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Use of Gas Chromatography for Determining Catabolic Products of Arginine by Bacteria

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A rapid and sensitive procedure for determining catabolic products of arginine metabolism by bacteria was developed. The method consists of inoculating a solution of L-arginine with a heavy cell suspension of the test organism. After a 2-hr incubation period, dissimilation products (citrulline, ornithine, agmatine, putrescine) are converted to volatile derivatives and analyzed by gas-liquid chromatography. Compared with conventional microbiological tests, the new procedure is rapid and can be used for sensitive quantitative measurements of specific metabolites from arginine.

Tests for the dissimilation of L-arginine are frequently included in the battery of biochemical reactions used to characterize and identify microorganisms (2, 3, 14, 15). The arginine decarboxylase test has been used for a number of years to differentiate among members of the *Enterobacteriaceae* (1, 7, 8) and the arginine dihydrolase test is particularly valuable for the *Pseudomonas* and other closely related bacteria (3, 10, 14, 15). Conventional laboratory tests for these enzyme reactions are made on the basis of a color reaction in a test medium containing added L-arginine and a *p*H-sensitive indicator (7), and, as such, fail to provide specific information about the nature of the reaction process. For example, the arginine dihydrolase system consists of arginine desimidase (L-arginine iminohydrolase), which breaks down arginine to citrulline and ammonia, and citrulline desimidase (carbamylphosphate:L-ornithine carbamyltransferase), which converts citrulline to ornithine, ammonia, and carbon dioxide (4, 13, 15). Because the ammonia produced from either or both of these reactions effects a color change, it is impossible to distinguish between the reaction processes of this system by conventional color tests. This lack of specificity has led to the recent development of a thin-layer chromatographic technique (TLC) for detection of arginine dihydrolase activity in nonfermentative gram-negative bacteria (16). In our laboratory we have developed a procedure using gas-liquid chromatography (GLC) to determine the catabolic products from arginine by resting cell suspensions of bacteria. Compared with TLC and conventional color tests, the procedure is rapid and simple and can be used for sensitive quantitative measurements of amino acid and amine metabolites from arginine.

MATERIALS AND METHODS

Cultures. The cultures used in this study were obtained from stock at the Center for Disease Control. The identity of each of the cultures was confirmed by established cultural and biochemical procedures (2, 11). Cells for the arginine dissimilation tests were grown for 24 hr at 37 C on triple sugar iron (TSI, Difco) agar slants and used immediately.

Tests for arginine dissimilation were made by GLC and by conventional procedures. Moeller's method as described by Edwards and Ewing (2) was used as the conventional test. Those media and procedures (2) were followed, except that the tubes were overlayed with sterile, melted petrolatum rather than mineral oil. For GLC analysis, two loopfuls of growth from the TSI slant were inoculated into 0.3 ml of a 2.5 mM solution of L-arginine (158 μg) in distilled water (*p*H 6.8). This dense cell suspension in substrate was mixed and incubated for 2.5 hr in a 37 C water bath. After incubation, the cells were removed by brief centrifugation; the remaining clear solution which contained arginine, citrulline, ornithine, or other reaction products was transferred to a high-temperature acylation tube (Regis Chemical Co., Chicago, Ill.) and dried at 100 C under a stream of nitrogen. The dried amino acids were then converted to *N*-heptafluorobutyryl (*N*-HFB)-*n*-propyl ester derivatives for analysis by GLC. The propylation step was carried out by adding 0.5 ml of an 8 M HCl-1-propanol mixture to the dried material and heating for 10 min at 100 C (9). The excess 1-propanol and HCl were then removed by evaporation to dryness under nitrogen at 100 C. At this step, 0.2 ml of heptafluorobutyric anhydride (Pierce Chemical

Co., Rockford, Ill.) and 0.1 ml of ethyl acetate were added, and the acylation tube was sealed. The tube was placed in an oil bath at 150 C for 10 min and then cooled to room temperature. The contents of the tube were evaporated just to dryness under a gentle stream of dry nitrogen. The dried *N*-HFB-*n*-propyl ester derivatives were dissolved in 0.1 ml of ethyl acetate, and 3 μliters was injected into the GLC apparatus.

Gas chromatographic analyses were made with a Perkin-Elmer model 900 instrument (Perkin-Elmer, Norwalk, Conn.) equipped with flame ionization detectors. Samples were analyzed on a 12 ft (ca. 366 cm) by 0.625 cm OD coiled glass column packed with 3% OV-1 (methyl silicone polymer) coated on 80 to 100 mesh, acid-washed, dimethylchlorosilane-treated, high-performance Chromosorb W (Applied Science Laboratories, State College, Pa.). The injection port temperature was 250 and the detector temperature 290 C. The column was maintained at 150 C for 5 min after injection of the sample and then temperature-programmed to 255 C at 5 C/min. The electrometer range was 10 with an attenuation of 16. This range resulted in a full-scale deflection of 3×10^{-10} *A* with a 1-mv recorder. Helium was used as the carrier gas at a flow rate of approximately 50 ml/min.

RESULTS AND DISCUSSION

Most clinical microbiology laboratories use Moeller's method (7) as the standard reference procedure for determining arginine dihydrolase and arginine decarboxylase activity. This method, however, is based only on a change in a *p*H-sensitive indicator and thus does not specifically identify or differentiate the possible reaction products from arginine. In addition to the arginine dihydrolase enzyme system which converts L-arginine to L-ornithine, arginine can be converted to ornithine and urea by arginase (L-arginine amidinohydrolase) and to agmatine and CO_2 by arginine decarboxylase (L-arginine carboxy-lyase). The agmatine from the decarboxylase reaction can be further degraded to putrescine and urea or to putrescine, CO_2, and NH_3. The alkaline products from either of these reactions (urea, NH_3, agmatine, putrescine) may produce a positive test by Moeller's method. Because of the reported absence of arginase in *Pseudomonas* (13), it appears that the principal mechanism for the conversion of arginine to ornithine in these organisms is the arginine dihydrolase pathway. The three enzymes involved in this pathway have, in fact, been firmly established in *P. aeruginosa* (12, 13), *P. fluorescens* (12, 13), and in *Streptococcus faecalis* (10). The reaction mechanism for catabolism of arginine by *Enterobacteriaceae* is not as well defined and may involve multiple pathways (8).

The GLC procedure used in this study provided specific information of the products from arginine catabolism. Figure 1 shows a GLC chromatogram of standards of the three amino acids involved in the arginine dihydrolase reaction. It is apparent from the chromatogram that the GLC procedure provides a means whereby the enzyme substrate (arginine) and breakdown products (citrulline and ornithine) can be determined simultaneously. The derivatives of each of these amino acids formed sharp, symmetrical GLC peaks which were well resolved from each other on the 3% OV-1 column. The retention time for citrulline was 11.5 min, for ornithine 13.5 min, and for arginine 19.5 min.

An additional advantage of the GLC procedure is that it can be used for the simultaneous determination of amine metabolites from arginine as well as amino acids. Shown in Fig. 2 is a GLC chromatogram of standards of putrescine, cadaverine, and agmatine which are the direct decarboxylation products of ornithine, lysine, and arginine, respectively. With the same derivatization procedure described for amino acids, the derivative (acylation product) of each of these amines showed only one sharp, symmetrical peak by GLC. When analyzed on the 3% OV-1 column, the retention times of these compounds (putrescine, 7.0 min; cadaverine, 10.0 min; agmatine, 14.5 min) were different from those of the amino acids run under identical GLC conditions (Fig. 1).

With the GLC procedure, those organisms reported to dissimilate arginine by the arginine dihydrolase pathway gave definitive results. Both *P. aeruginosa* and *S. faecalis* showed a large peak for ornithine, a small peak for citrulline, and a concomitant decrease in the concentration of arginine. The conversion of arginine to ornithine was essentially complete with these two organisms. Organisms reported to be arginine dihydrolase-negative [e.g., *P. acidovorans* (3)] failed to metabolize arginine, and only one peak appeared on the chromatogram. Uninoculated substrate and heat-treated cells added to the substrate served as controls; these also showed only one large peak (arginine). Nevertheless, care was taken to avoid contaminating the arginine substrate with medium associated with cells transferred from the TSI slants.

The dissimilation of arginine by various organisms was determined with the GLC procedure and with Moeller's test (Table 1). Strains of *P. putida*, *P. aeruginosa*, *S. faecalis*, and *Aeromonas hydrophila* gave a moderate to

strong color change in Moeller's medium after 24 hr of incubation. Each of these strains was also strongly positive by the GLC procedure as evidenced by the appearance of a large peak for ornithine and the essentially complete disappearance of the arginine peak (substrate). With the GLC procedure, citrulline produced by these strains could be detected even though only small to trace amounts (15 to 30 μg) were produced. A 3- to 5-day incubation period was required for a positive Moeller's test with *P. alcaligenes* and *P. pseudoalcaligenes*. However,

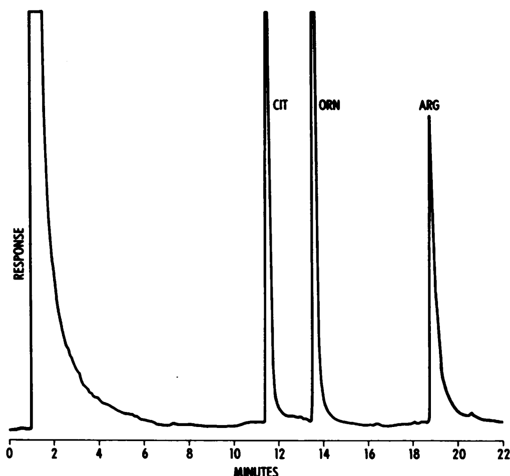

FIG. 1. *Gas chromatogram of the N-heptafluorobutyl-n-propyl ester derivatives of citrulline (4 μg), ornithine (4 μg), and arginine (3 μg) run on a 3% OV-1 column.*

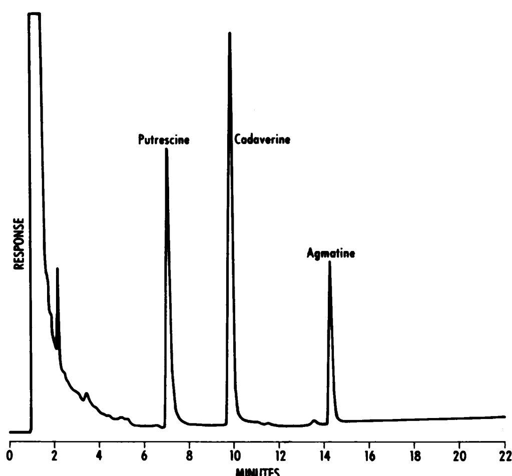

FIG. 2. *Gas chromatogram of the acylated derivative (heptafluorobutyl) of putrescine (3 μg), cadaverine (4 μg), and agmatine (2 μg) run on a 3% OV-1 column.*

TABLE 1. *Results obtained by Moeller's test and a gas-liquid chromatographic (GLC) procedure for determining dissimilation products from arginine by various microorganisms*

Organism	No. of strains	Reactions[a] by Moeller's test	Products from L-arginine detected by GLC		
			Putrescine	Citrulline	Ornithine
Pseudomonas putida	1	1 (m)	–[b]	+	++++
P. aeruginosa	2	1 (s)	–	T	++++
P. pseudoalcaligenes	1	3 (w)	–	T	++++
P. alcaligenes	1	5 (w)	–	T	+++
P. acidovorans	1	Neg	–	–	–
P. stutzeri	1	Neg	–	–	–
P. testosteroni	1	Neg	–	–	–
P. cepacia	3	Neg	–	–	–
P. maltophilia	4	Neg	–	–	+
Streptococcus faecalis	1	1 (s)	–	T	++++
Aeromonas hydrophilia	1	1 (m)	–	+	++++
Escherichia coli	2	2 (m)	T	–	+
Arizona hinshawii	1	4 (m)	T	–	+
Citrobacter freundii	1	2 (w)	T	–	+
Salmonella anatum	1	2 (w)	T	–	+
S. enteritidis bioserotype paratyphi A	1	5 (w)	T	–	+
S. enteritidis serotype typhimurium	1	3 (m)	T	–	+

[a] No. of days of incubation at 37 C for a positive test. Neg = negative test after 7 days of incubation. Letter in parenthesis indicates intensity of reaction; w = weak, m = moderate, s = strong.

[b] Symbols refer to the general magnitude of GLC peaks. T = peak within less than 10% full-scale deflection; + = 10 to 39%; ++ = 40 to 69%; +++ = 70 to 90%; ++++ = peak with full-scale deflection or greater; – = not detected.

with resting cells a large ornithine peak was produced after only 2 hr. These data indicate that the GLC procedure enables one to avoid the extended incubation period required with Moeller's test to detect slow or weak positive reactions. Agmatine and putrescine were not detected from any of the strains which produced large amounts of ornithine.

Each of the four strains of *P. maltophilia* examined showed no change in Moeller's medium after 7 days of incubation (Table 1). However, a small peak for ornithine was observed with the GLC procedure indicating that these strains possessed a very limited, but definite, activity for dissimilation of arginine. The production of ornithine by these strains was approximately 5% of that of *P. alcaligenes* and approximately 2% of that of *P. aeruginosa*. Strains of *P. acidovorans*, *P. stutzeri*, *P. testosteroni*, and *P. cepacia* were negative both by GLC and with Moeller's test. Thus, our results in Moeller's medium with *Pseudomonas*, except *P. maltophilia*, are consistent with those of other workers (3, 11, 14). In a recent study, Goldschmidt and Lockhart (4) reported that a strain of *P. maltophilia* contained an active arginine decarboxylase and gave a weak positive reaction by Moeller's test. The decarboxylase activity was confirmed by differential extraction of agmatine from the medium with 1-butanol and subsequent analysis of this compound with a modified diacetyl reagent which was reported to be specific for guanidino-containing compounds (5). Each of our four strains was extracted with 1-butanol after 7 days of incubation in Moeller's medium (plus arginine), and the 1-butanol fraction was tested with the diacetyl reagent and with the GLC procedure. Results with the diacetyl reagent were negative; no putrescine (less than 1 μg) or agmatine (less than 0.8 μg) was detected from either strain by GLC.

Cultures of representative members of the family *Enterobacteriaceae* showed weak to moderately positive reactions in Moeller's medium (Table 1). Initially each of these cultures gave a strong acid reaction which progressively decreased in intensity to a basic reaction at varying periods of incubation. Products from the resting cells of these bacteria were markedly reduced compared with the quantities produced by *P. aeruginosa* and *S. faecalis*. However, small amounts of L-ornithine and trace amounts of putrescine were detected by GLC. A 1-butanol extraction of Moeller's medium was carried out when each culture became alkaline, and the extract was tested with the diacetyl reagent and with GLC. The diacetyl test was positive for each culture; the GLC analysis showed relatively large amounts of putrescine and moderate to small amounts of agmatine. The presence of agmatine and putrescine is consistent with the known pathways of arginine metabolism in *Escherichia coli* as reported by Morris and Pardee (8). These workers demonstrated the presence of arginine decarboxylase, which decarboxylates arginine to agmatine, and agmatine amidinohydrolase, which converts agmatine to putrescine and urea. They further showed that putrescine was also produced by the direct decarboxylation of ornithine by L-ornithine carboxy-lyase. The formation of ornithine from arginine in *E. coli* could occur from one or more reactions including arginine dihydrolase. The limited activity observed with resting cells of *Enterobacteriaceae* could be due to a number of factors such as *p*H (1, 4, 6, 8), cofactors (4, 6), oxygen tension (4, 6), and temperature (1, 4). The effect of these parameters on the resting cell GLC procedure is being investigated.

The GLC procedure described in this report provides a means for rapid detection and sensitive quantitative measurements of amines and amino acids from arginine metabolism. The method alone does not provide sufficient information to identify specifically the enzyme reaction or enzyme system producing the product(s). This, however, is of secondary importance to the clinical microbiologist whose principal concern is rapid, accurate, and specific identification. The GLC procedure is not limited to arginine but could easily be adapted to study catabolic products of other amino acids.

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