Simplified Gas Chromatographic Procedure for Identification of Bacterial Metabolic Products

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A rapid and simple procedure is described for analysis of fermentation products from anaerobic bacteria grown in glucose broth. A 1-ml sample of the culture is drained through cation-exchange resin in a Pasteur pipette. The effluent fluid is directly analyzed isothermally in a gas chromatograph for volatile fatty acids (C_2 to C_6) as well as for lactic, pyruvic, and succinic acids. This procedure is considered to be suitable for routine use in clinical bacteriology.

Analysis of fermentation products from glucose was successfully used in identification of anaerobic bacteria (1). The fermentation products were separated isothermally in a gas chromatograph after volatile fatty acids were extracted from the culture broth with ether, and nonvolatile acids were methylated and extracted with chloroform. The time in the chromatograph was about 40 min (1).

Rogosa and Love (3) showed that the volatile fatty acids in fermentation media could be separated directly on the porous polymer PAR-1 with programmed heating of the gas chromatograph. Recently, various column materials for analysis of volatile fatty acids in aqueous solution were surveyed, and the porous polymer Chromosorb 101 in a glass column was found to separate volatile fatty acids, C_2 to C_6, isothermally in about 5 min (2).

By using the porous polymer Chromosorb 101, a time-saving procedure has now been worked out for analysis of fermentation products in glucose broth. The fatty acids are analyzed in aqueous solution, and lactic, pyruvic, and succinic acids can be separated together with the volatile fatty acids without any methylation step.

MATERIALS AND METHODS

Apparatus. The apparatus used was a dual-column gas chromatograph (model 5751G, Hewlett-Packard GmbH, Würtemberg, Germany) equipped with hydrogen flame detectors and a 2-mV span recorder (Servogor, AB Transfers, Stockholm, Sweden). Sensitivity of the electrometer: 1.0 × 10^{-18} A gave full-scale output on a 1-mV recorder at range 1 and attenuation 1. Coiled glass columns (ca. 1.83 m by 2 mm inner diameter) were used. The columns were packed with a porous polymer (Chromosorb 101, 80/100 mesh; Johns-Manville, Denver, Colo.) with a minimal amount of fine-grade glass wool in both ends. The columns were conditioned overnight at 250 C and repacked from the effluent end of the column, leaving 4 cm of space on the inlet side. The columns were then reconditioned at 250 C for 2 h and then run isothermally at 200 C, with inlet at 200 C and detector at 240 C.

A Hamilton syringe of 5-microliter capacity (75-N) (Hamilton Bonaduz AG, Bonaduz, Switzerland) was used to inject 0.5-microliter samples. The "dead space" of the syringe was filled with water, and the sample was injected directly into the column immediately above the column packing. Carrier gas flow was 10 ml of nitrogen per min, and the flow rates of hydrogen and air were optimized for acetic acid response.

Culture methods. The organisms were grown in prereduced, anaerobically sterilized (PRAS) media as described by Holdeman and Moore (1). Fermentation products from glucose were studied in a broth containing per liter: 10 g of neutralized bacteriological peptone (L34, Oxoid Ltd., London, U.K.); 10 g of yeast extract powder (L21, Oxoid Ltd., London, U.K.); 10 g of glucose; 0.5 g of cysteine HCl; H_2O and resazurin and salt solution as described by Holdeman and Moore (1).

Preparation of sample for gas chromatography. A sample of a culture in the glucose broth was applied on 1 ml of packed cation-exchange resin (AG 50W-X4, 200 to 400 mesh, hydrogen form, washed in water; BioRad Laboratories, Richmond, Calif.) on glass wool in a Pasteur pipette. The sample was allowed to drain through the resin, and the resin was then washed twice with 0.5 ml of water. All fluid from the pipette was collected in a test tube, and this fluid was directly analyzed in the gas chromatograph for volatile fatty acids as well as lactic, pyruvic and succinic acids.

For analysis of other nonvolatile acids, 1 ml of the fluid from the previous step was treated with metha-
nol and sulfuric acid as described by Holdeman and Moon (1).

One milliliter of the reaction mixture was then applied on 1 ml of packed anion-exchange resin (AG 1-X8, 200 to 400 mesh, formate form, washed in water; BioRad Laboratories, Richmond, Calif.) on glass wool in a Pasteur pipette. The sample was allowed to drain through the resin, and the resin was then washed twice with 0.5 ml of water. All fluid was collected in a test tube, and the methylated products in the fluid were analyzed in the gas chromatograph.

RESULTS AND DISCUSSION

The volatile fatty acids, C₂ to C₄, in mixture with lactic and succinic acids were separated in about 15 min in the gas chromatograph (Fig. 1). The recovery of these fermentations products from a glucose-broth culture was quantitative. In most clinical samples, caproic acid and succinic acid are not expected and the actual time of analysis is usually less than 10 min.

The relative retention times of the volatile fatty acids compared to acetic acid (1.0) were: propionic acid, 1.5; isobutyric acid, 2.0; butyric acid, 2.4; isovaleric acid, 3.3; valeric acid, 4.0, and caproic acid 6.5. Pyruvic, lactic, and succinic acids were eluted in asymmetric peaks with some tailing. Compared to the retention time of acetic acid (1.0), these acids started to be eluted at 2.1, 4.9, and 12.9, respectively. Ethanol, propanol, butanol, and pentanol had the following retention times relative to acetic acid (1.0): 0.5, 0.9, 1.4, and 2.4, respectively. The methyl esters of the acids had the following retention times compared to acetic acid (1.0): acetate, 0.8; propionate, 1.2; isobutyrate, 1.5; butyrate, 1.8; pyruvate, 2.0; isovalerate, 2.4; lactate, 2.4; valerate, 3.0; caproate, 4.9; fumarate, 8.5, and succinate 9.5.

Pentanol was not separated from butyric acid. However, the acid could be removed from the sample by passing the sample through the anion-exchange resin. If a sample contained a high amount of pyruvic acid, this acid was not separated from butyric acid. By methylating the sample, a separation of these acids was obtained. Lactic acid was usually eluted in only one peak (z, Fig. 2a). However, two other peaks (x and y, Fig. 2a) appeared if the injection of the sample was delayed after the needle of the syringe reached the top of the column packing. To get a quantitative response of lactic acid, the sample had to be in the needle and not in the glass part of the syringe during the injection procedure.

The fermentation products of a homofermentative lactobacillus, Lactobacillus acidophilus, are shown in Fig. 2b. The acetic acid in the chromatogram is a component of the glucose broth. Of various peptones and yeast extract powders tested for presence of lactic, pyruvic, succinic, and volatile fatty acids, only Trypti-case (BBL, Cockeysville, Md.) was found to contain significant amounts of acetic acid, which makes this peptone unsuitable for use in the fermentation media. Chromatograms of

![Fig. 1. Gas chromatographic separation of a mixture of 0.01 M acetic (A), propionic (P), isobutyric (iB), butyric (B), isovaleric (iV), valeric (V), and caproic acids (C), and 0.1 M lactic (L) and succinic acids (S) in aqueous solution. Glass column (ca. 1.83 m by 2 mm inner diameter) with Chromosorb 101 (80-100 mesh). Column temperature, 200 C. Injection port, 200 C. Flame detector, 240 C. Flow rate, 10 ml/min. Sample volume 0.5 μl. The signal attenuated 200 x.](image)

![Fig. 2. a, Chromatography of 0.1 M lactic acid. The injection of the sample was delayed after the needle of the syringe reached the top of the column packing. Lactic acid gave three peaks: x, y, and z. Conditions of chromatography were the same as in Fig. 1. b, Chromatogram of fermentation products in a glucose-broth culture of Lactobacillus acidophilus (NCDO 929). Conditions of chromatography were the same as in Fig. 1.](image)
fermentation products in glucose-broth cultures of *Eubacterium limosum* and *Bifidobacterium eriksonii* are shown in Fig. 3.

The stability of the porous polymer column was very good. More than 500 samples were separated on the column when the chromatograms presented were run. The separation characteristics of this column were the same as those of newly packed columns. The excellent stability of porous polymers was also noticed by Rogosa and Love (3), when they injected fermentation media directly on such columns. The purification of the sample by cation-exchange chromatography suggested in the present study improved the quality of the gas chromatograms and may also extend the life of the columns.

All the fermentation products used in primary identification of anaerobic bacteria (1) could be separated in just one isothermal run in the gas chromatograph. This means that almost any gas chromatograph can be used for this analysis. The simplicity and rapidity of the procedure justify the routine use of this method in clinical bacteriology.

**LITERATURE CITED**

1. Holdeman, L. V., and W. E. C. Moore (ed.). 1972. *Anaerobe laboratory manual*. The Va. Poly. Inst. and State Univ. Anaerobe Lab., Blacksburg, Va.

2. Ottenstein, D. M., and D. A. Bartley. 1971. Separation of free acids C3-C5 in dilute aqueous solution column technology. J. Chromatogr. Sci. 9:673-681.

3. Rogosa, M., and L. L. Love. 1968. Direct quantitative gas chromatographic separation of C1-C6 fatty acids, methanol, and ethyl alcohol in aqueous microbial fermentation media. Appl. Microbiol. 16:285-290.