APPLICATION OF CIRCULAR PAPER CHROMATOGRAPHY TO THE DIFFERENTIATION OF BACTERIA BY ENZYMIC TESTS

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The study of arginase and arginine dihydrolase in bacteria has shown that the presence of these arginine-degrading enzymes can serve as a differential test for certain species and bacterial types[1–5].

Our earlier investigations[6] have shown that arginase, the enzyme that degrades arginine to ornithine and urea, is present in very few bacterial species. For staphylococci we[5] found that a correlation exists between the presence of arginase and the pathogenicity of the various bacteria, which indicates that arginase has a special significance for these microorganisms. In the case of streptococci it was found that the enzymic system known as the arginine dihydrolase system, which converts arginine to ornithine, citrulline and ammonium, is only present in the haemolytic group, being absent in the virulent one. The degradation process of arginine can therefore serve as an important enzymic criterion for differentiating between the various bacterial species, and especially for purposes of differentiation within a group of the similar species. Therefore, a precise and at the same time simple method for determining the products of the enzymic degradation of arginine will be very useful.

Partition chromatography on filter paper, in the form of circular chromatography[7], possesses all the qualities of such a method. It was applied by us for purposes of differentiation, using an arginase test[5] in the case of staphylococci and an arginine dihydrolase test[4] in the case of streptococci.

PROCEDURE

The bacteria used were in the "*resting*" form. Bacteria from 18 to 20 hour-cultures on gelose were washed once with an isotonic saline solution and twice with distilled water; the final suspension had a microbial density of about 20–50 mg/ml (expressed as dry residues). This suspension was incubated at 37°, under a toluene layer, with a 1% solution of L-(+)-arginine hydrochloride in a buffer medium at pH 9.0 for the arginase test and at pH 8.0 for the arginine dihydrolase test. In the case of both enzymes the presence of Mn^{+2} was necessary for enzymic activity. The incubation time was 12 to 24 h. Control systems containing bacteria inactivated by keeping at 100° for 60 min, were treated in the same manner. Each experiment comprised the following systems:

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System I: 1 ml bacterial suspension (20–50 mg/ml)
1 ml buffer solution (*M*/15)
1 ml L-(+)-arginine monohydrochloride (1% soln.)
1 ml toluene

System II: 1 ml inactivated bacterial suspension (60′, 100°)
1 ml buffer solution (*M*/15)
1 ml L-(+)-arginine monohydrochloride (1% soln.)
1 ml toluene

After incubation (12 to 24 h at 37°) both systems were centrifuged and the supernatants submitted to qualitative and quantitative chromatographic determination on the same circular paper (diameter 30 cm).

The samples to be tested were spotted on different points on the circumference of a circle (about 5 cm diameter) drawn from the center of the circular paper. The test samples and known quantities of standard amino acid solution (0.005 ml of *M*/100 solution of arginine, citrulline, ornithine) as well as urea solution, were spotted on the same paper. The spotting of the solution was done with a micrometer syringe. The circular paper was placed on the surface of a Petri dish (25 cm diameter) containing the solvent mixture in a small dish at the center. A filter paper wick going through the center of the circular paper established contact between the solvent mixture and the circular paper disc.

The circular paper was held on the surface of the Petri dish by the lid, a space being kept between the paper and the lid. The whole set-up was covered with a bell jar.

The solvent mixtures used, were those that give a distinct separation of the amino acids being investigated[8] (namely arginine, ornithine, citrulline and urea). They were: *n*-Butanol–acetic acid–water (40:10:50); pyridine–isoamyl alcohol–acetic acid–water (80:40:10:40); phenol–isopropyl alcohol–water (70:50:25).

After the chromatograms had been irrigated for 24 hours the filter paper was dried at 90° and sprayed with a 0.2% ninhydrin solution in chloroform for the detection of all amino acids, with 8-hydroxyquinoline reagent for arginine[9] and with the Ehrlich-Dent reagent for urea.

For the quantitative determination of the amino acids identified by the qualitative test, the filter paper was sprayed with the following solution according to BARROLLIER[10]: 73 mg $CdCl_2$, 6 ml H_2O, 0.3 ml glacial acetic acid, 100 ml acetone, 2 g ninhydrin. After heating the chromatograms at 60° for 20 min the spots were cut out and put into separate test tubes containing 4 ml of methanol for extraction of the colour. The coloured extracts were determined by photometric reading against standard amino acid solutions that had been submitted to the same treatment.

RESULTS

(*a*) *Staphylococci* (*arginase test*)

Pathogenic staphylococci. Chromatographic investigation of the corresponding systems after incubation with arginine showed that arginine had completely or partially

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disappeared and that ornithine as newly formed amino acid was always present (spot A). The intensity of the enzymic activity was directly proportional to the amount of ornithine formed and to the amount of arginine that had disappeared from the same chromatogram (spots A and B).

Non-pathogenic staphylococci. Chromatographic investigation of the systems incubated with arginine revealed the absence of ornithine and of all other amino acids with the exception of the arginine initially added (spot D). The concentration of arginine on these chromatograms was the same as that found with the inactivated test system. The difference between the pathogenic and the non-pathogenic staphylococci

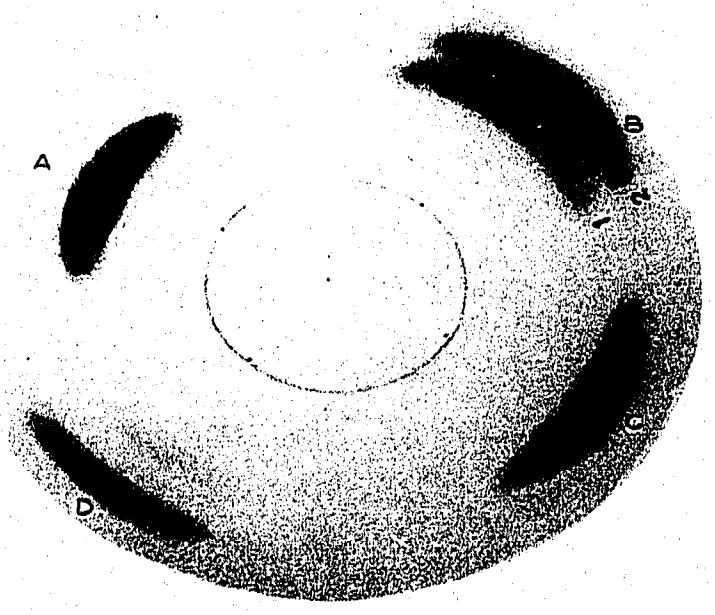

Fig. 1. Chromatographic differentiation between pathogenic and non-pathogenic staphylococci by an arginase test. A. Pathogenic staphylococci (Arginase-positive strain). Complete conversion of arginine to ornithine (spot of ornithine). B. Pathogenic staphylococci (arginase-positive strain). Partial conversion of arginine to ornithine (spots of ornithine (1) and arginine (2)). C. Pathogenic staphylococci (arginase-positive strain). Inactivated by heat (60′, 100°). Arginine not transformed (spot of arginine). D. Non-pathogenic staphylococci (arginase-negative strain). Arginine not transformed (spot of arginine).

was clearly illustrated by the appearance of ornithine in the case of the former group, and the absence of ornithine in the case of the latter. The presence of urea, identified on the chromatograms with the Ehrlich-Dent reagent and confirmed by the precipitation reaction with xanthydrol, indicates that the metabolism of arginine in pathogenic organisms proceeds via a ureogenic route. The arginase test (based on establishing the presence of ornithine and urea) has been shown, by comparison to classic tests of pathogenicity (coagulases, haemolysins, etc.) to be a valid criterion for the pathogenicity of staphylococci. Fig. 1 illustrates the chromatographic aspect of the metabolism of arginine in staphylococci.

(*b*) *Streptococci* (*arginine dihydrolase test*)

The chromatographic investigation of the metabolism of arginine in streptococci

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reveals the following facts: in the case of haemolytic streptococci arginine disappears completely or partially and concomitantly ornithine, or ornithine and citrulline, appear. Chemical assay indicates that ammonia is formed in these organisms and not urea, therefore the reaction is catalysed by arginine dihydrolase, as has been shown by various investigators. Recently[11], it has been established that arginine dihydrolase consists of two distinct enzymic systems: arginine desamidase, which hydrolyses arginine to citrulline and NH_3 and citrullinase which decomposes citrulline to orni-

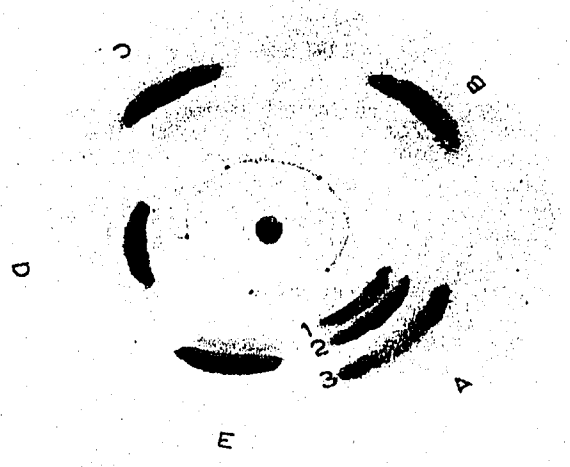

Fig. 2. Chromatographic differentiation within the streptococcus group by the arginine dihydrolase test. E. Viridans group: intact arginine spot. B and C. Haemolytic group: formation of citrulline and traces of ornithine. D. Haemolytic group: formation of ornithine. A. Solution containing known amounts of ornithine (1), arginine (2) and citrulline (3).

thine, NH_3 and CO_2; in the case of haemolytic streptococci two different groups can be distinguished on the basis of the intensity of their citrullinic action. Under identical experimental conditions one subgroup transforms citrulline into ornithine much more rapidly than the other; this is shown on the chromatogram (Fig. 2) by the complete disappearance of citrulline from the system (spot D). In the second subgroup, chromatographic analysis reveals the presence of citrulline and only traces of ornithine (spots C and B).

In the case of the viridans group of streptococci, neither ornithine nor citrulline is found to be present and there is no alteration of the initial concentration of arginine added as substrate to the system (spot E).

The chromatographic picture of the metabolism of arginine in streptococci shows that there are three distinct groups:

(a) Strains that do not degrade arginine (the viridans group).

(b) Strains that degrade arginine, forming citrulline and eventually also ornithine.

(c) Strains that degrade arginine, forming ornithine. These last two groups are subdivisions of the haemolytic streptococci.

Fig. 2 shows the chromatographic aspect of the metabolism of arginine in streptococci.

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SUMMARY

This paper presents the results of the application of circular filter paper chromatography to the differentiation of pathogenic and non-pathogenic staphylococci by means of an arginase test, and to the differentiation of haemolytic streptococci and viridans streptococci by an arginine dihydrolase test.

Chromatographic assay indicates that in the group of staphylococci only the pathogenic and not the non-pathogenic staphylococci metabolize arginine to ornithine and urea (presence of arginase).

Chromatographic assay indicates that in the group of streptococci only the haemolytic streptococci and not the viridans streptococci metabolize arginine to ornithine and citrulline, or to ornithine or citrulline only (presence of arginine dihydrolase in the haemolytic group).

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Received January 20th, 1958