Technical methods

Use of cellulose acetate gel (Cellogel) for the demonstration of lactic dehydrogenase isoenzymes

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The demonstration of lactic dehydrogenase isoenzymes is not a particularly easy procedure, the difficulties falling into two categories: (1) obtaining a suitable electrophoretic separation and (2) the subsequent staining of the preparation to demonstrate enzyme activity.

The earliest electrophoretic separations (Vesell and Bearn, 1957) were carried out using starch gel as a support medium (Smithies, 1959). This is probably the best medium to use but the technique is difficult, the electrophoretic run takes 18 hours, and staining the rather bulky blocks requires quite large volumes of the expensive reaction fluid. It is, therefore, not surprising to find that micro methods have been evolved, using agar gel on microscope slides (Wieme, 1959; Blanchaer, 1961) and cellulose acetate strips (Barnett, 1964). Agar gel slides are not particularly easy to handle and require careful preparation and storage, whilst the cellulose acetate strip method involves a transference procedure for staining. While seeking a better support medium, we used cellulose acetate gel (Cellogel) and after a few minor adaptations in technique found it to be an excellent medium for isoenzyme work, without any of the previously mentioned disadvantages, and capable of giving a very rapid separation in about one-and-a-half hours.

METHOD

The Cellogel blocks are manufactured by Chemetron of Milan and are marketed by Shandon in this country. They are supplied in packets of five, each block measuring 6×17 cm., and are received in a moist condition inside a waterproof bag. They must at all times be kept moist, and it is recommended that they be stored in 50% methanol. When so stored it is best to wash them in running water for a few minutes before use, but the new blocks work quite well without previous washing.

If a block is cut to give two 6×8.5 cm. pieces, each piece will be sufficient for at least four isoenzyme investigations. The first step is to cut a fine slit for the inoculation. It is essential to cut or scratch the surface, otherwise the specimen will not migrate at all. After many experiments, including the use of paper wicks, it was found that it was best to make a slit with a modified safety razor blade, as shown in the sketch (Fig. 1). By pressing lightly

FIG. 1. *Slit cutter formed from safety razor blade.*

and sliding the razor from side to side a fine slit can be cut in the block without injuring the surface. The block can be rested on clean filter paper while cutting the slits, which are made parallel to the long edge and about 2 cm. from it. Four slits are cut, with equidistant spacing and the block then placed in buffer to soak. Filter paper wicks are cut from thick Whatman no. 4, two pieces 9×12 cm. and two pieces 9×8 cm. being made into double thickness wicks which fit each side of the block as shown (Fig. 2). It is easier to place the wicks in position when they have been soaked in buffer, the short wick being put on the edge 2 cm. from the slits and the long one to the edge 4 cm. from the slits. When ready the block is laid on top of the little pegs inserted in the central ridge of a Kohn electrophoresis tank. The compartments of the tank on the positive (anode) side are filled as fully as possible, whilst those on the negative (cathode) side are kept at as low a level as possible. The long and short wicks enable the block to be displaced from a central position in the direction of the cathode, and by this means a slight hydrostatic pull against the current flow enables the LD₅, travelling with the gamma globulin, to be positioned just to the cathode side of the slot and not on it. The Oxoid barbitone buffer pH 8.6, supplied by Oxo Ltd., is very easily prepared and has proved to be quite satisfactory and even superior to borate buffers for this separation. When the block is in place it is left for a few minutes to allow any superfluous buffer to drain off, and when a completely dry surface is obtained, the serum, etc., can be applied.

Each slit is inoculated by means of a very finely drawn-out Pasteur pipette. The application is in the region of 5 to 8 μ l., but in practice if about six drops of serum are

FIG. 2. *Section of Kohn tank showing Cellogel block and wicks in place.*

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applied with these fine pipettes, this is achieved. It is essential to obtain a very fine line of inoculum for the best results of narrow discrete isoenzyme bands. The serum sinks into the Cellogel and stains the surrounding surface slightly. When the four applications have been made, the current is switched on. At a constant voltage of 150 volts separation takes about one-and-a-half hours. A jaundiced serum is a good marker to use at first, the bilirubin travelling with the albumin and the run being terminated when the yellow band is about $\frac{1}{8}$ in. from the edge of the anode wick. After a little practice the weak bilirubin band of non-jaundiced serum can be followed by viewing the block obliquely.

For staining, the block is removed and will be found just to fit inside the lid of the normal sized petri dish. The most suitable reactant fluid was found to be that of Ressler (Ressler and Joseph, 1962). This can be slightly modified to give a larger volume of stain as follows: 1 Tris buffer 0.1 M (18.3 g./litre adjusted to pH 7.4). This usually requires the incorporation of 140 ml. N/HCl in each litre).

2 Sodium lactate 2.14 M

3 Potassium cyanide 0.06 M

4 Each day a 0.2% solution of N-methyl-phenazonium methosulphate is required. This solution is light sensitive and lasts only a few hours even in the dark. As only a little is required it can be prepared by dissolving 10 mg. N-methyl-phenazonium methosulphate in 5 ml. distilled water.

To prepare the staining solution, 35 ml. of (1) is placed in a beaker, 1 ml. of (2) is added, followed by 3 ml. of (3). Then, 25 mg. of diphosphopyridine nucleotide (N.A.D.) is added, followed by 19 mg. of p-nitro blue tetrazolium, mixed, and finally 0.4 ml. of (4) added.

Solution is easily brought about by leaving in the 37°C. incubator for a short time. If the stock solutions 1, 2, and 3 are ready, the whole stain can be prepared in a few minutes just before the end of the electrophoretic run. It is then poured on to the block in the petri dish and incubated at 37°C. Gentle agitation at intervals over half an hour is necessary to prevent unsightly deposition

FIG. 3. *Lactic dehydrogenase isoenzymes on Cellogel (left to right). 1 LD₁ and LD₂ bands in serum from a case of myocardial infarction. 2 Mixture of LD₁ and LD₂ run in Labtrol. 3 LD₃ run in Labtrol. 4 LD₁ run in Labtrol. 5 LD₁, LD₂, and weak LD₃ serum from a case of pernicious anaemia.*

of the dye which sometimes occurs when deeply staining bands develop.

The block is removed when maximum stain intensity has been achieved and washed in running water, when the Cellogel quickly reverts to its white colour. Control markers may be run by adding a drop of the pure LD₁ (heart) isoenzyme and pure LD₅ (muscle) isoenzyme to a little Labtrol control serum. (Both these and the N.A.D. can be obtained from Boehringer Ltd.). It is most important to use p-nitro blue tetrazolium and not one of the many tetrazolium blues.

This procedure has been of great value in diagnosing myocardial infarction (Warburton, Bernstein, and Wright, 1965) since the materials are always ready, and the procedure can be run concurrently with the SGOT and total SLD determinations, all three tests being accomplished within two hours.

All the five LD isoenzymes are separated by this method (Fig. 4) and if a Chromoscan integrating densitometer is available, the uncleared strips can be scanned by reflectance using a green filter (520 mμ) and a slit of 0.5 mm. × 5 mm.

FIG. 4. *Chromoscan tracings. 1 LD₁, LD₂, from a case of myocardial infarction. 2 LD₁, LD₂, LD₃, LD₄, and LD₅ from a case of carcinomatosis. 3 LD₁ and LD₂ in a normal erythrocyte. 4 LD₁ and LD₂ in a pernicious anaemia erythrocyte.*

Depending upon the intensity of the bands, the sensitivity of the instrument is adjusted by selecting one of the four cams of varying slope. The bands are sufficiently demarcated and deeply stained to show the difference in LD₁ and LD₂ isoenzyme patterns of normal red cells and pernicious anaemia red cells (Fig. 4).

SUMMARY

Cellulose acetate gel (Cellogel) blocks form an ideal medium for the separation of lactic dehydrogenase isoenzymes. Primarily intended for electrophoresis of plasma proteins, they can be used for isoenzyme separations if the slight modifications described are incorporated. No prior preparation of the medium is required; separation is very rapid and direct staining with minimal quantities of reactant fluid is possible. All five lactic dehydrogenase isoenzyme bands are separated clearly and intensely enough to allow of good reflectance scanning by the Chromoscan integrating densitometer.

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Low temperature storage container using carbon dioxide

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Electric refrigerators which will maintain temperatures of -70°C . are now readily available and reliable. However, the initial cost of such refrigerators is high and the danger of a mechanical or power failure cannot be completely eliminated. Liquid nitrogen is particularly valuable for preserving cells (Nagington and Greaves, 1962), but it is neither so easy to handle nor so readily available as solid carbon dioxide, which is satisfactory for preserving viruses.

Low temperature storage containers using solid carbon dioxide have recently been described by Ring (1964) and Busby, House, and MacDonald (1964). The success of such a container depends on adequate thermal insulation, good conduction between the solid carbon dioxide and the specimens, and the prevention of condensation in the insulating material used for its construction. In the container described below insulation was provided by expanded polystyrene, good conduction internally was assured by an inner aluminium lining, and condensation was prevented from penetrating the insulation by a complete covering of polyester resin reinforced with fibre glass. The latter material is easily moulded by hand to form a continuous inner and outer covering, and any ice which forms on the inner surface near the top of the box is easily removed. The expanded polystyrene provides a good barrier to conducted heat, but not to radiant heat, which is reflected by the internal aluminium container.

MATERIALS AND METHOD OF CONSTRUCTION

The container is constructed from 6 in. thick expanded polystyrene (Jablo Plastics, Jablo Group Sales, Ltd., Mill Lane, Waddon, Croydon, Surrey), as shown in the diagram (Fig. 1). The necessary pieces are cut from two standard sheets (one 4 ft. $\times$ 4 ft. and one 2 ft. $\times$ 6 ft.), and glued together with U.S. adhesive and hardener (also from Jablo Plastics). To provide a well-fitting lid which allows excess carbon dioxide to escape, the upper edge of the box and the lower surface of the lid are covered with $\frac{1}{2}$ in. plywood. Before the fibre glass and polyester resin (Filabond Polyester resin No. 8748A, James Beadel & Co. Ltd., 26 Old Bailey, London, E.C.4) are applied the box is given three coats of oil paint to prevent the styrene in the resin attacking the expanded polystyrene. This difficulty may alternatively be overcome by using polyurethane foam (Baxenden Chemical Co. Ltd., Baxenden, Lancs.) in place of expanded polystyrene, but the cost of

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