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Analysis of Bacterial Fermentation Products by Isotachophoresis

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Various carboxylic acids from bacterial fermentation could easily be separated by isotachophoresis. The analyses were performed on an LKB 2127 Tachophor, and under the conditions used the minimum amount of sample that could be quantitatively estimated was approximately 0.1 nmol. The reproducibility of the method was good (ca. 5%). The time of analysis using a 23-cm column was 12 min. No pretreatment of the samples was required.

The analysis of acid end products is necessary in fermentation studies, and it is an important aid in the identification of anaerobic bacteria (7). The determination of the aliphatic series, "volatile" fatty acids C_2 to C_5, on gas-liquid chromatographic columns filled with porous polymers beads has been described (6, 8). The principal difficulty in these analyses has been the interaction of the acids with the column, causing tailing of the peaks and loss of the sample, or ghosting. For the quantitative estimation of mixtures including formic acid, lactic acid, or dicarboxylic acids, derivative formation is required but difficult to achieve (9, 10).

Isotachophoresis is an electrophoretic method that can be used for the qualitative and quantitative analysis of ionic species. A comprehensive treatise of the theory, instrumentation, and applications of isotachophoresis has been published by Everaerts et al. (5). In isotachophoresis, ions are separated according to differences in effective mobilities. After a steady state has been reached, the zones of the various ions migrate with equal velocities behind each other. The zones can be detected by appropriate detection systems.

Isotachophoresis has been applied for analysis of fatty acids (3), using methanol as a solvent. Low-molecular-weight fatty acids, including those from bacterial fermentations, are sufficiently soluble in water and can easily be determined in water. Formic acid, which is difficult to estimate with gas chromatographic methods, gives no problems in isotachophoresis.

Because isotachophoresis generally does not require pretreatment of samples, the method was expected to be very suitable for rapid analyses of acids in biological samples.

MATERIALS AND METHODS

Apparatus. The apparatus used in this investigation was the LKB 2127 Tachophor. The separation was performed in a 23-cm Teflon capillary with 0.4-mm ID. The capillary was kept at a constant temperature of 23°C. Some of the characteristics of the LKB Tachophor, including the ultraviolet (UV) light and thermal detection systems, have been described by Arlinger (1). The resolution of the thermal detector is about 50 times lower than that of the UV detector. The thermal detector responds to differences in mobility and is used for identification of ionic species. The UV detector signals are used to measure zone lengths. The origin of the UV peaks arises from the highly concentrating effect of isotachophoresis, leading to a concentration of any ionic impurities in the system. UV-absorbing impurities present in small amounts show up as peaks. These peaks indicate the boundaries between zones of sample compounds and can be used as markers.

Electrolytes. The experiments were carried out at two concentrations of the leading ion, depending on the concentrations of acids in the samples. The leading ion was chloride (2 or 10 mM), and Σ-aminocaproic acid (6-aminohexanoic acid) was used as the buffering counter-ion at pH 4. Polyvinylalcohol (0.05%) was added to the leading electrolyte to sharpen zone boundaries by depressing electro-endosmosis. The terminating electrolyte was sodium capronate, 2 or 10 mM, at pH 7.

The current was stabilized at 28 or 140 μA, depending on the concentration of the leading ion (2 or 10 mM). The voltage increased from 1.8 to approximately 8 kV at the end of the run in both electrolyte systems.

The electrolyte system used is suitable for the separation of a range of low-molecular-weight carboxylic acids, including volatile acids, hydroxy acids, keto acids, and dicarboxylic acids. In isotachophoresis the concentrations in the zones are adapted to the concentration of the leading ion. At a lower concentration of the leading ion the zones following the leading ion will also be more diluted, and longer zone lengths can be expected.

A concentration of 2 mM chloride was suitable for analyses of dental plaque extracts. Bacterial culture fluids were analyzed at a higher (10 mM) concentration of the leading ion.

All reagents used in this study were of analytical purity grade. The polyvinylalcohol was run through a

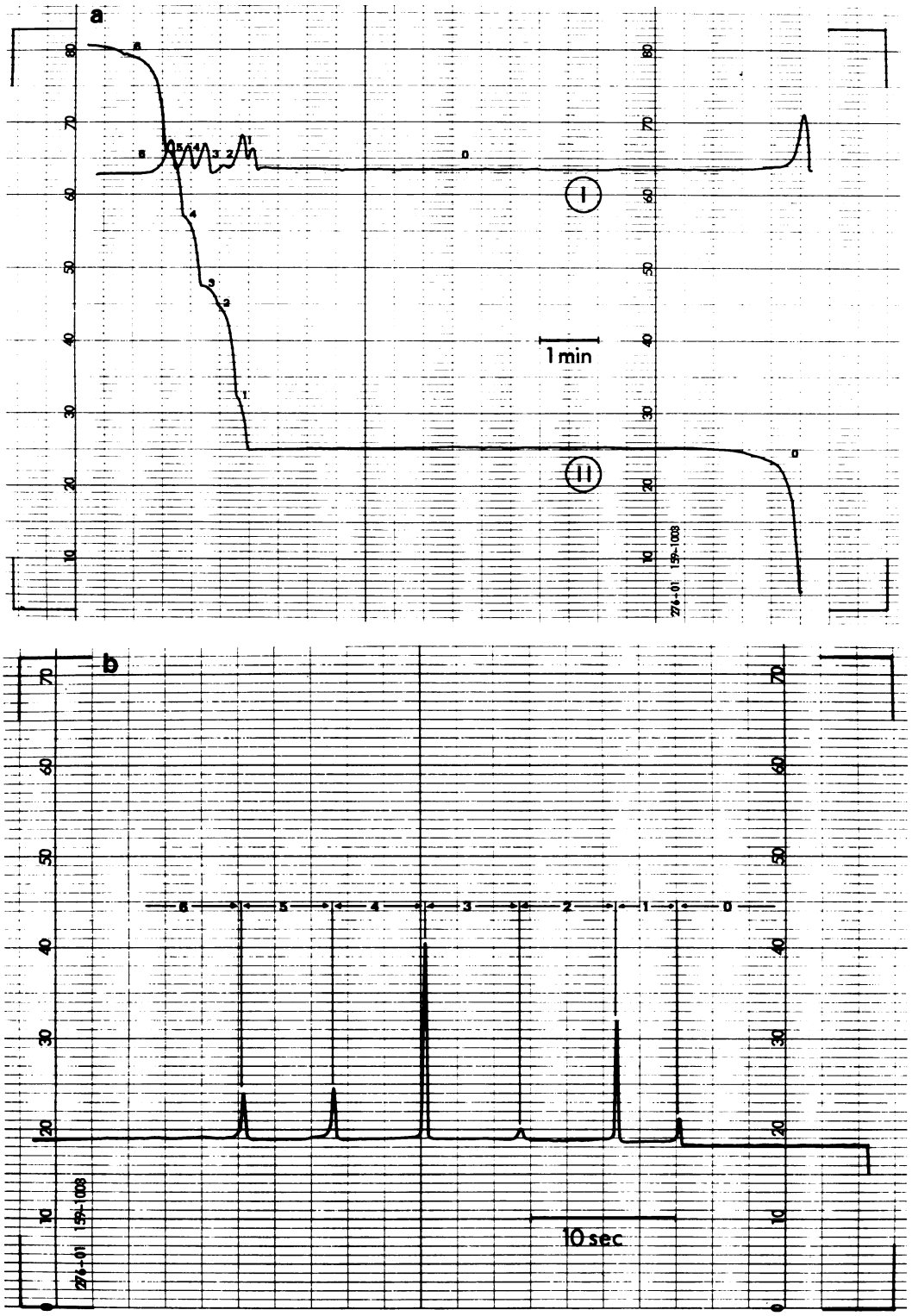

FIG. 1. *Isotachopherogram of a standard mixture (5 μl) containing 10.0 nmol of each of the following acids: 1, formic; 2, phosphoric; 3, lactic; 4, acetic; and 5, propionic. Zones 0 and 6 represent the leading and the terminator ions, respectively. Leading ion: chloride, 10 mM, at pH 4.0. (a) Thermal signal (I, 20 mm/min) indicating the steps of the various components. The differential signal (II) gives quantitative information. (b) UV detector signal (240 mm/min) showing the zones of the various components. To avoid wastage of recorder paper, a time clock switched on the paper drive at the end of the run just before the UV peaks are detected. Current stabilized at 140 μA.*

column filled with a mixed-bed ion exchanger to remove impurities.

After each run the separation column was cleaned by rinsing with leading and terminator electrolytes according to the instructions of the manufacturer. The rinsing procedure is very simple and only takes a few seconds.

Standard solutions. Standard solutions containing 0.5 or 2 mM formic, acetic, propionic, lactic, and phosphoric acids were prepared. Phosphate was included because it is present in significant quantities in dental plaque and bacteriological media.

Samples containing 1 to 5 μl were injected onto the column of the Tachophor, and calibration lines were constructed by plotting the zone length against the amount in nanomoles injected. Fatty acids higher than propionic acid (C_3) were not included in the tests.

Dental plaque. Gnotobiotic rats, inoculated with *Streptococcus mutans* C67-1, and conventional rats harboring complex microflora were fed on a powdered diet containing 16% saccharose. Dental plaque was removed with a probe and directly suspended in 100 μl of cold (4°C) 0.1 M histidine. The plaque was ultrasonically dispersed by using a Kontes Sonifier (K 881440) with a microtip for 30 s under cooling in ice. The suspension was centrifuged for 10 min at 20,000 × *g* in a Sorvall RC2B centrifuge at 4°C. The supernatant fluid (2 μl) was injected on the separation column.

Culture fluid. *S. mutans* C67-1 was grown in a chemostat under glucose limitation. The medium (PY) contained, per liter: peptone (Difco), 10 g; yeast extract (Difco), 10 g; and glucose, 10 g. Samples from the culture were centrifuged for 10 min at 20,000 × *g* (Sorvall RC2B) for removal of the cells. The supernatant

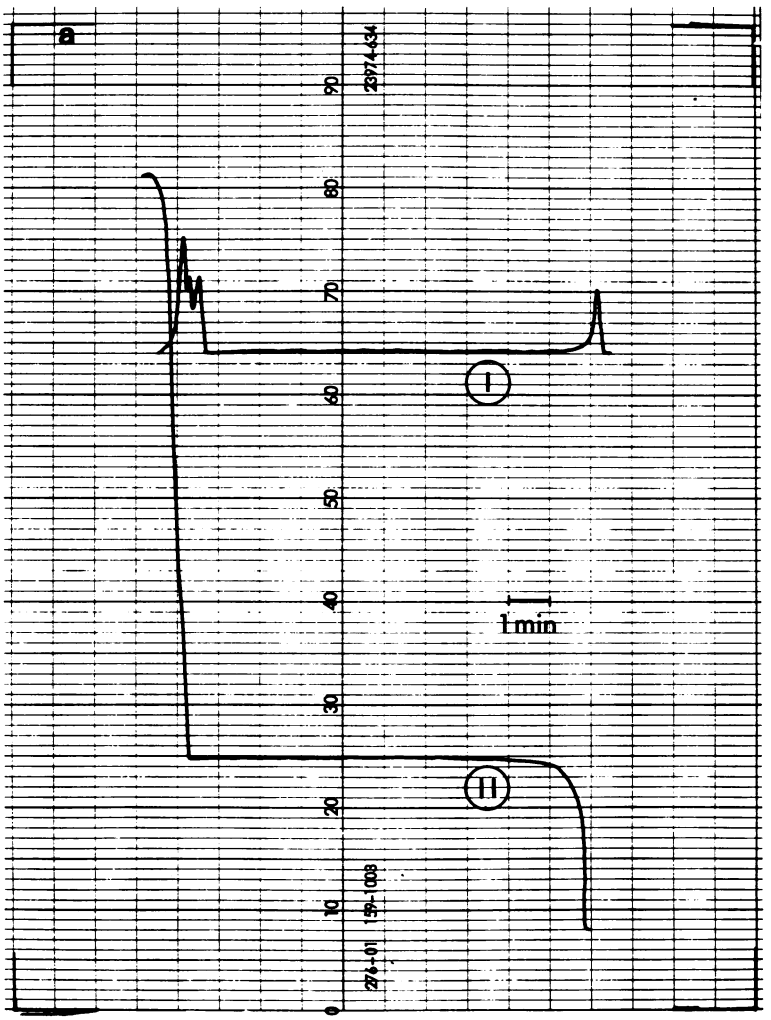

FIG. 2. *Isotachopherogram of a standard mixture (1 μl) containing 0.5 nmol of each of the following acids: 1, formic; 2, phosphoric; 3, lactic; 4, acetic; and 5, propionic. Zones 0 and 6 represent the leading and the terminator ions, respectively. Leading ion: chloride, 2 mM, at pH 4.0. (a) Thermal signal (I, 10 mm/min). (b) UV detector signal (240 mm/min). Current stabilized at 28 μA.*

fluid was 5× diluted in distilled water and injected onto the column of the Tachophor. The concentration of the leading ion was 10 mM.

RESULTS AND DISCUSSION

The separation of standard mixtures containing formic, acetic, propionic, lactic, and phosphoric acids in 2 or 10 mM leading ion is shown in Fig. 1 and 2. The time of analysis was 12 min. The minimum amount that can be estimated quantitatively is approximately 0.125 nmol, corresponding to a (minimum) zone length of 5 mm in the UV recording. The resolution of the thermal detector is much less than that of the UV detector. In the 2 mM electrolyte system, the UV recording reveals separation of the components, but no steps are seen in the thermal recording (Fig. 2a and b). In this concentration range, qualitative information must be obtained from the UV recording by the addition of reference substances to the sample. This procedure, however, is dependent on the presence of UV-absorbing contaminants marking the zones. The calibration line (Fig. 3) shows a linear relationship between zone length and sample size. The deviation in the slope of the line is less than 3%. The slope of the calibration line was similar for all components in the standard mixture. No difference was found in the zone length of an ion run either separately or in mixtures, which

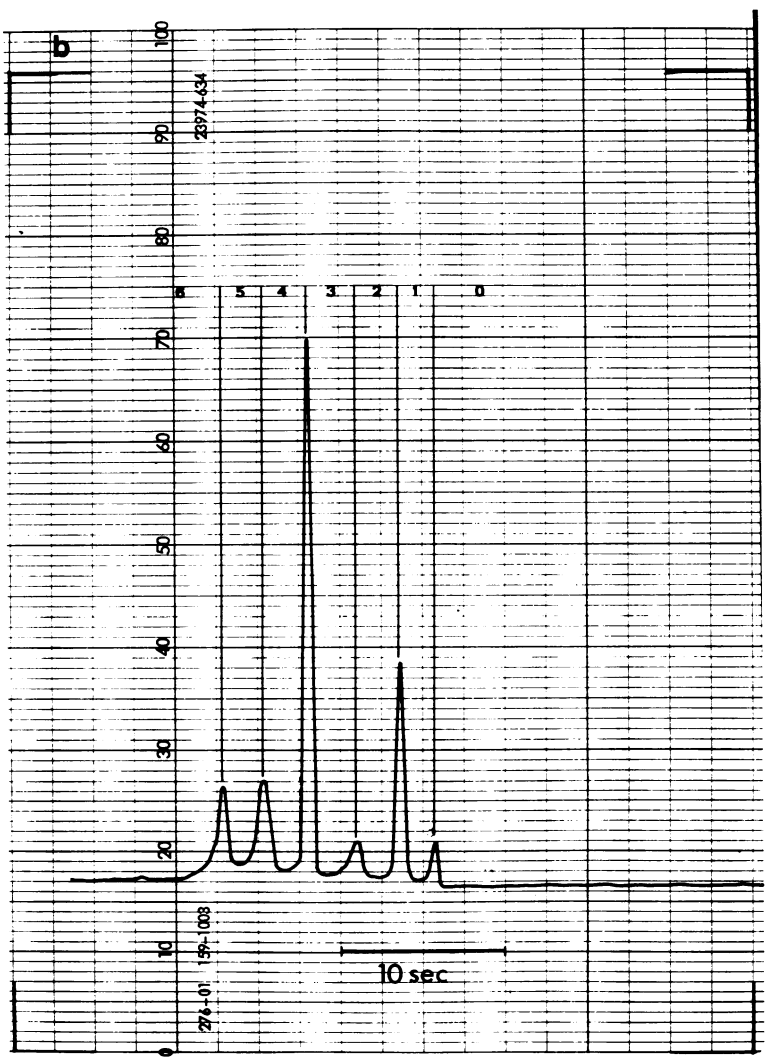

FIG. 2b.

confirmed that the zones do not have a mutual influence on each other (4). The reproducibility of the method is satisfactory. Deviations can be ascribed to the use of a 5-μl microsyringe for sample introduction.

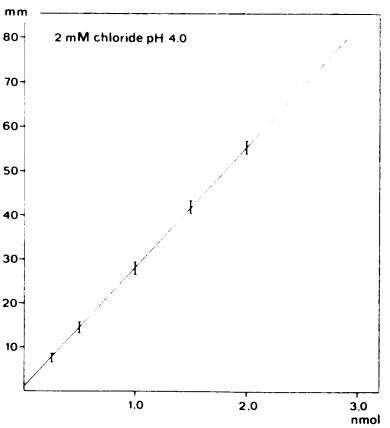

FIG. 3. *Calibration line for lactic acid. Zone length plotted against nanomoles injected. Analyses were performed with 2 mM chloride as the leading ion.*

The construction of calibration lines for each ionic species can be circumvented by using calibration constants (2).

Analyses of biological samples. Figure 4 shows the electropherogram of dental plaque consisting of *S. mutans* C67-1. Since dental plaque is only available in small quantities (0.1 to 1 mg, wet weight), the use of a low concentration of leading electrolyte (2 mM chloride) is required. As pointed out before, no qualitative information is then obtained from the thermal detector.

Instead, the identity of the ionic species in plaque was revealed by the addition of reference components to the sample. Elongation of a zone indicates the presence of the standard component added. This procedure should be repeated at a different pH of the leading electrolyte, which leads to changes in the electrophoretic mobilities and subsequent changes in the sequence of the zones.

By using this procedure, formate, phosphate, lactate, and acetate were identified in dental plaque from conventional rats (Fig. 5). In addition, some unidentified components were pres-

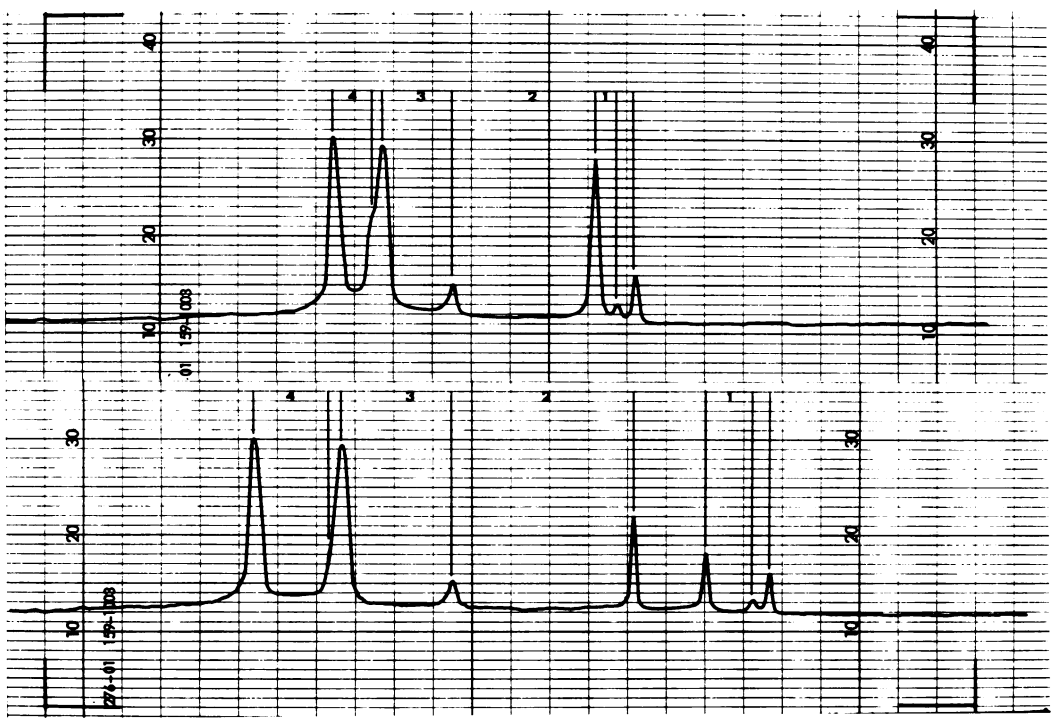

FIG. 4. *(Top) Isotachopherogram of dental plaque from gnotobiotic rats consisting of only S. mutans C67-1. The following anions were identified: 1, formate; 2, phosphate; 3, lactate; 4, acetate. Unidentified components not representing a common type of low-molecular-weight carboxylic acid are present (the zone before formate and another zone between lactate and acetate). (Bottom) Same sample plus standard solution containing formate, glucarate (between zones 1 and 2), phosphate, lactate, and acetate. Leading ion: chloride, 2 mM. Current stabilized at 28 μA.*

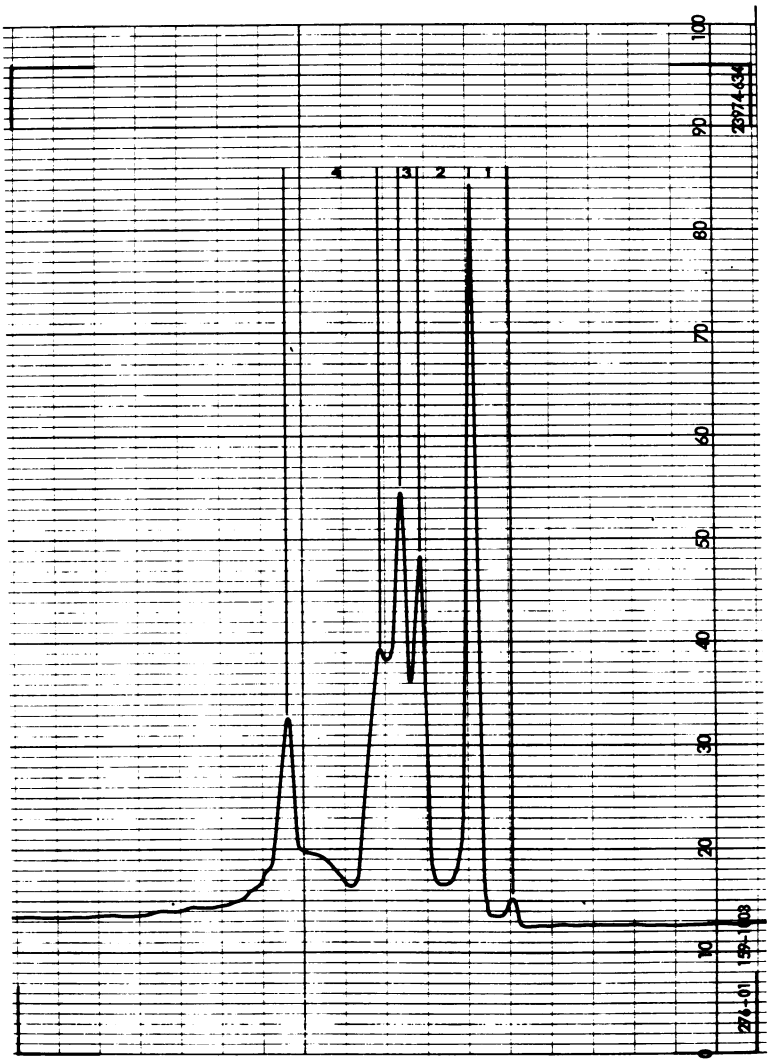

FIG. 5. *Isotachopherogram of dental plaque from conventional rats. The following anions were identified: 1, formate; 2, phosphate; 3, lactate; 4, acetate. Leading ion: chloride, 2 mM. Current stabilized at 28 μA.*

ent, including a UV-absorbing component which is only partially separated from acetate (Fig. 5). Acetate can be estimated accurately because the amount of the UV-absorbing component is very small.

The estimation of acid fermentation products in common broth cultures offers no problems. Figure 6 shows the UV recording of the culture fluid of *S. mutans* containing formate, lactate, and acetate and a small amount of pyruvate. Quantitative estimation in these samples is done by measurement of the zone length and using the calibration line.

No fatty acids higher than propionic acid were included in this study. However, the identification of butyric, isobutyric, valeric, and isovaleric acids is helpful in the identification of anaerobic bacteria. Using caproic acid as the terminating electrolyte, butyric acid is not always completely separated from the terminator. Instead, caprylic acid (*n*-heptanoic acid), which has a lower effective mobility at pH 4.0, can be used as a terminator electrolyte for the separation of fatty acids up to C_5 (valeric acid).

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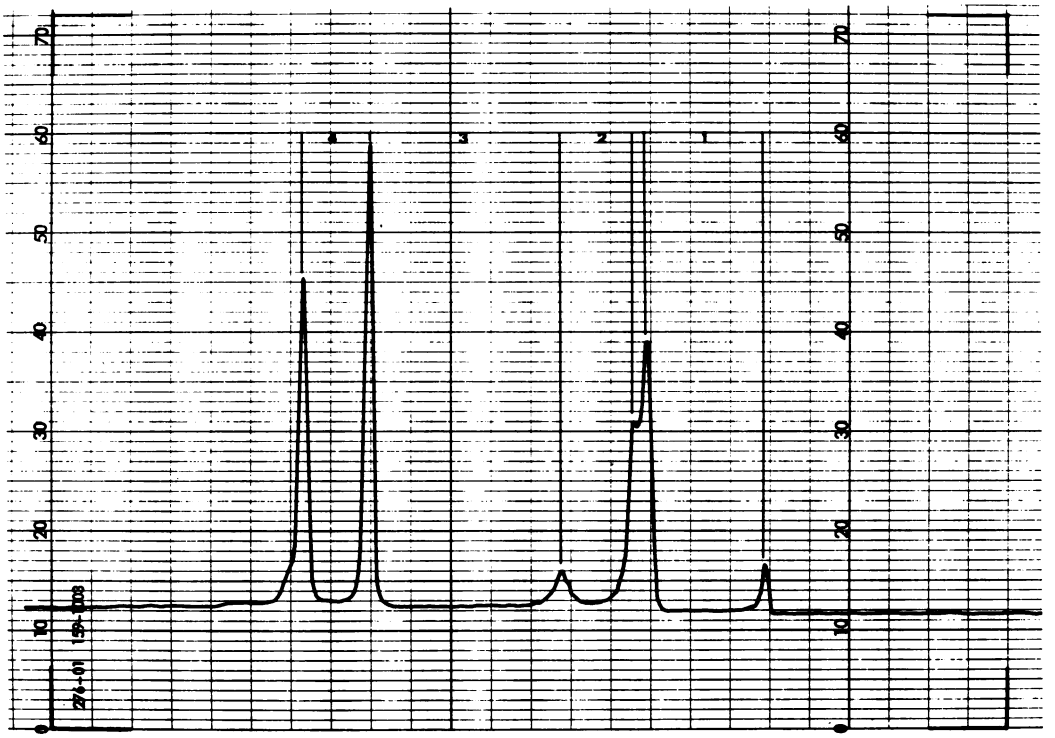

FIG. 6. *Isotachopherogram of the culture fluid of S. mutans C67-1 in PY medium. The following anions were identified: 1, formate; 2, phosphate; 3, lactate; 4, acetate. The small UV-absorbing zone between formate and phosphate represents pyruvate. Injection: 3 μl of 5×-diluted culture supernatant fluid. Leading ion: chloride, 10 mM. Current stabilized at 140 μA.*

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