A Novel Plate Assay Method for Amoebicides

For the *in vitro* assessment of the activity of amoebicidal drugs, laboratory strains of amoebae cultured in special media, together with a more or less well defined associated bacterial flora, have been widely used. A survey of the various techniques so far known was recently published by CAVIER[1].

Unless a given amoebicidal completely lacks antibacterial activity, it has so far been difficult to prove or disprove a direct amoebicidal action, because a mere inhibition of bacterial growth may also impair the viability of the amoeba. Considerable progress in culturing amoebae and the assay of amoebicides was recently achieved when it was found that amoebae[2] multiply on agar plates seeded and preincubated with suitable bacteria.

Methods. Plates prepared in Petri dishes with sterile nutrient agar containing 0.2% dextrose and 0.7% table salt are used throughout. *Escherichia coli* is isolated from human stool by passage on MacConkey's agar and suspended in suitable density for uniformly seeding the agar plates. After removing excess fluid and drying the surface of the agar, the plates are incubated at 37°C for 24 h. The plates are then exposed – after removing their cover – to an ultraviolet germicidal lamp at a distance of about 30 cm for a period sufficient to kill the bacteria (6 h, for example), or the bacteria may be killed by heating the plate to 60°C for 1 h in a hot air oven. The plates are then ready for inoculation by the amoeba in the centre of the plate or in 9 different foci widely separated all over. The plates are then incubated for a further 24 h, during which period the growing culture forms an increasing area of macroscopically altered surface from which amoebae can be isolated in abundance. The drugs to be assayed are dissolved in the desired concentration (1000 γ/ml, for example) in sterile water or other suitable solvent miscible with water and having no amoebicidal activity (propylene glycol, for example). Filter paper discs are soaked in the solution and put around the plate as in an antibiotic sensitivity test. Readings of inhibited amoebic growth and migration can be easily made at various periods of incubation.

Results. The amoebae multiply and spread actively; as the time of incubation increases, an ever-enlarging area is formed which is distinguished macroscopically by its greater transparency (Figure 1). As the active and multiplying amoeba will eventually cover the whole plate, inhibition of amoebic growth and migration become clearly visible within an area surrounding a disc impregnated with an amoebicide. In a typical experiment (Figures 2 and 3) emetin is clearly demonstrated to be active, whereas paromomycin, chlortetracycline and sigmamycin are devoid of any direct amoebicidal activity. None of 16 other different antibiotics inhibited the growth of the amoebae. A notable exception was offered by spiramycin and to a lesser extent erythromycin. These two antibiotics, as well as chloroquin, were effective against the amoeba.

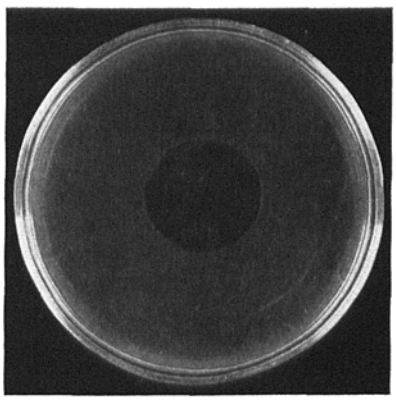

Fig. 1a

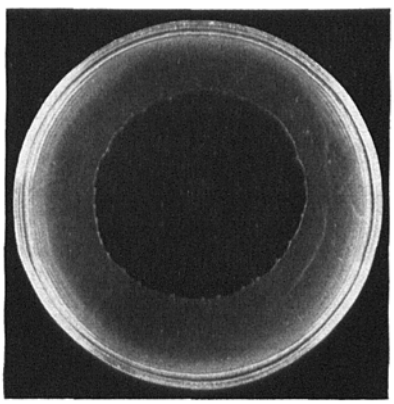

Fig. 1b

Surface growth of the amoeba repeatedly isolated from human stools containing *E. histolytica*. Cleared central area after 24 h (Figure 1a) and 40 h (Figure 1b) incubation. The amoeba was inoculated in the centre of the plate. Photographed by reflected light.

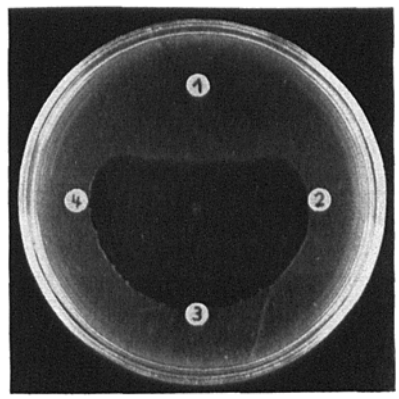

Figure 2. Assay of amoebicides against the surface growth of the amoeba mentioned above. 40 h incubation in the presence of emetin (1), paromomycin (2), chlortetracycline (3) and sigmamycin (4). The amoeba was inoculated in the centre of the plate. Photographed by reflected light.

[1] M. R. CAVIER, Ann. Pharm. Françaises *18*, 583 (1960).

[2] Many strains of amoeba could be cultivated with great success by the use of the method described. The strain reported under 'Results' was repeatedly isolated from human stools containing *E. histolytica*. A detailed description of this strain, as well as the method of its isolation, is given in another paper 'A Novel Plate Method for Mass Culture of Amoebae' to be published elsewhere. It has been submitted to the Journal of the Egyptian Medical Association.

The cysticidal activity of compounds. The method mentioned above is used to test the sensitivity of the trophozoites of the amoeba and is not applicable for cysts of the amoeba. Cysts of the amoeba, however, are readily obtained, and in abundance, after 48 h incubation at 37°C.

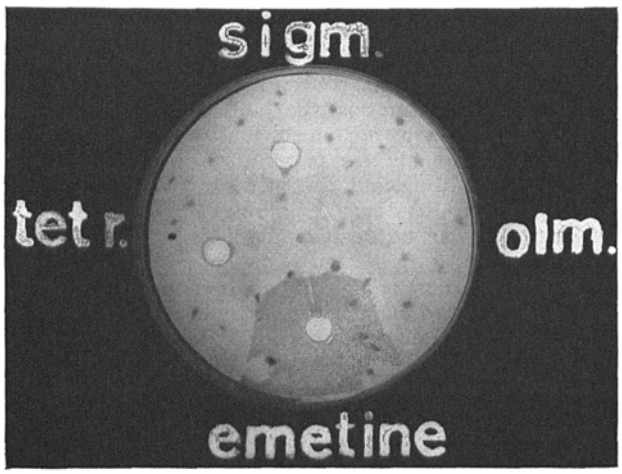

Fig. 3. Assay of amoebicides against the surface growth of the same amoeba in Figure 2. The amoeba was inoculated in many foci all over the plate marked by the opacities (due to bacterial growths). The plate was photographed by transmitted light. An 'inhibition zone' is noted within an area surrounding 'emetine'. The sectors of 'sigm' (sigmamycin), 'olm' (oleandomycin), 'tetr' (tetracycline) are devoid of any direct amoebicidal activity. The result was read here after 24 h incubation only, instead of 40 h as in Figure 2, because multifocal inoculation was used instead of the unifocal central inoculation used in Figure 2.

Saline is used to emulsify the cysts of the amoeba lying motionless on the surface of the agar with the aid of a platinum loop or a spreader as used in the preparation of bacterial vaccines. The suspension is then centrifuged at 1000 RPM for a few minutes and the supernatant is then discarded. Fresh saline is added to re-emulsify the cysts in the tube for recentrifugation. This is repeated 3–4 times or until the supernatant saline becomes perfectly clear. On a given cyst suspension (1,000,000 cysts/ml, for example) the cysticidal power of a test compound in a given concentration (1/1000, for example) after a known period of exposure and at a given temperature and the pH, can be determined. At the end of the exposure, the cysts are washed in saline and are tested for viability by staining and culture. Cysts that take the stain (eosin 1/1000) are considered non-viable. A loopful of the washed, exposed cysts is also inoculated on a fresh warm medium, prepared as usual, and watched for growth for 24–72 h before giving any report about the cysticidal power of the compound.

Growth stimulating substances. Whereas the spread of the amoeba towards a disc impregnated with an amoebicide is retarded, it was noted that the reverse is true, i.e. the spread is accelerated towards a growth stimulating substance.

Résumé. Une nouvelle méthode de culture d'amibes sur plaques d'agar (Figure 1) est avantageusement employée ici en vue de la détection rapide et facile de l'action antiamibienne directe des composés (Figures 2 et 3).

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Ministry of Public Health, Cairo (Egypt), February 10, 1964.

Continuous Optical Determination of Alkaline Phosphatase[1]

The possibility of a direct and continuous optical measurement of an enzymic reaction rate by recording the formation or disappearance of a chromogen with a recording photometer generally offers advantages in accuracy, precision and rapidity and allows a better control of kinetic behaviour.

A special substrate, phosphorylmonosalicylate, was proposed for measurement of phosphatase activity with direct and continuous optical methods[2,3]; but, at alkaline pH, a suitable substrate for such methods is also *p*-nitrophenylphosphate, very commonly used for phosphatase activity assay[4–6].

In the present paper a continuous optical procedure for the determination of alkaline phosphatase with *p*-nitrophenylphosphate as substrate is described in detail; this procedure has been applied to research concerning the adaptation of this enzyme in rat kidney[7].

Material and methods. The phosphatase source was a fraction of rat kidney homogenate, prepared as follows: the kidney was excised from the anaesthetized rat and immediately homogenized in 10 Vol of 300 m*M* sucrose, 2 m*M* EDTA, 10 m*M* triethanolamine buffer, at pH 7.2; the homogenate was centrifuged at 4000 *g* for 15 min and the supernatant fraction used as enzyme preparation. All operations were carried out at 1–3°C.

p-Nitrophenylphosphate was a B.D.H. product, glycerol-1-phosphate and glycerol-2-phosphate were Sigma products; other chemicals were from B.D.H. or Merck.

Phosphate was determined by the Fiske and Subbarow method[8]. Optical determinations were carried out with an Eppendorf recording photometer, operating with selected spectral lines from a mercury lamp[9].

[1] This research is a contribution from the 'Impresa Enzimologia' Group of the Italian Consiglio Nazionale delle Ricerche.

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