Separation of polymyxin B, D, E and M by thin-layer chromatography

The antibiotics of the polymyxin group are peptides with very similar structures[1,2], consequently their separation and identification presents quite a difficult analytical problem. Though investigations by paper chromatography of the amino acid components of their hydrolysates[1,2] affords a sure means of identification, this is a lengthy process and successful only with isolated products. The paper chromatography method with isopropylamine proposed by MISTRETTA[3] deserves special mention since it gives a good separation of intact polymyxin B and D, but the long time needed for development prevents quick evaluation, NASH AND SMASHEY[4] suggest the use of impregnated paper for this purpose.

Our object was to find a suitable method for the rapid and reliable separation and identification of polymyxin antibiotics when present as a preparation or in a nutrient medium.

Experimental

Experiments were carried out for the direct separation, and identification by TLC without hydrolysis of four types of antibiotic substances of the polymyxin group. For this a 15:5:1:2 mixture of acetone–water–acetic acid–2 *N* NH_4OH proved to be the most suitable, a 100 μ layer of Merck's Kieselgel G was used as the adsorbent. For the location of the spots, a ninhydrin spray and biological reactions were used. The standard preparations available were the following: polymyxin B sulphate (Pfizer); polymyxin D sulphate, isolated here; polymyxin E sulphate (Colistin, Laboratoire Roger Bellon); and polymyxin M sulphate from the U.S.S.R. These were also identified by their amino acid components, through paper chromatography according to the literature[1]. Table I lists the amino acid components of the four polymyxins investigated.

TABLE I

Polymyxin	*α,γ-Diamino-butyric acid*	*Phenyl alanine*	*Leucine*	*Serine*	*Threonine*
B	+	+	+	−	+
C	+	−	+	+	+
E	+	+	+	−	+
M	+	−	+	−	+

Investigation of the purified solid substances. In the first part of our work the R_F values of the four standard substances were determined separately, and then in a mixture. Glass plates 20 × 5 or 20 × 20 cm carried the Kieselgel G layer, and 20 to 50 μg samples of the polymyxin sulphates dissolved in water were spotted on them. Development with a 15:5:1:2 mixture of acetone–water–acetic acid–2 *N* NH_4OH took 90 min.

Spots were developed on the plates dried at 80°, with a 0.25 % solution of ninhydrin in *n*-butanol. R_F values, as shown in Fig. 1, were as follows: polymyxin B = 0.45; D = 0.51; E = 0.95; and M = 0.36.

Fig. 2 shows chromatograms, prepared in the same way, of the polymyxins used as standards, the spots being made visible on plates inoculated with a *Bordetella bronchiseptica* strain. Here the samples were 5 to 10 μg of polymyxin sulphates.

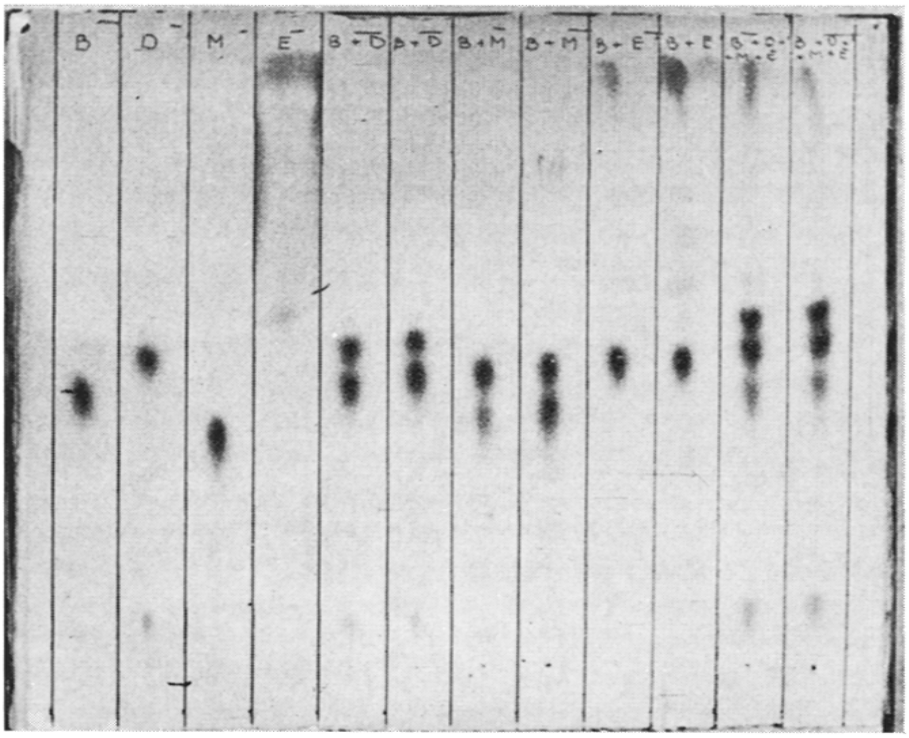

Fig. 1. Separation of polymyxin B, D, E, and M. Spots made visible through application of a 0.25% solution of ninhydrin in *n*-butanol saturated with water.

The figures show that pure polymyxin E dissolved in distilled water has two biologically active spots at lower R_F values than the principal component (R_F 0.95). One of these scarcely separates from the principal spot. No such phenomenon was observed with the other standard substances.

Identification of the active substance of a fermentation liquor containing polymyxin B. Since ninhydrin is positive to other substances present in fermentation broths the detection of the polymyxins was carried out by biological testing. Fermentation broths were acidified to pH 3 with solid oxalic acid and centrifuged for about 10 min. The supernatant liquid was neutralized with 2 *N* sodium hydroxide and its polymyxin B content was approximately determined biologically. A sample of this liquid,

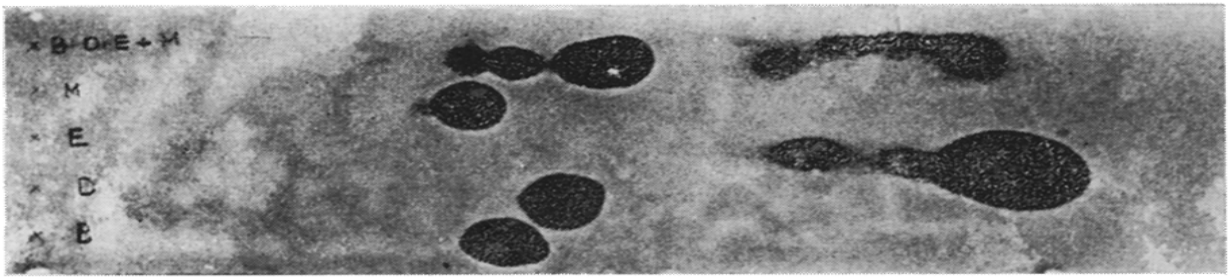

Fig. 2. Separation of polymyxin B, D, E, and M. Spots made visible on an agar plate inoculated with *Bordetella bronchiseptica*.

calculated as containing about 5 to 10 μg of polymyxin B was then spotted on to a plate at the starting point, together with similar samples of the standards. In addition to the fermentation liquor, mixtures of the fermentation liquor with several standard substances were also applied at the start line. Development was carried out as described in the preceding part. To ensure that no trace of solvent remained, the plates were dried at 80° for 1 h, then held in ammonia vapour for 5 min to neutralize any acetic acid possibly still present, since this would interfere with the biological testing on the agar plates inoculated with *Bordetella bronchiseptica*. A sheet of filter paper was

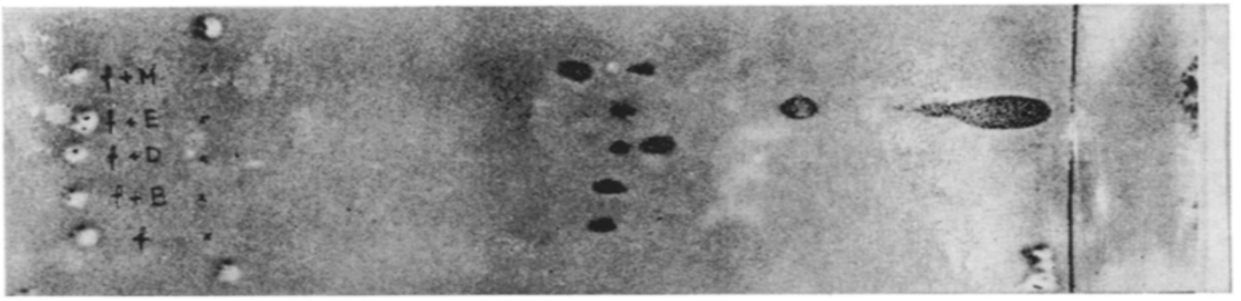

Fig. 3. Chromatography of fermentation broth containing polymyxin B, and of fermentation broths admixed with standard polymyxin substances. Spots made visible on an agar plate inoculated with *Bordetella bronchiseptica*.

laid on the agar plate and the chromatoplate was put on top. The latter and the filter paper were removed after 1 h and the agar plate was then incubated at 37° for 16 to 18 h. Fig. 3 shows that the active substance of the fermentation broths is distinct from all the standards, with the exception of polymyxin B. Thus the active substance produced is identical with it.

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Fingerprints of DNS-labeled protein digests on a millimicromole scale

The two-dimensional separation of protein hydrolysates on sheets of filter paper by combined electrophoresis and chromatography as developed by INGRAM[1] has become a standard technique for the study of similarities and differences among proteins. This so-called fingerprinting method has more recently been adapted to thin-