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Several Uses for Isobutyric Acid-Ammonium Hydroxide Solvent in Endotoxin Analysis

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Many steps in the analysis of rough and semirough endotoxins were found to be facilitated by the use of isobutyric acid-ammonium hydroxide solvent.

In 1975, Boman and Monner (1) observed that a solvent consisting of 5 parts isobutyric acid to 3 parts molar ammonium hydroxide, commonly used to separate peptidoglycan fragments, was useful for the paper chromatographic separation of different rough lipopolysaccharides (LPS) of *Escherichia coli*. Rosner et al. (8) later used this solvent in thin-layer chromatography (TLC) to characterize lipid fragments of one of the deep-rough LPS. In connection with our studies on endotoxins, for which few satisfactory chromatographic separations have been described, we found that the system could be extended to the determination of purity and of degree of heterogeneity (7) of LPS preparations and chromatographic characteristics of endotoxins of different rough and semirough bacterial strains and species, as well as for monitoring hydrolyses and derivatization reactions and column chromatography of LPS. The solvent was also modified for use with oligosaccharides with equal success.

Sources of endotoxin included *Neisseria meningitidis* serotype A, IM 1951; serotype B, IM 2228; and serotype C, IM 2135; *Haemophilus influenzae* Hi44; and *Bordetella pertussis* 1414 and 134. Cells or endotoxins of these strains were provided by the Institut Merieux, Lyon, France. Their LPS were extracted by either the phenol-water (10) or the phenol-chloroform-petroleum ether (3) method. To obtain their respective polysaccharide moieties, LPS were hydrolyzed for 1 h at 100°C in 1% acetic acid, followed by centrifugation at 400,000 × *g* for 45 min to remove the lipid moiety. LPS of *Salmonella minnesota* R595 and R7 and *E. coli* O111B4 were obtained from Sigma Chemical Co. LPS of *Vibrio cholerae* R mutant 1824 was a gift of B. Lindberg. For TLC, aluminum-backed, precoated Silica Gel 60 plates (E. Merck AG) were used. One to 1.5 h of development were usually sufficient to effect separations. Spots were most commonly detected by charring, which required about 5 μg of organic material. For ninhydrin-positive material, about 3 nmol was deposited. Excessive salt caused trailing. Detergents migrated close to the front and did not interfere.

LPS obtained from different bacterial strains can be characterized by migration upon electrophoresis in sodium dodecyl sulfate–15% polyacrylamide gels (Fig. 1) (4). When many of the same samples were developed on TLC with isobutyric acid-ammonium hydroxide as solvent, they were better separated (Fig. 2A and B) in 1 to 2 h. The silver stain method of detecting polysaccharides in gels (9) is very sensitive; it is, however, the only method in common use. TLC, on the other hand, has the great advantage that it allows the easy use of a number of nonspecific (e.g., carbonization by sulfuric acid) and specific detection methods for defined chemical functions (e.g., amines, phosphate, vicinal diols, radioactivity, etc.).

By altering the proportions of isobutyric acid and ammonium hydroxide, it was possible to separate many different polysaccharides, particularly those derived from endotoxic LPS. Figure 3 illustrates the migration patterns of several different polysaccharide preparations in the solvent isobutyric acid–1 M ammonium hydroxide, 3:5. The polysaccharide fraction of *E. coli* appears to be polydisperse, and those of *V. cholerae* and *N. meningitidis* contain two or three different molecular species of polysaccharide (6).

The method is also efficient for checking the purity of endotoxic preparations since common contaminants such as nucleic acids and phospholipids either did not migrate in the conditions given or migrated near the front. In fact, on a larger scale, all nonmigrating products could be removed

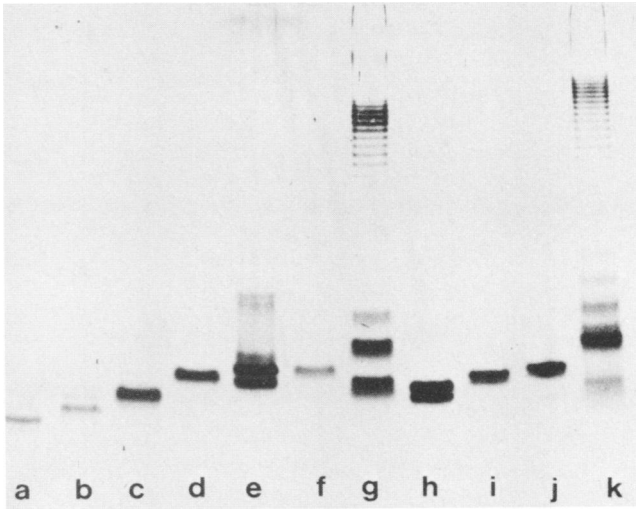

FIG. 1. Migration of the LPS of several bacterial strains upon electrophoresis in a sodium dodecyl sulfate–polyacrylamide gel. Lanes: a, *S. minnesota* R595 ($M_{r,app}$, 2,200); b, *S. minnesota* R7 ($M_{r,app}$, 2,600); c, *B. pertussis* 134 ($M_{r,app}$, 3,200); d, *B. pertussis* 1414 ($M_{r,app}$, 3,900); e, *H. influenzae*; f, *N. meningitidis* IM 2135; g, *E. coli* O111B4; h, *N. meningitidis* IM 1951; i, *N. meningitidis* IM 2228; j, *N. meningitidis* IM 2135; k, *S. minnesota*. Each well received 0.1 μg of an LPS. Molecular masses were calculated from references 5 and 7 and from unpublished data from this laboratory.

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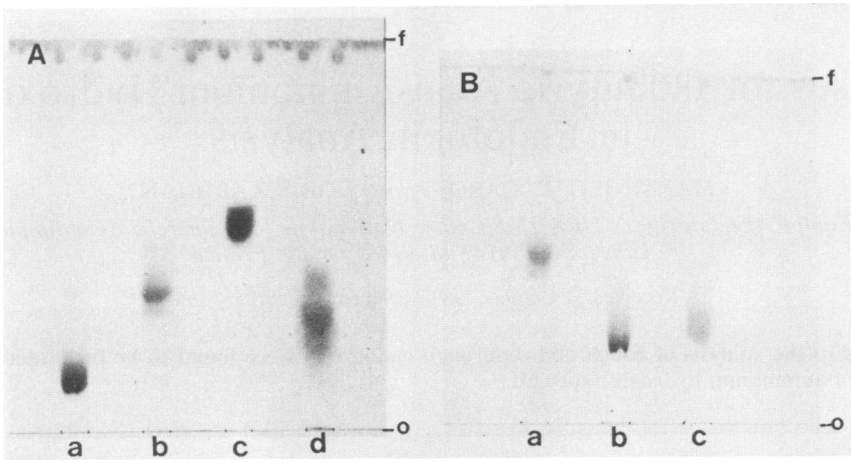

FIG. 2. Migration of LPS of several bacterial strains on TLC in isobutyric acid–1 M ammonium hydroxide, 5:3. Lanes: (A) a, *B. pertussis* 1414; b, *B. pertussis* 134; c, *S. minnesota* R595; d, *H. influenzae*; (B) a, *N. meningitidis* IM 1951; b, *N. meningitidis* IM 2228; c, *N. meningitidis* IM 2135. A 10- to 20-μg amount of LPS was deposited on each lane. Spots were revealed by charring. o, Origin; f, front.

from endotoxin samples by extraction with the solvent (10 mg of endotoxin per ml), followed by centrifugation (1,500 × *g*, 10 min). After removal of the excess ammonium hydroxide (rotatory evaporator, room temperature) and isobutyric acid (lyophilization) from the supernatant, endotoxin was obtained by trituration of the residue with ethanol and sedimentation at 1,500 × *g* for 10 min.

It is good laboratory practice to test that in radiolabeled endotoxin preparations the label is associated exclusively with the LPS and that the material has not undergone undesirable or uncontrolled chemical modifications. For this purpose, TLC in the solvent studied has proved to be very valuable.

The solvent and appropriate variants of it were also used for quick and easy monitoring of hydrolyses, derivatizations (N-acetylation, methylation, etc.), and other transformations of LPS and polysaccharide preparations.

Purification of endotoxic LPS and their derivatives usually involve liquid chromatography. We found that TLC in an appropriate mixture of isobutyric acid and ammonium hydroxide was eminently suitable to monitor column eluates and substituted perfectly for the laborious and time-consuming methods hitherto employed. For example, the polysaccharide fraction obtained by mild hydrolysis of *B. pertussis* 1414 endotoxin turned out to contain two polysaccharides, a dodecasaccharide, and a nonasaccharide (2). They were largely separable by chromatography on Bio-Gel P6 column. Figure 4 shows the fractionation profile obtained by TLC. The choice of fractions to be retained was much

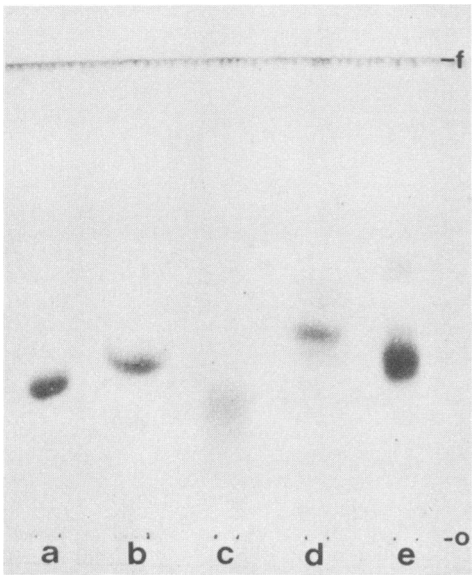

FIG. 3. Migration on TLC of endotoxin-derived polysaccharide obtained from several bacterial strains (see text). Solvent was isobutyric acid–1 M ammonium hydroxide, 3:5. Lanes: a, *B. pertussis* 1414; b, *B. pertussis* 134; c, *E. coli* K-12; d, *V. cholerae*; e, *N. meningitidis*. A 10- to 15-μg portion of polysaccharide was deposited on each lane. Spots were revealed by charring. o, Origin; f, front.

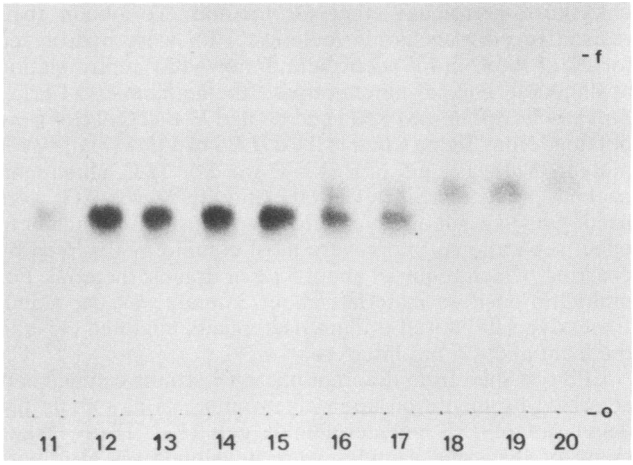

FIG. 4. Monitoring by TLC of chromatographic fractions from a column of Bio-Gel P6 (56 by 1 cm) loaded with the polysaccharide fraction (10 mg) of *B. pertussis* endotoxin. The flow rate was 3 ml/h. Numbers indicate fraction number after collection of an initial eluate (water) of 10 ml. A 5-μl portion of each fraction (0.5 ml) was deposited at the origin. The TLC solvent was isobutyric acid–1 M ammonium hydroxide, 3:5. Spots were revealed by charring. o, Origin; f, front.

more evident than it was by colorimetric analysis of the partially overlapping peaks.

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