Technical methods

Estimation of blood phenylalanine from a dried blood spot using the Guthrie test

This technical method is based on the principle and method of estimating blood phenylalanine by bacterial inhibition assay as described by Guthrie and Susi (1963). The test is now being used extensively in the United Kingdom. Some modifications resulting from practical experience are suggested. In addition advice and information regarding the supply of materials is offered.

Although the test is simple to perform, attention to a number of factors, such as concentration of inhibitor, time of incubation, and concentration of spores, will improve the sensitivity and readability of the test. The accuracy is also determined by the choice of collection paper and other materials.

Principle

The inhibition of growth of *Bacillus subtilis* (ATCC 6051) by β-2-thienylalanine in a minimal culture medium is prevented by phenylalanine. This finding has permitted the development of a convenient agar diffusion microbial assay for the detection of phenylketonuria (PKU), employing small filter paper discs, impregnated with blood, placed upon the surface of the agar culture medium.

Preparation of Specimens

A small amount of fresh blood, obtained by heel puncture preferably soon after the infant is 6 days old, is applied immediately to a piece of thick, very absorbent filter paper. The correct choice of paper is important and some samples commercially available have proved unsatisfactory.

Schleicher and Schuell 903 is used by most centres in Europe and the United States, but Whatman ET 31 has been found to be a practical alternative. The blood spot when air dried should be at least ½ inch in diameter (but not more than ⅓ inch) and close enough to the edge of the paper to facilitate punching out the disc. These papers are so absorbent that even very viscous blood from an infant spreads through the paper, so that the appearance of the blood spot is similar on both sides of the paper. These conditions must be met to obtain a uniform blood sample by means of the paper punch.

Arrangements have been made by the Department of Health and Social Security for the bulk purchase of paper. A printed specimen card is available through the Department and where this is not suitable for individual requirements the unprinted paper is available.

The air-dried samples may be enclosed in polythene envelopes for protection but this is not essential. Transmission by post has been approved by the Post Office.

Before assay, the individual filter papers are numbered, placed on pieces of metal screening or wire test tube racks so that each is separated from the next and autoclaved at 15 psi pressure for three minutes with dry steam. Where dry steam is not available a conventional laboratory autoclave may be used in the following manner thus avoiding dam cards due to condensed steam. The cards are placed in the autoclave when the water is boiling. The lid is closed and no free steaming is allowed. The pressure is raised to 5 psi and the autoclave is then allowed to cool without opening the exhaust valve until the pressure has reached zero.

Autoclaving prevents blood pigments from later diffusing from the paper discs into the agar during incubation, masking growth zones. It also reduces greatly the number of samples which would otherwise show interfering effects due to antibacterial substances in the blood. Prolonged autoclaving will destroy the phenylalanine. Standard samples must also be autoclaved with each batch of specimens.

A disc, ¼ in. (6·35 mm) in diameter, is punched from the centre of the blood spot for subsequent transfer to the culture plate. One hundred discs and controls may conveniently be accommodated on a 10 in. × 10 in. or on a 7 in. × 11 in. assay plate. The 'template' method described by Kennedy (1969) saves time when transferring the dried blood spots to the agar.

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1 The members of the Lemag working party on the standardization of the Guthrie test were R. L. Newman, Dr J. Stern, D. J. Starr (Queen Mary's Hospital, Carshalton); G. L. Gibson (Royal Victoria Hospital, Belfast); G. Lindsay, R. Kennedy (Stobhill Hospital, Glasgow); P. Lilly (Hospital for Sick Children, London); W. B. Obank and C. R. Watt (Department of Health and Social Security).

2 Laboratories wishing to screen for several amino acids may find it convenient to use 3 mm discs.
Technical methods

their location in the tray or template. Great care must be used to avoid confusion of specimens.

Duration of incubation affects the readability of the test and to bring the time of reading on the following day to a convenient hour, the discs are transferred to the assay plate in the late afternoon. The plate is placed in an incubator at 35°C-37°C and left overnight (approximately 16 hours). Results are read by comparing the diameter of growth zones with those of the standards.

Preparation of Assay Medium

The PKU medium supplied by Oxoid Ltd as a dehydrated powder has been found to be suitable. Those laboratories wishing to make their own medium should follow the method described in the original paper (Guthrie and Susi, 1963).

The Oxoid PKU agar may be prepared as follows:

Oxoid pku agar, without inhibitor, dehydrated powder

β-2-thienylalanine solution, 0.01 M, (mol. wt. 171) (stock solution) Divided into daily aliquots and stored frozen.

Sterile distilled water

Spore suspension Prepared as detailed later.

Of the inhibitor solution, 0.8 ml is added to 200 ml of sterile distilled water at 60°C in a conical flask. The quantity of inhibitor may be varied slightly to give optimal results but the concentration must not be increased to the point where no growth is obtained with normal samples. Nine g agar is added and the mixture allowed to stand for 15 minutes. It is slowly brought to the boil on a hotplate with gentle heat as overheating may spoil the medium. After approximately two minutes' boiling the medium becomes clear and boiling is continued for a further two minutes. When the medium has cooled to 60°C, 3.0 ml of spore suspension is added and the contents of the flask are well mixed. Care is taken to avoid bubbles and the medium is poured into the assay plate and allowed to solidify.

Preparation of Spore Suspension

Ten large plates of potato infusion agar are inoculated with spore suspension and grown for 48 hours at 37°C. They are then left at room temperature for four days. When the growth is seen on direct examination to contain at least 75% sporing organisms, it is scraped from the plates and suspended in 100 ml of sterile distilled water. The mixture is placed in a water bath at 60°C for half an hour. The spores are washed in sterile distilled water and resuspended in distilled water to an optical density of 0.9 measured at 550 mµ wavelength in a colorimeter. The spores are tested on a PKU plate to ensure that a suitable intensity of growth is obtained. If these requirements are met the spore suspension is distributed into bijou bottles in 4 ml amounts and stored at 4°C. This remains stable for several months.

The cost of commercially prepared spore suspensions is very high and laboratories unable to produce their own spores may obtain supplies from Carshalton.

Preparation of Standards

It is essential that standards are made on paper identical with that used for collecting the specimens and that standards in whole blood are used. Aqueous solutions of phenylalanine are not satisfactory. Outdated blood is obtained from the hospital blood bank and assayed for phenylalanine content by any available procedure, such as that of La Du and Michael (1960). L-Phenylalanine is then added to a series of aliquots of blood to make concentrations of 2, 4, 6, 8, 10, 12, and 20 mg/100 ml. With a pipette, the blood is spotted on the paper, to make spots of between 1/2 and 6 in. in diameter. After drying, these standards may be kept in a dessicator at 2 to 5°C but storage at room temperature in a dry atmosphere is satisfactory for as long as three months. These standards are suitable for routine use but should be checked against reference standards produced by a more refined method where the phenylalanine content of the stored blood has been reduced by dialysis. The Department of Health and Social Security has approved arrangements for the issue at intervals of sets of reference standards to regional laboratories from Carshalton.

The range of standards given above will allow routine screening and also monitoring of patients on treatment. Alternatively, for routine screening a set of 4, 6, and 8 mg/100 ml standards may be used. It should be noted that the standards should be autoclaved together with the test cards. The standards should be distributed on the plate so as to cover all representative areas as a control of the even distribution of the medium, inhibitor, and spores and should include a previously autoclaved 4 mg standard at each corner of the plate. The plastic trays as supplied by B.B.L. last for approximately six months if used daily, but metal and glass trays, although more expensive, will last longer and do not warp.

Interpretation of Results

When the test is carried out on babies 6 days old, a blood phenylalanine level of 4 mg/100 ml or less should be considered negative provided some growth is visible round the discs. Further investigations
should be carried out on other results. Between 0·5 % and 1 % of samples show a result between 4 and 6 mg/100 ml. These babies should be retested at about 14 days when most levels will have fallen to normal.

Antibiotics

The effect of antibiotics can occasionally be seen in Guthrie testing but, provided the samples have been autoclaved, levels of phenylalanine above 4 mg/100 ml will not be masked. Further details can be found in the paper by Newman and Starr (1971).

Commercial PKU Reagents and Equipment

All of the reagents mentioned in this paper may be purchased commercially. Care should be taken to ensure that any standards purchased from commercial sources are prepared on paper the same as that used for collecting samples. Standards made from aqueous solutions of phenylalanine are not comparable with those made in whole blood. A list of suppliers is appended whose products have been found to be satisfactory.

Suppliers

\[\text{\begin{tabular}{l}
\text{Hand punch: Mau Industries Ltd, Moor Lane, Mansfield, Notts.} \\
\text{Plastic trays: B.B.L., Empire Way, Wembley, Middlesex} \\
\text{Metal and glass assay plates: Mast Laboratories Ltd, 38 Queensland Street, Liverpool 7} \\
\text{PKU agar: Oxoid Ltd, Southwark Bridge Road, London, SE1} \\
\text{Inhibitor: B-2-thienylalanine: Sigma Ltd, 12 Lettice Street, London, SW6}
\end{tabular}}\]

Reference standards and spores

Sets of reference standards will be sent at regular intervals to screening laboratories on application direct to: Dr R. L. Newman, Regional Infant Screening Service, Pathology Department, Queen Mary's Hospital for Children, Carshalton, Surrey (Tel: 01-643-3300). Spores may be obtained from the same source.

References


A rapid silver impregnation technique for oligodendrocytes, microglia, and astrocytes

T. Scott From the Department of Pathology, Maida Vale Hospital, London

Marshall (1956) described the arrangement of the cells of the reticular tissue throughout the body using silver impregnation methods based on those of Rio-Hortega. The method he used for the demonstration of metallophilic cells in frozen or paraffin wax-embedded sections is a simple one and is based on the Weil-Davenport modification (Weil and Davenport, 1933; Weil, 1946) of Stern's method (1932) for demonstrating astrocytes and oligodendrocytes in cellloidin sections. We have found it to be of particular use in tissues of the central nervous system as a rapid and reliable method which can be employed to give useful impregnation of astrocytes, oligodendrocytes, and microglia. Minor variations of staining technique will heighten the degree of impregnation obtained in any one cell type. The method may also be used for the demonstration of microglia in cellloidin-embedded sections.

Method

Paraffin sections

Cut sections of 15-20 μ thickness and transfer directly into two successive xylol baths. Place sections in absolute alcohol then in 50 % alcohol. Wash twice in distilled water.

Frozen sections

Cut sections of formalin-fixed material of 20-25 μ thickness. Wash twice in distilled water. Impregnate sections in the silver bath for a variable time; usually three or four seconds is sufficient but often the shortest possible time is preferable. Transfer the sections rapidly to the formalin bath, moving them continuously while reduction takes place (approximately 30 seconds). When reduction is complete the sections should be an even, reddish brown colour. Wash the sections twice in distilled water and fix in 5 % sodium thiosulphate. Wash the sections twice in distilled water, fix on to glass slides, dehydrate, clear, and mount.

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