THE AGAR-ROPE TECHNIQUE FOR ESTIMATING PENICILLIN IN LIQUIDS

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Many methods for the estimation of penicillin have been published. Although chemical and physical methods may be used for its estimation in fairly pure preparations, in crude liquids and in low concentration the biological test is still necessary.

The following method is described because it can be carried out in a small clinical laboratory without special equipment and without the necessity of maintaining sterility. It is applicable to any body fluid, and it is possible to make a reading after three to four hours. The method is not suitable for the estimation of minimal detectable amounts.

Method

Serial dilutions of the material to be tested are placed in small vessels each of which contains a small block of agar containing bacteria, which, by their metabolism, produce metabolites in a short time. If glucose is added to the agar the change in pH is shown by an indicator such as phenol red. However, if the material to be tested shows any antibacterial activity, there will be no metabolites—i.e., no acid change. For an exact estimation of penicillin a series of dilutions of known strength are run in parallel with the test material. The results of our method were so constant that eventually we found it unnecessary to run a control series for each estimation.

Preparing the Agar Rope.—A rubber tube provided with a mouth-piece is connected to a glass tube of about 45 cm. in length by 6 mm. internal width, which is sterilized by flaming just before use or by autoclaving.

A penicillin-sensitive strain of *Staphylococcus aureus* is used. The standard Oxford strain H is suitable, but strains of different origin, when constant in their behaviour, will do as well. From an agar slope culture less than seven days old the coccus is seeded to two fresh agar slopes. The bacteria are grown at 37° for 16 hours and washed off with 2.5 ml. of sterile 0.8% saline. Both lots of bacterial suspensions are mixed and allowed to stand for sedimentation of the coarser particles. Then 1 ml. is pipetted into an ordinary test-tube and 5 ml. of glucose meat infusion broth is added. This broth must be brought to pH 7.6. Care should be taken that the broth has been sterilized carefully with regard to time and pressure.

Meanwhile a 4% block of agar is liquefied in water and cooled to 80° C.; 6 ml. of this is added to the 6 ml. suspension of bacteria in glucose broth, not the converse, and rapidly mixed. Then the agar mixture is drawn rapidly into the sterile 45 cm. glass tube, leaving about 1 ml. at the bottom of the test-tube, and the rubber tubing is closed with a clip. The long glass tube is quickly cooled in running tap water

without being withdrawn from the external test-tube, and the agar sets immediately. In a few minutes when the agar has solidified the long glass tube is taken out and wiped with a piece of cotton-wool. After removing the clip from the rubber tube the agar rope can easily be blown in a sterile petri dish. When the agar rope begins to form it will generally fall by its own weight. If a straight rope is preferred, it can be put in an aluminium dish 50 cm. long and covered by a lid; in this position it will be easier to cut the rope into small blocks. For purposes of sterility the first centimetre that comes out of the glass tube is cut off and discarded. The agar rope may be put into the ice chest at once and is ready for use. In practice it has been found that the rope should not be used before the first 24 hours nor after 10 days. After use the glass tube can be cleaned easily, sterilized, and prepared for the next rope.

Making Dilutions.—For making the dilutions the same slightly alkaline meat broth with 0.5% glucose is used. To each millilitre one drop of phenol red solution (1:1,000) is added. It is advantageous to use a drop technique for making the dilutions. When ordinary Pasteur pipettes are standardized this technique is no less accurate than measuring by calibrated pipettes. It is easy to standardize the external diameter by moving the capillary part of Pasteur pipette in a slit of exactly 1.0 mm. width cut in a small brass plate. When more exact figures are wanted the weight of the drops of the different liquids can be determined, and the viscosity and surface tension of the different liquids thus taken into account.

Preparing Agar Blocks.—It is necessary to have agar blocks of fairly equal dimensions, and to secure equality 11 thin razor blades are joined by two long screws through their holes, and the blades separated by brass rings to a distance of 1 mm. They are then fixed to a handle. By gently pressing the knives over the rope 10 equal disks will be obtained and will adhere between the blades, from which they can be pushed down by a small spatula.

FIG. 1.—Apparatus required for agar-rope technique of estimating penicillin in liquids.

be bactericidal. It is advisable to fit some cover or lid on the trays so that they can be piled up in the incubator and evaporation is suppressed. Aluminium as a material has the advantage of low heat capacity, high heat conductivity, and relative inertness.

Making Titration Trays.

—The tray can easily be made by impressing an aluminium plate of 2 mm. thickness with a male and female die of polished brass or iron. We used trays with 10 excavations each, each well (20 mm. diameter $\times$ 7 mm. deep) holding about 1 ml. of liquid. For practical purposes 12 drops per well is satisfactory as long as the agar disks are totally submerged. The trays are readily cleaned by rinsing. They should be well polished, as the lustre of the metal assists in reading the results. Take care to wash away traces of polish with soap or alcohol, as they may

Carrying out the Reaction.—A penicillin solution of known strength is diluted in phenol red glucose broth to a concentration of 1/20 unit per ml. In the wells 12, 11, 10, 9, 8, 7 drops of this penicillin solution and 0, 1, 2, 3, 4, 5 drops of phenol red glucose broth are added. The wells then contain successively 1/20, 1/22, 1/24, 1/27, 1/30, 1/34 unit per ml. Then 10 agar disks are cut with the cutting device from the agar rope and one disk dropped with the small spatula into each well. (Further dilutions can be tested if desired.) The tray is covered and put into the 37° C. incubator. After three to four hours the tray can be taken out and the result read. In practice we usually found that the liquid and disks in the third well (1/24 unit per ml.) were red whereas in the fourth (1/27 unit per ml.) they were yellow. Therefore 1/24 unit per ml. suppressed the metabolic processes of the staphylococcus.

Oblique illumination should be used for taking readings. In doubtful cases the agar blocks can be lifted from the wells on to the flat surface of the trays. Artificial light makes the readings somewhat more difficult, in which case a "daylight lamp" with a blue glass bulb may be used. Readings can be made by holding the tray at the level of the lamp and inspecting vertically.

The following examples may be useful.

Estimation of Penicillin in Culture Media.—Let it be supposed that the medium contains about 70 units per ml. and then dilute it 1/1,000 in phenol red glucose broth in a 3 ml. amount. Put 12, 10, 9, 8, 7, 6, 5, 4 drops in each well successively and add 0, 2, 3, 4, 5, 6, 7, 8 drops of phenol red glucose broth from the same pipette. Suppose that in the control series suppression of acid formation again takes place in the well containing 1/24 unit per ml. If suppression in the first well only has been observed then the penicillin content of the original liquid was 42 unit per ml. It would have been 50, 56, 63, 71, 83, 100, 125 unit per ml. respectively for each successive well showing red-coloured contents. If at the same time an original dilution of 1/500 or 1/2,000 had been prepared intermediate values could have been determined, so giving a more exact reading.

Estimation of Penicillin Content of Blood Serum.—A suitable way is to add to 2 ml. of serum 1 ml. of glucose broth pH 7.6 without phenol red. Put 11, 10, 9, 8, 7, 6, 5, 4, 3 drops into the wells and add 0, 1, 2, 3, 4, 5, 6, 7, 8 drops of glucose broth without phenol red and then one drop of phenol red 1:1,000 to each well. It may be advantageous to prepare the standard dilution of known penicillin content with normal human serum. Suppose that again the well containing 1/24 unit per ml. of standard penicillin is the first that shows lasting red colour, then each successive red-coloured well of the unknown serum indicates an original penicillin content of 0.06, 0.07, 0.08, 0.09, 0.11, 0.13, 0.15, 0.19, 0.25 unit per ml. serum. Shortly after an intramuscular injection of 40,000 units of penicillin the blood content may be so high that a dilution of 1:10 will be necessary. Then the readings will run from 0.27 to 1.00 unit per ml. serum or even higher if necessary.

As for serum determinations, we must admit that this is not a micro-method; in our opinion, however, this is fully counterbalanced by the advantages of the very simple bacteriological demands.

Discussion

The reason why this technique works so rapidly and easily is due to the fact that it uses material very rich in bacteria. Their fixation in the agar disk protects them from external influences. In the original cylinder plate method it was known that for reproducible results it is of some advantage to keep the ready poured plates with the staphylococci for 24 hours in the ice chest. We

found that this was also true for the agar rope; we have observed too that the properties of the rope do not alter until after 10 days. The number of ropes to be prepared each week can be calculated, as we know that one rope of 45 cm. will suffice for 35 to 40 determinations. The use of material is minimal. The rich "inoculum," the fact that the indicator organism is protected by the agar, and the rapid reading make sterile precautions unnecessary. The use of clean materials and sterile phenol red glucose broth will suffice. Some chance contamination of the utensils will be of no significance whatever, as the time of incubation is four hours at the most.

In principle the use of staphylococci is not obligatory. Any other organism sensitive to penicillin and able to produce a readable change in any indicator will be suitable. It should be possible to make estimations of other antibiotics (e.g., streptomycin) by this technique. The haemolytic streptococcus is more sensitive to penicillin and it can be used in the following way.

A suspension of suitable haemolytic streptococci is prepared, preferably so that no haemoglobin can stain the fluid. This can be done by growing the streptococci on Loeffler serum medium. To 5 ml. of this suspension are added 0.75 ml. of defibrinated horse blood and 5.75 ml. 4% agar in water as already described, and the agar rope is prepared. Readings are made on haemolysis in the agar disks—i.e., the change from turbid red to laked red, and often after only two hours' incubation. In this case a standard penicillin control must be set up as the haemolytic streptococcus is much more variable than the staphylococcus. In our experience haemolytic streptococci present another disadvantage not found in staphylococci. They are, in varying degree, sensitive to the bacteriostatic power of normal serum, thus giving rise to anomalous results (Kirby and Rantz, 1944; Dolkart, Dey, and Schwemlein, 1947). More than 50% of normal sera are endowed with some anti-streptococcal power. The same is true of *Bacillus subtilis*; 90% of normal sera show some bactericidal power towards these organisms (Elias, Merriam, and Speicher, 1945). For estimations in culture media the described technique is easily adapted to *B. subtilis* (Pratt and Dufrenoy, 1947), *Streptococcus dysgalactiae*, or other organisms, all its advantages being maintained.

Summary

A technique is described for the rapid determination (after two to four hours) of penicillin or other antibiotics in liquids such as serum, body fluids, or culture media. Accurately prepared agar disks, mechanically cut from an agar rope, containing heavy inocula of bacteria, are submerged in small quantities of graded dilutions of known, compared with unknown, penicillin-containing fluids. Staphylococci are most suitable; the method can be adapted for the use of other bacteria.

An indicator (phenol red, haemolysis, etc.) detects normal compared with suppressed bacterial activity.

The method is rapid, simple, and involves the use of a minimum of utensils which are to hand day and night and so can be used with the most rudimentary bacteriological equipment. It will suffice to have the basic bacteriological material supplied weekly from a better equipped institute.

The sensitivity of the method is 0.04 unit per ml. (serum). The quantity of material wanted for the test is 2 ml. (serum of 0.04 unit per ml.) or considerably less. With streptococci sensitivity can be augmented to 0.01 unit per ml. With practice this technique will save time, personnel, and materials.

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