 2 h before the injection of the amines. Six rats were used in each experiment.

Compounds administered	*Metanephrine excreted* μmoles	*Metanephrine glucuronide excreted* μmoles	*Total excreted* %
L-Epinephrine (5 μmoles)	0.14	1.08	25
D, L-Metanephrine (5 μmoles)	0.42	1.31	35
L-Epinephrine (5 μmoles) and iproniazide	0.18	2.55	55
D, L-Metanephrine (5 μmoles) and iproniazide	0.50	3.00	70
D-Epinephrine (5 μmoles)	0.06	0.86	18
L-Epinephrine (3 μmoles) §	0.05	0.47	18

§ This group of rats did not receive dibenamine.

* All solvents, reagent grade, were washed successively with 1/5 volume 1 *N* NaOH, 1 *N* HCl and twice with distilled water.